

# LIVER IMMUNOLOGY

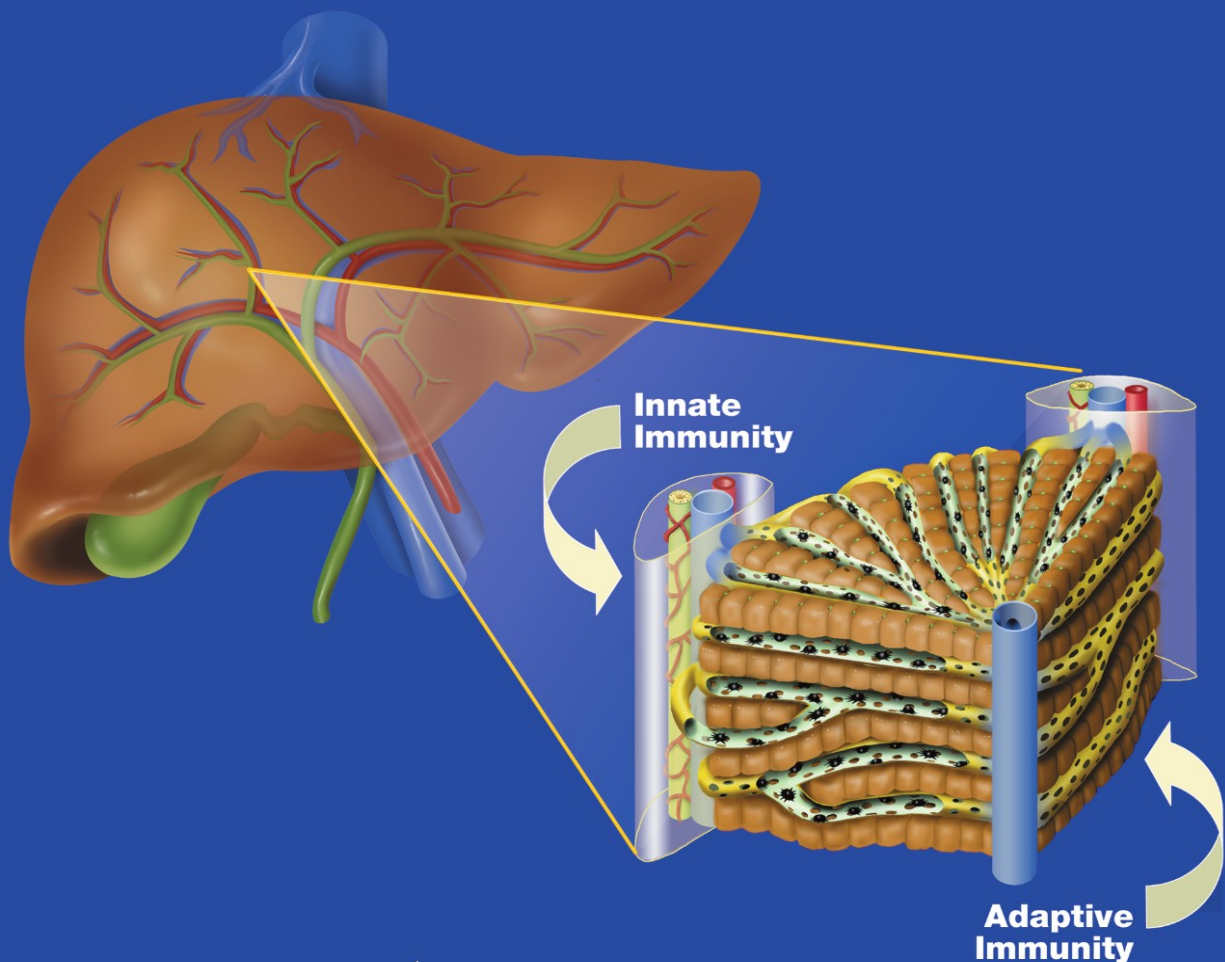
*Principles and Practice*

Edited by

M. Eric Gershwin, MD, FACP

John M. Vierling, MD, FACP

Michael P. Manns, MD



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*The editors and authors of this book dedicate the text and its contents in memory of Antony "Ryan" Moore (November 16, 1978 to March 12, 2005) "Fund for the cure and United through education, research and support." Together with the gracious help of PBC-ers, we will solve and cure not only primary biliary cirrhosis, but other immune-mediated liver diseases as well.*

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# Preface

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Recognition of the importance of the liver to health by Babylonians in the 19th century BCE stands in stark contrast to the relative obscurity of the liver in the minds of most educated adults today. Medical appreciation of the vital nature of the liver's diverse functions continues to evolve along with our efforts to better understand a multitude of hepatobiliary diseases caused by alcohol, xenobiotics, viruses, autoimmunity and genetic diseases. The unanticipated success of liver transplantation in the absence of histocompatibility matching between donor and recipient showed that the hepatic environment is immunosuppressive. Further studies proved that liver transplantation also protected other transplanted organs from being rejected, indicating that the liver is truly an immunologic organ. Recent data provide new insights into the physiological roles of hepatocytes, sinusoidal lining cells, activated macrophages (Kupffer cells), cholangiocytes and stellate cells, and their modulation of T cells, natural killer (NK) cells and NKT cells. Concurrently, studies of the pathogenetic mechanisms involved in hepatobiliary diseases have provided unequivocal evidence that the pathogenesis of virtually all hepatobiliary diseases involves inflammation involving the innate and/or adaptive immune responses.

Progress in our understanding of the liver as an immune organ and immunopathogenesis of diverse hepatobiliary diseases provides hope that this knowledge will rapidly be translated into more effective therapies in the near future. These factors were the impetus for the second edition of *Liver Immunology: Principles and Practice*, which is directed to clinicians, investigators and students. The editors are indebted to the all of the authors who have donated their talents, intellects and expertise to provide "state-of-the-art" contributions. All of us hope that this book will provide new perspectives of hepatobiliary physiology and pathophysiology and stimulate creative approaches to accelerate the pace of research progress in the field. Time has validated our belief that continued studies of immunology of the liver will ultimately improve the care and the prognosis of patients afflicted with a diverse array of hepatobiliary diseases. The editors have many people to thank, not the least of which are the contributors, all of whom worked very hard to have their manuscripts delivered on time and in the style we requested. However, we especially want to thank Nikki Phipps and Kathy Wisdom, our assistants at UC Davis, who worked so hard to make this book a reality.

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# Contemporary Liver Immunology and Immunopathology

## *Obstacles and Opportunities*

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IAN R. MACKAY

### KEY POINTS

- The liver is an important contributor to and prominent victim of the immunological reactivities of the body, thus providing a rationale for a second edition of this dedicated text on liver immunology. This introductory chapter identifies progress and problems among the immunoinflammatory liver diseases.
- For the autoimmune liver diseases, connectivities between the diagnostic “marker” autoantibodies and the damaging immune effector processes that impact on hepatocytes in autoimmune hepatitis (AIH) and cholangiocytes in primary biliary cirrhosis (PBC) remain obscure.
- For viral hepatitis, the same assembly of CD4+ and CD8+ T lymphocytes that eliminates virus-infected hepatocytes in recovery cases causes the futile damaging attack on hepatocytes in nonrecovery cases: better understanding of factors that determine this distinction is needed.
- Drug-induced liver diseases include various pathogenetic entities likely based on subtle gene polymorphisms. Animal models are scarce, and “human models” often emerge only with population exposures. Some types depend on drug metabolite interaction with a particular CYP450 isoform that eliminates the drug.
- Transplantation liver immunology, involving host-versus-graft or graft-versus-host disease, reveals elements of both immune privilege and immune vulnerability of liver cells. Considerations include the particular cytoarchitecture of liver, carry-over of functional passenger immunocytes of the donor in a hepatic allograft, and unique interactions of cholangiocytes with the immune system.
- Alcoholic hepatitis, and nonalcoholic steatohepatitis (NASH) with its creep to “cryptogenic cirrhosis,” add broader (and mysterious) dimensions to the immunoinflammatory liver diseases, dependent on the innate immune system,

production of proinflammatory cytokines, and stellate cell-induced fibrogenesis. Human studies and mouse models tell us to “remove the fat, cure the disease.”

### INTRODUCTION

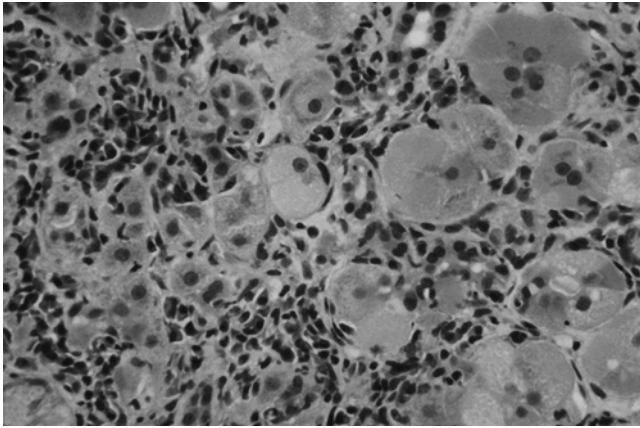
The relatively young science of immunology is just past its first centennial, and its detachment from microbiology was only 50 yr back. Since then remarkable progress has taken place in medical immunology, and hybrid disciplines have emerged: first neuroimmunology and, later, even osteoimmunology (1). The claim for liver immunology is amply justified by the role of the “lymphoid liver” as a constitutive part of the general immune system and in being the seat of several diseases because of particular immunological malfunctions (2,3). Indeed the liver, according to Knolle, Chapter 2, is a “unique immunological organ.” It is highly enriched in elements particular to the immune system, including cell systems with innate immune capacities such as Kupffer cells and sinusoidal epithelial cells, and cells participating in adaptive immune responses.

An “aerial” survey is provided here of the various immune-mediated liver pathologies: autoimmune diseases that destroy liver parenchyma or biliary ductular cells; virus-dependent diseases in which futile host immune responses provoke inflammatory damage to virus-harboring liver cells; immunologically mediated drug-induced liver diseases associated with faulty enzymatic degradation/disposal of medicinal drugs; alloreactive hepatitis or biliary ductulitis resulting from histocompatibility differences, as either host-versus-graft (HVG) or graft-versus-host (GVH) reactions; and finally diseases that are provoked independently of adaptive immunity by innate responses to noxious cytoplasmic inclusions, particularly lipids, with generation of damaging cytokine fluxes, notably nonalcoholic steatohepatitis (NASH). This introductory conspectus provides a rationale for liver immunology and a preface for the ensuing expert chapters.

### AUTOIMMUNE LIVER DISEASES

#### AUTOIMMUNE HEPATITIS

Knowledge on autoimmune hepatitis (AIH) has accumulated for over 50 yr such that readers could readily believe that all



**Fig. 1.** Histological appearances of liver in an acute phase of autoimmune hepatitis showing ballooned hepatocytes and pericellular lymphoplasmacytic infiltration. What is the operative effector agent(s)? Where lies the hepatocellular target? HE×800 (Photomicrograph kindly provided by Dr Nigel Swanson, University of Western Australia, Perth, Australia).

that should be known is at hand. Yet, since the first edition, published in 2003, many new insights have emerged and more are needed. There have been substantial benefits for liver immunology from the International Autoimmune Hepatitis Group (IAIHG) criteria for AIH, particularly in epidemiological settings (4). However, for clinical purposes, we look to a “streamlining” of the criteria, e.g., by an evaluation based merely on histological features, hypergammaglobulinemia, autoantibody responses, and absence of markers of hepatitis virus infection (5). Hepatologists have retained the concept of two types of AIH, -1 and -2, despite the difference being based mainly on serological expressions. However, the mutual exclusivity of these expressions at least dispels the idea that disease-defining hepatitis-associated autoantibodies (*see below*) occur merely as a consequence of liver cell destruction and antigen spillage; even so, an element of hepatic immunoreactivity does actually appear to be damage-dependent (*see below*). We urgently need to redress our insufficient knowledge on pathogenesis of AIH including both the inductive and executive/effector processes that result in the striking histological appearances shown in Fig. 1, drawing on modern concepts and technologies.

Extreme polyclonal hypergammaglobulinemia and particular autoantibodies are hallmarks of AIH (6). Hypergammaglobulinemia is in part dependent on the disease activity to the degree that it is a useful laboratory marker of response to treatment but the components of this response are unknown. The major autoantibodies detectable by indirect immunofluorescence (IIF) include in AIH-1 homogeneously reactive antinuclear antibody (ANA) and smooth muscle antibody (SMA) and in AIH-2 liver–kidney microsomal antibody (LKMAb).

In AIH-1 ANA could align the disease, despite its usual liver restriction, with the multisystem rheumatic disorders. The nuclear reactant is likely to be nucleosomal, nuclear chromatin, histones (7), but, in contrast to systemic lupus erythematosus

(SLE), the homogeneous ANA pattern fades during remission to unveil indeterminate speckled reactivities. The autoantibody demonstrable on smooth muscle substrates (SMA) is known to be reactive with various filamentous elements of the cellular cytoskeleton: the reactant specific for AIH-1 is microfilaments representing polymerized F-actin (8,9). However, discriminating assays are needed to analyze the multiple reactivities that constitute SMA, namely antibodies to actin (microfilaments), vimentin, desmin, etc. (intermediate filaments), and tubulin, and to assign disease specificities to these. Anti-F-actin can be assessed by IIF reactivity of serum with F-actin in renal glomeruli and tubules, SMA-g, and SMA-t (10), and with actin microfilaments in cultured cells (8). Notably, anti-F-actin serologically separates AIH-1 from SLE with which it was once allied. The autoantigenic properties of F-actin have been neglected given that this is a functionally important molecule with binding sites for over 70 cytoplasmic proteins (11), not least of which is its essential motility partner, myosin. Indeed myosin may contribute as an antigenic reactant for SMA and, like actin, is abundant in hepatocytes (12). Analyses of the actin autoantigen could include fine epitope mapping, additional to the single report of an epitope site within the C-terminus of  $\alpha$ -actin (13), and functional studies based on actin motility *in vitro* (14). A further reactant in AIH-1 is a cytoplasmic molecule first specified as liver–pancreas/soluble liver antigen (LP/SLA), now molecularly characterized as UGA-serine transfer (t)-RNA protein complex; its detection can identify patients with AIH-1 that are otherwise seronegative and, more controversially, those with likely severe or progressive disease (15), but no pathogenetic role for LP/SLA antibody has been ascertained. The antineutrophil cytoplasmic antibody (ANCA) is yet another interesting specificity, described in the 1960s at high prevalence and titer as “granulocyte-specific ANA” (16), so predating the use of “ANCA” by several years. However, these ANCA are not reactive with the usual substrates, myeloperoxidase and proteinase 3; the evidence that reactivity is with a neutrophil nuclear antigen prompted the acronym ANNA (17) (also used for the different paraneoplastic antibody, antineuronal nuclear antibody). Finally there is the autoantibody described in the 1980s as reactive with the asialoglycoprotein receptor; this has not received much attention lately because of either the difficulty in preparation of “assay-quality” antigen or an insufficient specificity for diagnosis of AIH (6,18).

AIH-2 versus AIH-1 has more of the features of a true organ-specific autoimmune disease and is mostly seen in childhood. The distinguishing LKM reactant has been molecularly identified as the 2D6 isoform of the large multifunctional cytochrome P450 enzyme family (CYP450 2D6), enriched in but not specific to liver. Various linear epitopes have been mapped using synthetic peptides; however, as for other autoantigenic molecules, most may be parts of complex conformational epitope structures (19). Also, as for other enzyme autoantigens, antibodies inhibit enzymic activity *in vitro*. Also recognized are various other anti-LKM-like specificities, mostly in drug-induced forms of hepatitis, with reactivity often directed

against the P450 isoform that hydroxylates the culprit drug, as described below. Is there then some undetected molecule that, in the course of its disposal by P450 2D6, initiates the spontaneously occurring form of the disease? Some 5% of hepatitis C virus carriers give low titer positive tests for anti-CYP450 2D6 but clinical expressions in such cases do not simulate those of an AIH; these autoantibodies in spontaneous and HCV-associated cases show a degree of epitope overlap (18) but more data are needed. The other frequent autoantibody in AIH-2 is the liver cytosol antigen type 1 (LC-1), now molecularly identified as formiminotransferase cyclodeaminase; this has greater specificity but lower sensitivity for diagnosis than anti-LKM. The identified autoantigens for AIH-2 have not been implicated in pathogenesis, yet these have proved effective as immunogens in generating an experimental model of AIH-2, as described below. There are various other autoantibodies described in AIH, so amounting to a real plethora of reactivities, and this is matched by multiple components discernible when sera are tested by immunoblot on extracts of hepatocytes (20,21); the fluctuation in intensity of signal according to disease activity (22) suggests these occur in response to antigen spillage.

T-cell studies remain rudimentary, even though T cells predominate in the liver infiltrates and are presumed to be the effectors of liver cell damage. In AIH-1 there are neither characterized autoantigen preparations nor cytotoxic assay systems available for T-cell analyses—but T-cell investigators in some other autoimmune diseases do not fare much better. The situation is better in AIH-2 and the capacity of T cells from blood has been demonstrated to respond to synthetic peptides derived from the sequence of the characterized autoimmune reactant CYP450 2D6. This study disclosed several dominant peptides that were stimulatory in proliferation assays using autologous T cells, thus representing likely T-cell epitope regions (23).

Models of AIH generally have been uninformative, although the model in C57/BL6 mice by immunization with a cDNA construct encoding murine CTLA-4 and human CYP450 2D6 and FTCD resulted in hepatic inflammation and production of the autoantibodies characteristic of human AIH-2 (24). The point of interest is that experimental induction of an immune response to the reactants associated with AIH-2 has hepatitis-inducing effects, suggesting involvement of these in the pathogenesis in the spontaneous human counterpart.

### PRIMARY BILIARY CIRRHOSIS

Primary biliary cirrhosis (PBC) stands as a paradigm and paradox for autoimmunity (25) because of the tightly specific association between the serologic antimitochondrial antibody (AMA) reaction and disease, yet with no explanation for connectivity between AMA and specificity of damage to the biliary epithelial cell (BEC).

Considerable optimism, as yet unrealized, followed the eventual identification in the 1980s of AMA as enzymes of the 2-oxo-acid dehydrogenase complex (2-OADC) family, and the localization of autoepitopes within their E2 subunits.

The major autoantigen is the E2 subunit of the pyruvate dehydrogenase complex (PDC-E2), and an immunodominant region for B and T cells resides in the inner lipoyl domain, residues 128–227. This contains a conserved linear sequence (residues 167–186, AEIETDKATIGREVQEEVGL) that includes lysine (<sup>173</sup>K) to which is attached the lipoyl cofactor, and this sequence is thought to encompass the B-cell autoepitope (26); however, epitope mapping by antibody screening of phage-displayed peptide libraries indicates a conformational epitope within the lipoyl domain with contact sites for the antibody paratope that include residues <sup>131</sup>MH<sup>132</sup> and <sup>178</sup>F...V<sup>180</sup> (27); eventually a solved crystal structure of a monoclonal anti-PDC-E2 in a complex with purified PDC-E2 will give a clearer picture. Some peculiar features of the antibody response in PBC include increased levels of IgM, a bias to production of the IgG3 subclass of IgG, overall and for AMA, and reactivity of PBC sera with an apically located reactant in the BEC that is unidentified but likely related to PDC-E2 (28).

AMA as the dominant autoantibody in PBC has tended to “blinker” the vision of a second set of autoantibodies to nuclear antigens, present in up to 40% of cases. Thus whilst their sensitivity for the diagnosis of PBC is low, these autoantibodies occur so rarely in other diseases that their specificity is high. Moreover these ANA unlike those in some other diseases are not “nondescript” but have well-defined patterns on IIF and are molecularly characterized. Their existence, in the absence of AMA, was once thought to mark a unique syndrome called autoimmune cholangitis, now reassigned as a serological variant of PBC (29). The ANAs in PBC include reactants for (a) the speckled dot (Sp100) and the related promonocytic leukemia (PML) antigens, (b) the nuclear pore complex (gp210 and gp63), and (c) centromeric protein (CENP) as detected also in limited cutaneous systemic sclerosis and which can co-occur with PBC (28). These ANAs provide no clue to a provocative cause of disease and their occurrence places PBC in a gray zone between the usually Th1-dominant organ-specific and the usually Th2-dominant multisystem autoimmune diseases. Finally these ANAs in PBC direct attention to faulty peripheral tolerogenesis and regulatory T cells (T-regs), as discussed below.

T-cell studies in PBC on reactivity to PDC-E2 have been informative in defining a linear T-cell epitope in the same lipoyl region of PDC-E2 as the B-cell reactant (26). As would be expected, there was a very high enrichment, 150-fold, of these PDC-E2 epitope-reactive CD4+ T cells in liver infiltrates compared with blood. But are these T cells pathogenic? The question is posed in view of the questionable access of T cells to their mitochondrially located, reactant and immunohistochemical evidence in PBC of invasion and destruction of biliary ductular cells by cytolytic CD8+ effector T cells that could be targeting an antigen in the BEC other than PDC-E2. Notwithstanding this marked T-cell autoreactivity, the earlier literature records a concurrent T-cell anergy in the cutaneous response to an extrinsic antigen, tuberculin (30) (*see below*).

## UNTANGLING THE ETIOPATHOGENESIS OF PBC—STILL GOOD OPPORTUNITIES

“The only certainty” according to a recent commentator is “the consensus driven hypothesis that PBC develops from an interaction between environmental factors and inherited genetic predisposition” (31); no surprises there. The strong genetic component does not depend on HLA risk alleles that are prominent in most other autoimmune diseases, but is evidenced by a high concordance rate for PBC in monozygotic twins (~60%) and high intrafamilial co-occurrences (~6%) (32). The very high female predisposition to PBC is universal in clinical and epidemiological studies (~10:1) and is likely genetic (estrogenic hormones), but are females in some way, socially or occupationally, overexposed to a ubiquitous environmental determinant? A study based on congenic manipulation of the highly autoimmunity-tilted NOD mouse, involving exchange of segments of chromosomes 3 and 4 from B6 mice, revealed that the inflammatory autoimmune process could be diverted from pancreatic islets to biliary ductular epithelium, with PBC-like histological lesions of bile ducts and serologic AMA and ANA reactivity (33). Genetic dissection identified an autoimmune biliary disease locus (*abd1*) on chromosome 4 for which an ortholog in humans may exist.

Coming to environment, epidemiological studies earlier incriminated water sources and later toxic waste sites (34), whilst case-control studies pointed to urinary infections and cigarette smoking (35). Sources of a possible environmental epitope mimic of the PDC-E2 lipoyl domain autoantigen could include microorganisms that utilize homologs of the PDC enzyme that can closely resemble the mammalian counterpart or xenobiotics/chemicals that can attach to and/or modify the attachment site of the lipoyl cofactor, so creating a mimicking neoepitope. Tolerance to PDC-E2 is broken by immune cross-reactivity to the mimic, whether microbe or chemical, after which the disease is perpetuated by ongoing exposure to the native autoantigen as discussed in Chapter 18—provided of course that PDC-E2 is in fact the pathogenic autoantigen! Here, animal models have been insufficiently informative (36). Moreover relatively few autoimmune diseases illustrate fulfillment of desired criteria for the mimicry hypothesis: (a) a credible epitope mimic that can be matched to the autoantigenic determinant, (b) reliable evidence for natural environmental exposure to this mimic, and (c) data showing that animals exposed to the mimic develop appropriate reactivity involving T and/or B cells, with ensuing disease. The alternative is the idea that spillage of native autoantigen during tissue degradation, whether by apoptosis or necrosis and under conditions of deficiency of natural immune tolerance, provides both the initiating and perpetuating autoimmunogenic stimulus.

One of the currently promising lines of enquiry for PBC relates to defects in peripheral (dominant) tolerance mediated by T-regs. There are various subsets of Treg, with major interest in that which expresses the FOXP3 transcription factor and the interleukin (IL)-2a receptor (CD25) and operates via the cytokine, transforming growth factor (TGF)- $\beta$ , and its receptor.

Mouse models in which these elements are genetically disrupted display inflammatory/ autoimmune phenotypes, including biliary ductulitis and AMA positivity (37). A study in human PBC showed a reduction in T-regs in blood and in infiltrates in portal tracts (38). Returning briefly to T-cell anergy in PBC (*see above*) and noting past comment on resemblances between PBC and sarcoidosis wherein anergy and inflammatory granulomata are prominent (39), there is a recent study suggesting that Tregs may be in functional excess in sarcoidosis (explaining T-cell anergy), but dysfunctional in failing to control release of inflammatory mediators (explaining granulomata) (40). Might a similar scenario be envisioned for PBC wherein T-cell anergy (30) and granulomata in portal tracts are features?

A final point is the possibility of a contribution to pathogenesis by the BEC itself, given that end-organ susceptibility has entered discussion as a component of pathogenesis of several autoimmune diseases. In the NOD.c3.c4 mouse model described above, susceptibility appears to reside to some degree at least in the target tissue, the biliary epithelium (33).

## PRIMARY SCLEROSING CHOLANGITIS

There are undoubted immunological accompaniments to this mysterious liver disease, outlined by Cullen and Chapman in Chapter 19. Those suggestive of autoimmunity include a high frequency (~88%) of ANCA (pANCA, but not of proteinase3 specificity), a tendency to overlap with AIH-1 seen occasionally in the later stages of AIH in adults, but more especially in childhood as noted by Vergani and Mieli-Vergani in Chapter 21, and a high association with the autoimmune HLA haplotype B8 DRB1\*0301. However, contrary to the idea of autoimmunity, PSC impacts more frequently on males than females, inflammatory elements including lymphocytes are usually sparse in the lesions, the periductular fibrogenesis component is not wholly in keeping, and the major disease association is with ulcerative colitis, itself now under doubt as a true autoimmune condition. One explanation for PSC could be that it results from an aberrant low-grade proinflammatory response to generally innocuous and normally tolerated microorganisms resident in the intestinal tract, with cytokine-mediated activation of an exuberant periductular myofibroblast response, and perhaps a contribution to the process from the BEC itself.

## CHRONIC VIRAL HEPATITIS

Of many viruses with hepatotropic potential, only hepatitis viruses B and C (HBV, HCV) are capable of establishing a non-cytopathogenic chronic infection of hepatocytes. The ensuing ineffective host immune response to epitopes of intracellular virus exposed on the surface of virus-infected cells provokes inflammation and rounds of liver cell necrosis, regeneration, and fibrosis: the culmination is cirrhosis and eventually hepatocellular carcinoma. In these respects the nature of HBV and HCV infections is similar. However, in other respects including virus lifestyle and infectivity and capacity to establish persistent infection, infection with HBV and HCV differs substantially, as described by Bertolotti in Chapter 14 and Wedemeyer in Chapter 15. Finally the immune system can be involved in two ways in



hepatitis virus infection: first, it determines clearance of the infection and, second, it determines the characteristics of the host inflammatory response in established chronic infections.

### CHRONIC HEPATITIS B

The widely different carrier rates globally for HBV depend on differences in racial-genetic background, socio-cultural lifestyles, and routes of viral transmission. In high-prevalence regions, transmission is frequently by vertical infection, mother to fetus, or by close perinatal contact, whereas in low-prevalence regions transmission is parenteral in the setting of intravenous drug use or sexual promiscuity. Failure of clearance of infection has host-related causes, mainly deficient immunity, and virus-related causes that include route of entry, dose of inoculum, and genotype of the virus. Viral gene mutations that encode structural changes in the pre-S region of the surface coat (HBs) are frequent during evolution of chronic disease, but their role in evasion of host immunity by the virus is not established (41).

Innate immunity would be involved in initial resistance to infection but the vigor of adaptive immunity has the major influence, such that healthy individuals clear the infection in some 95% of instances, with contributions from humoral antibody against the surface coat (HBs) and CD4+ and CD8+ T-cell responses against the core particle. The presence in blood of the e antigen (HBe) of the core particle is indicative of ongoing viral replication and reflects failure of T-cell responses. Immune deficiency states that favor chronic infection are immunological immaturity as in the fetus or neonate and associated with vertical or perinatal transmission by a carrier mother, or immunodeficiency associated with general debility as in renal failure, noting past outbreaks of HBV infection in renal dialysis units, and malnutrition associated with alcohol or drug abuse. Such debility-associated immune deficiency is readily demonstrable by simple antigenic challenge tests for humoral immunity using antibody response, or cellular immunity using tests for cutaneous delayed-type hypersensitivity (42). Immunosuppression associated with cytotoxic therapies for solid tumors or lymphomas may allow reactivation of an immunologically well-controlled HBV infection, providing a sharp challenge for the therapist (43). An important element of the lowered T-cell responsiveness to HBV is limitation in the capacity for engagement of the multiple antigenic epitopes presented by the virus, with only a few engaged by the immunoincompetent individual. With failure of viral clearance a default option for the host is tolerogenesis; this can occur initially with infection *in utero* or neonatally, and probably in adult infection as well, resulting in a “healthy carrier” state that can transition to an active (HBeAg+ve) response or to quiescent inflammation and anti-HBe (44). The worst outcome is a persisting but futile and damaging proinflammatory immune response seen clinically as “chronic active hepatitis B.” However, with current improved regimens of antiviral therapy, or even spontaneously, immunity can still prevail such that, among chronically infected individuals, there is a 2% per annum viral clearance rate (cure) with appearance in blood of HBV-reactive T cells and anti-HBs (45).

Immunogenetic factors influence the occurrence or outcome of infection with HBV and the response to HBV vaccine (46), and different HLA alleles appear protective or proinflammatory among different populations (47). Some studies suggest that the frequency of HLA B35 is increased in chronic HBV infection (42). HLA class II alleles are involved in viral clearance and vaccine responsiveness, as judged by binding affinities of peptides from the core particularly, and the surface protein of HBV (46). Other immunogenetic factors likely operate as well since, among Koreans, there were reports of small effects of polymorphisms of the promoter for particular cytokine genes, IL-10 and TNF- $\alpha$ , on outcome of HBV infection (42,47).

The determinants of liver pathology in chronic hepatitis B include the same T-cell system that normally clears the acute infection (48). Why is this? Viral load, balanced against T-cell “availability” (particularly CD4 T cells and injurious cytokines), seems an important factor. At least, a direct correlation has been drawn between viral load and propensity to progress to cirrhosis (49), and therapeutic reduction of viral load is clearly beneficial. However, the relative participation of CD4 and CD8 T cells in hepatocyte injury requires more study. B cells enter the picture in chronic HBV infection in the context of ongoing stimulation by noneliminated viral antigens, with ensuing immune complex disease and/or essential mixed cryoglobulinemia (50).

### CHRONIC HEPATITIS C

The HCV is less complex genetically and structurally than HBV but is just as illustrative of the immunologic complexity of interactions between a “survival-adapted” virus and its human host (51). Acute infection can be acquired at any age, is often silent, is less readily cleared than HBV, in only approx 30 versus approx 90–95% of infected individuals, and debility-related immune deficiency predisposes to but is not necessary for persistence. There is not a tolerance option as with HBV infection, since all carriers of HCV have some level of hepatic inflammatory response. Hepatic comorbidities are a feature, since chronic HCV hepatitis often coexists with other liver diseases, either because of alcohol or steatosis, noting a propensity of HCV itself to induce fat deposition in liver cells (52). The problems of cultivation of HCV *in vitro* and limited animal hosts have impeded the study of adaptive immune responses and vaccine development, but this is expected to change.

Innate immunity provides the first response to HCV infection, based on the capacity of phagocytic cells to recognize a pathogen (virus)-associated molecular pattern (PAMP) via Toll-like receptors (TLR); the RNA of HCV particularly engages TLR3 and so activates signaling pathways for induction and expression of proinflammatory and antiviral cytokines, particularly interferons, and primes for adaptive immune responses (51). Whilst interferon-gamma expression results in some reduction in levels of HCV in liver cells, full clearance requires additionally a rapid and effective adaptive immune response involving engagement by T cells and likely B cells to multiple antigenic epitopes of the virus polyprotein. For T-cell responses, there has been good progress in ascertaining important epitopes on structural and nonstructural proteins of

the HCV particle, their relative capacity for presentation by different HLA molecules, and their capacity for activating protective CD4 and CD8 T-cell responses which, although critical, are often delayed (53,54). Comparably with HBV infection, outcome depends on the quality and number of HCV epitopes initially engaged and efficient development of effector/memory T cells (48,53).

There are many explanations for the capacity for escape of HCV from immune attack: ongoing development of immunologically variant quasispecies that “outrun” the repertoire of available T-cell specificities; suppression of T-cell activities by HCV proteins; tardiness of primed T cells to move rapidly to the newly infected liver; defective engagement of critical HCV epitopes such as NS5A that favors viral persistence by antiapoptosis effects on hepatocytes (55); and depletion of CD8 T-cell responsiveness during evolution of infection (53). Debility-related impairment of immune function impacts on T-cell and NK-cell activities and as well is limiting for efficient interferon- $\gamma$  responses. Another possibility is that the first encounter between naïve T cells and HCV occurs in the tolerogenic milieu of the liver rather than in the immunogenic milieu of a regional lymph node (56). Among genetic influences, HLA class I and class II alleles influence clearance (48), well illustrated for the highly protective class I allele HLA B27 that engages an epitope within the NS5B protein of HCV; however, structural polymorphisms of HCV evolve to circumvent this (57).

Events in the chronic liver-damaging phase of HCV infection are interesting, in that CD4 and CD8 cytolytic T cells (CTLs) are operative. Initially, good control of viremia is associated with greater evidence of histological liver damage (58) whereas later in the infection T-cell activity wanes; however, even then CTL activity is still demonstrable among T cells in liver, although not in blood. B cells have received relatively less comment in the host interaction with HCV, although antibody to HCV is clearly demonstrable and is directed to multiple components of the HCV polyprotein.

Anti-HCV has neutralizing capacity, at least in infected chimpanzees, and likely serves to limit cell to cell transfer of virus in the liver. However, the B-cell response is more relevant to the liver immunologist in the late pathology of HCV infection in being responsible for many of the numerous extrahepatic manifestations (59,60), including type 2 mixed cryoglobulinemia seen at high frequency in endemic regions of infection. The cryoglobulins contain HCV, anti-HCV, and oligoclonal IgM rheumatoid factor, are proinflammatory causing arthralgia, vasculitis, cutaneous purpura, and membrano-proliferative glomerulonephritis, and production is antigen (HCV)-driven since therapy with IFN- $\alpha$  reduces viral load and, concurrently, ameliorates clinical expressions (61). Another B-cell feature, seen more in the later stages of infection, is production of autoantibodies, albeit to relatively low titer, including either AIH-1-type antibodies, ANA approx 10%, SMA approx 7%, and rheumatoid factor (60) or AIH-2-type antibodies, anti-CYP450 2D6 and anti-LC-1 (62); the nexus between these autoantibodies and associated autoimmune expressions is unclear. And further, B cells can undergo lymphoproliferative

expansion resulting in non-Hodgkin B-cell lymphoma, attributable to chronic antigen drive complicated by lymphomagenic chromosomal translocations such as translocation of the apoptosis inhibitory gene *BCL-2* from chromosome 16 to the IgH locus on chromosome 14 [t(14;18) (q32;q21.3)], although in a recent study on human HCV-infected liver tissue this translocation was not demonstrable (63).

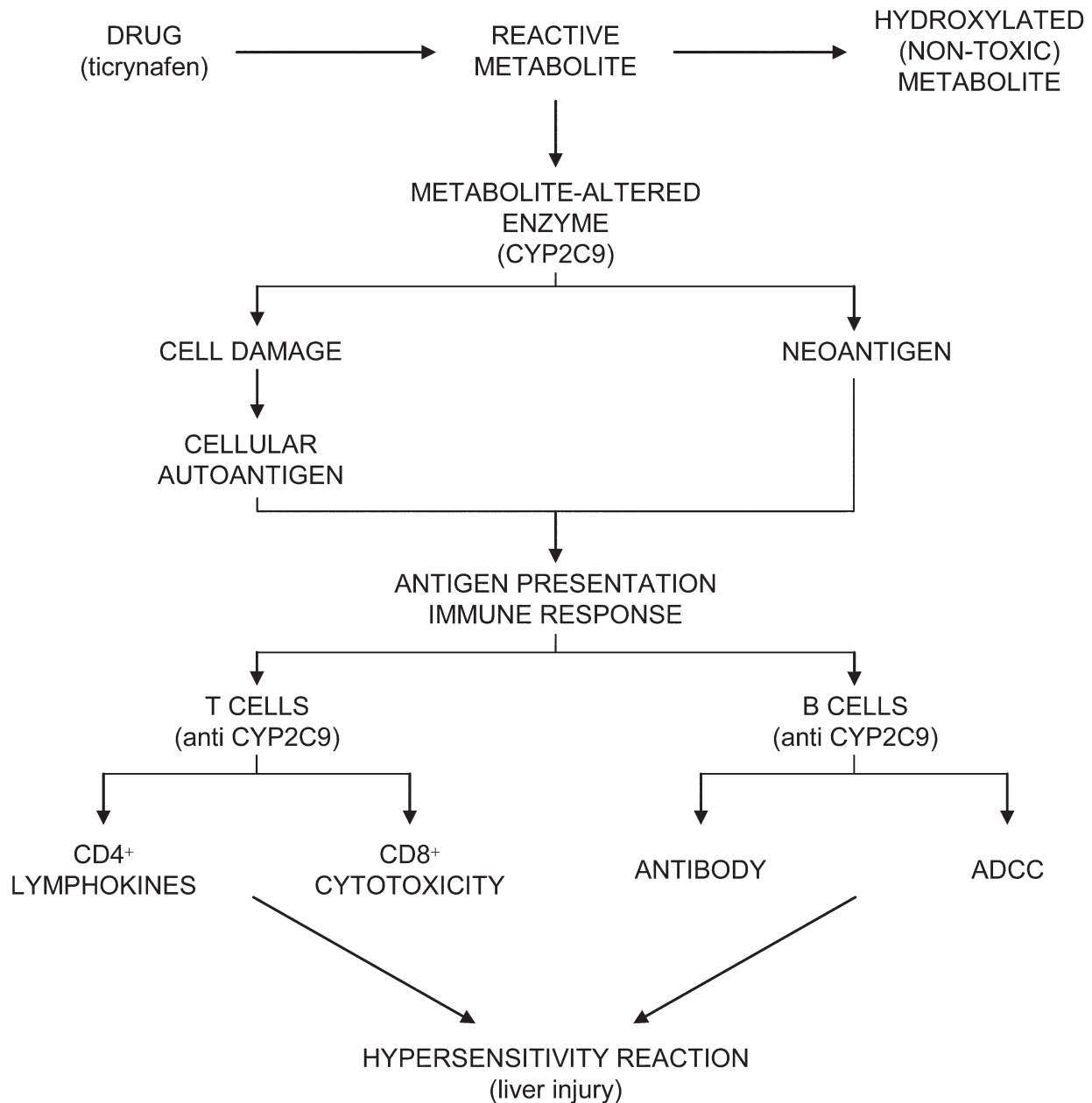
As a final point, it is heuristic that the treatment of chronic hepatitis C with a type 1 interferon can “reactivate” an AIH or, more usually, provoke autoimmune reactions *de novo* in other tissues, particularly the thyroid gland (64).

## IMMUNE-MEDIATED DRUG-INDUCED LIVER INJURY

An estimated frequency of hepatotoxic effects of medicinal drugs is 40–60 events per million exposures, a seemingly low risk, but considerable given the high frequency of drug usage in contemporary societies. Predictable toxicities and nonpredictable but purely pharmacological idiosyncrasies, for example to troglitazone (66), account for a high proportion of these events while, for the remainder, the immune system is an essential accomplice. Immune-mediated drug-induced liver injury (im-DILI) is itself diverse in clinical and histological expressions and also in pathogenesis. Susceptibility to drug-induced immune pathology varies widely among different tissues, with high rates attributable to the constituent cells/tissue being readily exposed to immune effectors, e.g., blood, vascular endothelium; being rich in APCs, e.g., skin, liver; or being a participant in the metabolism/excretion of the drug, e.g., liver, kidney. The complex issues relating to im-DILI are explored by Kaplowitz and Liu in Chapter 28 and van Pelt et al. in Chapter 29.

Historically the first definitive analysis of immune-mediated drug-induced tissue injury in the 1940s was that of Ackroyd on sedormid-induced thrombocytopenic purpura (65) and his conclusions remain generally applicable today. Thus, in the case of the liver, drug-induced immune injury to hepatocytes would depend on conjugation of a reactive metabolite of the drug to a host protein and likely an enzyme responsible for disposal of the drug (67). This generates a “self + X” neoantigenic moiety which, according to the scheme shown in Fig. 2 and predictably on a permissive genetic background, promotes the inductive phase of an immune response resulting in immunization (sensitization) to the drug as a hapten, with often an accompanying autoimmune response. The site of immune induction, whether within the liver or more likely within a perihepatic lymph node, is not established, since animal models for idiosyncratic reactions seldom replicate the human counterpart. The executive/effector phase of the response may be antibody or T-cell dominant and is either predominantly “allergic” with overt eosinophilia in liver tissue and blood, or cell-mediated and presumably dependent on Th1 CD4+ T cells and inflammatory cytokines. At present, *in vitro* or *in vivo* test systems in patients are not well sufficiently developed to define precisely the mechanisms in most cases. im-DILI is highly specific for the particular culprit drug since in most cases there is

## A mechanism for im-DILI



**Fig. 2.** One of the several possible pathways to IM-DILI involving the sensitizing drug ticrynafen and CY P4502C9 that hydroxylates this drug. A reactive metabolite of this drug may generate a cell damage directly or by creation of a neoantigen. The ensuing immune response is expressed as hepatocellular damage, and production of antibodies to LKM, here called LKM-2 and identified as anti-CYP450 2C9 ADCC, antibody-dependent cellular cytotoxicity (*see ref. 67*).

fading of the reaction and clinical recovery when the culprit drug is withdrawn and recurrence in accelerated fashion on re-exposure or after direct challenge—a rather risky albeit sometimes necessary clinical diagnostic procedure.

im-DILI can be accompanied by production of autoantibodies that simulate those of spontaneous AIH, either ANA/SMA as in AIH-1 or anti-LKM as in AIH-2. The occurrence of

the latter would be intuitive since drugs are enzymatically disposed of by hydroxylation by enzymes of the CYP450 family, and the notable point here is that the specificity of the LKM antibody is to the CYP450 isoform that hydroxylates the drug. For example ticrynafen (a uricosuric, no longer marketed) which is degraded by CYP450 2C9 often provoked im-DILI accompanied by anti-CYP450 2C9 and, similarly,

hydralazine (an antihypertensive drug) which is degraded by CYP450 1A2 provoked im-DILI accompanied by anti-CYP450 1A2 (67). Unfortunately, cases of im-DILI accompanied by AIH-1 type antibodies (ANA/SMA) are not so neatly explained. Previously these were seen after exposure to oxyphenisatin (a laxative, no longer marketed) and alpha methyl dopa (an antihypertensive, now obsolete), and currently with other drugs in this category that include antibiotics, minocycline and flucloxacillin. Explanations, but without good evidence, include interference by the drugs with processes of peripheral tolerance.

The pathogenesis of DILI in general is still opaque since genetic polymorphisms can influence pharmacokinetics, enzymatic degradation, and/or immunologic reactivity to drug adducts. Immunogenetic factors are implicated since HLA class II alleles influence the pattern of expression, at least of liver injury (68). Moreover, collateral factors such as intrahepatic inflammatory stress can potentiate hepatic reactivity to drugs. These and other issues are critically examined in a recent wide-ranging conspectus on the topic (69).

## ALLOIMMUNE INFLAMMATORY LIVER DISEASES

Alloimmune liver disease occurs as HVG or GVH reactions, in the setting of allogeneic liver or bone marrow/hemopoietic stem cell transplantation (HSCT). In earlier days, the vigorous immunologic responses to allografts bearing foreign (non-self) MHC/HLA molecules supported the notion of “immunological self” to the degree that pessimism was expressed on a future for human tissue transplantation (69a), but this was soon disproven, first for kidney and then for liver allografts. Today transplantation immunology is a thriving specialty that makes prolific contributions to immunological theory and practice and particularly to liver immunology and immunopathology, as evident from Chapter 32 from Neuberger and Chapter 31 from McDonald and Shulman.

### HOST-VERSUS-GRAFT DISEASE

The demanding technical needs of liver transplantation fortunately are offset by a more tolerogenic response of the host to a liver allograft compared with, say, a skin or kidney allograft. In fact for some species (pig) and for some rodent strain combinations, a liver allograft will succeed without immunosuppression across an MHC barrier. Although this applies only occasionally in humans, the demand for immunosuppression is generally less than for other allografted tissues (70). This leads to the consideration whether donor-specific tolerance is “measurable” as a prerequisite for tolerogenesis regimens in humans (71). In any event the liver certainly could not be regarded as “immunologically privileged” since it is accessed by two circulations, portal and arterial, and the constituent cells, hepatocytes, sinusoidal endothelial cells, Kupffer cells, and BECs, all abundantly express MHC Class I, and for some, Class II as well. What then is the explanation for the claimed “tolerogenic milieu” that prevails within the liver? Answers include the special cytoarchitectural features (absence of a blood tissue barrier); preferential non-costimulatory (and therefore tolerogenic) activation of T cells; exit from the graft of long-surviving donor leukocytes that maintain chimerism

(thereby promoting tolerance); and recruitment of different subsets of regulatory T cells. On the other hand, studies in mice indicate that liver allografts can induce robust intrahepatic CTL responses (72); tolerance could develop later along with recruitment of Treg cells. Thus, in humans, rejection reactions will occur despite immunosuppression in some 80% of instances, either acute or chronic.

Two types of alloreactivity are distinguished: direct, wherein host T cells recognize native donor MHC molecules on graft-associated APCs, and indirect wherein host T cells recognize (various) allogeneic donor peptides present on host APCs (70). Acute rejection reactions, usually the direct type, are expressed as portal leukocytic (granulocyte and mononuclear) infiltration, interface hepatitis, biliary ductulitis, and venous endothelitis, and chronic rejection reactions, usually the indirect type, are expressed particularly by biliary ductopenia and obliterative arteritis. While an eventual stable tolerance is the hoped-for outcome, the threat of a rejection reaction is ever-present; the role here of pathogen, usually virus-induced alloreactivity, was discussed in the context of T-cell receptor degeneracy, virus-induced lymphopenia, and homeostatic expansion of T cells including alloreactive memory T cells (73).

It is intriguing for the liver immunologist to confront a recurrence, or the occurrence *de novo*, of an AIH in an allografted liver, given that recipient hepatocytes will likely carry nonhost HLA alleles. However, there are well-documented examples (74), validated by histological appearances and serological evidence such as increased levels of  $\gamma$ -globulin and AIH-relevant autoantibodies. The recurrence, or *de novo* occurrence, in a liver allograft of an autoimmune disease, whether AIH or PBC—if such indeed do occur—raises interesting pathogenetic considerations for autoimmune disease in general, discussed by Ishibashi in Chapter 34.

### GRAFT-VERSUS-HOST DISEASE

The several applications of HLA-matched allogeneic hemopoietic stem cell (bone marrow) transplantation (HSCT) include immunodeficiencies, hematological malignancies, aplastic anemia, and, increasingly, intractable autoimmune diseases. GVH disease can be expected in 30–50% of allogeneic HSCT from HLA-matched siblings and is caused by mature T lymphocytes of the donor, protected by immunosuppression of the recipient, reacting with “foreign” (non-HLA) cell-surface minor histocompatibility alloantigens of host provenance. The tissues predominantly affected by GVH disease are skin, intestinal tract, mucosal surfaces, and liver (75), and the expressions in many respects, and not surprisingly given the similar modes of pathogenesis, resemble those of multisystem autoimmune disease. In the liver, comparably with HVG disease, the lesions can be hepatic with histologic resemblances to AIH, cholangitic with some histologic resemblances to PBC, or even vascular and partly simulating those of systemic sclerosis. There does seem to be a particular vulnerability of the cholangiocyte in HVG and GVH disease, the nature of which has been recently reviewed in some depth (76). Particular comment has been directed to resemblances between cholangitic GVH disease and PBC since in both conditions there is destructive

invasion of BEC by activated T lymphocytes (77). Although AMA were claimed to be demonstrable in GVH disease in humans and in an animal model, a subsequent report found no such instance among 95 human examples (78).

### AUTOINFLAMMATORY (IMMUNOINFLAMMATORY) HEPATITIS—STEATOTIC LIVER DISEASE

Autoinflammatory or immunoinflammatory diseases include cytokine-mediated inflammatory responses to products of cellular injury caused by various cytoplasmic inclusions, e.g., resulting from protein misfolding diseases, that are insufficiently eliminated by chaperone pathways, autophagy, or other mechanisms. In the case of the liver, alcoholic abuse or fatty liver associated with the metabolic syndrome can result in potentially injurious accumulations of fat and Mallory bodies in liver cells. The associated innate immune processes lead on to neutrophilic inflammatory reactions, release by T cells and NKT cells of proinflammatory cytokines, and progressive fibrosis culminating in cirrhosis. Indeed the judicious inclusion in this volume (Chapters 24–26) of alcoholic hepatitis and NASH acknowledges the positioning of these entities at the intersect of hepatology, metabolism, immunology, inflammation, and genetics.

The disease in question was first recognized in the early 1980s as NASH, within a wider category of nonalcoholic fatty liver disease (NAFLD) (79); the “nonalcoholic” component of the title is a residue from earlier days when fat in the liver was regarded as pathognomonic of alcohol abuse. Although immune-inflammatory responses of adaptive type to protein adducts of metabolites of alcohol have been described, mechanisms related to innate immunity are now more favored (80), and likewise the pathogenetic process in NASH seems more likely attributable to activation of cells of the innate immune system with release of inflammatory mediators. In as many as one third of cases, there is progressive fibrogenesis and cirrhosis and, interestingly, obesity rather than inflammation appeared to be the determinant of this (81). The basis for fat accumulation in the liver in the first instance, described as the “first hit” in NASH (82), is in some 85% of cases the genetically multifactorial and mysterious metabolic syndrome, characterized by central obesity, hypertriglyceridemia, hypertension, type 2 diabetes, and insulin resistance (79). Food overload contributes an environmental element and accentuates obesity and fatty liver. The “second hit” is postulated to be delivered by oxidative stress (82). Notably, fat in the liver *per se* is not necessarily injurious, since in many instances the response is bland. What determines the adverse reaction to fat in the liver in NASH? One idea is that a genetically based capacity for overproduction of leptin by adipocytes could contribute to attraction into adipose tissue of macrophages (83) with production of inflammatory cytokines such as TNF- $\alpha$ . However, in ob/ob mice and in nutritionally obese C57BL/6 mice, there is deficiency of leptin and hepatic NKT cells, yet NASH develops under the influence of prolonged Th1 responsiveness (84). A further genetic determinant could be predisposition to excessive fibrogenesis, with the profibrogenic cytokine TGF- $\beta$

presumably acting via stimulation of stellate cells in the liver (85). Obviously more will need to be learnt about the pathogenesis of NASH in future years in the context of the current global “epidemic” of obesity. Meanwhile studies in humans and mouse models support the mantra: “remove the fat, cure the disease.”

### CONCLUDING REMARKS AND OPEN QUESTIONS

The past 50 yr have been witness to remarkable advances in knowledge on the nature of diffuse inflammatory diseases of the liver. Former preoccupations with morphologic types of cirrhosis (macronodular, micronodular) and microscopic patterns of hepatocellular necrosis stand in contrast with the pathogenetic insights of the post-2000 era. Yet for each of the current delineated causes of inflammatory liver injury, whether autoimmune, viral infection, drug sensitization, allograft reactivity, or the newly recognized metabolic fat-induced inflammation and fibrosis as in NASH, obstacles to understanding are readily discerned (see Key Points), with each providing novel research opportunities for the future. These are discussed in this introductory chapter as a “curtain raiser” to the more detailed analyses in the next chapters of Liver Immunology, Second Edition.

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# INTRODUCTION

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# 1 A Short Primer on Fundamental Immunology

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CLIONA O'FARRELLY AND DEREK G. DOHERTY

## KEY POINTS

- Cells of the innate immune system, such as monocytes, macrophages, neutrophils, dendritic cells, and natural killer cells, recognize microbial products and host molecules expressed by pathogen-infected and tumor cells.
- Recognition of danger by the innate immune system is followed by the release of chemokines that direct inflammatory cells to the site of the danger and removal of the danger by the combined action of phagocytic cells, cytotoxic cells and cytokines, acute phase proteins, and complement.
- Activation of the adaptive immune system requires the activation of T lymphocytes. T cells express clonotypic antigen receptors that recognize peptide fragments of protein antigens presented by major histocompatibility complex molecules on antigen-presenting cells.
- Activation of a naïve T cell requires a signal through its antigen receptor (signal 1) as well as a danger signal through a costimulatory receptor (signal 2). This causes it to differentiate into an effector cell capable of subsequently mediating its effector function upon receipt of signal 1 alone.
- Adaptive immune responses to danger can be either inflammatory responses involving cytotoxic T cells, Th1 cells, and natural killer cells or antibody responses involving Th2 cells and B cells, mast cells, and eosinophils. Antibodies can neutralize toxins and viruses, opsonize pathogens for phagocytosis, cytotoxicity, and directed histamine release, and activate complement.
- Th1/Th2 cell differentiation, effector functions of the adaptive immune system, and termination of adaptive immune responses are controlled by cytokines released by T cells and cells of the innate immune system.
- The innate and adaptive immune systems interact with and regulate each other. Dendritic cells and macrophages are central to both innate and adaptive immune responses. Some T cells have predominantly innate immune functions.

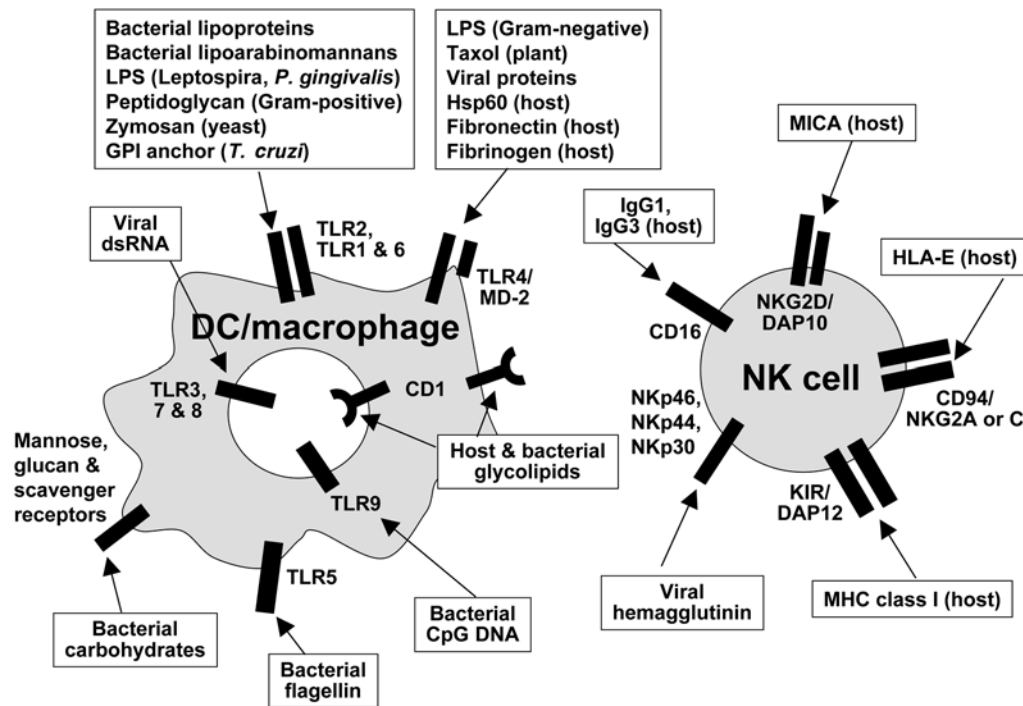
## INTRODUCTION

Mammals protect themselves against exogenous pathogens (viruses, bacteria, fungi, parasites, and toxins) and endogenous danger (malignancy) with a complex, interacting set of defence mechanisms. These include primordial “identify and destroy” strategies (innate immunity) as well as sophisticated detection and targeted killing processes that display exquisite specificity, multiple layers of regulation, and memory (adaptive immunity). In this chapter, the fundamental concepts of innate and adaptive immunity and how they interact are briefly reviewed. Further details on individual topics can be obtained in the in-depth reviews cited.

## RECOGNITION OF DANGER BY THE INNATE IMMUNE SYSTEM

Primordial defence strategies began to evolve with the appearance of multicellular organisms. They rely on cells with killing potential, such as monocytes, macrophages, neutrophils, dendritic cells (DCs) and natural killer (NK) lymphocytes, as well as hard-wired detection systems involving cell-surface molecules that detect microbial products or changes in host cells that signal danger. Such *pattern-recognition receptors* (PRRs) include receptors for bacterial carbohydrates and the Toll-like receptors, which recognize various components of microorganisms (including lipopolysaccharides, lipoproteins, glycolipids, flagellin, viral RNA, and bacterial DNA), as well as endogenous ligands (heat shock proteins released by damaged or necrotic host cells) (1,2) (Fig. 1). Engagement of these molecules initiates the activation of monocytes, macrophages, neutrophils, and/or DCs. The result is the targeted destruction of the activating organism, infected cell, or tumor cell by phagocytosis or the release of cytotoxic agents.

A second type of detection system in the innate immune system is a variety of activating receptors on NK cells, which recognize changes to host cells that signify danger such as infection or tumor transformation. Such “natural cytotoxicity receptors” include NKG2D, which recognizes the stress-inducible molecule MICA (which is upregulated on tumor and



**Fig. 1.** Recognition of danger by the innate immune system. Conserved pathogen associated molecules and host cell-surface changes that signify danger are recognized by dendritic cells (DCs), macrophages, and natural killer (NK) cells. Toll-like receptors (TLRs) on DCs and macrophages recognize viral and bacterial products and stress inducible molecules released by host cells. Natural cytotoxicity receptors on NK cells recognize viral products, changes in major histocompatibility complex (MHC) class I expression that signify danger, Fc portions of IgG1 and IgG3 antibodies, and the stress-inducible molecule MICA. LPS, lipopolysaccharide; GPI, glycosphosphatidylinositol; KIR, killer immunoglobulin-like receptor; hsp, heat shock protein.

virus-infected cells), and NKp46, which appears to recognize viral hemagglutinin (3). Ligation of these receptors results in immediate killing of the infected or tumor cell by the NK cell. NK cells also express stimulatory and inhibitory receptors (killer immunoglobulin-like receptors [KIRs] and CD94 in humans; Ly49 in mice) that detect changes in the levels of major histocompatibility complex (MHC) class I molecules, which occur during times of abnormal protein synthesis such as tumor transformation or viral infection (4,5) (Fig. 1).

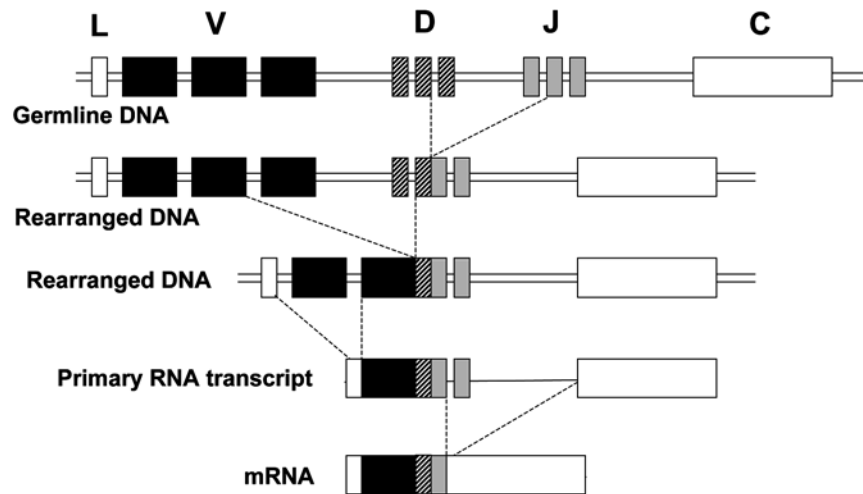
## THE INFLAMMATORY RESPONSE

Inflammation is a general term given to the mobilization and effector activity of the innate immune system in response to signals of "danger." It is initiated by the release of a variety of chemical messengers from activated cells of the innate immune system and from pathogen-infected and tumor cells. These chemical messengers include chemokines (e.g., macrophage inflammatory protein- $\alpha$  [MIP-1 $\alpha$ ], MIP- $\beta$ , interleukin-8 [IL-8], and regulated on activation, normal, T-cell expressed and secreted [RANTES]) and cytokines (granulocyte-monocyte colony-stimulating factor [GM-CSF], tumor necrosis factor- $\alpha$  [TNF- $\alpha$ ], the interleukins IL-1, IL-6, IL-12, and IL-18, and the interferons IFN- $\alpha$  and IFN- $\beta$ ), which diffuse rapidly through the tissues and into the circulation.

A key function of this activity is the recruitment of additional inflammatory cells from other sites of the body. Chemokines

direct monocytes, neutrophils, and lymphocytes bearing the appropriate chemokine receptors to the site of infection or metastasis (6,7). Cytokines activate the synthesis and release of soluble antimicrobial agents, such as complement and acute-phase proteins (C-reactive protein and mannose-binding lectin). Cytokines also stimulate the growth, differentiation, and activation of effector cells of the innate immune system. The result is a tightly focused, effective series of physical assaults on the activating structure (8,9). Neutrophils and macrophages (tissue-infiltrating monocytes) internalize and eliminate bacteria by phagocytosis. NK cells directly kill virus-infected and tumor cells by inducing apoptosis. Acute-phase proteins and complement bind to microorganisms, targeting them for destruction and phagocytosis. Interferons disrupt viral replication. These effector functions continue until the stimulating structure is destroyed or removed, at which time anti-inflammatory cytokines, such as IL-10 and TGF- $\beta$ , induce the resolution of innate immune responses and the activation of tissue repair and remodeling enzymes and proteins (10,11). In some situations, these immune effector functions fail to be resolved, and chronic inflammation results in permanent scarring, tissue damage, or fibrosis, such as joint destruction in rheumatoid arthritis or fibrosis and cirrhosis in chronic hepatitis.

Innate immune strategies are activated within seconds of detection of danger. It is likely that such innate defence functions are regular events in the healthy individual, occurring throughout



**Fig. 2.** Generation of diversity in T-cell and B-cell antigen receptors. Genomic (germline) DNA coding for the T-cell antigen receptor (TCR) and B-cell antigen receptor (immunoglobulin; Ig) consists of multiple gene segments coding for the variable (V), diversity (D), joining (J), and constant (C) portions of these molecules. TCR  $\alpha$ -chains and Ig light chains contain no D gene segments. During T-cell or B-cell maturation, somatic recombinations result in the joining of D and J gene segments and excision of the intervening DNA (shown by dotted lines), followed by the joining of a V and the DJ gene segments. Splicing of the primary RNA transcript results in the joining of the VDJ segment with the C gene segment. L, leader sequence. Imprecise joining of gene segments, random addition of nucleotides at the junctions of the gene segments, somatic hypermutations, and differential pairing of TCR  $\alpha$ - and  $\beta$ - chains or Ig heavy and light chains generate further diversity in these receptors.

the body but perhaps more frequently at sites of high cell turnover (where there is likely to be a higher incidence of mutation) and increased exposure to foreign antigens (such as the gastrointestinal tract, liver, lungs, and uterus). However, it remains impossible to determine how frequently these events occur and whether certain tissues are more likely to be sites of frequent inflammatory events.

## ADAPTIVE IMMUNITY

If a microorganism or tumor is able to evade the innate defense mechanisms and succeed in expressing a threshold level of antigen, inflammation is not resolved and the adaptive immune system is initiated. The first and crucial step is the activation of T lymphocytes. Naïve, antigen-inexperienced T cells circulate between the blood and peripheral lymphoid tissues as small inactive cells with condensed chromatin, few organelles, and minimal metabolic and transcriptional activity. They remain in this inactive state until they encounter an infectious agent or danger signal, which usually occurs in the lymphoid tissues. Recognition of an antigen or danger signal results in their proliferation and differentiation into effector lymphocytes capable of responding to the infection or danger.

### T-LYMPHOCYTE RECOGNITION OF ANTIGEN

Naïve T cells can only be activated by “professional” antigen-presenting cells (APCs), which are capable of capturing, processing, and displaying antigen on their cell surface (12). These functions are performed by DCs, macrophages, and B cells, and DCs have the additional ability to transport antigens to the T-cell-rich lymphoid tissues. APCs digest protein antigens into short peptides and present them on their cell surface complexed with MHC molecules. MHC molecules are highly

polymorphic and can thus present a diverse range of different peptides. T cells recognize peptide/MHC complexes by highly specific clonotypic T-cell receptors (TCRs). During T-cell development, a great diversity of TCR specificities is generated by the rearrangement of multiple germline gene segments that code for different regions (variable, diversity, joining, and constant) of the molecules. This is followed by the variable addition of nucleotides and hypermutation of antigen receptor genes at positions that generate further diversity in the antigen-recognition sites of these molecules. Thus, T cells display extreme diversity in antigen recognition, with up to  $10^{16}$  possible specificities of TCRs, providing the immune system with an enormous anticipatory repertoire of antigen-specific effector cells (13,14) (Fig. 2). However, this number is greatly reduced by the removal of T cells whose TCRs are either unable to recognize self-MHC molecules (positive selection) or whose TCRs are potentially autoreactive (negative selection). The processes of positive and negative selection occur during T-cell maturation in the thymus.

### T-CELL ACTIVATION

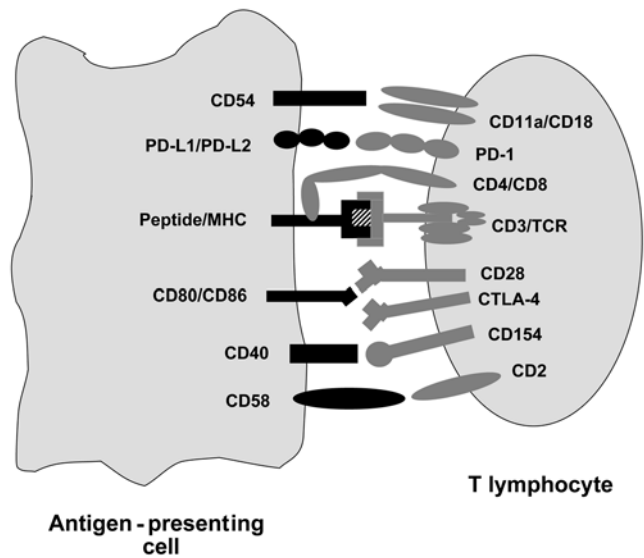
Distinct classes of T cells recognize intracellular and extracellular antigens. Peptides derived from endogenously synthesized antigens, such as self-peptides or viral peptides (in infected cells), are loaded onto MHC class I molecules in the endoplasmic reticulum and presented on the cell surface to  $CD8^+$  T cells, which typically kill the infected or tumor cell by Fas- or granzyme-mediated induction of apoptosis and the release of  $IFN-\gamma$ , which disrupts viral replication (15,16). Peptides derived from extracellular antigens, which are internalized by APCs, are loaded onto MHC class II molecules for presentation to  $CD4^+$  T cells, which, in turn, activate other cells of the adaptive immune response (17).

Engagement of the TCR by peptide/MHC complexes, in the absence of additional signals, is insufficient for the activation of naïve T cells. Instead, it induces T-cell inactivation, a process known as anergy, which protects against unwanted immune responses against harmless or self-antigens. Full activation of a naïve T cell requires the simultaneous engagement of a series of accessory molecules on the T cell with corresponding costimulatory molecules on the APC that are induced by danger signals from the innate immune system (18,19). The B7 family of molecules, CD80, CD86, and B7-homolog on an APC transduce costimulatory signals to T cells through CD28 and inducible costimulatory receptors (ICOS). Additionally, CD40 on the APC interacts with its T-cell ligand, CD154, upregulating B7 expression. Further nonspecific interactions between adhesion molecules on the APC and the T cell strengthen the physical association between the two cells (Fig. 3). If the interaction between the TCR and the peptide/MHC is maintained over a threshold amount of time, the naïve T cell is activated, and it undergoes clonal proliferation and differentiation into effector T cells. Full activation of naïve T cells takes 4 to 5 d and is accompanied by changes in cell-surface adhesion molecules that direct effector T cells from the lymphoid tissues to the sites of infection or danger in the periphery. Effector T cells can then respond in a variety of ways to the same peptide/MHC complexes, alone, without the need for costimulation.

### EFFECTOR FUNCTIONS OF THE ADAPTIVE IMMUNE SYSTEM

The differentiation of naïve T cells into functional effector cells is controlled by signals from the innate immune system (20,21) (Fig. 4). Release of IL-12 and IL-18 by macrophages and DCs and IFN- $\gamma$  by NK cells promotes the development of CD8<sup>+</sup> cytotoxic T cells and CD4<sup>+</sup> T-helper 1 (Th1) cells. Release of IL-4 and IL-6 promotes the development of CD4<sup>+</sup> Th2 cells. Th1 cells are generally induced by viruses and intracellular bacteria, whereas Th2 cells are induced by allergens and helminth pathogens. Th1 cells secrete IFN- $\gamma$  and TNF- $\beta$  and activate macrophages but also provide helper function for B-cell production of complement-fixing and virus-neutralizing antibodies of the IgG2a isotype in mice. In contrast, Th2 cells secrete IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13 and are considered to be the true helper cells, activating differentiation and class switching of B cells to secrete IgE, IgA, and IgG1 (20,21). A third population of CD4<sup>+</sup> T cells with regulatory function, termed Th3 or T-regulatory 1 (Tr1) cells, produces IL-10 and transforming growth factor- $\beta$  (TGF- $\beta$ ). They suppress Th1 responses and have been implicated in the maintenance of immunological tolerance at mucosal surfaces (10).

Antibodies, like TCRs, are coded for by sets of rearranging gene segments and thus possess as much diversity and specificity for antigen as the TCR (13) (Fig. 2). Antibodies released in soluble form can neutralize toxins and viruses and opsonize pathogens for phagocytosis by macrophages, cytotoxicity by NK cells, and directed histamine release by mast cells and basophils (22). Antibodies can also activate complement for the lysis of bacteria (23). Cell-surface antibodies, expressed by B cells, can specifically bind antigens, leading to their

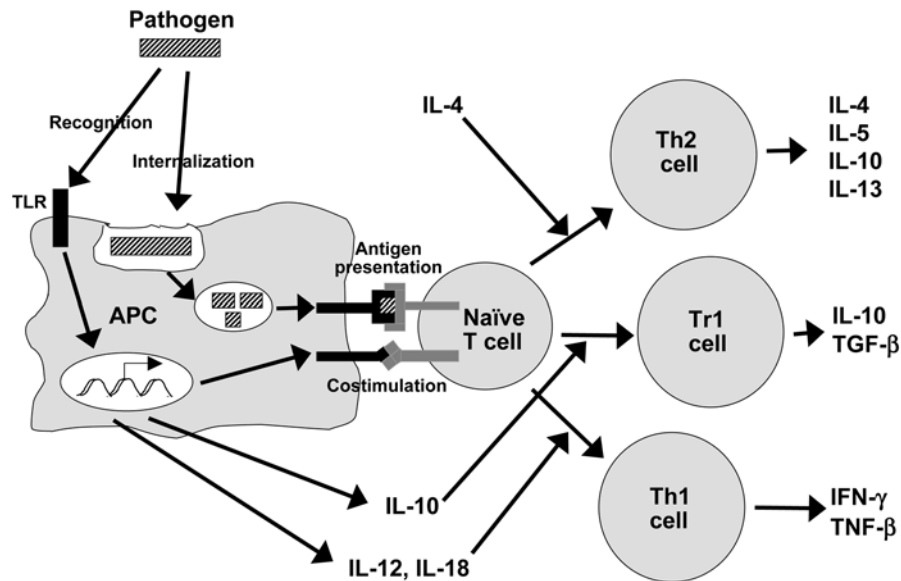


**Fig. 3.** Molecular interactions that mediate naïve T-lymphocyte activation by antigen presenting cells (APCs). Antigen recognition is mediated by ligation of the T-cell receptor (TCR) and the CD4 or CD8 coreceptor with a peptide/major histocompatibility complex (MHC) on the surface of the APC. Costimulation of T-cell activation generally involves the ligation of CD28 on the T cell with CD80 (B7-1) or CD86 (B7-2) on the APC. Ligation of the TCR is associated with upregulation of CD154 expression by the T cell, which binds to CD40 on the APC, thereby increasing expression of CD80 and CD86. Nonspecific interactions between the adhesion molecules CD54 (intracellular adhesion molecule-1 [ICAM-1]) on the APC and CD11a/CD18 (lymphocyte function antigen-1 [LFA-1]) on the T cell and between CD58 (LFA-3) on the APC and CD2 on the T cell strengthen the physical association between the two cells. T-cell activation results in the upregulation of cytotoxic T-lymphocyte antigen-4 (CTLA-4), which competes with CD28 for CD80 and CD86 binding and downregulates T-cell activation. Antigen-specific interactions with APCs lacking costimulatory or adhesion molecules can result in inactivation of naïve T cells by anergy, whereas effector T cells do not need costimulation for their activation. Ligation of programmed death receptor-1 (PD-1) by its ligands PD-L1 or PD-L2 inhibits T-cell activation and regulates tolerance and autoimmunity.

internalization and presentation to T cells. Adaptive immune responses are terminated by anti-inflammatory cytokines, such as TGF- $\beta$  and IL-10, which can be secreted by a range of APCs and by antigen-specific T cells (Tr1 cells and Th3 cells) (10,11). These cytokines inhibit and downregulate inflammatory responses effects and initiate tissue repair. Resolution of both T-cell and B-cell immune responses is associated with the generation of antigen-specific memory cells, which can be rapidly reactivated by the same antigens.

### INTERACTION AND INTERDEPENDENCE OF INNATE AND ADAPTIVE IMMUNE SYSTEMS

Until recently, innate and adaptive immunity were thought of (and certainly taught as) two independent, almost mutually exclusive systems. However, innate and adaptive immune systems are in continuous dialogue, with each regulating the other. Macrophages and DCs of the innate immune response act as APCs for T cells in the initiation of adaptive immune responses



**Fig. 4.** Activation and regulation of naïve  $CD4^+$  T cells. Pathogens are internalized by antigen-presenting cells (APCs) by phagocytosis, endocytosis, or receptor-mediated endocytosis, processed into peptides within the APC, and presented to naïve T cells complexed with major histocompatibility complex (MHC) class II molecules. Through recognition of pathogenic molecules (*see* Fig. 1), Toll-like receptors (TLRs) signal the production of cytokines, such as interleukin (IL)-10, IL-12, and IL-18 and the expression of costimulatory molecules on the APC cell surface. Antigen presentation in the presence of costimulation results in the activation of naïve T cells, and the APC-derived cytokines instruct the naïve T cells to differentiate into T-helper 1 (Th1) or T-regulatory 1 (Tr1) cells. IL-4 from other sources promotes the differentiation of naïve T cell into Th2 cells. TEF-transforming growth factor; TNF, tumor necrosis factor.

(12). The selective differentiation of naïve T cells into Th1, Th2, or Th3/Tr1 cells is controlled by signals from cells of the innate immune system, such as DCs and macrophages (Fig. 4). Immature DCs internalize antigens in the tissues and migrate to the lymph nodes, where they act as APCs for the activation of T cells (24,25). DCs are capable of directing T-cell maturation into distinct T-cell subtypes (26). The nature of the antigen influences the pattern of cytokines produced by the DCs, which in turn determines the type of T cell expanded from naïve precursors. The release of IL-12 and IL-18 by DCs stimulates Th1 induction, whereas IL-10 production by DCs stimulates the generation of Tr1 cells (20,21). Recent evidence suggests that PRR ligation of immature DCs can cause them to mature into one of two mutually inhibitory DC subsets, DC1 or DC2 cells, which promote Th1 or Th2 responses, respectively (24,25). NK cells also can regulate Th1 or Th2 cell differentiation by the selective production of IFN- $\gamma$ , IL-5, or IL-13.

In addition to the cross-talk between the cells of the innate and adaptive immune systems, many cells of the adaptive immune system have evolved antigen recognition and effector mechanisms that are characteristic of the innate immune system. Several subsets of T and B cells can recognize non-protein antigens, which are not subject to antigenic drift and are therefore relatively conserved between classes of pathogens. Natural killer T (NKT) cells possess TCRs that recognize glycolipid antigens presented by the nonclassical antigen-presenting molecule CD1 (27).  $\gamma\delta$  T cells can directly recognize small metabolite molecules (prenyl pyrophosphates, thymidine metabolites, alkylamines, and glycoproteins) and stress-inducible proteins (nonclassical MHC class I molecules and heat shock

proteins) without the need for MHC restriction (27).  $\gamma\delta$  T cells can also recognize glycolipid antigens presented by CD1 (27,28). Upon activation, NKT cells and  $\gamma\delta$  T cells can rapidly kill tumor cells, regulate Th1/Th2/Tr1 cell differentiation by the selective production of IFN- $\gamma$ , IL-4, or IL-10, and induce maturation of DCs into APCs.

## APPENDIX 1: CLUSTER OF DIFFERENTIATION (CD) ANTIGENS

CD1	MHC class I-like lipid presenting molecules expressed by APCs and other cells
CD2	Adhesion/costimulatory molecule expressed by T cells and NK cells
CD3	TCR-associated molecular complex necessary for TCR-mediated signal transduction
CD4	Coreceptor for MHC class II molecules found on T cells, monocytes, and macrophages
CD8	Coreceptor for MHC class I molecules found on T cells and some NK cells
CD11	Family of adhesion molecules found on lymphocytes, granulocytes monocytes, and macrophages
CD14	Receptor for lipopolysaccharide and other molecules found on DC and macrophages
CD16	Immunoglobulin Fc receptor found on neutrophils, macrophages, and NK cells
CD18	Adhesion molecule found on leukocytes that associates with CD11
CD19	Costimulatory receptor found on B cells
CD20	Costimulatory receptor found on B cells

CD25	High-affinity IL-2 receptor ( $\alpha$ -chain) found on activated T cells, B cells, and monocytes	CD158	Stimulatory/inhibitory receptor (KIR) found on NK cells
CD28	Naïve T-cell receptor for costimulatory molecules CD80 and CD86	CD161	Costimulatory receptor found on NK cells and some T cells
CD34	Adhesion molecule found on hematopoietic precursors	<b>APPENDIX 2: CYTOKINES</b>	
CD35	Complement receptor found on most leukocytes	<b>INFLAMMATORY CYTOKINES</b>	
CD40	B-cell receptor for costimulatory molecule CD154	IL-1 $\alpha$ , - $\beta$	Stimulates T-cell and macrophage activation and increases body temperature
CD44	Leukocyte adhesion molecule	TNF- $\alpha$	Tumor necrosis factor- $\alpha$ : induces local inflammation, activation of macrophages, and nitric oxide production
CD45	Signaling molecule that augments signals through T-cell and B-cell antigen receptors	IFN- $\alpha$ , - $\beta$	Interferons- $\alpha$ and - $\beta$ : stimulate MHC class I expression and inhibit viral replication
CD49	Family of adhesion molecules found on leukocytes	IFN- $\gamma$	Interferon- $\gamma$ : stimulates Th1 cell, NK cell, and macrophage activation and MHC expression by APCs; inhibits Th2 cell differentiation
CD50	Family of adhesion molecules found on leukocytes	IL-6	Stimulates lymphocyte growth and acute-phase protein production by the liver
CD54	Family of adhesion molecules found on hematopoietic cells	IL-8	Chemotactic factor for leukocytes
CD56	Adhesion molecule found on NK cells	IL-12	Activates NK and NKT cells and promotes Th1 cell differentiation
CD58	Adhesion molecules found on hematopoietic cells	IL-18	Promotes Th1 cell differentiation
CD64	Immunoglobulin Fc receptor found on monocytes and macrophages	<b>Th1 CYTOKINES</b>	
CD69	Lectin of unknown function found on activated T cells, B cells, NK cells, and macrophages	IL-2	Stimulates T-cell growth and proliferation and cytotoxicity by NK cells
CD74	MHC class II chaperone molecule found in APCs	TNF- $\beta$	Tumor necrosis factor- $\beta$ : mediates cell killing
CD79	B cell antigen receptor-associated molecular complex required for Ig-mediated signal transduction	IFN- $\gamma$	Interferon- $\gamma$ : stimulates Th1 cell, NK cell, and macrophage activation and MHC expression by APCs; inhibits Th2 cell differentiation
CD80	Costimulatory molecule found on APCs	<b>Th2 CYTOKINES</b>	
CD81	B cell coreceptor	IL-4	Stimulates production and class switching of IgG1 and IgE and growth of mast cells
CD86	Costimulatory molecule found on APCs	IL-5	Stimulates IgA production and growth of eosinophils
CD94	Stimulatory/inhibitory receptor for HLA-E found on NK cells and some T cells	IL-6	Stimulates lymphocyte growth and acute-phase protein production by the liver
CD95	Apoptosis-inducing molecule found on a wide variety of cells (Fas)	IL-9	Enhances mast cell activity
CD102	Adhesion molecule found on resting lymphocytes, monocytes, and endothelial cells	IL-10	Suppresses Th1 cell and macrophage activity and costimulates mast cell growth
CD106	Adhesion molecule found on endothelial cells	IL-13	Stimulates B-cell growth and differentiation and inhibits macrophage activity
CD116	Receptor for granulocyte-macrophage colony stimulating factor found on myeloid cells	<b>Tr1 CYTOKINES</b>	
CD117	Stem cell factor receptor found on hematopoietic cell precursors	IL-10	Suppresses Th1 cell and macrophage activity and costimulates mast cell growth
CD119	IFN- $\gamma$ receptor found on macrophages, monocytes, and B cells	TGF- $\beta$	Transforming growth factor- $\beta$ : inhibits Th1 cells
CD120	TNF- $\alpha$ and - $\beta$ receptor found on many cell types	<b>HEMATOPOIETIC GROWTH FACTORS</b>	
CD121	IL-1 receptor found on T cells, B cells, macrophages, and monocytes	IL-3	Growth factor for hematopoietic progenitor cells
CD122	IL-2 receptor $\beta$ -chain found on NK cells and some T cells and B cells	GM-CSF	Granulocyte-macrophage colony-stimulating factor: stimulates growth and differentiation of myeloid cells
CD124	IL-4 receptor found on mature T cells and B cells	IL-7	Induces lymphocyte differentiation, induces RAG1 and RAG2 expression, which is required for TCR and Ig gene rearrangement
CD125	IL-5 receptor found on eosinophils, basophils, and activated B cells	IL-15	Induces differentiation of NK and NKT cells
CD132	Common $\gamma$ -chain receptor for IL-2, IL-4, IL-7, IL-9, and IL-15		
CD134	Costimulatory molecule found on activated T cells (OX40)		
CD152:	Negative regulator of T-cell activation that interacts with CD80 and CD86 (CTLA4)		
CD154	Costimulator of B-cell activation found on activated T cells		

## APPENDIX 3: GLOSSARY

**Accessory cell:** A cell that aids an adaptive immune response but does not mediate specific antigen recognition.

**Acute-phase proteins:** A series of blood proteins that participate in the early phases of host defense against infection.

**Adaptive immune response:** The response of antigen-specific lymphocytes to antigen and the development of immunological memory.

**Adhesion molecules:** Mediate the binding of one cell to another.

**Adjuvant:** A substance that enhances the immune response to an antigen with which it is mixed.

**Alleles:** Variants of a single gene.

**Allergy:** An immune response to an innocuous antigen.

**Alloreactivity:** The stimulation of T cells by non-self MHC molecules.

**Anergy:** A state of T-cell nonresponsiveness to antigen.

**Antibody:** Plasma proteins (immunoglobulins) that bind specifically to antigens and mediate neutralization, opsonization, and complement activation.

**Antigen:** Molecules that are recognized by T cells or B cells.

**Antigen presentation:** The display of peptide fragments of protein antigens bound to MHC molecules for T-cell recognition.

**Antigen-presenting cells (APCs):** Specialized cells that can internalize, process, and present antigens to T cells.

**Antigen processing:** The intracellular degradation of proteins into peptides for presentation to T cells.

**APC:** *See* antigen presenting cell.

**Apoptosis:** Programmed cell death.

**Autoimmune disease:** Pathology caused by immune responses to self-antigens.

**Basophils:** White blood cells with functions similar to those of mast cells.

**B cells:** Lymphocytes with antigen-specific immunoglobulin receptors.

**B7:** *See* CD80 and CD86 (Appendix 1).

**Bone marrow:** The site of hematopoiesis.

**CD:** Cluster of differentiation (*see* Appendix 1).

**Cell-mediated immunity:** Immune responses involving cytotoxic T cells and NK cells.

**Chemokines:** Cytokines that attract cells to a site of inflammation.

**Clonal expansion:** Proliferation of antigen-specific lymphocytes, allowing rare cells to increase in number.

**Complement:** A set of plasma proteins that attack extracellular pathogens.

**Complement receptors:** Cell-surface receptors that bind pathogen-bound complement, resulting in their phagocytosis.

**Complementarity-determining regions:** The regions of the T-cell receptor or immunoglobulin molecules that make contact with antigens.

**Coreceptor:** Cell-surface proteins that participate in antigen recognition by lymphocyte antigen receptors.

**Costimulation:** A signal from an APC required in addition to antigen for full activation of lymphocytes.

**C-reactive protein:** An acute-phase protein that binds to phosphatidylcholine on bacteria and opsonizes them for phagocytosis

**C gene segment:** Constant gene segment, coded for by Ig and TCR genes.

**CTLA-4:** *See* CD152 (Appendix 1).

**Cytokines:** Proteins secreted by cells that affect the behavior of other cells (*see* Appendix 2).

**Cytokine receptors:** Cellular receptors for cytokines.

**Cytotoxic:** T cells T cells that can kill other cells.

**D gene segment:** Diversity gene segment, coded for by Ig and TCR genes.

**DC:** *See* dendritic cell.

**Dendritic cell:** Cells of the innate immune system that capture antigens and present them to T cells and direct T-cell subtype differentiation.

**Diapedesis:** Movement of cells from blood across blood vessel walls into tissues.

**Effector cells:** Lymphocytes that mediate the removal of pathogens from the body without the need for further differentiation.

**ELISA:** *See* enzyme-linked immunosorbent assay.

**ELISpot assay:** An adaptation of ELISA in which individual cells are placed over a bound antibody or antigen that trap the cells' secreted products and are detected with an enzyme-coupled antibody.

**Endotoxin:** A bacterial toxin that is released when the cell is damaged.

**Enzyme-linked immunosorbent assay (ELISA):** Serological assay in which bound antigen or antibody is detected by a linked enzyme that converts a colorless substrate to a colored product.

**Eosinophil:** White blood cells involved in immunity against parasites.

**Epitope:** The region on an antigen that is recognized by a lymphocyte.

**Fas:** *See* CD95 (Appendix 1).

**Fc receptors:** Cellular receptors for the constant portions of immunoglobulins.

**Flow cytometry:** Characterization of cells with regard to cell size, cell granularity, and fluorescence owing to bound fluorescent antibodies.

**Gene segments:** Segments of TCR and immunoglobulin genes that undergo somatic recombination resulting in the generation of diversity of antigen recognition.

**Germinal centers:** Sites in secondary lymphoid tissues of B-cell proliferation, selection, and maturation.

**Granulocytes:** Polymorphonuclear leukocytes.

**Haplotype:** A set of genes associated with one haploid genome.

**Helper T cells:** CD4<sup>+</sup> T cells.

**Hematopoiesis:** Generation of all blood cells from their precursors.

**Histamine:** A vasoactive amine stored in mast cell granules that is released upon antigen binding to IgE molecules on mast cells.

**Histocompatibility:** The ability of tissues to coexist without eliciting immune responses.

**HLA:** Human leukocyte antigens encoded by the MHC.

**Humoral immunity:** Specific immunity mediated by antibodies.

**Hypersensitivity:** Immune responses to innocuous antigens that occur repetitively.

**ICOS (inducible costimulatory receptors):** Molecules found on the surface of T cells required for T cell activation after engagement of the TCR.

**Ig:** *See* immunoglobulin.

**Immunization:** The deliberate provocation of an immune response by introducing antigen.

**Immunoblotting:** A technique in which proteins are separated by electrophoresis and detected by antibodies.

**Immunofluorescence:** A technique for detecting molecules using antibodies labeled with fluorescent dyes.

**Immunoglobulin (Ig):** B cell-surface and secreted antigen receptors (*see* antibodies).

**Immunoglobulin superfamily:** Receptor proteins with shared structural features to immunoglobulins.

**Immunohistochemistry:** A technique employing enzyme-labeled or fluorescent antibodies to detect specific molecules in tissue sections.

**Immunological memory:** The ability of antigen-specific effector T cells and B cells to persist for years.

**Immunoprecipitation:** Detection of soluble proteins using specific antibodies.

**Immunoreceptor tyrosine-based activation motifs (ITAMs):** Tyrosine residues on the cytoplasmic domains of signaling proteins that upon phosphorylation trigger cell activation.

**Immunoreceptor tyrosine-based inhibitory motifs (ITIMs):** Similar to ITAMs except they signal inhibition of cellular functions.

**Inflammation:** Early phase of an immune response involving the local accumulation of plasma proteins and leukocytes at a site of infection.

**Innate immunity:** A variety of defense mechanisms that nonspecifically target pathogens in the early stages of an immune response.

**Integrins:** A family of adhesion molecules.

**Interferons:** A family of cytokines with antiviral activity.

**Interleukins:** Cytokines produced by leukocytes (*see* Appendix 2).

**J chain:** Protein used to hold the pentamer of IgM and the dimer of IgA together, coded for by a nonimmunoglobulin gene.

**J segment:** Joining gene segment, coded for by Ig and TCR genes.

**Knockout mice:** Mice with heritable targeted disruptions of specific genes.

**Kupffer cell:** Specialized phagocytic cells found in the liver.

**Leukocyte:** General term for white blood cells.

**Lymphatic system:** A series of channels that drain fluid from the tissues to the blood.

**Lymph nodes:** Secondary lymphoid organs where adaptive immune responses are initiated.

**Lymphocytes:** Mononuclear leukocytes that mediate adaptive immune responses.

**Lymphokines:** Cytokines produced by lymphocytes.

**Macrophage:** Myeloid cell of the innate immune system with APC function found in the tissues (e.g., Langerhans cells in the skin; Kupffer cells in the liver).

**Major histocompatibility complex (MHC):** Highly polymorphic gene complex found on chromosome 6 in the human; codes for class I and class II antigen-presenting molecules as well as other molecules of immunological importance.

**Mannose binding lectin:** Acute-phase protein synthesized in the liver early in inflammation.

**Mast cells:** Histamine-releasing cells of myeloid origin with IgE receptors found fixed in tissues.

**Membrane attack complex:** Complement components that can disrupt membranes of pathogens.

**MHC:** *See* major histocompatibility complex.

**MHC restriction:** Recognition of peptide antigens presented by MHC molecules by T cells.

**MICA, MICB:** MHC class I-related stress proteins expressed by epithelial cells recognized by NK cells and some T cells.

**Minor histocompatibility antigens:** Antigens that can lead to graft rejection when recognized by T cells.

**Minor lymphocyte stimulatory (Mls) loci:** Mammary tumor virus genes integrated into the mouse genome that code for superantigens.

**MIP-1 $\alpha$  and - $\beta$ :** Macrophage inflammatory proteins  $\alpha$  and  $\beta$  chemokines.

**Monoclonal antibodies (MAbs):** Antibodies produced by a single clone of B cells.

**Monocyte:** Myeloid phagocytic cell found in the circulation.

**Myeloid cells:** Macrophages and granulocytes.

**N nucleotides:** Nucleotides that are inserted into the junctions between gene segments of TCR and Ig DNA to create further diversity.

**Naïve lymphocytes:** Lymphocytes that have never encountered antigen.

**Natural cytotoxicity:** Spontaneous killing of cells by NK cells.

**Natural killer (NK) cells:** Lymphoid cells of the innate immune system that kill virus-infected and tumor cells.

**Natural killer T (NKT) cells:** Cells that combine the phenotypic and functional characteristics of NK cells and T cells.

**Necrosis:** Death of cells owing to physical or chemical injury, as opposed to apoptosis.

**Negative selection:** Intrathymic deletion of developing T cells that recognize selfantigens.

**Neutralization:** Inhibition of infectivity of a virus or toxicity of a toxin by antibodies.

**Neutrophil:** Polymorphonuclear, phagocytic leukocyte; most numerous in the circulation.

**NK cell:** *See* natural killer cell.

**NK1.1<sup>+</sup> T cell:** T cells that express the NK cell stimulatory receptor NK1.1.

**NKG2D:** Activating receptor found on NK cells and some T cells.

**NKp46:** Natural cytotoxicity receptor found on NK cells that recognizes viral hemagglutinin.



**NKT cells:** See natural killer T cells.

**Nude mice:** A mutant strain of mice with no hair and defective thymic formation so they have no mature T cells.

**Opsinization:** Alteration of the surface of a pathogen so that it can be recognized and ingested by phagocytes.

**Pathogen-associated molecular patterns (PAMPs):** Conserved antigenic structures present on microorganisms that are recognized by the innate immune system.

**Pattern recognition receptors (PRPs):** Receptors on cells of the innate immune system that recognize common structures (PAMPs) found on infectious agents.

**Perforin:** A protein produced by T cells and NK cells that can polymerize to form a pore in a target cell as part of cell killing.

**Peyer's patches:** Aggregates of lymphocytes in the small intestine.

**Phagocytosis:** Engulfment of particles and cells by cells of the myeloid lineage.

**Plasma cell:** A terminally differentiated B cell.

**Polygenic:** Several gene loci code for multiple proteins of similar function.

**Polymerase chain reaction (PCR):** A technique for amplifying specific sequences of DNA.

**Polymorphic:** A gene locus with multiple alleles.

**Positive selection:** Selective maturation of T cells that can recognize self-MHC molecules in the thymus.

**Priming:** Initial interaction between an lymphocyte and an antigen.

**Professional APC:** Cells that are capable of presenting antigen to naïve T cells.

**Programmed death receptor-1 (PD-1):** A receptor on activated lymphocytes that mediates inhibition of lymphocyte effector functions.

**Proteasome:** A multifunctional protease that degrades antigenic proteins into peptides for antigen presentation.

**Radioimmunoassay (RIA):** A technique in which an antigen or antibody is bound to a solid support and specific radio-labeled antibody or antigen in a preparation is quantified by binding to these molecules.

**RAG1 and RAG2:** Recombinase activating genes that are critical to TCR and Ig gene rearrangement.

**RANTES (regulated on activation, normal, T-cell expressed and secreted):** A chemokine responsible for influencing the migration of T lymphocytes.

**Receptor-mediated endocytosis:** Internalization of molecules by cells using specific receptors for the molecules.

**Receptor repertoire:** The totality of lymphocyte receptors present in an individual.

**Regulatory T cells (Tr cells):** T cells that suppress the activity of effector T cells.

**Secondary immune response:** A more rapid and potent lymphocyte response induced by second exposure to antigen.

**Second signal:** A costimulatory signal required for lymphocyte activation.

**Selectins:** A family of adhesion molecules.

**Seroconversion:** The phase on an infection in which antibodies are produced.

**Serology:** The use of antibodies to quantify antigens.

**Somatic recombination:** Rearrangement of TCR or Ig gene segments.

**Superantigens:** Molecules that stimulate whole families of T cells by binding to MHC class II molecules and V $\beta$  domains of the TCR.

**Suppressor T cells:** See regulatory T cells.

**Syngeneic:** Between two genetically identical individuals.

**T cell:** Lymphocytes that mature in the thymus and recognize antigen by a TCR associated with the CD3 protein complex.

**T-cell clone:** Cultured T cells expanded from a single cell.

**T-cell line:** Cultures of T cells grown by repeated stimulation.

**T-cell receptor (TCR):** Antigen-specific receptors on T cells.

**T lymphocyte:** See T cell.

**TCR:** See T-cell receptor.

**TGF- $\beta$ :** See Appendix 2.

**Th1 cells:** CD4<sup>+</sup> T cells that secrete IFN- $\gamma$ , TNF- $\beta$ , and IL-2 and activate macrophages and promote inflammation.

**Th2 cells:** CD4<sup>+</sup> T cells that secrete IL-4, -5, -9, -10, and -13 and promote B-cell differentiation.

**Th3 cells:** CD4<sup>+</sup> T cells that secrete TGF- $\beta$  and suppress Th1 cell responses.

**Thymus:** Organ where T cells differentiate from bone marrow-derived hematopoietic stem cells.

**TNF (Tumor necrosis factor):** A family of inflammatory cytokines (see Appendix 2).

**Tolerance:** The failure of the immune system to respond to antigen.

**Toll-like receptors:** Receptors on macrophages and dendritic cells that recognize common components of microorganisms and mediate signaling pathways analogous to the Toll receptor in *Drosophila*.

**Transgene:** Introduction of foreign genes to the genome of an organism.

**V gene segments:** Variable gene segment, coded for by Ig and TCR genes.

**Vaccination:** The deliberate induction of immunity against a pathogen by immunization with a dead, attenuated, or defective form of the pathogen.

**Western blotting:** A technique for detecting proteins separated by gel electrophoresis using labeled antibodies.

**Xenogeneic:** Between organisms of different species.

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# 2 Role and Function of Liver Sinusoidal Endothelial Cells

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PERCY A. KNOLLE

## KEY POINTS

- The liver is involved in induction of peripheral immune tolerance, as evidenced by acceptance of liver allografts across MHC barriers, by split tolerance to further organ transplants from the same donor, and by intraportal application of antigen, leading to antigen-specific immune tolerance.
- Although the liver is composed of many different cell types, the sinusoidal cell populations, predominate, i.e., the Kupffer cells and the liver sinusoidal endothelial cells (LSECs), which are in direct contact with cells of immune system passing the liver with the bloodstream. The sinusoidal cells physically separate hepatocytes from passenger leukocytes in the sinusoidal lumen.
- LSECs express many different pattern recognition receptors, which allow these cells to fulfill a dual function: (1) scavenging of macromolecules from the circulation and (2) sensing of “dangerous” or “foreign” agents leading to cell activation and release of soluble mediators. These two functions of LSECs are required for hepatic clearance function and for coordination of complex hepatocellular functions, such as generation of acute-phase proteins.
- The scavenger function of LSECs, in particular expression of certain pattern recognition receptors, is targeted by hepatotropic viruses in order to leave the vascular compartment and to infect hepatocytes. Experimental evidence exists for a role of LSECs in infection with hepatitis C virus, duck hepatitis B virus, and human immunodeficiency virus.
- LSECs bear a unique immune phenotype expressing markers typical for cells of myeloid origin (CD1, CD4, CD11c), although these cells repopulate from hepatic progenitor cells. LSECs constitutively express costimulatory molecules necessary to interact with T cells in an antigen-specific manner (CD80, CD86, CD40, MHC I, MHC II). With regard to their phenotype, LSECs resemble immature dendritic cells rather than typical microvascular endothelial cells from other organs.
- Interaction of passenger leukocytes is facilitated by the narrow lumen of the hepatic sinusoid, slow and intermittent sinusoidal blood flow, and constitutive expression of adhesion-promoting molecules on the surface of LSECs. Aberrant expression of gut-homing molecules on LSECs may provoke recruitment of memory T cells to the liver that were initially activated in the gut. If these T cells recognize their antigen in the liver, they may initiate liver damage.
- LSECs have the capacity to act constitutively as antigen-presenting cells. MHC class II restricted presentation of soluble antigens by LSECs is controlled by factors of the hepatic microenvironment. Naive CD4<sup>+</sup> T cells primed by antigen-presenting LSECs fail to differentiate toward effector Th1 cells but express high levels of immune-suppressive mediators. Furthermore, LSECs contribute to allospecific immune tolerance in liver transplantation. Thus, antigen presentation by LSECs contributes to induction of immune tolerance in the liver by tolerizing CD4<sup>+</sup> T cells.
- Presentation of soluble, exogenous antigens on MHC class I molecules, termed cross-presentation, occurs with high efficiency in LSECs. However, naive CD8<sup>+</sup> T cells primed by cross-presenting LSECs lose their ability to respond to their specific antigen upon restimulation, i.e., failure to express effector cytokines (IFN- $\gamma$ ) and failure to develop specific cytotoxicity. In this way, LSECs contribute to induction of CD8 T cell tolerance toward oral antigens and toward antigens contained in apoptotic cell material.
- In contrast to professional antigen-presenting cells such as dendritic cells, LSECs represent a new type of organ-resident antigen-presenting cell. Sessile antigen-presenting LSECs clearly serve different functions than professional motile dendritic cells. These are: (1) immune surveillance of hepatocytes in case of the presence of effector T cells, and (2) induction of immune tolerance to soluble exogenous antigens in naive T cells. This presumably results in protection of hepatocytes from immune responses and may contribute to confinement of systemic immune responses.

## INTRODUCTION

The liver holds a unique position with regard to the blood circulation. It receives venous blood draining from almost

the entire gastrointestinal tract via the portal vein and from the systemic circulation via the hepatic artery. More than 2000 L of blood stream daily through the human liver, and peripheral blood leukocytes pass through the liver on average more than 300 times per day. These simple facts clearly demonstrate that the liver is a “meeting point” for antigens and leukocytes circulating in the blood.

Among the many functions of the liver, clearance of the blood from macromolecules and its metabolization are important for the understanding of the liver as an immunoregulatory organ. Nutrients have to be extracted from portal venous blood and further used for hepatocellular metabolism, but at the same time the liver must eliminate from the blood toxic waste products and proinflammatory agents (such as endotoxin or other bacterial degradation products derived by translocation from the gut) without eliciting an immune response to all these antigens.

Induction of immune tolerance in the liver was reported in 1967 by Cantor et al. in 1969 by Calne et al. (1,2), and since then by many other groups. Three main points demonstrate the ability of the liver to induce antigen-specific immune tolerance.

1. Liver transplants are accepted by recipient's immune systems despite MHC discrepancies and even in the absence of immune suppression (1,2).
2. Simultaneous transplantation of the liver and another organ from the same donor leads to increased graft acceptance of the cotransplanted organ. Further organ transplants from another donor lead to graft rejection, demonstrating antigen-specific induction of immune tolerance by the transplanted liver (3).
3. Drainage of an organ transplant directly into the portal vein or direct application of donor cells into the portal vein leads to increased acceptance of the graft (4–7).

This implies that antigen delivered to the liver leads to induction of tolerance by local immune-regulatory mechanisms. It became clear that almost every cell population in the liver is involved in induction of immune tolerance (8–11). However, most studies concentrated on the induction of immune tolerance toward transplantation antigens but not soluble antigens. Although immune tolerance toward organ transplants is important for transplantation medicine, immune tolerance to soluble antigens is most relevant for everyday life. Several reviews have covered the features of hepatic immune tolerance extensively (12), in particular with relevance to persistent viral infection with hepatitis B virus (HBV) and hepatitis C virus (HCV) (13). This chapter focuses on the role of a particular hepatic cell population, the liver sinusoidal endothelial cells (LSECs), in the regulation of immune responses, as these cells are strategically positioned within the liver to interact with immune cells and bear all necessary functions to stimulate T cells.

## MICROANATOMY OF THE LIVER

The liver is optimally structured to function as a metabolic organ, i.e., to clear blood from macromolecules and to release metabolic products from hepatocytes into the blood stream. Nutrient-rich blood from the gastrointestinal tract enters the

liver via the portal vein, which drains after extensive ramifications into the so-called portal field, which is comprised of one portal venous vessel, one arterial vessel, and a bile duct surrounded by connective tissue. Portal-venous and arterial blood drain into the hepatic sinusoids, which form a 3D meshwork of vessels generating a mixed arterial-venous perfusion of the liver. Blood flows from the portal tract to the central veins, which convene to hepatic veins draining into the inferior vena cava. The hepatic sinusoids are composed of several cell populations (Table 1).

Although hepatic sinusoidal cell populations contribute to only 6.3% of the total liver volume, they represent approx 40% of the total number of hepatic cells, 26% of the total membrane surface (mainly LSECs), 58% of total endocytotic vesicles (mainly LSECs), and 43% of the total lysosomal volume (mainly Kupffer cells and LSECs) (14).

LSECs form a thin but continuous cell layer physically separating leukocytes passing the liver within the bloodstream from hepatocytes (15). In contrast to endothelial cells in other organs, there is no basement membrane. It is controversial whether LSECs physically separate hepatocytes from leukocytes circulating in the blood or whether constitutive interaction of circulating leukocytes is possible via hepatocellular extensions protruding through endothelial fenestrae (*see next paragraph*). The space between hepatocytes and LSECs is called the space of Dissé, which contains abundant extracellular matrix produced by LSECs and is populated by the stellate cells, which surround the LSECs and control sinusoidal blood flow by contraction, leading to reduction of the sinusoidal diameter (16). Kupffer cells are located predominantly in the periportal region and are in close contact with LSECs. The blood flow in the liver is peculiar, being rather chaotic in the sinusoid (17), which is ideal for clearance of macromolecules from the blood and initiation of contact between hepatic sinusoidal cells and passenger leukocytes.

LSECs have pores, so-called fenestrae, approx 100 to 150 nm in size (18), which can be dynamically regulated by the actin cytoskeleton upon contact with substances like alcohol or nicotine (19,20). Blood cells passing through the narrow hepatic sinusoids exert a “sinusoidal massage,” causing improved exchange of fluid between the sinusoidal lumen and the space of Dissé (15). Flexible macromolecules larger than 100 nm in diameter or rigid macromolecules larger than 12 nm are excluded from access to the space of Dissé via diffusion through fenestrae, resulting in a “sieve” function of LSECs (15). Larger molecules such as chylomicrons, exceeding 100 nm in size, first have to be metabolized by membrane-associated lipase (21) before they can pass through fenestrae (22). Alternatively, molecules may gain access to hepatocytes through receptor-mediated uptake by LSECs and subsequent transcytosis (*see next section*) (23). Loss of endothelial fenestrae in liver cirrhosis may contribute to loss of hepatic function as a consequence of impaired exchange between sinusoidal blood and hepatocytes (20).

Liver-associated lymphocytes form a heterogeneous population of hepatic lymphocytes showing an unusual repertoire

**Table 1**  
**Sinusoidal Cell Populations**

<i>Hepatic cell population</i>	<i>% of liver volume<sup>a</sup></i>	<i>% of liver cells</i>
Kupffer cells	2.1	15
Liver sinusoidal endothelial cells	2.8	19
Stellate cells	1.4	5–8
Liver-associated lymphocytes/NK (T) cells	n.d.	n.d.
Hepatocytes	78	60
Dendritic cells	n.d.	n.d.

<sup>a</sup>Sinusoidal lumen 10.6%, space of Dissé 4.9%.  
Adapted from ref. (14).

**Table 2**  
**Receptors Associated with Scavenger Function**

<i>Molecules expressed by LSECs</i>	<i>Reference</i>
Scavenger receptors	35
Mannose receptor	36
CD14	37
TLR4	38
TLR9	39
L-SIGN	40
CD36	41
Fcγ receptors	42,43
Stabilin 1/2	44
LSEctin	45

of surface molecules and a restricted, T-cell receptor (TCR) repertoire (24). These cells are found in close association with LSECs and Kupffer cells, engaging in concert with these cells in local defense mechanisms against invading pathogenic microorganisms or tumor cells (25). Further studies revealed that the liver harbors a large population of CD1- and MHC I/II-restricted T cells bearing natural killer (NK) cell markers, so-called NKT cells, which have an activated phenotype and rapidly release substantial amounts of soluble mediators upon TCR-induced activation (26). NKT cells patrol hepatic sinusoids and arrest upon recognition of their cognate antigen on sinusoidal cells, suggesting the presence of a local intravascular immune surveillance system (27).

Within the periportal region, a rather specialized population of dendritic cells is found, which together with Kupffer cells is ideally situated to scavenge pathogenic agents from portal venous blood (28). The liver is connected to the lymphatic system, as particles injected via the portal vein are found within a few hours in retroperitoneal lymph nodes inside dendritic cells, suggesting that dendritic cells had ingested the particles and had migrated to lymphatic tissue (29,30). Certainly, liver dendritic cells play a key role in regulating immune responses to antigens delivered via the bloodstream to the liver (31–33).

## SCAVENGER FUNCTION OF LIVER SINUSOIDAL ENDOTHELIAL CELLS

Besides their strategic anatomic position in the hepatic sinusoid and the optimal local conditions of slow sinusoidal blood flow, LSECs are equipped with surface receptors that enable them to scavenge macromolecules and pathogenic agents from sinusoidal blood (Table 2). Because of their extraordinary ability to eliminate macromolecules from the circulation, these cells were called scavenger endothelial cells (34).

Efficient receptor-mediated uptake is accomplished by very fast kinetics of receptor recycling in LSECs, as exemplified by a fast turnover for the mannose receptor, which is only 15 s for ligand binding and delivery into endosomal compartments (36). Approximately 25,000 mannose receptor molecules are detected on average on the surface of LSECs. Together with the fast internalization rate of receptor molecules, this renders LSECs

most efficient in uptake of soluble material (36) even compared with professional scavenging cells such as macrophages and dendritic cells (46). LSECs even engage in phagocytosis of particles smaller than 200 nm (47) and receptor-mediated uptake of apoptotic bodies (48). In contrast to other scavenger cell populations, LSECs fail to employ macropinocytosis as a means of ingesting antigenic material.

Most of the receptors described in Table 2 are pattern recognition receptors that recognize pathogen-associated molecular patterns (PAMPs). This may ensure that preferential scavenging of pathogenic agents or cellular debris occurs through LSECs. Indeed, uptake of endotoxin, which is a physiological constituent of portal venous blood derived from translocation of bacterial products from the gut lumen into the blood circulation (49,50), occurs through both cell populations, Kupffer cells and LSEC (51–53). Elimination of advanced glycation end products from the circulation occurs also mainly by uptake via scavenger receptors on LSECs (54,55). Furthermore, LSECs are the predominant cell population involved in uptake of collagens and hyaluronic acid from the circulation (35,56,57). This enormous scavenger activity of LSECs is found in many vertebrates, which underlines the importance of this cell population for elimination of waste molecules (58).

It is assumed that LSECs process the molecules ingested by receptor-mediated endocytosis and deliver the degradation products by release into the space of Dissé, where directly adjacent microvilli of hepatocytes allow for uptake and further hepatocellular metabolization. Transcytosis of endocytosed ligands through LSECs has been demonstrated for transferrin and coeruleoplasmin as well as for mannose/galactose-coated beads (23,59,60). The extraordinary scavenger capacity allows LSECs to function as a funnel, directing blood-borne macromolecules toward hepatocytes. In a way, LSECs appear to “fuel” hepatocytes with substrates destined either for destruction and elimination via the bile or for further metabolization (34).

## CONTRIBUTION OF SCAVENGER LSECs TO VIRAL INFECTION OF THE LIVER

The molecular mechanisms underlying efficient infection of the liver by blood-borne viruses, such as HBV or HCV,

have been suspected to be related to expression of specific receptors exclusively expressed by hepatocytes. Alternatively, blood-borne viruses may abuse the scavenger activity of LSECs to escape from the hostile environment within the bloodstream and to target the liver. Infection of the ultimate target cell—the hepatocyte—would need then to occur after transit of the virus through LSECs. Such infection of a target cell *in trans* through another cell type that initially bound the virus was first reported for HIV (61). This principle also seems to apply to hepatotropic viruses. Using a model HBV, it was first shown that not hepatocytes but rather LSECs took up blood-borne virus and that infected hepatocytes were often observed in the vicinity of LSECs (62), suggesting a model of primary uptake into LSECs as a general mechanism by which blood-borne hepatotropic agents are targeted to the liver. Indeed, HCV was found to use liver-specific ICAM-3-grabbing non-integrin (L-SIGN) on LSECs as a liver-specific capture receptor (63), which mediates trans-infection of hepatocytes (64,65). HCV glycoproteins mediating binding to L-SIGN have been identified (66,67) and also appear to be responsible for viral escape from lysosomal degradation (68). HIV has also been shown to bind to L-SIGN, and thus LSECs are likely to contribute to infection of passenger CD4 T cells with HIV locally in the liver (40,69). HIV even leads to low levels of infection by HIV (70), similar to the low-level infection observed in dendritic cells (61).

LSEC may be particularly well suited for *trans*-infection of other cell populations, because transcytosis is a fast process (60), whereas lysosomal degradation occurs at rather slow rate (71). However, it is unclear how trans-infection from LSECs to hepatocytes occurs at the molecular level and whether it involves a virus receptor or whether it is a membrane fusion process. A recent publication suggests that coculture with LSECs induces a differentiated phenotype in hepatocytes characterized by expression of low-density lipoprotein (LDL) receptor and increased uptake of LDL. The development of this differentiated phenotype was further accompanied by hepatocellular uptake of HCV particles (72). These observations support the hypothesis that some hepatotropic viruses abuse the physiological scavenger function, which is operative to increase delivery of macromolecules to hepatocytes, in order to target the liver.

However, the scavenger function of LSECs does not seem to be the sole mechanism contributing to hepatocellular viral infection. Upon intravenous injection, adenoviruses, which are often used as viral vectors for gene therapy, efficiently target the hepatocytes, although these cells do not express the relevant viral receptor coxsackie adenovirus receptor (CAR) (73). Adenoviruses also fail to infect LSECs (74). The critical parameter underlying hepatocyte transduction with adenovirus rather seems to be size of endothelial fenestrae, as pharmacological “widening” of endothelial fenestrae induced increased viral transduction rates of hepatocytes *in vivo* (75). These results demonstrate that blood-borne viruses use complex molecular mechanisms to target hepatocytes.

## INNATE IMMUNE FUNCTION OF LIVER SINUSOIDAL ENDOTHELIAL CELLS

Expression of pattern recognition receptors not only enables LSECs to function as most efficient scavenger cells but also allows these cells to respond directly to encounters with pathogenic agents with the expression of a number of soluble mediators (Table 3).

Release of proinflammatory mediators such as interleukin-1 (IL-1) and IL-6 from Kupffer cells and LSECs is required to induce expression of acute-phase proteins in hepatocytes (81), as hepatocytes themselves are not directly responsive to many pathogenic agents owing to the lack of expression of pattern recognition receptors (Limmer, unpublished observation). As little as 10 pg/mL of endotoxin are sufficient to lead to activation of LSECs *in vitro*, demonstrating the high sensitivity of these cells toward endotoxin and underlining their importance in generating systemic innate immune responses to infection through indirect induction of acute-phase proteins (82). Moreover, release of prostanoids from Kupffer cells and LSECs following exposure to endotoxin triggers glycogenolysis in hepatocytes (83). Release of nitric oxide (NO) from LSECs potentiates calcium signaling in surrounding hepatocytes (84). The release of soluble mediators from endothelial cells may present a mechanism by which they contribute to the coordination of hepatocellular cellular functions. Furthermore, increased expression of surface molecules such as P-selectin or CD54 following contact with endotoxin results in increased adhesion of passenger leukocytes and platelets to LSECs (85,86), which is a prerequisite for induction of inflammation. It is important to note that LSECs do not depend on other immune cell populations in the initiation of an inflammatory reaction but display cell-autonomous innate immune cell function as a virtue of constitutive expression of pattern recognition molecules. However, coordinated action between sinusoidal cell populations and passenger leukocytes, especially neutrophils, is necessary to mount a fast and efficient immune response against infecting microorganisms (87).

On the other hand, endotoxin is a physiological constituent of portal venous blood as a result of bacterial translocation from the gut (49,50). Both cell populations (LSECs and Kupffer cells) have been reported to develop a hyporesponsive state to endotoxin as a result of the unique microenvironment of the liver or intrinsic regulation of endotoxin sensitivity (38,82,88). This may ensure that physiological concentrations of endotoxin do not induce activation and cytokine release from LSECs or Kupffer cells and thus fail to induce an acute-phase response or local inflammatory reactions in the liver during the physiological situation.

Constitutive exposure to gut-derived bacterial degradation products in portal venous blood contributes to the unique hepatic microenvironment. Endothelial cells from mice raised under germ-free conditions do not express CD54 constitutively. After bacterial colonization of the gut, rapid induction of CD54 expression is observed (89). Furthermore, endotoxin not only induces release of proinflammatory mediators from

**Table 3**  
Soluble Mediators Released From LSECs

Mediator	Reference
IL-1	76
IL-6	76
MCP-1	Knolle et al., unpublished data
IP-10	77
MIP-1 $\alpha\beta$	77
NO	78,79
PGD <sub>2</sub>	80
PGE <sub>2</sub>	80
TXA <sub>2</sub>	80
PGF <sub>2<math>\alpha</math></sub>	80
PGI <sub>2</sub>	80

Abbreviations: IL, interleukin; IP-10, interferon- $\gamma$  inducible protein; MCP-1, monocyte chemoattractant protein-1; MIP-1 $\alpha$ , macrophage inflammatory protein-1 $\alpha$ ; NO, nitric oxide; PG, prostaglandin; TXA<sub>2</sub>, thromboxane A<sub>2</sub>.

hepatic sinusoidal cell populations but at the same time leads to expression of a number of potent antiinflammatory, immune-suppressive mediators such as IL-10 (90), transforming growth factor- $\beta$  (TGF- $\beta$ ) (91), and certain prostanoids such as prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) (80,92). The presence of these mediators contributes to a local environment that rather favors suppression of immunity and induction of immune tolerance, similar to the unique microenvironment found in the gut and intestinal lymphatic tissue (93).

The innate immune functions of LSECs raise the question of how these cells respond to contact with blood-borne viruses. At present, little is known about whether LSECs recognize the presence of virus after endocytosis or during transcytosis and whether there is an antiviral immune response triggered by such recognition, e.g., through expression of type I interferon. LSECs isolated from human liver are productively infected by HIV (70). LSECs constitutively express functionally relevant Toll-like receptor 3 (TLR3) and TLR7 molecules (Schumak and Scholz, ms. in preparation) and thus should in principle bear the ability to recognize RNA viruses.

Apart from sentinel function LSECs also display innate effector activity. This becomes most evident when one looks at the, antitumor effect of sinusoidal cells (94). During interaction with tumor cells, LSECs showed an increased expression of NO which exerted antitumor effects *in situ*. Central to the ability of LSECs to produce NO is the interaction with mature T cells (95). Furthermore, LSECs constitutively express CD95L at the cell surface. Expression levels of CD95L can be further increased by incubation of LSECs with endotoxin. Importantly, LSECs bear the capacity to induce CD95-dependent apoptosis in hepatocytes and lymphocytes by shedding CD95L from their surface (96). TRAIL is another apoptosis-inducing effector molecule, which is also constitutively expressed by LSECs (Limmer and Knolle, unpublished observation). In combination with the phagocytic activity (47) and the ability to ingest apoptotic cell material (48), these results strengthen the notion that LSECs represent an unusual population of endothelial

cells that has evolved to fulfill the unique functional requirements within the hepatic microenvironment.

## INTERACTION OF LIVER SINUSOIDAL ENDOTHELIAL CELLS WITH PASSENGER LEUKOCYTES

LSECs are strategically positioned in the hepatic sinusoid to establish interaction with passenger leukocytes in the blood flowing through the liver. As already mentioned, the small diameter of the hepatic sinusoid (7–12  $\mu$ m) and the slow and intermittent sinusoidal blood flow support the establishment of physical interaction with leukocytes in the blood. Studies with macrovascular endothelial cells and *in vitro* adhesion assays have demonstrated that the first steps of leukocyte-endothelial interaction depend on binding of carbohydrates with molecules of the selectin family (such as CD62E) expressed on endothelial cells that slow down leukocytes and lead to leukocyte rolling on endothelial cells (97). As direct contact of leukocytes with endothelial cells in the hepatic sinusoid exists already, there appears to be no need for expression of CD62E (98). However, challenge with endotoxin in high concentrations leads to induction of CD62E expression on LSECs *in vivo* (99), although no upregulation of CD62E gene expression was observed in isolated LSECs *in vitro* following exposure to endotoxin (A. Uhrig and P. Knolle, unpublished results). As already mentioned, upregulation of CD62P is pathophysiologically important in the induction of neutrophil-mediated liver injury during exposure to high concentrations of endotoxin (85,100). Expression of chemokines further enables LSECs to attract T cells to the liver. Since expression of chemokine receptors on T cells distinguishes functional T-cell subsets, it is assumed that expression of certain chemokines by LSECs promotes T-cell recruitment during viral infection of the liver (86).

Intravital microscopy of the liver revealed that there is constitutive interaction of LSECs with passenger leukocytes *in vivo* (38). This may be related to the constitutive expression of adhesion-promoting molecules such as CD54 (ICAM-1) and CD106 (VCAM-1) on LSECs, which are known to stabilize the adhesion of leukocytes to endothelial cells (101). The constitutive surface expression of adhesion molecules on LSECs appears to be related to the presence of bacterial degradation products in portal venous blood. Germ-free mice show much lower levels of CD54 on liver sinusoidal cells, which can be changed back to normal levels following intestinal colonization with bacteria (89). Furthermore, morphological changes in LSECs following exposure to endotoxin have been described that lead to narrowing of the sinusoidal diameter as well as increased contact with leukocytes (102). Constitutive CD54 expression by LSECs is required for selective retention of activated CD8 T cells in the liver under physiological conditions (103,104). In summary, the unique hepatic microenvironment favors constitutive interaction of LSECs with passenger leukocytes, a feature most likely linked to the immune-regulatory function of LSECs (*see below*).

However, in comparison with postcapillary endothelial cells, LSECs have a distinct phenotype lacking expression of CD31, CD34, VE-cadherin, and E-selectin. Furthermore, blockade of molecules typically involved in leukocyte adhesion, such as integrins and selectins, fails to abrogate leukocyte adhesion in the sinusoids (105,106). LSECs show constitutive expression of an important molecule mediating recruitment of lymphocytes into tissue, i.e., the ectoenzyme amine oxidase termed vascular adhesion protein-1 (VAP-1), which is upregulated during inflammatory reactions in the liver (101,107). VAP-1 serves two functions, as an adhesion-mediating molecule and as an ectoenzyme catalyzing oxidative deamination leading to generation of hydrogen peroxide, which in turn leads to cell activation (108). VAP is needed for leukocyte extravasation in vivo by mediating slow rolling and firm adhesion (109,110). Importantly, adhesion of CD4 Th2 cells in the liver occurs via VAP-1, whereas CD4 Th1 cells employ  $\alpha 4\beta 1$ -integrin adhesion (111). Blocking of VAP-1 activity leads to a reduction in lymphocyte adhesion and similarly results in improvement of immune-mediated hepatic inflammation (111,112). Interestingly, the cross-talk with hepatocytes enables LSECs to promote lymphocyte adhesion via CD54, CD106, and VAP-1 (113). This identifies VAP-1 as an interesting molecular target to modulate immune-mediated disease processes.

Detailed knowledge of the molecular mechanisms orchestrating hepatic lymphocyte adhesion is important to understand the pathogenesis of certain liver diseases. It has been demonstrated that expression of the chemokine CCL25 by liver endothelial cells leads to recruitment of CCR9<sup>+</sup> gut-homing lymphocytes to the liver in patients with primary sclerosing cholangitis (114). Together with the expression of sinusoidal expression of Mucosal address in cellular adhesion molecule-1 (MadCAM-1) in chronic inflammatory liver disease (115), the hypothesis was put forward that long-lived lymphocytes originally activated in the gut are recruited to the liver via aberrantly expressed gut-homing molecules, including CCL25 and MadCAM-1. If these T cells encounter their antigen in the liver, they may cause liver damage (116).

LSECs further contribute to development of hepatic metastasis of melanoma and lymphoma cells. Interaction of tumor cells with LSECs via pattern recognition receptors, in particular the mannose receptor, leads to local release of soluble mediators that subsequently result in upregulation of those adhesion molecules critical for tumor cell adherence to LSECs such as CD54 and CD106 (117–119). LSECs contribute to the development of hepatic metastasis through increased expression of adhesion molecules and angiogenesis, and they also participate in antitumor defense through release of mediators like NO and hydrogen peroxide, as described above (78,94,120).

### IMMUNE PHENOTYPE OF LIVER SINUSOIDAL ENDOTHELIAL CELLS

Compared with endothelial cells from other organs, LSECs have an unusual expression pattern of surface molecules (Table 4) that was investigated by immunohistochemistry or

**Table 4**  
**Immune Phenotype of Murine LSEC**

Surface molecule expressed	Constitutive expression level	Reference
CD1d	Intermediate	Wingender, unpublished results
CD4	Low	121
CD11c	Low	122
CD14	Low	121
CD54 (ICAM-1)	High	121
CD102 (ICAM-2)	Intermediate	Knolle et al. unpublished data
CD62E	Low to absent	
CD62P	Intermediate	85,86
CD106 (VCAM-1)	High	101
VAP-1	High	107
CD40	Intermediate	122
CD80 (B7-1)	Low	123
CD86 (B7-2)	Low	123
B7H1 (PD-L1)	Intermediate	124
MHC-I	High	
MHC-II	Low	125
CD95	Intermediate	96
CD95L	Low	96
TRAIL	Low	Limmer et al. unpublished data

flow cytometric analysis of LSECs after isolation, which allows sensitive detection of expression levels.

LSECs express a number of receptors, suggesting a myeloid origin of these cells, such as CD1, CD4, and CD11c. However, careful investigation of LSECs from male recipients of female liver allografts clearly demonstrated that LSECs did not derive from the bone marrow but presumably repopulated from a cell population present within the liver (126). Considering the hematopoietic function of the liver early in life and the ability of transplanted liver allografts to establish microchimerism (127,128), it is not surprising to find repopulation of LSECs from liver-intrinsic “stem” cells. In contrast, endothelial cells of the portal field or of the central venous area were replaced by the recipient’s endothelial cells as were splenic endothelial cells (126), showing that LSECs markedly differ from other endothelial cells in the liver and in other organs.

Furthermore, LSECs constitutively express MHC class I and II molecules and all cosignaling molecules required to interact successfully with T cells. Different isolation techniques for LSECs may give results that conflict with those described above (129). Together with the expression of CD11c and CD4, LSECs bear resemblance to immature dendritic cells rather than endothelial cells from other organs. A comparison between the functional phenotype of LSECs and dendritic cells will be given below in Antigen presentation of LSECs to CD4<sup>+</sup> T cells. Following induction of inflammation during acute liver failure or ischemia/reperfusion injury, massive upregulation of surface expression of adhesion molecules (CD54) and costimulatory molecules (CD80/CD86)



is observed on LSECs (130,131). This implies that LSECs have the capacity to act as accessory cells for T cells locally in the hepatic sinusoid.

### ROLE OF LSECs IN LIVER INJURY

Because LSECs form the inner lining of cells in the hepatic sinusoid separating hepatocytes from the bloodstream, breakdown of this barrier after contact with toxic agents or damaging immune cells may result in development of liver injury. Damage to LSECs was identified in a number of experimental systems.

Liver failure is observed when mice are intravenously injected with antibodies to CD95 that are able to induce apoptosis (132). It was believed that antibodies to CD95 directly bound to hepatocytes and led to development of fulminant hepatocellular apoptosis and liver failure (132). However, ultrastructural analysis at early time points after antibody injection revealed that antibodies to CD95 did not bind to hepatocytes but were almost exclusively found on the surface of LSECs (133). In time-course experiments, it became evident that apoptosis of CD95 expressing LSECs and development of sinusoidal thrombosis preceded hepatocellular apoptosis (133). Antibodies to CD95 mediate apoptosis in LSECs (134). Apoptotic death of circulating immune cells or even hepatocytes is considered to be a silent process, whereas apoptosis of endothelial cells is accompanied by disruption of their barrier function and therefore leads to microvascular perfusion disturbance (135). These findings demonstrate that hepatocyte apoptosis is rather a secondary phenomenon following injection of antibodies to CD95 resulting from initial damage to LSECs.

Initial damage to LSECs as the cause for subsequent liver injury is not restricted to this artificial system but is also observed in acetaminophen-induced hepatic necrosis. In addition to direct hepatocellular damage through metabolic activation of acetaminophen, centrilobular microvascular congestion and infiltration of erythrocytes through large gaps in LSECs into the space of Disse is observed within a few hours (136,137). Acetaminophen is directly toxic to LSECs, and was dependent on cytochrome P450 expression; compared with hepatocytes, toxicity for LSECs was more pronounced (138). The deleterious effect on sinusoidal lining LSECs contributes to acetaminophen-induced liver damage.

Neutrophil extravasation and activation are critical steps in the acute inflammatory organ damage that also affects the liver, e.g., during sepsis and endotoxemia (139). Efficient extravasation of neutrophils in the liver requires expression of adhesion-promoting molecules on LSECs (100) and further interaction with Kupffer cells (87). However, recent findings indicate that additional damage to LSECs, such as formation of gaps in sinusoidal lining within 4 h after endotoxin challenge, are instrumental in neutrophil extravasation and subsequent organ damage. Interestingly, the increased expression of hepatic matrix metalloproteases is causally involved in disruption of the sinusoidal barrier (140), indicating that tissue remodeling in the liver not only is relevant for fibrosis

after chronic inflammation but also has a critical influence on organ integrity during acute inflammatory conditions.

Intravenous injection of a T-cell mitogen, concanavalin A (Con A), leads to development of fulminant hepatic injury with death of mice ensuing from liver failure (141). The local release of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) from activated T cells and other cells in the liver plays a pivotal role in the mediation of liver injury, as neutralizing antibodies to TNF- $\alpha$  prevent induction of liver injury (142). Similarly, TNF-R knockout mice fail to develop ConA-mediated liver injury (143). It was shown that ConA localized to LSECs following intravenous injection and that LSECs served as competent accessory cells to induce T-cell activation and TNF- $\alpha$  release (144). As a consequence of accessory function, LSECs were deleted by activated T cells. This suggests that T-cell-mediated injury to LSECs is a precipitating factor for liver injury, as destruction of this sinusoidal cell population abrogates the anatomic barrier and allows unrestricted access of now activated T cells to hepatocytes (144). Moreover, sinusoidal microcirculatory failure owing to development of intrasinusoidal thrombosis and tissue hypoxia further worsens liver injury.

Furthermore, in liver transplantation, LSECs are the hepatic cell population most sensitive to damage from ischemia/reperfusion, and injury to liver endothelium is considered as a first sign of graft rejection (145). After ischemia/reperfusion, widespread denudation of hepatic sinusoids from LSECs is observed, leading to severe microcirculation problems (146,147). Subsequent studies clearly identified the susceptibility of LSECs to ischemia-reperfusion injury (148). Numerous cellular and molecular mechanisms contribute to damage to endothelial cells including CD4 T cells (149), platelets (150), and Kupffer cells (151). However, a number of mechanisms are operative in LSECs to protect this cell population from damage. Expression of granzyme B inhibitors protects from induction of apoptosis (152), and various forms of preconditioning induce hyporesponsive states in LSECs that render them resistant to damage (38,153,154).

Taken together, these experimental findings provide evidence that the barrier function of LSECs is instrumental in maintaining hepatic organ integrity. Destruction of this barrier leads to microcirculatory disturbances and exposure of other hepatic cell populations to circulating leukocytes, all of which contributes to hepatocellular damage either by tissue hypoxia resulting from perfusion failure or direct immune-mediated attack. This makes LSECs attractive targets for pharmaceutical intervention strategies. In fact, numerous successful novel treatment strategies to prevent liver damage may rely on improved LSEC survival, as most RNA- or DNA-based pharmaceutical agents are first taken up from LSECs by virtue of their scavenger activity (155,156).

### ANTIGEN PRESENTATION OF LSECs TO CD4<sup>+</sup> T CELLS

MHC class II-restricted presentation of antigen to CD4<sup>+</sup> T cells is believed to be restricted to so-called professional antigen-presenting cells (APCs), such as dendritic cells, macrophages, and B cells. However, studies by Rubinstein et al suggested that hepatic sinusoidal cell populations were able

to present MHC class II antigen restricted to CD4<sup>+</sup> T cells but did not allow the distinction between antigen presentation by Kupffer cells or LSECs (125,157). In vitro studies employing pure cultures of LSECs demonstrated that these cells have the capacity to present MHC class II antigen restricted to previously activated CD4<sup>+</sup> T cells, resulting in cytokine release and proliferation of CD4<sup>+</sup> T cells (123). Antigen presentation by LSECs was almost as efficient as antigen presentation by Kupffer cells or bone marrow-derived APCs. Therefore, LSECs are similar to dendritic cells or macrophages with regard to MHC class II restricted presentation of antigen to previously activated CD4<sup>+</sup> T cells. However, MHC class II-restricted presentation by LSECs occurs only at high antigen concentrations (129), which is in stark contrast to professional APCs such as dendritic cells that require only minute antigen amounts to generate T-cell responses. The ability to present antigen to CD4<sup>+</sup> T cells attributes a new function to LSECs: immune surveillance. But how does antigen presentation by LSECs correlate with prevention of immune activation or with tolerance induction in the liver?

MHC class II-restricted presentation of antigen by LSECs is efficiently controlled by factors of the unique hepatic microenvironment. Endotoxin as a physiological constituent of portal venous blood induces release of the anti-inflammatory mediator IL-10 from Kupffer cells (90). IL-10 release from Kupffer cells is controlled by a negative autoregulatory feedback loop (158). As IL-10 is washed away from Kupffer cells, which are located predominantly in the periportal region, it is likely that (once activated through endotoxin) Kupffer cells release substantial amounts of IL-10, which then distributes along the sinusoids (158). IL-10 potently inhibits antigen presentation of LSECs to CD4<sup>+</sup> T cells by downregulation of costimulatory molecules and reduced receptor-mediated uptake of antigen (159). Furthermore, contact of LSECs with endotoxin in the absence of other cell populations directly reduces MHC class II-restricted antigen presentation through diminished antigen processing and downregulation of costimulatory molecules (37). Furthermore, inhibition of cyclooxygenase improves antigen presentation by LSECs to CD4<sup>+</sup> T cells in vitro, suggesting that intrinsic generation of prostanoids continuously controls APC function in LSECs (P. Knolle, unpublished data). However, control of APC function of LSECs by the hepatic microenvironment as a sole mechanism still does not explain induction of immune tolerance in the liver.

Similar to dendritic cells, LSECs bear the capacity to prime CD4<sup>+</sup> T cells, i.e., stimulation of cytokine release from naive CD4<sup>+</sup> T cells that have not encountered their specific antigen before (122). Although dendritic cells require maturation and signals from the highly specialized lymphatic microenvironment in order to function as potent APCs for naive CD4<sup>+</sup> T cells (160), LSECs do not require maturation or migration into lymphatic tissue in order to gain APC function. This function of LSECs as sessile, organ-specific, and constitutively active antigen-presenting cells is not shared by endothelial cells from other organs. Microvascular endothelial cells from the skin or the gut are unable to act as APCs for naive CD4<sup>+</sup> T cells unless they are stimulated with proinflammatory cytokines such as interferon- $\gamma$  (IFN- $\gamma$ )

(161–163). In contrast to antigen presentation by dendritic cells, however, CD4<sup>+</sup> T cells stimulated by antigenpresenting LSECs fail to differentiate into effector Th1 CD4<sup>+</sup> T cells but rather gain an immunoregulatory phenotype (122). CD4<sup>+</sup> T cells primed by LSECs release large amounts of IL-4 and IL-10 following triggering via the T-cell receptor (122), which efficiently downregulate ongoing T-cell-mediated immune responses (P. Knolle, unpublished results). However, LSECs do not induce development of regulatory CD4<sup>+</sup> T cells, which have a most important function in the mediation of peripheral immune tolerance (164). LSECs also function as tolerancepromoting APCs for alloreactive T cells. Stimulated by the finding that livers transplanted across MHC barriers in rodent models are normally accepted without immune suppression of the recipient, LSECs were investigated for their ability to induce tolerance in alloreactive CD4<sup>+</sup> T cells. In addition to the induction of immune-suppressive mediator release from CD4<sup>+</sup> T cells, LSECs prevented CD4<sup>+</sup> T-cell proliferation once T cells had transmigrated through LSECs (165). Central to development of CD4<sup>+</sup> T-cell tolerance in this model system was, first, recognition of alloantigen on LSECs and, second, expression of FAS-L by LSECs, because liver allografts from FAS-L-deficient mice were rejected (165,166). In conclusion, antigen presentation by LSECs to naive CD4<sup>+</sup> T cells downregulates Th1-type cell-mediated immune responses.

Endothelial cells from other sites equally fail to lead to development of fully differentiated effector Th1 CD4<sup>+</sup> T cells (163,167). It is important to note that these endothelial cells lack the capacity to engage actively in immune modulation as either endothelial cells or T cells have to be prestimulated with cytokines such as type II interferon or TNF- $\alpha$  in order to observe functional interaction, thus requiring other cell populations that drive the developing immune response. Together with the observation that intraportal injection of antigen leads to development of T cells that release IL-4 and IL-10 upon restimulation (168), it can be assumed that LSECs, unlike endothelial cells in other organs, are involved in tolerance induction toward intraportally applied antigens.

#### PRESENTATION OF EXOGENOUS ANTIGEN ON MHC CLASS I MOLECULES TO CD8<sup>+</sup> T CELLS BY LSECs

Cytotoxic CD8<sup>+</sup> T cells are of crucial importance for successful immune response against infection with intracellular pathogens and against development of cancer cells. Presentation of antigen on MHC class I molecules to CD8<sup>+</sup> T cells was believed to be restricted to those antigens synthesized *de novo* within the same cell. Although this allows for immune surveillance of parenchymal cells by CD8<sup>+</sup> T cells, it is difficult to envisage how professional APCs, not infected by the pathogenic microorganism or not transformed into a neoplastic cell, could induce a protective and efficient CD8<sup>+</sup> T-cell-mediated immune response in the first place. Thus, presentation of exogenous antigens on MHC class I molecules (termed cross-presentation) is obviously required. The phenomenon was initially identified by Bevan et al. (169), and it was recently demonstrated that cross-presentation occurs in bone marrow-derived APCs

such as dendritic cells and macrophages and in some instances in B cells (170,171). Cross-presentation by dendritic cells was shown to be necessary in order to mount an efficient CD8<sup>+</sup> T-cell-mediated immune response against virus infection, although not all infections by viruses appear to require cross-presentation by myeloid APCs for induction of immunity (172).

It is therefore surprising to find that LSECs can efficiently cross-present exogenous antigens on MHC class I molecules to CD8<sup>+</sup> T cells (173). Cross-presentation by LSECs is characterized by a number of features: efficient uptake of antigen by receptor-mediated endocytosis, shuttling of antigen from endosome to cytosol for proteasomal degradation, transporter associated with antigen processing (TAP)-dependent loading of processed peptides on *de novo* synthesized MHC class I molecules in the endoplasmic reticulum, and transport to the cell surface (173). LSECs require only 60 to 120 min to complete cross-presentation and to express peptide-loaded MHC class I molecules on the surface. Minute amounts of antigen, i.e., in the low nM range, are sufficient for cross-presentation by LSECs, suggesting an important role of cross-presenting LSECs in the hepatic immune response (173).

#### INDUCTION OF IMMUNE TOLERANCE IN CD8<sup>+</sup> T CELLS BY LSECs

LSECs not only cross-present antigen to armed effector CD8<sup>+</sup> T cells but have in fact the capacity to stimulate naive CD8<sup>+</sup> T cells (173). Following an encounter with cross-presenting LSECs, naive CD8<sup>+</sup> T cells release cytokines and start proliferation *in vitro*. However, antigen-specific restimulation of these T cells revealed that they lost the ability to express effector cytokines such as IL-2 and IFN- $\gamma$  and that they lost their cytotoxic activity (173). *In vivo* it has been demonstrated that LSECs cross-present antigen to naive CD8<sup>+</sup> T cells outside the lymphatic system. So far, stimulation of naive T cells was believed to occur exclusively in the highly specialized lymphatic microenvironment.

Following stimulation by cross-presenting LSECs, naive CD8<sup>+</sup> T cells start to proliferate locally in the liver. However, the outcome of cross-presentation by LSECs *in vivo* is the induction of systemic immune tolerance. Similar to CD8<sup>+</sup> T cells stimulated by cross-presenting LSECs *in vitro*, CD8<sup>+</sup> T cells *in vivo* lose the capacity to express effector cytokines and to exert cytotoxic activity against their specific target antigens once stimulated by cross-presenting LSECs (173). Deletion of antigen-specific CD8<sup>+</sup> T cells occurs to some extent but is not the main mechanism of immune tolerance induced by LSECs. Mice rendered tolerant by LSECs cross-presenting a model antigen fail to develop an immune response against a tumor carrying this model antigen, which constitutes the prime target of the immune response in nontolerant littermates, leading to immunity and tumor rejection in control animals (173).

The induction of CD8 T-cell tolerance by cross-presenting LSECs has relevance for two physiological situations: immune tolerance toward oral antigens and immune tolerance toward antigens associated with apoptotic cells. In contrast to the

common knowledge that orally ingested antigens remain localized to the gut or the gut-associated lymphatic tissue, a rapid systemic distribution of oral antigens is observed within less than 2 h after ingestion (174,175). Immune reactions mounted after oral ingestion of antigen may even lead to development of autoimmunity (176). Portal-venous drainage of gut-derived antigens into the liver may therefore play an important role in the control of systemic immune reaction toward oral antigens. Besides the tolerogenic function of hepatic dendritic cells (177), LSECs also contribute to oral tolerance by cross-presentation of gut-derived antigens to CD8<sup>+</sup> T cells and rendering CD8<sup>+</sup> T cells tolerant (178). Passage of oral antigens through the liver appears to control immunity at early time points after ingestion of antigen, whereas regulatory T cells generated in gut-associated lymphatic tissue arise at later time points and ensure continuation of immune tolerance toward oral antigens. The liver is further involved in elimination of apoptotic cells from the circulation (179). LSECs also contribute to removal of apoptotic cell material (48). Antigens contained within apoptotic cell material are cross-presented by LSECs and subsequently induce tolerance in CD8 T cells (Berg et al., submitted). Although this mechanism may ensure that removal of apoptotic cell material from the circulation does not lead to induction of immune reactivity, i.e., autoimmunity, the same mechanism may enable tumor cells metastasizing via the bloodstream to induce CD8 T-cell tolerance. Indeed, it was observed that intravenous dissemination of tumor cells leads to removal of tumor cell material by LSECs, cross-presentation, and subsequent induction of tumor-specific CD8 T-cell tolerance toward tumor antigens. Taken together, experimental data suggest that the liver, in particular LSECs, acts as a tolerogenic organ to ensure immune tolerance toward systemically circulating antigens.

#### CONCLUDING REMARKS AND OPEN QUESTIONS

LSECs represent a new type of organ-resident APCs, a type that is organ specific. To establish organ-specific control of immune responses—as is observed in the liver—local presentation of antigen by resident APCs has a number of advantages.

1. Dendritic cells take up antigen in the peripheral organs and following appropriate stimuli migrate to draining lymph nodes. During this journey they undergo functional maturation, rendering them potent APCs after arrival in the highly specialized and structured microenvironment of lymphatic organs. In contrast, LSECs perform simultaneously all salient functions of an APC, i.e., uptake, processing, and presentation of antigen, without the requirement for maturation. This ensures that antigen presentation of blood-borne antigens by LSECs occurs within a short time frame.
2. Although LSECs preclude access of blood-borne antigen-specific T cells to hepatocytes presenting the cognate antigen in the absence of local inflammation (180), it has been shown that armed effector cells can gain access to hepatocytes (181) once LSECs can present the cognate

antigen. Depending on the presence of sufficient numbers of armed effector T cells, antigen presentation by LSECs then apparently allows for immune surveillance of the liver. The liver-resident population of NKT cells that continuously patrols hepatic sinusoids appears to fulfill intravascular immune surveillance function via interaction with CD1-expressing LSECs (27).

3. Continuous culture of T cells (182) or professional APCs such as dendritic cells with immune-suppressive mediators such as IL-10 or TGF- $\beta$  in vitro gives rise to APCs that induce T-cell tolerance rather than immunity (183). Situated in the hepatic sinusoid, sessile LSECs are continuously exposed to the unique hepatic microenvironment, which is especially rich in immune-suppressive mediators. Incorporation of signals from an organ-specific microenvironment is clearly more prominent in sessile LSECs than in conventional APCs that stay only for short periods in peripheral organs before migration into lymphatic tissue. The unique hepatic microenvironment may thus gain considerable influence on the way immune responses are modulated by sessile LSECs.
4. Systemic distribution of antigen leads to development of immune tolerance (184,185). Given the dual function of LSECs, fast and efficient presentation of blood-borne antigens and induction of immune tolerance, timing, and distribution of an antigen appears to determine the outcome of the ensuing immune response critically. As dendritic cells require time for migration, maturation, and induction of T-cell immunity in the lymphatic system (160), tolerance induction by LSECs can occur in a much shorter time frame. Immune tolerance ensues if antigen is first presented by LSECs in the liver (173). Given the ever-changing nature of antigens released from metabolizing hepatocytes, tolerance induction by LSECs appears to be a useful mechanism to prevent immune attack against innocuous antigens released from hepatocytes. However, it is possible that LSECs contribute to persistence of viral infection in hepatocytes, as abundant viral proteins are released from infected hepatocytes and can be taken up and presented by LSECs to T cells. Local presentation of antigen by LSECs may thus constitute a mechanism to balance the immune response in the liver and protect hepatocytes from immune-mediated damage.

LSECs are ideally positioned in the hepatic sinusoid to scavenge blood-borne antigens and to present these antigens to passenger T cells. Given the large volume of blood—containing both T cells and antigens—passing daily through the liver and the large cumulative surface of LSECs, the liver sinusoid appears to be a perfect “meeting point” where immune responses toward blood-borne antigens can be shaped.

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# 3 Innate Immune Mechanisms in the Liver

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## KEY POINTS

- The liver is under constant immunological challenge, often requiring tolerance and response simultaneously.
- Hepatic immunity is dominated by innate immunological components including macrophages, dendritic cells (DCs), natural killer (NK) and natural killer T (NKT) cells, inflammatory cytokines, complement components, acute-phase proteins, and chemokines.
- Adult liver is an important site of production of factors of innate immunity.
- Systemic inflammation is regulated by the liver.
- A primary focus of innate immune mechanisms in the liver is tumor surveillance.

## SETTING THE SCENE: DOMINANCE OF INNATE IMMUNITY IN THE LIVER

Having been ignored by immunologists for years, the liver is now known to be a site of complex immune activity and to play a key role in some of the most important pathologies, including septicemia, metastases, and hepatotropic infections. Even in its healthy state, the liver is presented with an intricate combination of immunological challenges for which it is surprisingly well equipped. These challenges include massive antigenic loads of harmless dietary and commensal products borne by the portal tract, which must be immunologically tolerated, but which may be laced with pathogens or toxins, requiring a swift response. Its blood supply of approx 1.5 L per minute ensures that the liver is the organ most frequently exposed to blood-borne metastatic stimuli, while products of hepatic metabolism may be carcinogenic. The liver immune system must therefore provide protection against pathogens, transformed liver cells, and metastatic cells while at the same time tolerating harmless self and foreign antigens.

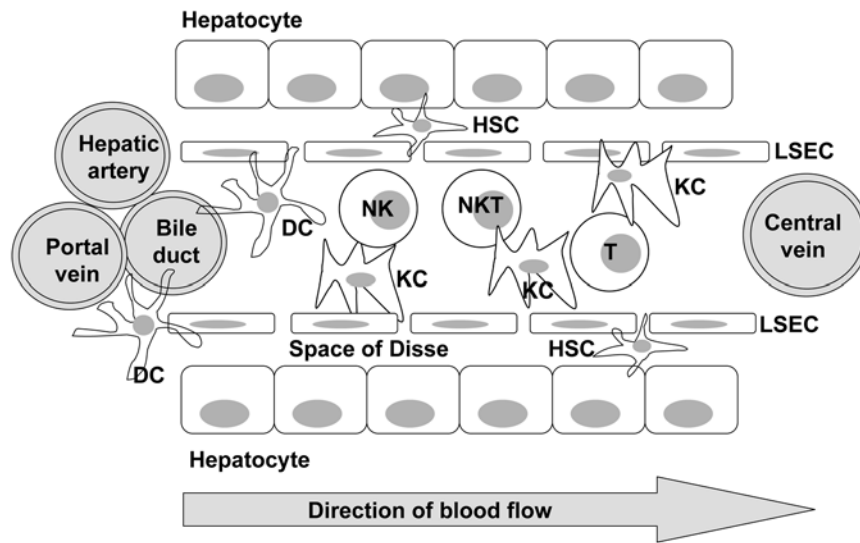
Local cellular and molecular components of innate and adaptive immunity combine with elements of the circulating immune system to provide the liver with its own powerful regional immune system. Blood from the portal and systemic circulations enters the liver at the portal triads, passes through

a network of liver sinusoids, and leaves the liver via the central hepatic vein (Fig.1). The portal tracts and liver sinusoids are interspersed with multiple cell types capable of phagocytosis, antigen presentation, and/or cytotoxicity. Hepatic immunity is dominated by innate immunological components, which rapidly detect and respond to foreign infectious agents and infected or transformed self. Components of innate immunity that are active in the liver include reticuloendothelial (Kupffer) cells, DCs, NK cells, NKT cells, inflammatory cytokines, complement, acute-phase proteins, and chemokines; many of these are triggered by signals from hepatocytes, sinusoidal endothelial cells, and bile duct epithelial cells. The liver is an important site of synthesis as well as activity for many of these components and therefore plays an important role in regulating systemic inflammation as well as local hepatic immune responses.

## CENTRAL ROLE OF THE LIVER IN SYSTEMIC INNATE IMMUNITY

Innate immunity is the initial, rapid response to potentially dangerous stimuli, including pathogens, tissue injury, stress, and malignancy, and it is central to the inflammatory response (1,2). Innate immune mechanisms are ancient and critical to species survival, having appeared early in the evolution of multicellular organisms and being present in invertebrates as well as vertebrates (Table 1; ref. 3). Localization of many of these components of innate immunity in the vertebrate liver, together with the ability of this organ to produce many of these factors, suggests a central immunological function for this organ and emphasizes its role in systemic as well as regional defense.

Innate immune mechanisms are initiated by activation of cells by potentially harmful factors that stimulate activation of local phagocytes and production of inflammatory cytokines, acute-phase proteins, and antimicrobial peptides (Fig. 2). If local inflammation fails to clear the stimulus, inflammatory mediators induce the synthesis and release so many of these proteins and peptides into the circulation that the red cell sedimentation rate is altered (4). Production, activation, and release of inflammatory cells from the bone marrow, such as neutrophils, are also driven by liver-synthesized cytokines (Fig. 2). The cells are targeted to the primary site of stimulation by their expression of chemokine receptors that guide



**Fig. 1.** Immune cells in the liver. Blood enters the liver at the portal triads, passes through a network of liver sinusoids, and leaves the liver via the central hepatic vein. The liver sinusoids are lined by a fenestrated layer of sinusoidal endothelial cells (LSECs). The portal tracts and liver sinusoids are interspersed with Kupffer cells (KCs), dendritic cells (DCs), natural killer (NK) cells, natural killer T (NKT) cells, and T cells. The space of Disse contains the hepatic stellate cells (HSCs).

**Table 1**  
**Innate Immune Components in Certain Invertebrates**

<i>Immune component</i>	<i>Invertebrate</i>
Inducible inflammatory system (Toll, NF- $\kappa$ B)	Insects; arthropods; <i>C. elegans</i>
Acute-phase proteins: pentraxins, complement	Insects; arthropods
Mannose binding lectins	<i>C. elegans</i> ; tunicates
Antimicrobial peptides	Insects; molluscs; worms
NK-like cells with CD homologs	Sipunculids; annelids; molluscs
Phagocytic cells	Annelids; starfish; <i>Daphnia</i>

From ref. 3.

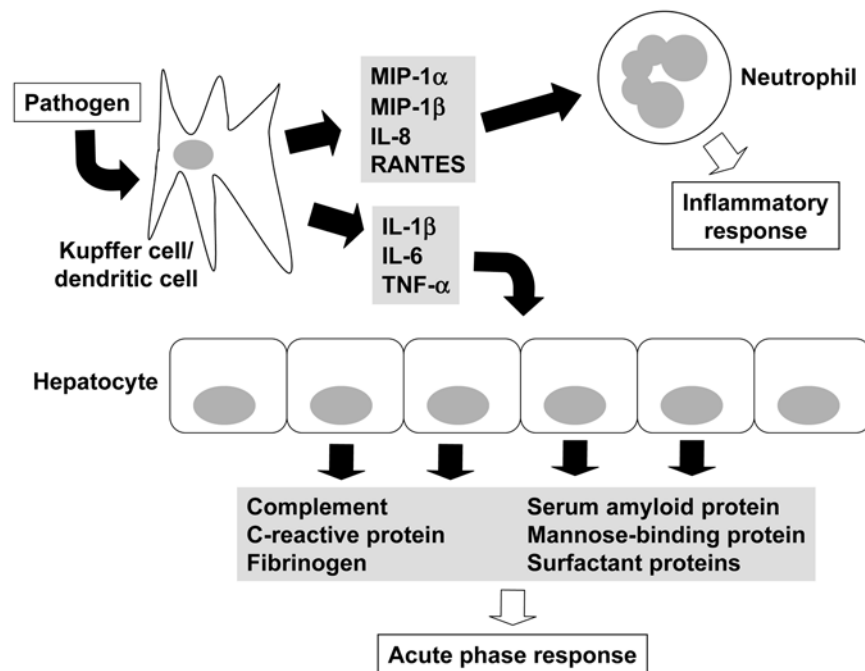
the cells along chemokine concentration gradients. Many factors required for systemic inflammation are synthesized by hepatocytes, Kupffer cells, and hepatic DCs (5,6). Upregulated expression of receptors for inflammatory cytokines and bacterial constituents by activated hepatocytes, DCs, and Kupffer cells amplifies the response to systemic inflammatory stimuli by driving autocrine synthesis and secretion of type 1 interferons, inflammatory cytokines, acute-phase proteins, and antimicrobial peptides. In response to proinflammatory cytokines (tumor necrosis factor- $\alpha$  [TNF- $\alpha$ ] and IL-6, in particular) or microbial constituents, hepatocytes increase the synthesis of plasma acute-phase reactants such as the pentraxins (including C-reactive protein) as well as amyloid, fibrinogen, and transforming growth factor- $\beta$  (TGF- $\beta$ ), which mediate systemic inflammation and facilitate tissue repair and regeneration (Fig. 2). Hepatocytes also produce serum mannose-binding lectin, which recognizes microbial-specific sugar motifs, leading to activation of innate immunity and microbial clearance through opsonization (7). Hepatocytes are primary producers of complement proteins,

key molecular mediators of the innate immune response. Hepatocytes and Kupffer cells also mediate liver regeneration by releasing TGF- $\beta$ , TNF- $\alpha$ , IL-6, and granulocyte-monocyte colony-stimulating factor (GM-CSF) (8,9).

### TOLL-LIKE RECEPTORS: SENTINELS OF INNATE IMMUNITY IN THE LIVER

Innate immune signal sensors activated by pathogenic molecules are key to driving inflammatory responses. Toll-like receptors (TLRs), an evolutionarily conserved group of molecular pattern recognition receptors, are the best defined innate immune signal sensors (10,11). TLRs are expressed at the cell surface and intravesicularly by DCs, Kupffer cells, and some lymphocytes. They bind various microbe-derived molecules and activate these cells through receptor-associated kinases (12), leading to their maturation into antigen-presenting cells (APCs) and their release of proinflammatory cytokines. TLRs may associate with other non-TLR cell-surface receptors (such as CD14 in TLR4 binding of lipopolysaccharide [LPS]) or may form heterodimers with other TLRs and adaptor molecules to achieve unique binding and signaling specificities. In the prototypic LPS-driven activation of TLR4 signaling in macrophages, the transcription factor NF- $\kappa$ B is activated, leading to production of proinflammatory cytokines (TNF- $\alpha$ , IL-10, IL-12, interferon- $\gamma$  [IFN- $\gamma$ ]), the upregulation of microbicidal mechanisms, such as the production of reactive oxygen and nitrogen species, and an enhanced capacity to activate lymphocytes of the adaptive immune system.

Little is known about TLR expression in healthy human liver, although Kupffer cells and DCs are thought to be primary expressors of these innate sensors. Kupffer cells may represent a unique population of tissue-resident macrophages in that they are normally subjected to unusually high basal levels



**Fig. 2.** Innate immune responses in the liver are initiated by phagocytes. Upon phagocytosis of pathogenic material, Kupffer cells and dendritic cells release a variety of chemical messengers that initiate the acute-phase response and inflammation. Interleukin-1 $\beta$  (IL-1 $\beta$ ), IL-6, and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) stimulate the release of complement and acute-phase proteins by hepatocytes, which bind to components of pathogens and opsonize them for phagocytosis. Chemokines (macrophage inflammatory protein [MIP]-1 $\alpha$ , MIP-1 $\beta$ , IL-8, and RANTES) recruit neutrophils and other cells of the immune system to the site of danger.

of gut-derived TLR ligands such as LPS. As a consequence of chronic LPS stimulation, Kupffer cells are thought to produce IL-10 constitutively, leading to the establishment of the predominant antiinflammatory cytokine milieu characteristic of the liver (14). Comparatively low levels of expression of TLR4 by liver DCs has been demonstrated, perhaps explaining their limited response to specific ligands, resulting in reduced or altered activation of hepatic adaptive immune responses and contribution to the tolerogenic milieu of the liver (15). Hepatocytes are also likely to be important TLR expressors. Hepatocyte cell lines and mRNA extracts from murine liver have been shown to express TLR9 constitutively and to respond to CpG DNA (16).

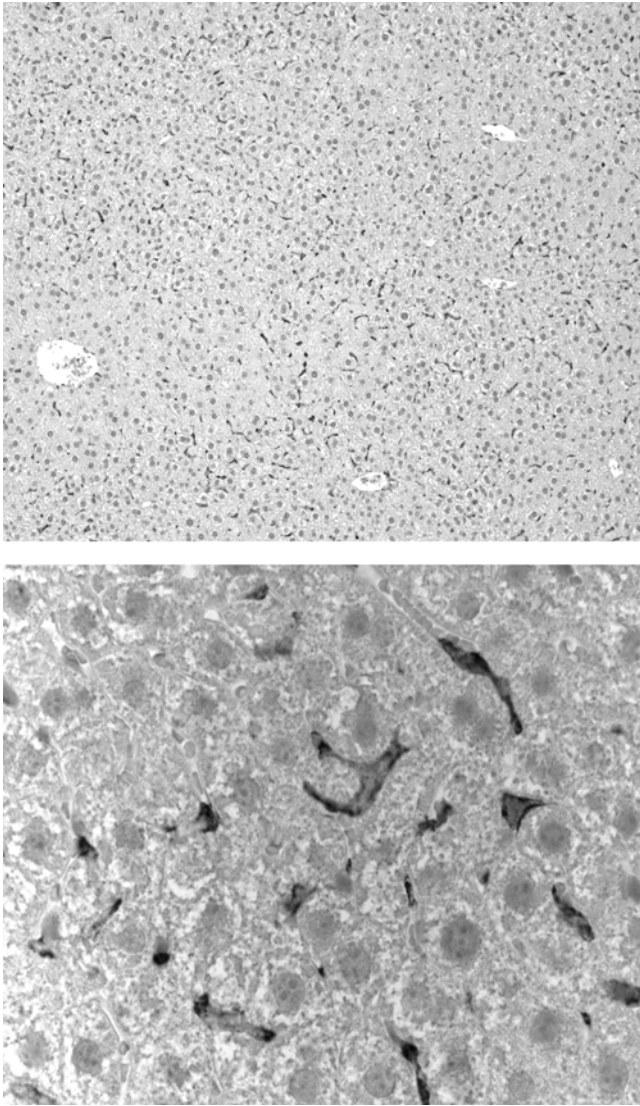
### IMMUNE SURVEILLANCE BY HEPATIC RETICULOENDOTHELIAL CELLS

Kupffer cells represent the largest population of tissue resident macrophages in the body and are interspersed with fenestrated liver sinusoidal endothelial cells in a mosaic fashion to make up the sinusoidal lining (17; Figs. 1 and 3). Kupffer cells are active phagocytes and important secretors of inflammatory cytokines, in particular interleukin-1 (IL-1), IL-6 and TNF- $\alpha$  as well as GM-CSF and chemokines such as macrophage inflammatory protein (MIP-1 $\alpha$ ), and Regulated on Activation, Normal, T-cell Expressed and Secreted (RANTES) (Fig. 1). They play an important role in surveillance and uptake of intravascular debris, including dead bacterial cells and other blood-borne particulates (18). However, overproduction

of inflammatory mediators by Kupffer cells can lead to liver injury (19,20).

Kupffer cells express several cell-surface receptors and receptor complexes involved in immune stimulation (21). These include complement receptors (CRs), Fc-receptors, receptors for lectin-containing opsonins such as plasma mannose-binding lectin, adhesion receptors including those that bind intracellular adhesion molecule-1 (ICAM-1), TLRs, and receptors for polysaccharides of microbial and host origin (22). Kupffer cells express both high-affinity Fc $\gamma$  receptors, which facilitate phagocytosis of IgG-coated particles, as well as receptors for IgA (23). This ability to bind IgA-coated particles is thought to represent an important “second line of defense” in the case of a breach of lower gastrointestinal mucosal immune barriers. In addition to opsonin receptors, Kupffer cells express galactose and mannose receptors (24) and scavenger receptors, which are capable of directly binding microbial surface components such as sugars and polyanionic moieties as well as receptors for bacterial *N*-formylmethionine-containing peptides.

Kupffer cells appear to be derived from circulating bone marrow-derived monocytes, but they may also be capable of limited self-renewal (25). It is likely that there is considerable overlap between Kupffer cells and “newly recruited” monocytes/macrophages, or other closely related myeloid-derived cell types such as liver dendritic cells (DCs). In mice and rats, the presence of F4/80 antigen (which becomes expressed as monocytes maturing into tissue resident macrophages) on sinusoidal



**Fig. 3.** Kupffer cells in the murine liver. Original magnification  $\times 100$ ;  $\times 600$ .

liver cells has been used to “define” Kupffer cells, but low levels of F4/80 have also been reported to be expressed on DCs as well. Hematopoietic stem cells that express myeloid markers have been demonstrated in murine (26,27) and human adult liver (28,29), suggesting that regional development of some local populations of phagocytic cells of myeloid origin takes place.

### HEPATIC DENDRITIC CELLS

DCs are phagocytic cells thought to represent the critical APCs required for the stimulation of naïve T cells. Morphologically, DCs show thin membranous projections and are currently believed to be derived from both myeloid and lymphoid lineages, although the latter is controversial. Immature DCs can be found residing within the epithelial compartment of organs such as the gut, skin, and lungs, well positioned to intercept microbial antigens. After capturing antigen, DCs begin to mature and transport the antigens to draining lymph nodes to initiate

an adaptive immune response (Fig. 4; 30). The regional lymph nodes draining the liver to which the DCs presumably migrate include the hilar, hepatoduodenal ligament, and caval lymph nodes, which include the hepatic artery and portal vein nodes.

The maturation of DCs depends on signals from the environment, including cytokines and the engagement of pattern recognition receptors that bind to conserved structural motifs of microorganisms. Thus, LPS from Gram-negative bacteria engages TLR4, whereas structural features of microbial DNA (CpG) engage TLR9 expressed by DCs. In the presence of these signals, full maturation of DCs occurs. In contrast, if such signals are absent, DCs may differentiate to a semimature state, in which they will interact with T cells to promote abortive T-cell differentiation or suppressor rather than effector T-cell function (Fig. 5).

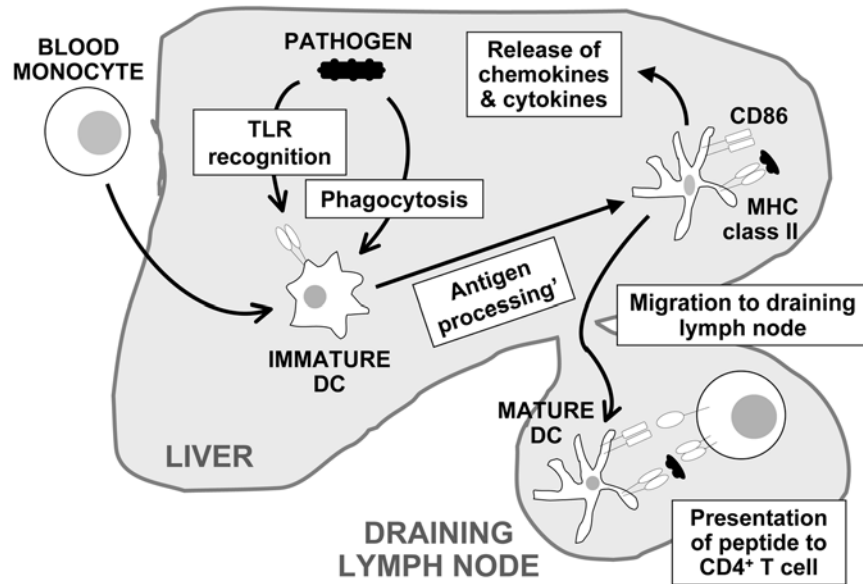
Only recently have investigators begun to focus on liver-specific DC populations, and evidence suggests that distinct subpopulations of liver DCs bias the immunological micro-environment of the liver toward tolerance (31). Hepatic DCs are present in very low numbers in fresh tissue but can be expanded upon stimulation with Flt3-ligand. They are then found to have the phenotype of immature DCs, expressing low levels of major histocompatibility complex (MHC) class II molecules as well as low levels of the costimulatory molecules CD80 and CD86. These cells are likely to induce tolerance rather than activation of CD4<sup>+</sup> cells (32,33). A recent study comparing human DCs obtained from surgical explants of skin and liver demonstrated that liver-derived DCs lack CD1a, produce IL-10, and are less efficient than skin DCs at stimulating naïve T cells (34).

There is evidence to suggest that hepatic DCs may differentiate from the hepatic hematopoietic stem cells mentioned above. Cytokines required for hematopoietic lineage cell proliferation, including IL-7, IL-10, and IL-15, are also found in adult liver and GM-CSF. The normal liver therefore has a cytokine milieu conducive to DC differentiation; however, TGF- $\beta$ , expressed by hepatocytes, inhibits the maturation of DCs, thus preventing them from becoming activating APCs.

### NATURAL KILLER CELLS

NK cells, key cellular players in innate immune responses, are the predominant innate lymphocyte population in the livers of mice and humans, accounting for up to 50% of the total lymphoid pool in the healthy liver (35). The first description of hepatic NK cells used immunohistological analyses of liver tissue from rats to describe large granular cells, originally termed Pit cells, present in the liver sinusoids. More recently, flow cytometry has facilitated enumeration and phenotypic analysis of NK cells in healthy adult liver (35–39). NK cells are capable of spontaneously lysing various tumor cell lines *in vitro*, and they participate, in innate immune responses against viruses, intracellular bacteria, parasites, and transformed cells. The higher numbers of NK cells in liver compared with blood is reflected by higher levels of hepatic NK cytotoxic activity (35).

NK cells do not have antigen-specific receptors but detect changes in membrane glycoprotein expression on target



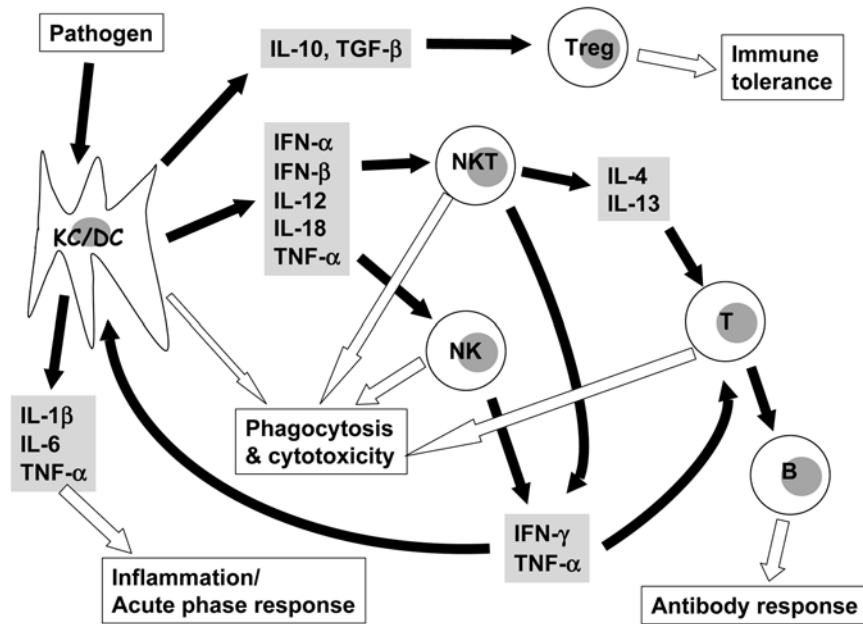
**Fig. 4.** Dendritic cells mature upon contact with pathogens. Immature dendritic cells (DCs) differentiate from blood monocytes in the liver. These cells express low levels of MHC class II and costimulatory molecules, such as CD80 and CD86 but are efficient phagocytes. The dual events of phagocytosis and toll-like receptor (TLR) ligation by microbial products induces the DC to mature into an antigen-presenting cell. The mature DC loses its phagocytic activity but expresses peptide fragments of protein antigens bound to MHC class II molecules, as well as costimulatory molecules required for the activation of naïve T cells, and secretes cytokines that mediate inflammation, the acute-phase response, and T-cell differentiation. Mature DCs laden with antigen then migrate through the afferent lymphatics to the T-cell areas of the draining lymph nodes, where they activate the adaptive immune response via antigen presentation to T cells.

cells—in other words, they detect “altered self” (2,40). Their activities are controlled by receptors that mediate activation or inhibition upon ligation of surface molecules on target cells and by cytokines in the environment such as IFN- $\alpha$ , IL-2, IL-12, and IL-15. Human NK receptors that mediate activation include CD16, the Fc receptor for IgG, responsible for antibody-dependent cellular cytotoxicity and cytokine secretion upon ligation by antibody-coated target cells, NKG2D, which binds to the stress-inducible molecule MICA on target cells, and a variety of “natural” cytotoxicity receptors, whose ligands are mostly unknown but include some viral proteins. In addition, several other costimulatory and adhesion molecules have been implicated in NK cell activation. NK surface molecules, the killer immunoglobulin-like receptors (KIRs), are ligated by MHC class I molecules on target cells, resulting in either activation or inhibition of NK cells with the inhibitory signal exerting a dominant effect over the activating signal. The complex interactions between NK receptors and MHC class I molecules allow NK cells to respond to subtle changes in MHC class I expression and antigen presentation, which may occur in tumor and virus-infected cells. A primary role for the liver, with its rich complement of NK cell populations, is therefore likely to be immune surveillance for tumors, metastatic cells, and virally infected cells, and evidence is accumulating that this function is compromised in tumor-bearing liver tissue (41–44). Two populations of NK cells have been described: those expressing high levels of CD56, which are characterized by their ability to release high levels of IFN- $\gamma$  but which display weak natural cytotoxic activity, and those that express

low levels of CD56, which display potent natural cytotoxicity and low IFN- $\gamma$  secretion (Fig. 6A). These two populations are easily quantified in the human liver (Fig. 6B), and we have preliminary evidence that in hepatitis C infection, the relative proportions of these two populations are altered and can predict how a patient will respond to subsequent infection. Evidence that NK cells play a critical role in hepatotropic viral immunity is suggested by observations that they are targets of several viral evasion strategies (45,46).

#### HEPATIC T CELLS WITH NK CELL RECEPTORS

Several populations of hepatic lymphoid cells coexpress NK cell and T-cell receptors. In mice, up to 50% of hepatic T cells express the NK stimulatory receptor, NK1.1, and a T-cell receptor consisting of an invariant  $\alpha$ -chain, V $\alpha$ 14J $\alpha$ 18, which pairs with one of a limited number of  $\beta$ -chains. These “invariant NKT cells” recognize bacterial and autologous glycolipids presented by the MHC-like antigen-presenting molecule CD1d, display rapid MHC-unrestricted cytotoxic activity, prompt T helper type 1 (Th1) and Th2 cytokine secretion, and have the capacity to induce DC maturation into APCs (47,48). Therapeutic activation of invariant NKT cells promotes effective tumor rejection, prevention of autoimmune disease, and immunity against infection in murine disease models (48,49); however, immunotherapy involving invariant NKT cells in humans has been less efficacious (50,51). Human invariant NKT cells appear to be structurally and functionally similar to murine invariant NKT cells (50); however, they are found at approx 100-fold lower numbers in liver and blood



**Fig. 5.** Dendritic cells (DCs) and Kupffer cells (KCs) control innate and adaptive immune responses. Interactions between liver DC and KC result in the secretion of cytokines, which can activate and polarize innate and adaptive immune responses. Type 1 interferons (IFN- $\alpha$  and IFN- $\beta$ ), interleukin [IL]-12, IL-18, and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) can stimulate natural killer (NK) and natural killer T (NKT) cells which mediate cytotoxicity against virus-infected and tumor cells and release cytokines (IFN- $\gamma$ , IL-4, IL-13) that can polarize naive T-cell differentiation. IFN- $\gamma$  and TNF- $\alpha$  also stimulate phagocytic functions of macrophages. The release of IL-10 and transforming growth factor- $\beta$  (TGF- $\beta$ ) by KCs, DCs and other cells can inhibit T-cell differentiation and suppress adaptive immune responses. The release of IL-1 $\beta$ , IL-6, and TNF- $\alpha$  promotes inflammation.

(52,53). However, T cells expressing the NK cells receptors (NKR) CD56, CD161, CD94, and KIRs are substantially enriched in adult human liver (35). Like NKT cells, they display potent MHC-unrestricted cytolytic activity and prompt cytokine secretion, but they do not carry invariant T-cell receptor chains. Particular populations of NKR<sup>+</sup> T cells, including invariant NKT cells, CD56<sup>+</sup> T cells, and  $\gamma\delta$  T cells, are expanded or depleted in the livers of patients with various diseases (37,44,52–55).

### CYTOKINES AND CHEMOKINES CREATE A RICH IMMUNOLOGICAL ENVIRONMENT IN THE LIVER: ACTUAL AND POTENTIAL IMMUNOTHERAPEUTIC TARGETS

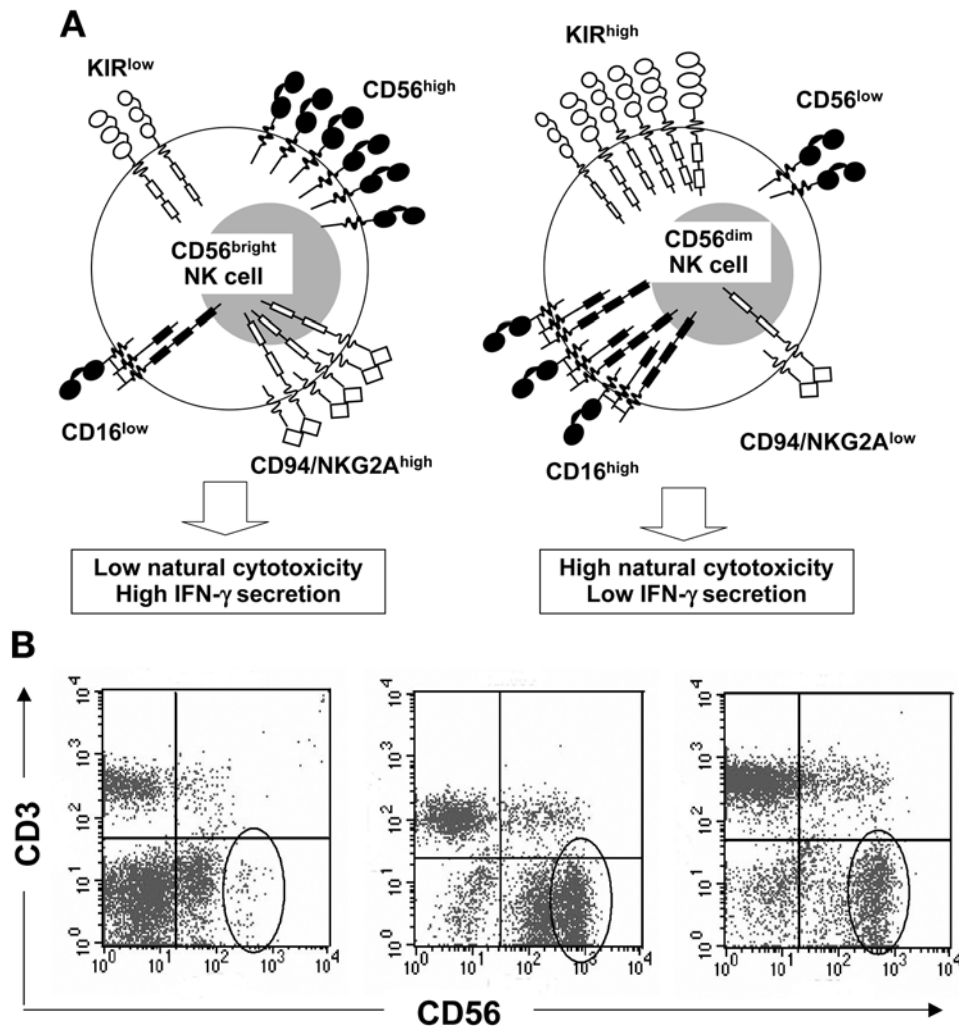
Hepatocytes, NK cells, DCs, and Kupffer cells produce pro- and anti-inflammatory cytokines as well as molecules such as prostaglandins, lipoxins, reactive oxygen and nitrogen species, and chemokines that are critical for regulating local immunity and inflammation. DCs from healthy liver induce IL-10 and IL-4 secretion by mononuclear cells, which keep IFN- $\gamma$  levels low and promote tolerance (32). Early in innate immune responses, particularly against viral infection, type 1 interferons are produced that promote NK cell activity and suppress IFN- $\gamma$  production. In chronic hepatitis C virus (HCV) infection, therapeutic doses of IFN- $\alpha$  are required to stimulate effective antiviral activity. We have found relatively low levels of endogenous IFN- $\alpha$  but high levels of IFN- $\gamma$  in HCV-infected liver. In this high IFN- $\gamma$ -rich environment, local populations

of NK cells are more likely to produce more IFN- $\gamma$  and induce pathology rather than kill virally infected cells.

IL-12 produced constitutively by hepatic myeloid cells, including monocytes, Kupffer cells, and DCs, influences the maturation of NK cells, CD8<sup>+</sup> T cells, and NKT cells, all of which have potent tumoricidal activities. IL-12, therefore, is critical for promoting tumor surveillance. The high levels normally found in healthy liver are upregulated in tumor-bearing tissue (56), and this cytokine is under investigation as a novel antitumor therapy (57). Suppression of cytokine expression is also being promoted as a therapeutic strategy to promote antitumor immunity. IL-10 is thought to be key to the tolerogenic potential of the liver but appears to be overexpressed in tumor-bearing liver.

### FUTURE PERSPECTIVES

In the healthy liver, the regional immune system is dominated by innate immune components and mechanisms. These are not quietly dozing, waiting for some signal to become stimulated but are continuously in action, distinguishing harmful from harmless stimuli, and providing protection against infection and malignancy, while simultaneously tolerating dietary, commensal, and self-antigens. Successful defence against pathogenic challenge requires specific changes in local production of inflammatory and regulatory cytokines and chemokines, significant proliferation of local cell populations, and influx of circulating cells. The failure to return to homeostasis allows chronic disease to flourish. Defects in the hepatic



**Fig. 6.** Two populations of natural killer (NK) cells are found in the liver. **(A)** CD56<sup>bright</sup> NK cells express high levels of the adhesion molecule CD56, high levels of CD94/NKG2A, and low levels of killer immunoglobulin receptors (KIRs) and CD16. These cells are potent secretors of interferon- $\gamma$  but display weak cytotoxicity. CD56<sup>dim</sup> NK cells express low levels of CD56, low levels of CD94/NKG2A, and high levels of KIRs and CD16. CD56<sup>dim</sup> NK cells are potent cytolytic effectors but display low IFN- $\gamma$  secretion. **(B)** Flow cytometric analysis of CD56 expression by hepatic NK cells shows that human liver can contain low (left), medium (center), or high (right) ratios of CD56<sup>bright</sup>/CD56<sup>dim</sup> NK cells.

innate immune system therefore contribute to tumor growth, chronic infection (e.g., hepatitis C), hepatic insulin resistance, and nonalcoholic steatohepatitis. Targeting these dysregulated pathways of innate immunity is providing a whole new therapeutic strategy for major diseases of the liver.

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# 4 Antigen Processing and Presentation in the Liver

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## KEY POINTS

- The unique structural organization of the liver has profound implications for its immune function.
- The flow of blood from the intestines to the liver results in continuous exposure of hepatic leukocytes, endothelial cells, and other cells to bacterial endotoxin.
- The liver contains at least three types of antigen-presenting cells (APCs), and their phenotypes and functions differ considerably.
- Kupffer cells (KCs) represent the largest group of macrophages in the body. They play a role in the elimination of endotoxin and presentation of antigens.
- Liver sinusoidal endothelial cells (LSECs) play an important role in filtration, endocytosis, and regulation of sinusoidal blood flow. They have the capacity to present antigen and play an important role in the induction of hepatic immune tolerance.
- In the liver, dendritic cells (DCs) reside as immature APCs. They express low levels of surface MHC and accessory molecules necessary for T-cell activation. These DCs are extremely well equipped for antigen capture.
- Liver DCs consist of several subsets, in both humans and rodents.
- Functional changes in DCs in human liver disease, such as viral hepatitis, autoimmune liver disease, and cancer, have been reported.
- There are important mechanistic roles for liver DCs in determining the outcome of organ transplantation.
- DC-based immunotherapies have been shown to be effective in animal models and are currently being tested in clinical trials.

## INTRODUCTION

The liver is an important site of infectious, parasitic, autoimmune, and malignant diseases. Immune responses and their modulation within the liver are critical to the outcome of these conditions and also in liver transplantation. Immune

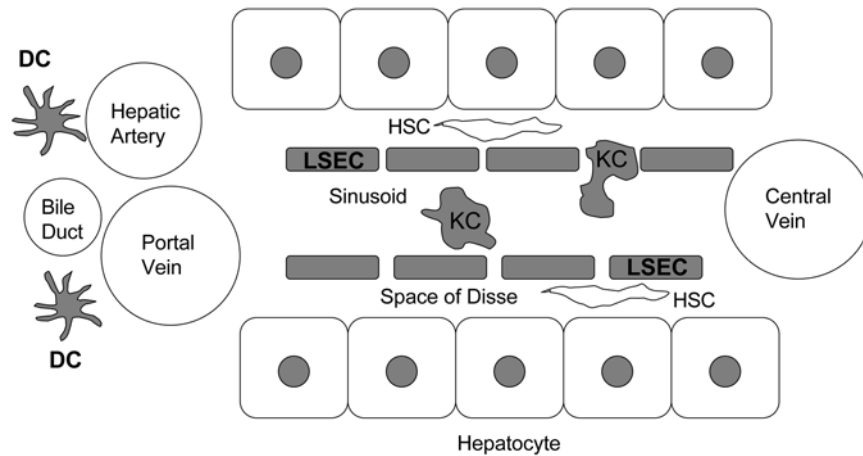
responses in, or elicited by, the liver can result in tolerance rather than immunity. Hepatic tolerance was demonstrated initially by the acceptance of liver allografts across major histocompatibility complex (MHC) barriers, without immunosuppressive therapy. In addition, the liver appears to play an important role in the induction of oral tolerance, as well as in the development/persistence of certain viral infections and cancer. Underlying mechanisms of this comparative immune privilege have not been validated convincingly. However, in addition to its unique anatomical structure, hepatic APCs might be involved in this process (1). APCs exist in several forms within the liver and exhibit a spectrum of abilities to capture, process, and present antigen to immune effector cells.

The microenvironment in which APCs develop or are activated influences their function and their effect on T-cell populations. For example, the liver is rich in the anti-inflammatory cytokines interleukin (IL)-10 and transforming growth factor (TGF)- $\beta$ . KCs and LSECs constitutively express IL-10 and TGF- $\beta$ , whereas hepatocytes secrete IL-10 in response to autocrine and paracrine TGF- $\beta$  (2,3). Lipocytes, another liver-specific cell population, that includes Ito and stellate cells, also express increased TGF- $\beta$  on activation (4). These cytokines not only affect T-cell differentiation directly (skew to T helper [Th]2) but they can also confer tolerogenicity on APCs by inhibiting their maturation and T-cell stimulatory function. In addition, the flow of blood from the intestines to the liver results in continuous exposure of hepatic leukocytes, endothelial cells, and other cells to bacterial endotoxin, which can modulate the function of APCs.

In this chapter, we focus on the functions of APCs within the liver under normal physiological and pathogenic conditions. In addition, we review potential (or experimental) APC-based immunotherapies for patients with liver diseases.

## APCS IN THE LIVER

The normal liver contains several types of APCs (Fig. 1). LSECs constitute the wall of the liver sinusoids, and KCs are located in the sinusoidal lumen. DCs typically reside around portal areas in normal physiological conditions. All three APCs internalize antigen by phagocytosis, receptor-mediated



**Fig. 1.** Antigen-presenting cells in the liver. DC, dendritic cell; HSC, hepatic stellate cell; KC, Kupffer cell; LSEC, liver sinusoidal endothelial cell.

endocytosis, or pinocytosis, but their phenotypes and functions differ considerably (2,5).

#### KUPFFER CELLS

KCs account for the major portion (80–90%) of resident macrophages in the entire body. They compose approx 20% of hepatic nonparenchymal cells (NPCs) (6). Physically, KCs protude from inside the sinusoidal wall, a position that enables them to perform easily their endocytic role for blood-borne materials entering the liver. One of the most important functions of KCs is the clearance of circulating endotoxin. In addition, however, they effectively clear viruses, bacteria, fungi, and parasites, as well as immune complexes, tumor cells, liposomes, lipid microspheres, iron, and various other microparticles. The Toll-like receptor (TLR) 4 ligand, endotoxin (lipopolysaccharide [LPS]), is a potent stimulator of KCs; its binding leads to the production of inflammatory mediators, such as IL-1 $\beta$  and tumor necrosis factor (TNF)- $\alpha$ , as well as oxygen radicals and proteases. KCs are present throughout the liver, but there is a variation in the population density, cytologic characteristics, and physiologic functions of KCs in different zones of the hepatic lobule.

KCs have been shown to present antigen and to activate effector CD4<sup>+</sup> T cells *in vitro*, but they do so less efficiently than either spleen- or bone marrow-derived macrophages (7). KCs express IL-10 in response to physiologic concentrations of LPS, which derives from the gut under normal healthy conditions (7). IL-10 expression in KCs is regulated at the transcriptional level by a negative autoregulatory feedback loop (8). IL-10 suppresses CD4<sup>+</sup> T-cell activation by LSECs and KCs through downregulation of receptor-mediated antigen uptake and inhibition of cell-surface expression of MHC class II and costimulatory molecules (9). In addition, KCs constitutively express TGF- $\beta$  and prostanoids, the expression of which is further increased by contact with LPS.

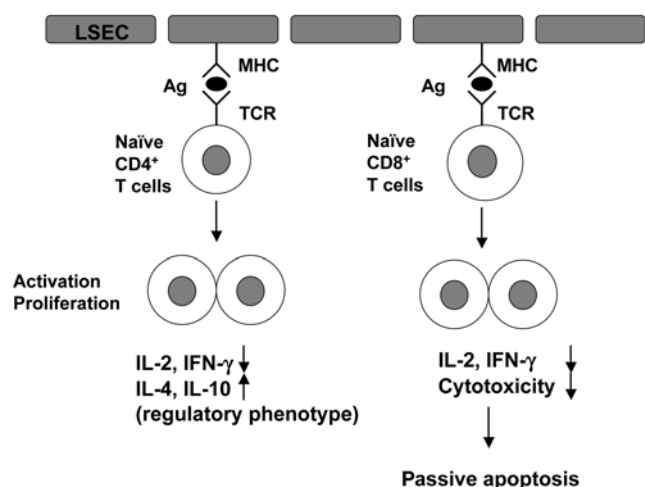
Inhibition of KC activity abrogates the prolonged survival of allografts induced by portal vein infusion of allogeneic donor cells (10). In addition, KCs have a role in oral tolerance; blockade of KCs prevents the induction of oral tolerance (11).

#### LIVER SINUSOIDAL ENDOTHELIAL CELLS

LSECs are resident cells that line the hepatic sinusoidal wall and therefore are in close contact with leukocytes passing through the liver. Physiologically, the LSECs play an important role in filtration, endocytosis, and regulation of sinusoidal blood flow. They possess fenestrations (approx 100 nm); however, they separate hepatocytes from passenger leukocytes in sinusoidal blood efficiently (12).

LSECs express surface molecules necessary for efficient antigen uptake by receptor-mediated endocytosis, such as mannose and scavenger receptors. LSECs also express molecules necessary for establishment of the interaction of T cells. This interaction is dependent on the constitutive surface expression of various adhesion molecules, such as CD54 (intercellular adhesion molecule [ICAM]-1) and CD106 (vascular cell adhesion molecule [VCAM]-1). Moreover, LSECs constitutively express surface costimulatory molecules (CD40, CD80, and CD86) as well as MHC class I and II molecules necessary for presentation of antigen to T cells. Thus, LSECs are endowed with a set of surface molecules that renders them competent for both recruitment of T cells and antigen presentation to T cells. In addition, LSECs express apoptosis-inducing molecules, such as Fas ligand (L) (CD95L), TNF receptor apoptosis-inducing ligand (TRAIL), and membrane TNF- $\alpha$ , which may contribute to intrahepatic T-cell death (13).

Unlike vascular endothelial cells in other organs, such as the skin, gut, or lung, LSEC can modulate proliferation and cytokine production in CD4<sup>+</sup> and CD8<sup>+</sup> T cells *in vitro*, without the need for stimulation with inflammatory stimuli, such as interferon (IFN)- $\gamma$  or TNF- $\alpha$ . Antigen presentation by LSECs to T cells is stringently controlled by mediators in the local microenvironment, such as prostaglandin E2 and IL-10 (9), which are expressed by other hepatic populations. In addition, pre-treatment with physiological concentrations of LPS reduces antigen presentation to CD4<sup>+</sup> T cells considerably (14), indicating that portal blood flow directly influences the APC function of LSECs.



**Fig. 2.** Antigen presentation by liver sinusoidal endothelial cells (LSEC). LSECs can promote tolerance by modulating cytokine production by CD4<sup>+</sup> and CD8<sup>+</sup> T cells and promoting the apoptosis of CD8<sup>+</sup> T cells. IFN, interferon; IL, interleukin; TCR, T-cell receptor.

Murine LSECs can stimulate naïve CD4<sup>+</sup> T cells; however, these T cells fail to differentiate toward a Th1 phenotype. Instead, the CD4<sup>+</sup> T cells adopt a regulatory phenotype, expressing IL-4 and IL-10 upon restimulation (15). In addition, murine LSECs can stimulate CD4<sup>+</sup>CD25<sup>+</sup> T regulatory cells (T-reg) to suppress the proliferation of CD4<sup>+</sup> T cells (16). LSECs also have the capacity to present exogenous antigen on MHC class I molecules to CD8<sup>+</sup> T cells (cross-presentation). CD8<sup>+</sup> T cells primed by LSECs become activated and proliferate but fail to differentiate into cytotoxic effector cells, followed by passive cell death (17) (Fig. 2). Onoe et al. (18) recently demonstrated that murine LSECs inhibit the proliferation of allogeneic T cells in vitro and in vivo and that the Fas/FasL pathway is involved in this process. Collectively, these data strongly indicate that LSECs contribute to the induction of hepatic immune tolerance.

### DENDRITIC CELLS

DCs are rare, ubiquitously distributed, migratory leukocytes, derived from CD34<sup>+</sup> hematopoietic stem cells (19). Many new insights into the origin and differentiation of these uniquely well-equipped APCs and their role in the induction and regulation of immune responses have been gained recently. DCs convey antigen from peripheral sites, such as liver and other nonlymphoid tissues, via afferent lymphatics or blood to T cells in secondary lymphoid organs. The morphology (veil-like processes and dendrites) and motility of DCs are well suited to their roles in antigen capture, processing, and presentation to rare T cells expressing specific receptors that recognize antigen peptides bound to MHC molecules. In nonlymphoid tissues, DCs reside as “immature” APCs. When freshly isolated, they express low levels of surface MHC and accessory molecules necessary for T-cell activation (e.g., CD40, CD80, and CD86) and are at best poor stimulators of naïve T cells (Fig. 3A). These immature DCs, however, are

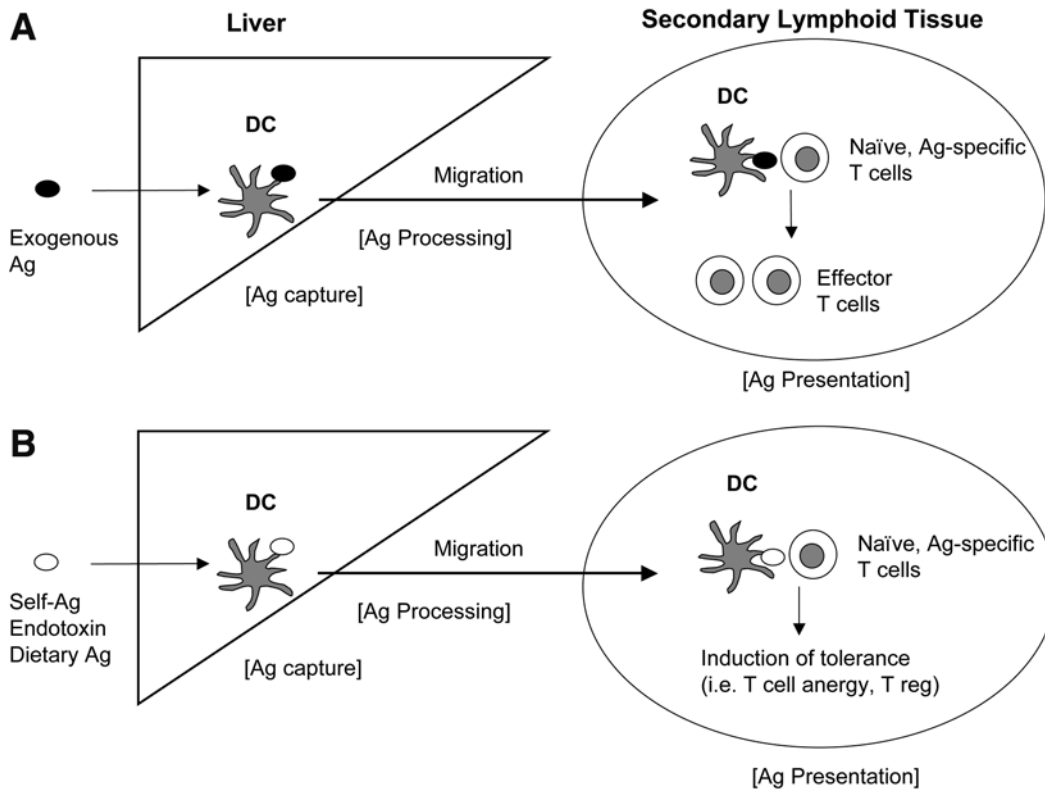
extremely well equipped for antigen capture and the efficient loading of foreign antigen fragments onto MHC class II molecules for export to the cell surface. DCs present antigen peptides bound to MHC class II molecules to CD4<sup>+</sup> T cells. To generate cytotoxic T lymphocytes (CTL), DCs must present antigen peptides complexed with MHC class I to CD8<sup>+</sup> T cells. DCs are able to “cross-prime” or “cross-tolerize” T cells to self-antigen, endotoxin, and dietary antigen (Fig. 3B).

DC maturation is essential for the initiation of acquired immune reactivity and is stimulated by microbial products (e.g., LPS, CpG-oligodeoxynucleotides [ODNs]), proinflammatory cytokines (TNF- $\alpha$ , IL-12), and cyclooxygenase metabolites. Nuclear translocation of the transcription factor nuclear factor (NF)- $\kappa$ B, induced by signaling through TNF receptor (R) family members and ligation of TLR-2, -3, -4, -7 and -9, triggers the phenotypic and functional maturation of DCs. Upon maturation, DCs synthesize high levels of IL-12 and are rich in MHC products, and adhesion and costimulatory molecule expression. Upregulation of expression of the CC chemokine receptor (CCR) 7 allows trafficking of DCs to T-cell areas of secondary lymphoid tissues in response to the CCR7 ligands CCL19 or CCL21. By secreting bioactive IL-12 p70, mature DCs induce CD4<sup>+</sup> Th0 cells to differentiate into IFN- $\gamma$ -producing Th1 cells; with IL-4, DCs induce differentiation of IL-4/IL-5-producing Th2 cells. In turn, ligation of TNFR family members on DCs by activated/memory T cells upregulates costimulatory molecules and the release of IL-12 and chemokines (e.g., CCL3, CCL4, and CCL5) by the DCs. IL-10 blocks IL-12 synthesis by DCs, downregulates their expression of costimulatory molecules, and accelerates their apoptotic death.

### PHENOTYPE OF LIVER DCs

The normal murine liver has a relatively high total interstitial DC content, about two- to fivefold greater than that of other parenchymal organs, such as kidney and heart (20). However, when the density of MHC class II<sup>+</sup> DCs between organs is compared, the liver ranks as the lowest (20).

Various markers have been used to identify rodent and human DCs. Although none are specific to liver DCs, variations occur in the level of expression of certain markers compared with others. CD11c is a common but universal marker for DC detection in the murine system. In addition, other markers, such as CD205, have also been used to identify DCs. OX62, an integrin molecule, is commonly used to detect rat DCs. In humans, DCs are identified as lineage-HLA-DR<sup>+</sup> cells in most studies. In addition, dendritic cell-specific ICAM-3 grabbing non-integrin (DC-SIGN), a c-type lectin receptor, is used as a marker for immature DCs. Recently, human DC-specific markers, such as blood dendritic cell antigen (BDCA)-1, -2, -3, and -4, have been identified. Bosma et al. (21) have reported the characteristics of human liver DCs using BDCA-1 monoclonal antibody (MAb), which identifies an antigen expressed on both immature and mature myeloid (m) DCs. Both freshly isolated and liver perfusate mDCs have a more immature phenotype than mDCs from blood and hepatic LNs in normal humans.



**Fig. 3.** Antigen capture, processing and presentation by dendritic cells (DC). In the normal liver, DCs reside as immature antigen-presenting cells. These immature DCs are extremely well equipped for antigen (Ag) capture. **(A)** When exogenous Ag is captured by DCs, they migrate to secondary lymphoid tissue and can prime naïve, Ag-specific T cells. **(B)** By contrast, when self-Ag, endotoxin, or dietary Ag are captured by DCs, they can induce Ag-specific T-cell tolerance, such as induction of T-cell anergy and regulatory T cells (T reg).

DCs have been generated *in vitro* from mouse liver stem/progenitor cells in response to granulocyte-macrophage colony-stimulating factor (GM-CSF). The liver-derived DC progenitors (22–24) exhibit high surface expression of CD45, CD11b, CD24, and CD44 and moderate expression of CD11c and CD205. Lu et al. (25) have shown that culture of mouse liver NPCs with IL-3 and CD40L yields a unique population of DC-like cells that are CD205<sup>hi</sup>/CD11c<sup>-</sup>/B220<sup>+</sup>/CD19<sup>-</sup>.

Three principal DC subsets have been identified in mouse liver, as well as in lymphoid tissue (26–29). Myeloid (CD8 $\alpha$ <sup>-</sup>/CD11b<sup>+</sup>) and “lymphoid-related” (CD8 $\alpha$ <sup>+</sup>/CD11b<sup>-</sup>) DCs were thought initially to represent distinct lineages and to fulfill distinct functions. Because of their *in vitro* functional properties, it was suggested that murine CD8 $\alpha$ <sup>+</sup> might be DCs specialized for tolerance induction, but other findings conflicted with this view. In addition, there is evidence that these subsets derive from a common precursor and that rigid lineage affiliations between these subsets do not exist. Moreover, there is no known phenotypic counterpart of murine CD8 $\alpha$ <sup>+</sup> DCs in humans.

Pre-plasmacytoid DCs (pDC) have also been identified in mouse liver (27–30). They are CD11c<sup>lo</sup>/B220<sup>+</sup>/Ly-6C<sup>+</sup>/CD11b<sup>-</sup> and produce a large amount of type-I IFN in response to bacterial or viral stimuli. Mouse liver contains more pDCs than spleen (29). In humans, Kunitani et al. (31) have shown that CD123<sup>+</sup> (IL-3R<sup>+</sup>) cells are found in portal areas in the liver

of patients with liver diseases. The percentage of CD123<sup>+</sup> cells is lower in the liver than in the peripheral blood of patients with chronic hepatitis due to hepatitis C virus (HCV) (CH-C).

### POPULATION DYNAMICS OF LIVER DCs

In the rat, isolation of DCs from lymph draining the liver following bromodeoxyuridine feeding indicates that the DC migration rate from the liver is approx 10<sup>5</sup> DC/h (32,33). About half of the DCs leaving the liver via the lymph have arisen by division within the previous 5.5 d (33). In rats given latex particles, particle-laden DCs appear quickly (within 1 h) in lymph draining the liver. It has been suggested that these particle-laden DCs may not be derived from DCs resident within the liver, but from a marginated circulating pool that translocates rapidly via hepatic sinusoids to lymph vessels (34). Thus, the liver may represent an important site in which circulating DCs that ingest particles can gain access to lymph draining to the celiac lymph nodes. Adoptively transferred allogeneic DCs, which appear to migrate from blood to celiac lymph nodes via this pathway, can induce proliferation of alloreactive T cells in paracortical regions of celiac nodes (34). These observations suggest that celiac lymph nodes may be important sites for the induction of immune responses to blood-borne pathogens, particularly as the rate of DC migration via this route appears to increase following intravenous

administration of particulates (35). Liver DCs normally travel to the celiac lymph nodes via lymph (34), and possibly to the spleen via blood. If the lymphatic vessels are disrupted, as occurs in liver transplantation, large numbers of donor-derived DCs are detected in both the spleen and celiac lymph nodes (36).

## FUNCTIONS OF LIVER DCs

### PHAGOCYTOSIS

In the rat, Matsuno et al. (33) have shown that micro-particulate carbon-laden DCs localize in the celiac nodes within 2 h of intravenous administration of carbon particles. Furthermore, it was determined that immature DCs were the major population of particle-laden cells that entered the hepatic lymph. It was suggested that these phagocytic DCs were recruited from the systemic circulation and were not part of the resident DC population. Iyoda et al. (37) have reported that in mice, only the liver-resident CD8 $\alpha$ <sup>+</sup> DC subset exhibits phagocytic properties *in situ*.

### T-CELL STIMULATION

Murine liver DC progenitors are weak stimulators of naïve allogeneic T cells (22–24); however, these cells induce proliferation of antigen-specific memory T cells (23). Khanna et al. (38) found that administration of liver DC progenitors to allogeneic recipients resulted in a selective increase in IL-10 production within secondary lymphoid tissue. By contrast, mature bone marrow-derived DCs elicited increased IFN- $\gamma$  but not IL-10 production. Liver-derived DC-like cells propagated with IL-3 and CD40L (25) can induce T-regulatory 1 type cells (IL-10<sup>+</sup>/IFN- $\gamma$ <sup>+</sup>) *in vitro* and promote alloreactive T-cell apoptosis.

Several groups (28,29,39) have reported that liver DCs are less immunostimulatory than spleen DCs in mice. This might be explained by the facts that liver DCs (CD11c<sup>+</sup>) exhibit lower MHC class II and costimulatory molecule expression and induce less naïve allogeneic T-cell proliferation and Th1-type cytokine production than spleen DCs. In addition, the liver microenvironment appears to play an important role in this phenomenon. Interestingly, human monocytes differentiated into DCs direct Th2 responses when cocultured with rat liver epithelial cells or liver-conditioned media (21,40). These DCs produce IL-10 but not IL-12p70 (40). As mentioned, the liver is rich in IL-10 and TGF- $\beta$  (3,4). In addition, because the liver is located downstream of the gut, it is constantly exposed to endotoxin. We have shown recently that liver DCs express comparatively low levels of TLR4 mRNA and poorer ability than spleen DCs to activate allogeneic T cells in response to LPS (39). Th1 responses induced by LPS-activated liver DCs were lower than those induced by similarly activated spleen DCs. In addition, adoptive transfer of LPS-activated liver DCs induced Th2 skewing.

Freshly isolated mouse liver pDCs (CD11c<sup>lo</sup>/B220<sup>+</sup>/Ly-6C<sup>+</sup>/CD11b<sup>-</sup>) exhibit very weak allostimulatory capacity. Following stimulation with the TLR9 ligand CpG-ODN, these pDCs induce naïve allogeneic T-cell proliferation (27). In addition, pDCs produce a large amount of IFN- $\alpha$  in response to CpG or viral stimulation (27,28).

### CHEMOTAXIS

Migration of DCs to and from peripheral tissue depends on the production of chemokines and expression of specific chemokine receptors. Most chemokine receptors are promiscuous and can ligate a variety of different chemokines.

Uniquely in the liver, KCs trap blood-borne DC precursors via *N*-acetylgalactosamine-specific sugar receptors (41). During infection with *Propionibacterium acnes*, blood-borne mDC precursors expressing CCR1 and CCR5 form intra-hepatic granulomas in response to CCL3. After maturation, the mDCs express CCR7 and become responsive to CCL21, which promotes their migration to lymphoid tissue (42). By contrast, pDC precursors directly enter lymph nodes via high endothelial venules in a CXCL9- and E-selectin-dependent manner and rarely enter the liver in a short-term homing assay (43). However, this finding is inconsistent with the observation that liver contains pDCs in the normal steady state.

With respect to liver DCs, Drakes et al. (44) investigated expression of chemokines and their receptors on liver DC progenitors. There were no striking differences in CC and CXC chemokine mRNA expression between liver DC progenitors and bone marrow-derived DC. In addition, CCR1-5 mRNA expression showed no discernible difference between these two populations. CCL3 expression was greatly increased upon liver DC maturation and stimulation by LPS or allogeneic T cells (44). We have also reported on CC chemokine receptor expression and the migratory capacity of mouse liver DC subsets in response to chemokines (30). The levels of expression of CCR by liver pDCs were similar to those of liver myeloid and “lymphoid-related” DCs. Stimulation with GM-CSF and CpG induced upregulation of CCR7 expression and significant CCL19-mediated migration by liver mDCs and pDCs. CCR7 expression by each liver DC subset was strongly enhanced in response to maturation; however, the *in vitro* migratory response of pDCs to CCL19 was lower than that of myeloid and “lymphoid-related” DCs.

## DCs IN LIVER DISEASE

### AUTOIMMUNE DISORDERS

DCs may play essential roles in both the initiation and perpetuation of autoimmunity and autoimmune disorders. The mechanisms underlying the breakdown of self-tolerance and the induction of autoimmunity are not well understood; however, several observations implicate the involvement of DCs in autoimmunity.

DCs have been observed frequently in portal areas in the early phases of primary biliary cirrhosis (PBC) but are much less common in advanced disease, where they may often be located periductally (45). Mature DCs expressing CD83 have also been found in liver tissues of PBC patients (46), suggesting a role for DCs in different activation states in disease pathogenesis. The functions of DCs have been evaluated in PBC. The capacity of monocyte-derived (Mo-) DC to stimulate allogeneic T cells is significantly decreased compared with control subjects (47).

In autoimmune hepatitis (AIH) patients, the number and nature of circulating DCs was evaluated by flow cytometry.

The numbers of mDCs and pDCs did not differ between AIH patients and controls, but the expression of HLA-DR on both mDCs and pDCs was decreased in AIH patients (48).

### GRANULOMATOUS LIVER DISEASE

Granulomatous inflammation is a characteristic of persistent infection. Hepatic granuloma formation involves not only a macrophage component but also the participation of DCs recruited in response to specific chemokines, as mentioned above.

In patients with HCV infection, plasma cells and B cells are found in association with DCs within hepatic portal areas, as in lymphoid tissue (49). Similarly, portal inflammation and portal-associated lymphoid tissue (PALT) development have been identified in primary sclerosing cholangitis (PSC), and CCL21 appears to be involved in this process (50). These findings suggest that there may be important immune cell interactions occurring within portal tracts, perhaps circumventing the need for DC migration to lymphoid tissue.

### VIRAL HEPATITIS

Several hepatitis viruses infect humans and nonhuman primates. Infection with HBV and HCV causes acute hepatitis or the infection remains asymptomatic and usually resolves after an acute attack. However, some individuals infected with these viruses cannot clear the virus and become chronic viral carriers. Chronic HBV and HCV infection is also associated with progressive liver diseases, including liver cirrhosis and hepatocellular carcinoma. DCs have attracted the attention of hepatologists and immunologists in the context of chronic infection of HBV and HCV, because these patients constitute a major public health problem.

### DCs IN TRANSGENIC ANIMAL MODELS

Transgenic (tg) mice have contributed greatly to elucidation of the immunopathological processes involved in chronic viral hepatitis. By microinjection of entire (or selected) portions of the viral genome into fertilized eggs of inbred mice, several laboratories have developed unique lines of tg mice that express products of the viral genome and exhibit signs of viral replication. Most studies of DCs in chronic HBV carriers have been conducted using HBV-tg mice. In a report by Akbar et al. (51), immune responsiveness of HBV-tg mice to keyhole limpet hemocyanin (KLH), a T-cell-dependent antigen, was evaluated. This study demonstrated that the cellular and humoral immune response to hepatitis B surface (HBs) antigen and also to KLH was impaired in these animals. A series of coculture experiments showed that functional impairment of DCs contributed to defective immune responses of HBV-tg mice. In subsequent studies, they demonstrated that DCs from HBV-tg mice expressed lower levels of MHC class II than those from controls (51). Expression was normalized by the treatment of DCs from HBV-tg mice with IFN- $\gamma$  (52). In addition, treatment of tg mice with IFN- $\gamma$  resulted in reduced levels of HBV DNA, both in the liver and the serum. In a similar study of HBV-tg mice, a reduced ability to mount an antibody response against HBs antigen has been reported (53). In this study, it was demonstrated that defective APC functions of spleen DCs in HBV-tg mice were responsible for their inability to produce anti-HBs antigen. Impairment

of functions of liver DCs in HBV-tg mice have also been reported (54).

Similar findings have been reported for murine DCs transfected with HCV genes. Spleen DCs expressing HCV antigen showed impaired allostimulatory capacity and low IL-12 production (55). Further study revealed that MHC class I expression was impaired in tg mice expressing HCV structural proteins in DCs (56), indicating that HCV also affects the APC function of DCs.

### DCs IN CHRONIC HBV CARRIERS

Arima et al. (57) demonstrated that Mo-DCs from patients with chronic hepatitis B (CH-B) harbored HBV DNA and RNA, using polymerase chain reaction (PCR) and reverse transcription (RT)-PCR *in situ* hybridization methods. Other groups (58,59) have reported similar findings. In addition, Mo-DC from healthy volunteers inoculated with HBV *in vitro* exhibited impaired allostimulatory capacity and Th1 responses (60), indicating that the presence of intracellular HBV particles was associated with impaired APC function of DCs.

Mo-DCs in patients with CH-B exhibit less stimulatory capacity for allogeneic T cells and produce lower levels of IL-12 compared with healthy controls (57–59). With respect to circulating DCs in peripheral blood, reduction in mDC and pDC numbers has been reported in patients with CH-B (61). mDCs from CH-B patients have impaired allostimulatory capacity and produce low levels of proinflammatory cytokines (IL-12 and TNF- $\alpha$ ), and pDCs from CH-B patients also induce decreased levels of IFN- $\alpha$  (61,62). These findings indicate that impairment of DC function may contribute to viral persistence and disease chronicity.

### DCs IN CHRONIC HCV CARRIERS

The binding of HCV to DCs is thought to be mediated by several receptors. Among them, DC-SIGN is a major receptor on DCs (63). In addition, the presence of both positive-strand HCV RNA and its replicative intermediate, negative-strand HCV RNA, can be detected in DCs (64). These findings indicate that there is active replication of the HCV genome within DCs.

Several studies have shown that HCV proteins can modulate DC functions. Dolganiuc et al. (65) have demonstrated that HCV core and nonstructural protein 3 inhibit the differentiation and allostimulatory capacity of DCs and induce production of IL-10. Others have shown that DCs infected with adenoviral vectors encoding HCV core and E1 proteins exhibit poor allostimulatory and autologous recall capacity (66).

Several groups have studied the functions of DCs in patients with CH-C (Table 1). Most of these studies have demonstrated that allostimulatory capacity is impaired in Mo-DCs from CH-C patients (67–69). In addition, defective IL-12 production by Mo-DCs in CH-C patients has been reported (67). Bain et al. (68) have shown that patients who respond to antiviral therapy do not show any impairment of the allostimulatory capacity of Mo-DCs, indicating that HCV may be the cause of the defect. Mo-DCs from CH-C patients do not activate NK cells adequately in response to IFN- $\alpha$  stimulation, as a result of defective upregulation of

**Table 1**  
**Dendritic Cell Functions in Chronic HCV Infection**

Reduced frequency of circulating DCs  
Impaired IL-12 production by DCs  
Impaired IFN- $\alpha$  production by pDCs  
Impaired stimulation of allogeneic, naïve T cells  
Reduced natural killer cell stimulation

Abbreviations: DCs, dendritic cells; IFN, interferon; IL, interleukin; pDCs, plasmacytoid dendritic cells.

the natural killer-activating ligands MHC class I-related chain A and B (70).

Recently, several groups have analyzed circulating DCs from patients with CH-C. Most of the studies have demonstrated that numbers of mDCs are reduced in CH-C patients (71–73) and that their IL-12 production is impaired (74). Impairments of allogeneic T-cell stimulatory capacity and IFN- $\gamma$  production by T cells (71,73,74) have also been reported. Defects in allostimulatory capacity have been restored after antiviral treatment (74).

Reduction of pDC numbers in CH-C patients has also been reported (71–73,75,76). In addition, IFN- $\alpha$  production by pDC is impaired in CH-C patients (71,73,76).

#### HEPATOCELLULAR CARCINOMA

In patients with hepatocellular carcinoma (HCC), Mo-DCs have impaired allostimulatory capacity and IL-12 production (77). These DCs remain immature in the presence of inflammatory cytokines (TNF- $\alpha$ ) that normally induce DC maturation. In addition,  $\alpha$ -fetoprotein (AFP), a tumor-associated antigen that is elevated in patients with HCC, impairs allostimulatory function, reduces CD40 and CD86 expression and proinflammatory cytokine (IL-12 and TNF- $\alpha$ ) production, and induces apoptosis in Mo-DCs (78). Beckebaum et al. (79) have shown that the frequency of circulating mDCs and pDCs is reduced and that HLA-DR and costimulatory molecule expression on both mDCs and pDCs is decreased in patients with HCC. These findings are associated with increased IL-10 concentrations in sera and with tumor progression, indicating that the tumor environment can affect DC function in patients with HCC.

Chen et al. (80) have shown that the numbers of CD83<sup>+</sup> mature DCs in liver tissues are significantly decreased in patients with HCC; more importantly, there are no CD83<sup>+</sup> DCs in cancer nodules in HCC, indicating that DCs may have an important role in surveillance and clearance of tumor cells in HCC.

#### TRANSPLANTATION

The immunobiology of liver transplantation has long been a field of intense study, as it may provide valuable insight into the mechanisms underlying transplant tolerance. Hepatic tolerance was demonstrated initially by the acceptance of liver allografts across MHC barriers, without immunosuppressive therapy. Interstitial donor leukocytes, including DCs, might play an important role in this phenomenon.

Microchimerism and associated two-way “silencing” of immune reactivity, linked to deletion of alloreactive T cells, is

associated with the persistence of donor hematopoietic cells within both lymphoid and nonlymphoid tissues of the host (81,82), including patients off all immunosuppressive therapy. Significantly, donor-derived DCs can be propagated from blood or bone marrow of liver transplant recipients, but not from murine heart transplant recipients who reject their grafts acutely (83).

There are several potentially important mechanistic roles for liver DCs in determining the outcome of transplantation. Alloreactive host T-cell apoptosis in mouse liver transplantation is associated with tolerance, whereas less apoptosis is seen with rejection (84). Conceivably, donor DCs may play a role in inducing apoptosis in host T cells via death ligand (Fas) pathways (85). Neutralization of IL-12 produced by liver-resident DCs and other APCs in murine livers transplanted from fms-like tyrosine kinase 3 ligand (Flt3L)-treated donors (that are rejected acutely) restores long-term graft survival and enhances alloreactive T-cell apoptosis (86). The immature state of normal liver DCs, associated with failure to provide adequate costimulation, may be important in inherent liver tolerogenicity. Administration of donor-derived liver DC progenitors prior to transplantation has been shown to increase pancreatic allograft survival (87).

In human liver transplantation, Mazariegos et al. (88) demonstrated that progressive weaning and operational tolerance in patients who underwent successful withdrawal of immunosuppression were associated with a higher incidence of circulating pDCs (relative to m- or Mo-DC) compared with that in patients receiving maintenance immunosuppression. The higher incidence of pDCs in the successful weaning patients could not be ascribed to reduced levels/absence of immunosuppressive drug therapy (89). Although further studies are warranted to clarify the role of pDCs, this study suggests that monitoring of DC subsets may aid in the identification of patients from whom immunosuppression can safely be withdrawn or weaned.

## VACCINATION WITH DCs IN LIVER DISEASE

### VIRAL HEPATITIS

The outcome of vaccine therapy is extremely heterogenous in both human and murine HBV carriers. Successful vaccination may be determined by the function of APCs, especially DCs, as evidenced by Akbar et al. (90). In two independent, placebo-controlled 12-mo vaccine therapy trials in HBV-tg mice, it was demonstrated that neither the pre-vaccine titer of viral markers nor the function of lymphocytes had a significant influence on the outcome of vaccine therapy. However, HBV-tg mice with potent DC function became completely negative for HBs antigen and HBe antigen and had reduced HBV DNA. HBV-tg mice with poor DC function at the start of the vaccine therapy became non-responders. Moreover, the effectiveness of DCs that expressed higher levels of MHC class II and CD86 has provided encouragement that a more effective vaccine therapy can be developed for chronic HBV carriers by injecting vaccine containing HBs antigen with modulator(s) of the APC function of DCs (91). These experiments illustrate the importance of DCs in vaccine therapy and also provide a rational basis for



upregulation of the function of DCs prior to vaccine therapy. DCs have also been shown to break CTL tolerance in HBV-tg mice. Immunization with cytokine-activated, bone marrow-derived DCs can break tolerance and trigger antiviral CTL responses in HBV-tg mice (92). These studies also suggest that immunization with activated DCs is more efficient in the case of HBV-tg mice.

The importance of DC function during vaccination was confirmed by vaccine therapy in CH-B patients (93). The CH-B patients received HBs antigen once every 2 wk for 24 wk (12 doses). Eight of 11 patients responded to vaccine therapy by showing normalization of transaminases and reduced HBV DNA. The activation of DCs by vaccine therapy may be responsible for its therapeutic effect.

The safety and efficacy of HBs antigen-pulsed, autologous DCs in human volunteers have been established (94). Chen et al. (95) showed that injection of HBs antigen-pulsed DCs subcutaneously (twice) reduced serum viral load and/or HBe antigen/anti-HBe seroconversion, in 11 of 19 patients with CH-B.

Recently, a therapeutic effect of DC vaccination against HCV has been reported by Encke et al. (96). In this study, immunization with mouse bone marrow-derived DCs pulsed with recombinant HCV core protein or core peptide induced humoral and cellular immune responses to HCV core protein. In addition, HCV core-pulsed DC vaccination showed therapeutic and prophylactic effects in a mouse model using an HCV core-expressing mouse myeloma cell line. This finding indicates that HCV core-pulsed DC vaccination might be useful for patients with CH-C.

### HEPATOCELLULAR CARCINOMA

DC-based immunotherapies have been shown to be effective in treating HCC in animal models (97) and are currently being tested in clinical trials (98). In a phase I clinical trial, the safety and feasibility of tumor lysate (TL)-pulsed DC-based immunotherapy for patients with advanced HCC was assessed (98). In this study, four vaccinations of TL-pulsed, TNF- $\alpha$ -activated Mo-DC were performed into inguinal lymph nodes at weekly intervals. Tumor size decreased in 1 of 10 patients, and serum tumor markers decreased in 2 patients after vaccination.

Because it is difficult to obtain sufficient quantities of tumor cell-loaded DCs *ex vivo* for therapy, the *in vivo* provocation of immunity by direct injection of DCs into tumors after apoptosis/necrosis-inducing therapy (which provides "danger signals" for DC activation, such as heat shock protein) would be more applicable. Recently two groups have demonstrated interesting findings. Chi et al. (99) have demonstrated the therapeutic effect of a combination of conformal radiotherapy and intratumoral injection of Mo-DCs. In this study, autologous immature DCs ( $5\text{--}50 \times 10^6$ ) were injected intratumorally, 2 d after conformal radiotherapy, and a second vaccination was performed 3 wk later. There were no side effects. Two and 4 of 14 patients had partial and minor responses, respectively. IFN- $\gamma$  secretion and NK cell activity were enhanced after the therapy. Kumagi et al. (100) showed that autologous,

immature DCs injected intratumorally 2 d after administration of 100% ethanol decreased tumor markers in one of the four patients.

Although DC-based immunotherapy for HCC might be promising, important questions remain regarding (1) type of DC, (2) loading DC with tumor antigen, and (3) dose, frequency, and route of administration. Further studies are necessary to establish optimal regimens for HCC treatment.

### CONCLUDING REMARKS AND OPEN QUESTIONS

The liver has an array of cells that possess the capacity for processing and presenting antigen under various conditions. These hepatic APCs are not only critical for the induction of innate and adaptive immune responses but are also important for regulation of the immune response in the liver and the induction of tolerance. The liver microenvironment appears to play a role in the control of immune responses. Although there is growing evidence that DC functions are altered in the pathogenesis of liver disease, most work to date has been performed on circulating DCs. The use of DC-based immunotherapy protocols to elicit immunity against liver cancer and infectious disease shows great promise. Increasing knowledge of liver DC biology is likely to improve our understanding of disease pathogenesis and resistance to and therapy of liver disease.

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# 5 Adaptive Immunity in the Liver

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JAMES D. GORHAM

## KEY POINTS

- *Adaptive immunity* describes lymphocyte-mediated host defense that adapts to the specific microbial invader. Lymphocytes express specific antigen receptors for antigens and are therefore the key mediators of adaptive immunity. Adaptive immunity can be classified into humoral immunity and cell-mediated immunity, mediated by B lymphocytes and T lymphocytes, respectively. B cells produce and secrete antibodies, and T cells are responsible for cell-mediated immunity. T cells recognize peptide fragments bound to specialized peptide display molecules (MHC) on antigen-presenting cells (APCs). T cells are further classified into CD8<sup>+</sup> T cells and CD4<sup>+</sup> T cells.
- Antigens are microbial structures recognized as foreign by B or T lymphocytes. Antigens promote specific responses from specific lymphocytes, such as cell division, and differentiation into specialized lymphocyte effector cell types.
- Important features of adaptive immunity that distinguish it from innate immunity include *specificity*, *diversity*, and *memory*.
- The composition of liver lymphocytes differs somewhat from that found in the circulation. The liver harbors large numbers of activated TCR $\alpha\beta$  T cells.
- CD4<sup>+</sup> T cells can differentiate into several types of effector cells that produce specific cytokines implicated in specific liver pathologies. These subsets include Th1, Th2, T-reg, and the newly described Th17 cells. Each of these T-helper cell types has been implicated in a variety of liver diseases.
- Specialized lymphocytes expressing both T cell and NK cell markers (NKT cells) are abundant in the liver and are implicated in the regulation of autoimmunity in the liver.
- CD8<sup>+</sup> T cells are important for the elimination of intracellular pathogens, particularly viruses.
- The adhesion and survival of T lymphocytes in liver sinusoids is regulated through specific molecular interactions between T cells and liver sinusoidal endothelial cells.

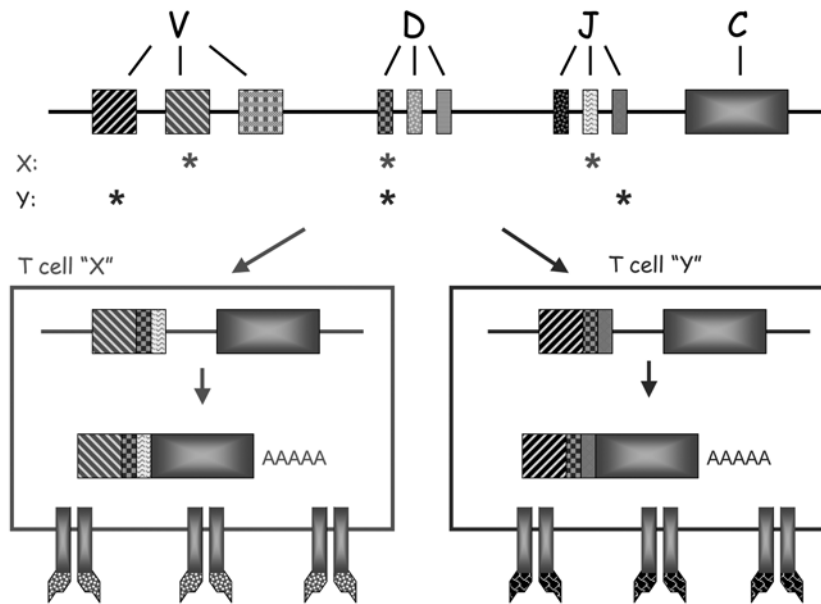
- The liver regulates the fate of effector T cells, suggesting an important role in modulating systemic T-cell-mediated immunity.

## INTRODUCTION

Whereas innate immunity can provide the initial defense against infections, completely effective immunity to an invading microbial organism typically requires an adaptive immune response specific to the invader. Adaptive immune responses in the liver contribute both to effective defense against invading microbes and to a variety of pathologic states. The term *adaptive immunity* refers to lymphocyte-mediated immune defense tailored to a specific microbial invader. Adaptive immunity can be classified into humoral immunity and cell-mediated immunity, mediated principally by B and T lymphocytes, respectively. *Antigens* are structures found on microbes that are recognized as foreign by B or T lymphocytes. Antigens elicit specific responses from the lymphocytes expressing cognate antigen receptors. Such specific responses include both clonal proliferation and lymphocyte differentiation into specialized effector cell types with important functions serving to fight microbes. Such functions include the release of antibody (B cells), the killing of infected cells (cytotoxic T cells), and extracellular release of signaling molecules (i.e., cytokines) that can act in an autocrine, paracrine, or endocrine fashion to elicit responses from other immune and nonimmune cells.

Important features of adaptive immunity that distinguish it from innate immunity include *specificity*, *diversity*, and *memory*.

- *Specificity* refers to the ability of each individual lymphocyte to recognize and respond to specific foreign antigen. The specificity of each lymphocyte is a consequence of antigen receptor rearrangement at the level of genomic DNA during lymphocyte development. B cells develop in the bone marrow, and T cells develop in the thymus. After development and emergence from the bone marrow or thymus, each newly generated B or T lymphocyte expresses on its cell surface only one unique antigen receptor.
- *Diversity* refers to the ability of the adaptive immune system to respond to nearly any foreign antigen. Like specificity, diversity is also achieved through antigen receptor rearrangement. The variable (or antigen-recognition) component of each antigen receptor is generated through



**Fig. 1.** Lymphocyte diversity is generated through genetic recombination at the DNA level during ontogeny. At the top is depicted a series of V, D, and J genes in the germline at a T-cell receptor genetic locus. During T-cell development in the thymus, one V gene, one D gene, and J gene recombine in thymocytes, the precursors to mature T cells. The bottom shows that the pattern of VDJ recombination is different between T cells. The combination that gave rise to T cell “X” is different from the combination that gave rise to T cell “Y.” This process underlies two of the important properties of the adaptive immune system: specificity and diversity.

differential assembly of a large number of individual gene segments during VDJ recombination (Fig. 1). Further diversity is created through the addition of “nontemplated” nucleotides at the junctional joining ends during T-cell receptor and B-cell receptor rearrangement. Each lymphocyte expresses a different, and unique, combination of gene segments. Newly generated lymphocytes are produced in the hundreds of millions. Since each lymphocyte expresses a unique antigen receptor, the potential antigen recognition repertoire of the adaptive immune system is huge.

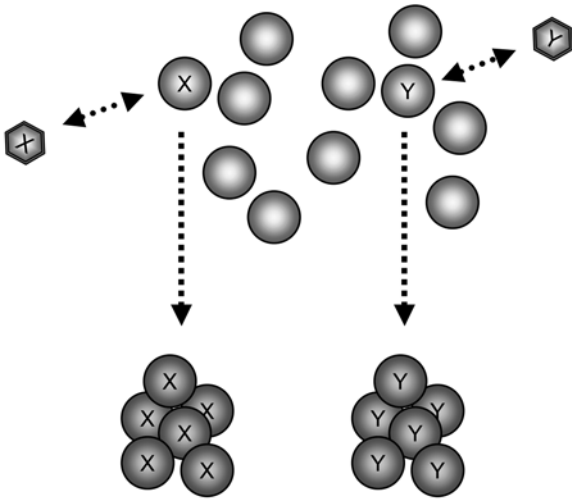
- *Memory* refers to the ability of the adaptive immune system to respond to a recurrent infection with a more rapid and more robust response than in a first infection by the same microbe. Unlike specificity and diversity, memory is not generated at the stage of lymphocyte development. Instead, memory develops after the first encounter of an adaptive lymphocyte with its antigen, i.e., following a first infection. Memory is best understood in the context of clonal selection during immune responses that is, each antigen elicits an immune response by selecting and activating only those (rare) lymphocytes that can recognize the antigen (Fig. 2).

Following encounter with its cognate antigen, the activated lymphocyte will repeatedly divide, forming a lymphocyte sub-population, a clonally derived battalion of lymphocytes with specificity for the invader. Most of the responding lymphocytes will, when the infection is eliminated, go on to die via apoptosis. However, during the course of infection, some small portion of these activated clonal lymphocytes will make the transition to become long-lived memory cells. Two important concepts

related to memory lymphocytes are: (1) for a given microbial infection, following primary infection, the memory pool has relatively higher numbers of specific lymphocytes than does the naïve pool; and (2) compared with naïve lymphocytes, which have never encountered their antigen, memory lymphocytes can be activated rapidly and easily in response to a reinfection. For both of these reasons, recall (memory) immune responses are more rapid and robust than initial (primary) immune responses to initial infection.

## LYMPHOCYTE SUBSETS AND FUNCTIONS

B lymphocytes produce antibodies. Plasma cells are fully differentiated B cells whose functions are to produce antibodies in large quantities. B cells/plasma cells are therefore the cells that mediate humoral immunity. B cells express membrane-bound forms of antibodies that serve as the B-cell receptor (BCR), which binds directly to soluble antigens or antigens on the surface of microbes. T lymphocytes are responsible for cell-mediated immunity. Their antigen receptors (TCRs) recognize peptide fragments bound to specialized peptide display molecules on antigen-presenting cells (APCs). Unlike BCRs, which recognize antigens without any other required molecule, TCRs recognize the combination of peptide with MHC-encoded proteins. CD8<sup>+</sup> T cells recognize peptides bound to class I MHC molecules, found on the cell surface of virtually all nucleated cells, whereas CD4<sup>+</sup> T cells recognize peptides bound to class II MHC molecules. Class II MHC molecules are much more narrowly expressed than class I MHC, being found typically only on “professional APCs,” such as dendritic



**Fig. 2.** Lymphocyte expansion during an adaptive immune response is a function of clonal selection. Each lymphocyte bears a distinct antigen receptor. Although the naïve lymphocyte repertoire is quite broad, cell division occurs only within the antigen-specific lymphocyte population. In this cartoon, one virus (hexagon “X”), elicits the expansion only of antigen-specific (i.e., “X”) lymphocytes. A different virus (hexagon “Y”) elicits the expansion only of lymphocytes that bear an appropriate (i.e., “Y”) antigen receptor.

cells, and also B cells. Fully differentiated  $CD8^+$  T cells are known as cytotoxic T lymphocytes (CTLs) and are primarily involved in the killing of infected cells.  $CD4^+$  cells have the important function of secreting cytokines, signaling molecules that strongly regulate and modulate responses of other immune cells.

### THE NORMAL HEPATIC LYMPHOCYTE REPERTOIRE

The healthy liver contains a collection of lymphocytes with a composition somewhat different from that found in blood (1). In the circulation, T cells expressing the  $\alpha\beta$  T-cell receptor (TCR $\alpha\beta$ ) are the most numerous. The remaining lymphocytes comprise largely B cells (approx 10%), natural killer (NK) cells (10–15%), and a few other lymphocyte subsets. In the liver, conventional  $\alpha\beta$  T cells are present in substantial numbers but make up less than half of the hepatic lymphocyte population. Among the remaining lymphocytes, B cells are under-represented compared with blood (3–6%), whereas the NK population is relatively expanded, accounting for nearly one-third of liver lymphocytes in mouse. Other important lymphocyte subsets found in relative abundance in the liver include T cells expressing a second type of TCR, the TCR $\gamma\delta$  receptor, and lymphocytes bearing both TCR $\alpha\beta$  receptors and NK markers, known as NKT cells; these two lymphocyte subsets are less frequent in the circulation. The biological basis for, or relevance of, these differences in lymphocyte subset distribution in the liver is not completely understood. Here, I focus primarily on  $CD4^+$  T cells in the liver and their roles in liver health and disease.

### $\alpha\beta$ T CELLS IN THE LIVER

Even among the “conventional” T cells (the TCR $\alpha\beta$  T cells), there is something unconventional about them. For example, in the peripheral blood, the ratio of  $CD4$  to  $CD8$  T cells is about 2:1. In the normal liver, however, this ratio is reversed (approx 1:2.5). There are increased numbers of  $CD4^-/CD8^-$  “double-negative” T cells compared with peripheral blood. Liver TCR $\alpha\beta$  T cells tend to have lower levels of expression of the TCR $\alpha\beta$  chains, the associated  $CD3$  signaling complex, and associated  $CD4$  or  $CD8$  coreceptors. Many T cells express cell surface markers indicating previous activation, such as elevated expression levels of  $CD44$  and  $CD25$  (2). Injection of cognate antigen into mice bearing TCR transgenic T cells leads to the activation of T cells and the accumulation of activated antigen-specific T cells in the liver, as well as in other organs (3). Thus, T cells in the liver reflect a combination of resident hepatic T lymphocytes, as well as T cells activated extrahepatically that migrate to the liver, where they are retained through interaction with specific cell-surface adhesion molecules (4). Many T cells trapped in the liver are eliminated through apoptosis, which may be an important mechanism by which the liver promotes immune tolerance. Taken together, these observations suggest that the liver harbors large numbers of TCR $\alpha\beta$  T cells that are not quiescent but show evidence of recent activation, proliferation, and apoptosis, as well as a high level of activity (5).

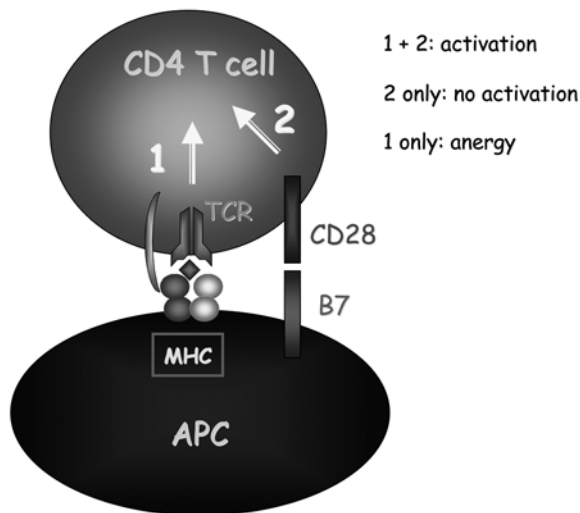
**$CD4^+$  TCR $\alpha\beta$  T Cell Subsets and Functions**  $CD4^+$  TCR $\alpha\beta$  T cells perform important functions in the immune response. A principal function is to secrete *cytokines* upon antigen stimulation. These cytokines serve as key signals to other cells involved in the immune response, and the types and quantities of cytokines produced have important consequences for both the generation of effective immune responses and for the development of immunopathology.  $CD4^+$  T cells that have never encountered antigen are referred to as *naïve*  $CD4^+$  T cells. They retain the potential to differentiate further into *effector*  $CD4^+$  T cells—which produce effector cytokines upon encounter with antigen—and into long-lasting *memory*  $CD4^+$  T cells (6).

#### NAÏVE $CD4^+$ T CELLS

The initial activation of naïve T cells by antigen is typically mediated by *dendritic cells* (DCs), specialized leukocytes that phagocytose protein antigens at peripheral sites, undergo physiologic maturation, and migrate to lymph nodes, where they present peptide antigens to naïve T cells. An important function of the lymph node is to enhance the probability of naïve T cells encountering their cognate antigen, presented by DCs. When a  $CD4^+$  T cell recognizes MHC class II/antigen with sufficient affinity and duration, along with stimulation through an accessory molecule such as the  $CD28$  coreceptor, the T cell is activated, proliferates, and gives rise to  $CD4^+$  T-cell effector cells (Fig. 3).

#### EFFECTOR $CD4^+$ T CELLS

By contrast with naïve  $CD4^+$  T cells, effector  $CD4^+$  T cells may be found at the sites of inflammation or pathogen challenge.



**Fig. 3.** Activation of naïve T cells requires two signals. T cells, through their T-cell receptor (TCR) recognize a peptide antigen (diamond shape) displayed in the context of MHC molecules on the surface of antigen-presenting cells (APC). This interaction elicits a signal to the T cell (signal 1) that is necessary but not sufficient for full cell activation. Full activation of the naïve T cell also requires a costimulatory signal (signal 2) that is typically delivered by the cell surface molecule CD28, when it interacts with its ligand B7 expressed on the surface of the APC. Delivery of signal 2 without signal 1 results in no T-cell activation, whereas delivery of signal 1 without signal 2 results in the development of T-cell anergy, a state in which T cells are refractory to subsequent antigen stimulation.

Effector CD4<sup>+</sup> T cells produce a variety of cytokines, in large quantities. Compared with naïve T-cell stimulation, the activation of effector CD4<sup>+</sup> T cells is relatively easier, requiring less sustained TCR signal and less costimulation through CD28. On the basis of the cytokines they produce, effector CD4<sup>+</sup> T cells have been traditionally classified into two well-established subsets, Th1 and Th2 (7). More recent findings clearly establish the existence of additional effector cell subsets, including regulatory T cells (T-reg) (8) as well as inflammatory CD4<sup>+</sup> cells characterized by the production of IL-17 (Th17 cells) (9,10). It is now well established that the cytokines interleukin-12 (IL-12) and IL-4 influence the development of antigen-stimulated naïve T cells into the Th1 and Th2 effector cell subsets, respectively (7). The factors that direct the differentiation of T-reg cells or Th17 cells are less well understood, but recent data point to roles for additional cytokines, including IL-23, IL-6, and transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) (Fig. 4) (11–13).

### TH1 CELLS

Upon encounter with antigen, Th1 cells produce large amounts of interferon- $\gamma$  (IFN- $\gamma$ ) and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ). These cytokines are important for arming cellular immunity and play important roles in immune defense against certain classes of pathogens, particularly intracellular bacteria such as *Listeria monocytogenes*, and *Mycobacterium* species. IFN- $\gamma$  strongly stimulates macrophage expression of nitric

oxide and reactive oxygen intermediates, through induction of the synthetic enzyme inducible nitric oxide synthase (iNOS). IFN- $\gamma$  augments antigen presentation by APCs and promotes Th1 development, in an important positive feedback loop. TNF- $\alpha$  activates macrophages and amplifies the inflammatory response pathway by inducing the expression of numerous cytokines and chemokines, the iNOS enzyme, and adhesion molecules, as well as the production of eicosanoids. TNF- $\alpha$  and IFN- $\gamma$ , particularly in concert, can be directly toxic to hepatocytes. Both cytokines are robustly produced by intrahepatic CD4<sup>+</sup> T cells isolated from biopsy samples from patients with active autoimmune hepatitis (14–16).

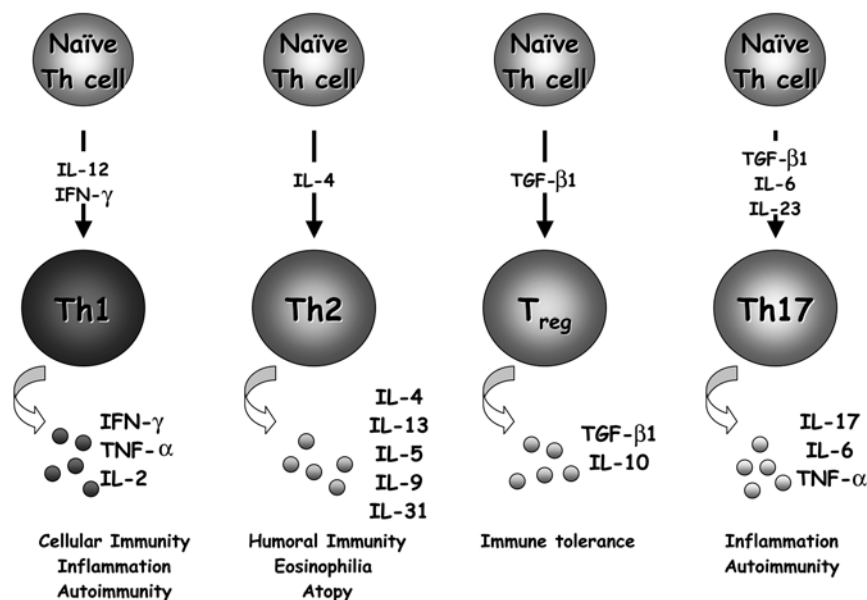
Experimental studies in mice demonstrate that both cytokines participate in inflammation and hepatocellular damage. TNF- $\alpha$  mediates hepatotoxicity in many animal models, such as following the administration of Concanavalin A (ConA) (17) or lipopolysaccharide (LPS) (18). TNF- $\alpha$  also may play a pathogenic role in patients with alcoholic liver disease (19) and viral hepatitis (20). TNF- $\alpha$  can be produced by both T cells and macrophages; a recent study in mice has clarified that TNF- $\alpha$  produced by T cells substantially contributes to liver injury following ConA administration (21). The biological role of TNF- $\alpha$  in the liver is complex, as it is required for normal hepatocyte proliferation during liver regeneration, functioning both as a mitogen and as an inhibitor of apoptosis, through induction of the antiapoptotic transcription factor nuclear factor- $\kappa$ B (22). Transgenic expression of IFN- $\gamma$  in the liver in mice leads to a chronic hepatitis (23). Mice deficient in SOCS-1, a key inhibitor of IFN- $\gamma$  signaling, develop fulminant IFN- $\gamma$ -dependent liver disease characterized by fatty degeneration and necrosis of hepatocytes (24). Finally, the interplay between the inflammatory Th1 cytokine IFN- $\gamma$  and counterregulatory cytokines, such as TGF- $\beta$ 1, is critical to the maintenance of immune homeostasis in the liver: BALB/c mice deficient in the cytokine TGF- $\beta$ 1, an important inhibitor of Th1 differentiation (25,26), rapidly develop hepatic Th1 lymphocytosis and necroinflammatory liver disease that is dependent on both CD4<sup>+</sup> T cells (27) and IFN- $\gamma$  (28).

### TH2 CELLS

Th2 cells produce the cytokines IL-4, IL-5, and IL-13. IL-4 strongly enhances B-cell proliferation, regulates Ig class switching, augments T-cell proliferation, and, in an important positive feedback loop, promotes the differentiation of naïve T-helper cells into Th2 cells. IL-4 is a principal cytokine responsible for B-cell switching to IgE; as a consequence, IL-4 has a critical role in the development of allergic responses. IL-5 is important for the recruitment and induction of eosinophils. IL-13 is similar in structure and activity to IL-4, enhancing B-cell responses and augmenting Th2 development.

Th2 cells are key participants in the immune response in the liver to infection by *Schistosoma mansoni*. *S. mansoni* parasites reside in mesenteric veins and lay hundreds of eggs per day. Some of these eggs become trapped in the liver microvasculature, where they induce a robust granulomatous response that leads ultimately to liver fibrosis. Early granuloma





**Fig. 4.** Naïve T cells differentiate into distinct effector cell subclasses. Concurrent with initial antigen stimulation through signals 1 and 2, additional cues in the T-cell microenvironment dictate the developmental fate of the effector T cell. Cytokines present during initial antigen stimulation are important for the differentiation of activated T cells into Th1, Th2, T-reg, or Th17 effector T cells. These effector cell subsets have distinct functions in the immune system, determined in large part by the cytokines secreted during subsequent encounters with antigen. IFN, interferon; IL, interleukin; TGF, transforming growth factor; TNF, tumor necrosis factor.

formation is associated with a Th1 response that quickly transitions to a Th2-type response (29). The Th2-type response plays a protective role in the initial stages of infection, but the same cytokines lead eventually to a severe fibrosis, with accompanying portal hypertension (30). Studies evaluating which Th2 cytokines are important for protection or pathology in response to *S. mansoni* infection revealed distinct roles for IL-4 and IL-13, showing that IL-4 is beneficial to survival, whereas IL-13 is detrimental. IL-13-deficient mice demonstrated significantly enhanced survival following infection, correlating with reduced hepatic fibrosis; in contrast, IL-4-deficient mice exhibited increased mortality and hepatocellular damage. Both IL-4 and IL-13 are necessary to develop a vigorous, eosinophil-rich granuloma response (31). Inhibition of IL-13 in vivo using a soluble inhibitor is effective in preventing *S. mansoni*-induced fibrosis in mice (32). The fibrogenic properties of IL-13 may be direct, as IL-13 can induce collagen synthesis in fibroblasts in culture (32).

IL-4 can be either beneficial or deleterious to the health of the liver, depending on the context. Whereas IL-4 is a protective cytokine in *S. mansoni* infection, it contributes to liver damage in response to ConA administration. Indeed, in vivo treatment with neutralizing anti-IL-4 monoclonal antibody prior to ConA administration attenuates hepatic injury (33). IL-4 appears to have at least a dual role in promoting pathogenesis. First, IL-4 produced by ConA-activated hepatic NKT cells augments the cytotoxic activity of these cells in an autocrine fashion (34). Second, IL-4 enhances expression of eotaxins in hepatocytes and sinusoidal endothelial cells and induces IL-5 expression, facilitating the recruitment of eosinophils and neutrophils (35).

Recent work has shown that the Th2 cytokine IL-5 is a critical mediator of ConA-mediated hepatotoxicity, acting through its potent eosinophil-recruitment activity (36).

### T-REG CELLS

The Th1/Th2 division has been useful in understanding immunity and immunopathology, but it is becoming increasingly apparent that this division is inadequate to describe the spectrum of immune responses in which CD4<sup>+</sup> T cells participate. Regulatory CD4<sup>+</sup> T (T-reg) cells have become objects of intense scrutiny in research laboratories. T-reg cells produce neither IFN- $\gamma$  nor IL-4, but rather the immunosuppressive cytokines IL-10 and TGF- $\beta$ 1. T-reg cells inhibit the proliferation and effector functions of other T cells, utilizing several mechanisms, including cell-cell contact and the elaboration of immunosuppressive cytokines such as IL-10 or TGF- $\beta$ 1 (37). As T-reg cells are important for regulating the onset and duration of T-cell-mediated immune responses, their deficiency or dysfunction may underlie autoimmunity or other immune pathologies. T-reg cells are typically identified by expression of CD4 along with the marker CD25 (38). FoxP3 is a transcription factor expressed in T-reg cells and may be the most specific marker for T-reg cells thus far identified. Mice deficient in FoxP3 spontaneously develop a fatal lymphoproliferative disorder (39), and ectopic expression of FoxP3 confers regulatory activity on conventional T cells (40). Thus, FoxP3 is both necessary and sufficient for the development of regulatory T cells. T-reg cells develop through at least two sources. *Natural* T-reg cells develop in the thymus as a function of high avidity positive selection (reviewed in ref. 41), whereas *induced* T-reg cells

arise as a consequence of antigen stimulation in the periphery. The factors that drive induced T-reg selection are not fully elucidated, but recent evidence suggests that the cytokine TGF- $\beta$ 1 is able to induce FoxP3 expression in CD4<sup>+</sup> T cells and confer regulatory activity on them (11,42).

The potential participation of T-reg cells in liver health and disease is an exciting new area for research that has begun to attract considerable interest. A deficiency in T-reg numbers or function appears to be associated with autoimmune liver disease. In patients with autoimmune hepatitis (AIH), peripheral T-reg numbers are depressed compared with controls, and they are lower in patients at the time of diagnosis than during remission (43). Moreover, the percentage of T-reg cells in blood inversely correlates with serum titers of anti-LKM antibodies (43). Similarly, T-reg cells in primary biliary cirrhosis (PBC) patients are lower in number, although not in function, compared with controls (44). Recent evaluation of the mechanism of action of T-reg cells in the context of AIH shows a requirement for cell-cell contact with target effector T cells (45). Coculture of T-reg cells with effector T cells enhanced the secretion of the immune regulatory cytokines IL-4, IL-10, and TGF- $\beta$ 1 (45, 46). These studies suggest a role for T-reg cells in maintaining immune tolerance in the liver.

Tumor-infiltrating lymphocytes are associated with hepatocellular carcinoma (HCC). FoxP3-staining cells can be found to infiltrate HCC diffusely and express cell surface TGF- $\beta$ 1 (47). In addition, HCC patients have a significant elevation in the percentage of T-reg cells in peripheral blood mononuclear cells (PBMCs) (48). The numbers of CD8<sup>+</sup> T cells at tumor margins are inversely proportional to CD4<sup>+</sup>/CD25<sup>+</sup> cells in the same region, implying a functional relationship *in situ* (49). Together, these results suggest that T-reg cells play a role in suppressing antitumor immune responses in HCC. Whether T-reg cell frequency or function predicts a poor prognosis in HCC deserves additional evaluation.

Recent studies implicate a role for T-reg cells in mediating hepatitis C virus (HCV) persistence. Peripheral CD4<sup>+</sup>/CD25<sup>+</sup> cells are present at higher frequency in patients with chronic HCV infection compared either with patients who have recovered or with normal controls (50). Additional studies have linked T-reg cells with functional inhibition of CD8<sup>+</sup> T cell responses both against HCV and against unrelated viruses (51, 52). The HCV-specific TGF- $\beta$ 1 response by CD4<sup>+</sup>/CD25<sup>high</sup> T cells is inversely correlated with ALT levels (53). Thus, T-reg cells may play important roles during HCV infection in limiting the immune response against both the virus and the infected hepatocytes.

### TH17 CELLS

CD4<sup>+</sup> cells producing IL-17, as well as TNF- $\alpha$  and IL-6 (54), are the most recent addition to the classification scheme of differentiated T-helper cells. IL-17 is a proinflammatory cytokine that stimulates other cells, including fibroblasts, endothelial cells, epithelial cells, and macrophages, to produce a variety of inflammatory mediators, including cytokines such as IL-1, IL-6, and TNF- $\alpha$ , chemokines, and metalloproteinases (54). IL-17 appears to contribute to the induction or development of

several allergic and autoimmune diseases, including rheumatoid arthritis, multiple sclerosis, inflammatory bowel disease, and asthma (55). IL-17 has an important role in the recruitment and activation of neutrophils, and an emerging model is that CD4<sup>+</sup> T cells, through IL-17 production, serve to enhance neutrophilic inflammation (55). Th17 effector CD4<sup>+</sup> T cells appear to constitute a separate developmental lineage from either Th1 or Th2 cells, and indeed the absence of IFN- $\gamma$  and IL-4 appears to be necessary to permit the development of the Th17 effector state (12,13). Whereas the IL-12-related cytokine IL-23 was initially considered key to the differentiation of Th17 cells, recent reports show that the combination of TGF- $\beta$ 1 and IL-6 is important for the initiation of the Th17 differentiation pathway, with IL-23 serving to enhance Th17 cell survival and proliferation (12,13,56). IL-6 is produced by APCs that have been stimulated through Toll-like receptors by pathogen-associated molecules such as LPS or leukotriene A (LTA). Thus, the presence of IL-6 appears to be the key switch that determines whether T-helper cells encountering antigen in the presence of TGF- $\beta$ 1 will develop into Th17 cells, rather than T-reg cells, and helps to ensure that Th17 cell development is linked to infection. The signals that result in pathologic development of Th17 cells remain obscure.

Most studies about IL-17 have not focused on the liver, and little is known about the role of IL-17 in the hepatic immune system. Mice deficient in IL-23, important for Th17 cell expansion and survival, exhibit delayed pathogen clearance from the liver after infection by the fungus *Cryptococcus neoformans* (57). Transgenic mice overexpressing the IL-23 p19 subunit exhibit neutrophilia and increased expression of acute-phase proteins in the liver (58). In a model of liver ischemia-reperfusion in mice, CD4<sup>+</sup> T cells are rapidly recruited to the liver following reperfusion and facilitate subsequent neutrophil recruitment via an IL-17-dependent mechanism (59). As liver immunologists begin to focus their attention on this interesting T-helper cell subset, we can expect further examples of the role of Th17 cells in liver health and disease.

### NKT CELLS

NKT cells are abundant in the liver (60). As the name implies, these lymphocytes express both TCR $\alpha\beta$  and NK cell-surface receptors; conceptually, they are perhaps appropriately considered to be at the interface between the innate and adaptive immune systems. Most hepatic NKT cells are CD4<sup>+</sup> but only express a very limited TCR repertoire. Each NKT cell expresses only a single type of TCR $\alpha$  chain (V $\alpha$ 24-J $\alpha$ Q in humans and V $\alpha$ 14-J18 in mice) and one of only a few TCRV $\beta$  chains. Whereas conventional TCR $\alpha\beta$  T cells recognize peptides presented by class II MHC molecules, NKT cells recognize *glycolipids* presented by the CD1 cell surface molecule.

Until very recently, the only ligand known to bind CD1 and activate NKT cells was  $\alpha$ -galactosyl ceramide ( $\alpha$ -GalCer) to which NKT cells respond by rapidly producing both Th1 and Th2 cytokines.  $\alpha$ -GalCer was originally extracted from sea sponge, and several laboratories have made advances recently in identifying more physiological ligands for NKT cells. These

include a natural ceramide, iGb3 (61), as well as structurally similar compounds isolated from *Sphingomonas* species (62, 63). It appears therefore, that NKT cells can be activated by both endogenous and exogenous lipid ligands.

The participation of NKT cells in immune responses is the focus of intense interest. Most work has been done in mice, so in extrapolating findings to humans, caution is advised. NKT cells may participate in tumor surveillance and are implicated in autoimmunity (64). In the liver, there is evidence that NKT cells can participate in the induction of autoimmune pathology. Injection of NKT ligands into mice results in rapid activation of intrahepatic NKT cells, with an associated transaminitis and histopathologically evident hepatocellular damage (65). Hepatocellular damage following ConA administration is greatly reduced in NKT cell-deficient mice compared with NKT cell-replete mice (66). Selective enrichment of NKT cells at the site of inflammation is observed in PBC, suggesting a role for these cells in the development of this autoimmune liver disease (67). Understanding the variables that determine the precise mechanisms by which NKT cells participate in health and disease in the liver is an important research goal.

### CD8<sup>+</sup> T CELLS

Like CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells may also be classified into naïve and effector/memory subsets. CD8<sup>+</sup> T cells participate in the immune response as effector cells and are important for the elimination of intracellular pathogens, particularly viruses. Indeed, CD8<sup>+</sup> T-cell responses are important for the elimination of hepatotropic viruses such as HBV and HCV. Effector CD8<sup>+</sup> T cells recognize peptides presented in the context of cell surface MHC class I molecules. Effector CD8<sup>+</sup> T cells, known as cytotoxic T lymphocytes (CTLs), classically mediate the killing of the antigen-presenting target cells through a variety of mechanisms. These mechanisms include the induction of programmed cell death, using cell-surface effector molecules such as FasL. CTLs can also kill through the insertion of perforin into target cells, creating holes used for the delivery of granzymes, resulting in the destruction of the target cell from within. Recent evidence suggests that CD8<sup>+</sup> cells may eliminate hepatotropic viruses such as HBV and HCV through mechanisms that do not involve killing of the target cell. Instead, release of cytokines such as IFN- $\gamma$  or TNF- $\alpha$  may be sufficient to prevent viral replication, while simultaneously sparing the hepatocyte (68,69).

### B CELLS

Compared with T cells, relatively little is known about the role of B cells in the liver or in liver diseases that intimately involve the immune system (70). B cells are found in small numbers in healthy liver (71) and can be found both in portal tracts as well as scattered throughout the parenchyma (72). During HCV infection, B-cell expansion in the liver can be observed associated with hepatic germinal center-like structures (73). The serological response to HBV and HCV is clinically invaluable in the diagnosis of infection by these viruses. Intrahepatic plasma cells are a prominent feature of autoimmune hepatitis (74), and the target specificity of the immunoglobulin

response is an important clinical aid in the diagnosis and classification of autoimmune liver diseases (75). However, direct evidence that the humoral immune response participates in either eradication of viral infection or, conversely, in the pathogenesis of inflammatory pathology during viral hepatitis or autoimmune hepatitis is scant. Although the serologic B-cell response is useful in diagnostics, the cellular (T-cell) response appears to be more significant in determining the outcome of liver diseases that involve a significant immune component.

### T-LYMPHOCYTE RECRUITMENT IN THE LIVER

A key step in the development of immune responses to invading pathogens is the egress of leukocytes from the circulation into the tissue parenchyma. Early work on this process involved analysis of high-flow tissues such as the cremaster muscle or mesentery (76–78) and revealed that leukocyte attachment to the endothelial lining involves two phases, rolling and adhesion. In high-flow tissues, members of the selectin family of adhesion molecules are important mediators of the initial rolling step. However, leukocyte adhesion in the liver, but not in the cremaster muscle, is intact in mice lacking functional selectins (78). Thus, in the liver, a slow-flow tissue, the requirement for rolling, and the selectins that mediate rolling, in the process of leukocyte adhesion and movement into the tissue, is significantly reduced. Liver endothelium lacks expression not only of P- and E-selectin, but also of CD34 platelet-endothelial cell adhesion molecule-1 (PECAM-1), and VE-cadherin (79). Liver endothelium is not devoid of cell-surface molecules that may mediate adhesion of lymphocytes, and both intracellular adhesion molecule-1 (ICAM-1) and vascular adhesion protein-1 (VAP-1) are constitutively expressed (80). The slow-flow movement of leukocytes through narrow sinusoids, combined with a relative paucity of expression of a variety of cell adhesion molecules on sinusoidal endothelium, has led to a model of lymphocyte recruitment mediated by physical trapping rather than adhesive interactions by specific cell-surface molecules. This model has been supported by studies showing little effect of neutralizing antibodies to a large variety of cell-surface molecules on the retention of T lymphocytes in liver (81), although requirements for ICAM-1 (82) and vascular cell adhesion molecule-1 (VCAM-1) (82) have also been reported.

A recent study challenges the physical trapping model and presents evidence that T lymphocytes do utilize specific molecular interactions to mediate adhesion to the liver sinusoidal and postsinusoidal endothelium. Using intravital microscopy of mouse liver to examine the dynamic behavior of infused lymphocytes, the study showed that Th1 cell adherence to liver sinusoids requires  $\alpha_4\beta_1$ -integrin, whereas Th2 cell adherence requires VAP-1 (83). This important study shows that not only are specific molecules required but the rules of engagement that govern lymphocyte-endothelial adhesion differ between Th1 cells and Th2 cells. NKT cell movement within sinusoids may be regulated by cell activation. Hepatic NKT cells “patrol” sinusoids in an apparently random fashion, with an equal number moving against as with the flow of blood, until they are

activated through their TCR by cognate ligand, at which point their movement ceases (84). The chemokine receptor CXCR6 is important for the survival but, interestingly, not the migration, of sinusoidal NKT cells (84). As CXCL16, the only known ligand for CXCR6, is expressed on liver sinusoids, sinusoidal endothelial cells may influence NKT cells by delivering a survival signal.

Together, these findings suggest that the biological response of T lymphocytes within liver sinusoids is carefully and specifically regulated by molecular signals expressed by sinusoidal endothelial cells. Importantly, it appears that the recruitment and/or survival of distinct hepatic lymphocyte subsets are regulated by distinct molecules. This raises the exciting possibility of targeted therapeutic interventions that may enhance or restrict the adhesive or survival properties of specific hepatic T-cell subsets in patients with T-cell-mediated inflammation of the liver.

### THE LIVER AS END-GAME OF THE T-CELL RESPONSE

One of the intriguing aspects of the liver is that it preferentially retains activated T cells compared with naïve T cells. In experimental models, the liver preferentially retains activated CD8<sup>+</sup> T cells through ICAM-1/ leukocyte function-associated antigen-1 (LFA-1) interactions between liver endothelial cells and T lymphocytes, perhaps explaining the reversed CD4/CD8 ratio. Many intrahepatic T cells are apoptotic (85), leading to the hypothesis that T cells activated in extrahepatic sites and transiting through the liver are preferentially eliminated via apoptosis. Thus, the liver may serve as a “graveyard” for spent effector T cells (86), suggesting that the liver has an important role beyond “local” immune responses, as a general regulator of T-cell-mediated immunity.

### CONCLUDING REMARKS

The adaptive immune system has the responsibility of generating effective and durable pathogen-specific immunity. In addition to specificity, diversity and the ability to generate memory are key components of the adaptive immune system. As the liver is continuously bathed by a variety of complex substances, including toxins, dietary antigens, and the byproducts of commensal organisms, the adaptive immune system in the liver is faced with the additional challenges of avoiding deleterious inflammation and autoimmunity and suppressing responses to benign foreign antigens. The liver participates in preventing the development of harmful immune responses to ingested substances (oral tolerance). Since the liver is also a favorite host tissue for a number of pathogens, including hepatotropic viruses and bacteria, the liver adaptive immune system must be under exquisite regulatory control. Distinct types of T-helper cells, such as Th1, Th2, T-reg, and Th17 effector cells participate in specific types of immune responses in the liver, some of which are beneficial and some of which are deleterious. How distinct T-cell subsets are generated in the liver and how their effector functions are regulated remain key questions for future discovery.

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# 6 Hepatic NK, NKT, and T Cells

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GOLO AHLENSTIEL AND BARBARA REHERMANN

## KEY POINTS

- The liver is an immunologically distinct organ that contains unique cell populations of the innate and adaptive immune response.
- The liver's location and unique architecture contribute to its role in the induction of tolerance and to its role as an effector site of immune responses to pathogens.
- About 30% of the total blood passes through the liver every minute, carrying about  $10^8$  peripheral blood lymphocytes in 24 h.
- Natural killer (NK) cells constitute a large proportion of liver-resident lymphocytes. Their function is regulated by both activating and inhibitory receptors, with inhibition as the dominant signal.
- Natural killer T (NKT) cells arise in the thymus, display a very restricted T-cell receptor repertoire, and recognize antigens in the context of the MHC class I molecule CD1d.
- The intrahepatic T-cell population includes conventional CD8 and CD4 T-cell subpopulations and large subpopulations of unconventional lymphocytes, such as CD4/CD8 double-negative T cells, CD4/CD8 double-positive T cells, and  $\gamma\delta$  T cells.
- Recruitment of T cells into the liver is a multistep process and is facilitated by the fenestrated sinusoidal membrane, slow blood flow, and high shear stress in the intrahepatic vascular bed.
- Infiltration of T cells into the liver parenchyma is enhanced by gradients of chemokines. Individual T-cell subsets respond to different chemokines. Tissue-specific migration is related to the activation status of T-cells but not necessarily to their antigen specificity.
- Fas-, TNF- $\alpha$ -, and perforin-mediated mechanisms have been implicated in T-cell-mediated hepatocyte death during inflammatory liver injury.
- A large percentage of liver-infiltrating T cells undergoes passive or activation-induced cell death within the liver.

## INTRODUCTION

The liver's unique location between the gastrointestinal tract and peripheral lymphoid organs and its fenestrated

endothelium allow contact with many antigenic substances. These consist of dietary proteins transported from the gut via the portal vein, products of intrahepatic metabolism, and bacterial and viral liver pathogens. According to the different origin of these antigens, the liver has the unique ability to induce either tolerance or inflammatory reactions (1,2) (Table 1). Furthermore, the liver can actively modulate ongoing immune reactions: the intrahepatic inflammatory infiltrate can be increased by chemotactic attraction and activation of leukocytes (3) and decreased by induction of apoptosis of activated intra-hepatic lymphocytes (4). These dual and apparently opposing functions are important to understand the mechanisms of tolerance to oral and allograft antigens and the pathogenesis of liver diseases caused by parasitic and viral pathogens. This chapter addresses the unique role of intrahepatic natural killer (NK), natural killer T (NKT), and T cells during this process.

## LYMPHOCYTE POPULATIONS IN THE HEALTHY LIVER

The uninfected, average liver weighs approximately 1200 to 1500 g and contains  $10^9$  to  $10^{10}$  lymphocytes. About 30% of the total blood passes through the liver every minute (5), carrying about  $10^8$  peripheral blood lymphocytes in 24 h (6). Blood enters the hepatic parenchyma via terminal portal vessels, then passes through a network of liver sinusoids, and leaves the parenchyma via the central hepatic veins. Because of the small diameter of the sinusoids, minimal increases in systemic venous pressure and perturbations of sinusoidal flow result in stasis and promote lymphocyte extravasation. Extravasation is further facilitated by fenestrations in the monolayer of sinusoidal endothelial cells (7) that allow lymphocytes to access the space of Dissé via cytoplasmic extensions and to "touch" the underlying extracellular matrix, stellate cells, and hepatocytes. The liver's lymphocyte population differs considerably from that of the blood and includes liver-resident subpopulations of the innate (NK and NKT cell) and adaptive (T- and B-cell) immune response (Fig. 1).

NK cells are present at a high frequency among liver-resident lymphocytes (8). Although they account for about 10 to 20% of the lymphocytes in the peripheral blood, they represent around 30% of the resident lymphocyte population

**Table 1**  
**The Liver as Target and Regulator of Cellular Immune Responses**

	<i>References</i>
<i>The liver as a mediator of:</i>	
Tolerance	1,2
Immune defense against bacterial and viral liver pathogens	103,118–121
Autoimmune liver disease	122–124
<i>The liver as the site of:</i>	
Priming of specific T cells	3
Effector functions of liver-infiltrating T cells	103,110,121
Elimination of activated T cells via inductions of apoptosis	17,125

in the liver (9). This percentage increases further during liver inflammation (10).

NKT cells express both the NK cell marker CD56 and the T-cell marker CD3 (11). NKT cells arise in the thymus, display a very restricted T-cell receptor (TCR) repertoire (typically consisting of TCR V $\alpha$ 24 and V $\beta$ 11 chains in humans), and recognize antigens in the context of the MHC class I molecule CD1d (12). Although their natural antigen is not known, the marine sponge antigen  $\alpha$ -galactosyl ceramide ( $\alpha$ GalCer) is used as a reliable experimental tool to activate all classical NKT cells. Classical NKT cells can be either CD4 positive or CD4/CD8 double negative. By contrast, non-classical NKT cells encompass TCR $\alpha\beta$  and TCR $\gamma\delta$  T cells, do not use the T-cell receptor V $\alpha$ 24 chain, and do not express the CD8 $\beta$ -chain (13). Classical and nonclassical NKT cells are more abundant in the liver than in other organs and constitute up to 30% of the intrahepatic lymphocyte population (14).

The intrahepatic T-cell population includes the conventional CD8 and CD4 T-cell subpopulations. Both subpopulations display a diverse TCR- $\alpha\beta$  repertoire and recognize antigens in the context of MHC class I and II molecules, respectively. CD8 T cells typically outnumber CD4 T cells in the liver, and the frequency of effector/memory cells is higher than in the blood. The T-cell population also includes a large percentage of unconventional lymphocytes (15), such as CD4/CD8 double-negative T cells (16–18), CD4/CD8 double-positive T cells (15), and  $\gamma\delta$  T cells (19).

## NK CELLS

NK cells are large granular lymphocytes that, unlike T cells, lack TCRs and, unlike B cells, do not express immunoglobulins. Furthermore, unlike T and B cells, their activation does not require prior sensitization. NK cells express activating and inhibitory receptors, and under noninflammatory conditions inhibition dominates over activation. Therefore, the threshold for NK cell activation is lowest in the absence of ligands that bind to inhibitory receptors (20) and in the presence of activating inflammatory cytokines (21).

### NK CELL FUNCTION

NK cells are best known for their ability to kill virus-infected cells (22) and tumor cells independent of MHC restriction (23).

Cytotoxicity is initiated by release of prestored perforin and granzyme B into the contact zone with the target cell. Other molecules that NK cells use to induce cell death include FAS ligand, tumor necrosis factor (TNF)- $\alpha$ , and TNF-related apoptosis-inducing ligand (TRAIL). NK cells also produce a number of cytokines with antiviral and immunostimulatory properties such as interferon- $\gamma$  (IFN- $\gamma$ ), TNF- $\alpha$ , and granulocyte-macrophage colony-stimulating factor (GM-CSF) (24) and modulate immune responses by interaction with other antigen-presenting cells (APCs) and T cells. Interaction between NK cells and dendritic cells (DCs), for example, leads to activation and cytokine production of both cell types, which results in maturation of DCs, proliferation of NK cells, and NK cell-mediated cytotoxicity against immature DCs (reviewed in ref. 25). Finally, NK cells contribute to the recruitment of T cells to the liver. They secrete chemokines such as macrophage inflammatory protein (MIP)-1 $\alpha$  and MIP-1 $\beta$  and release IFN- $\gamma$ , which stimulates hepatocytes and liver sinusoidal endothelial cells (26) to secrete the chemokine CXCL9 and thereby recruit T cells to the liver. As NK cells express costimulatory molecules such as CD40 ligand and OX40 ligand (27), they may also be important during the activation of the recruited T and B cells.

### NK CELL SUBSETS IN HUMANS

Human NK cells are defined as CD3 negative, but CD56 (N-CAM) and/or CD16 (Fc $\gamma$ RIII) positive lymphocytes. Whereas CD56 is an adhesion molecule, CD16 is a receptor for IgG, thus enabling NK cells to recognize and kill IgG-coated targets.

NK cells can be divided into three major subsets based on the CD16 and CD56 expression. Most (approx 90%) of NK cells in the peripheral blood express CD16 but only a relatively small number of CD56 molecules on their cell surface (CD3<sup>-</sup>/CD16<sup>+</sup>/CD56<sup>dim</sup>). They also express chemokine receptors such as CXCR1 and CX<sub>3</sub>CR1 and thus respond to chemokines released during inflammation (28). Once activated, the predominant effector function of CD3<sup>-</sup>/CD16<sup>+</sup>/CD56<sup>dim</sup> NK cells is cytotoxicity and only to a much lesser degree cytokine production (29).

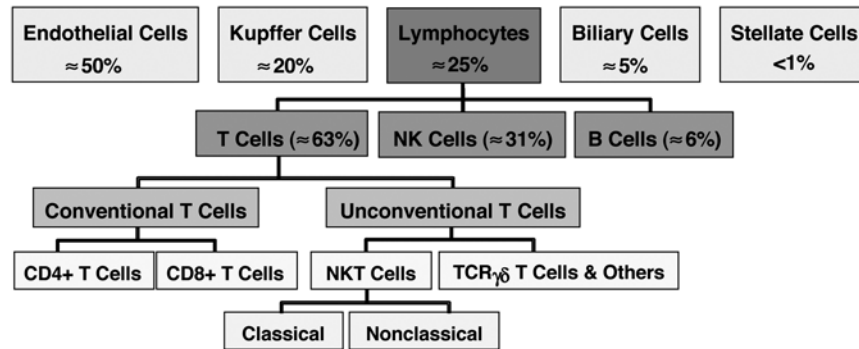
A significantly smaller subset of NK cells (approx 10%) is defined as CD16 negative and CD56 bright (CD3<sup>-</sup>/CD16<sup>-</sup>/CD56<sup>bright</sup>) (30). These NK cells express chemokine receptors such as CCR7 and CXCR3, which are known as lymph node and tissue homing markers (31). They are therefore predominantly found in lymph nodes (32) and in the liver (9). Upon activation, they release large amounts of IFN- $\gamma$  but exhibit only a little cytotoxicity.

The third NK cell subset is rare in healthy individuals and consists of CD16-positive and CD56-negative NK cells (CD3<sup>-</sup>/CD16<sup>+</sup>/CD56<sup>-</sup>) (33). These NK cells represent a rather dysfunctional subset and exert very little cytotoxicity. Expansion of this subset has been mainly reported in subjects with high levels of HIV viremia (34).

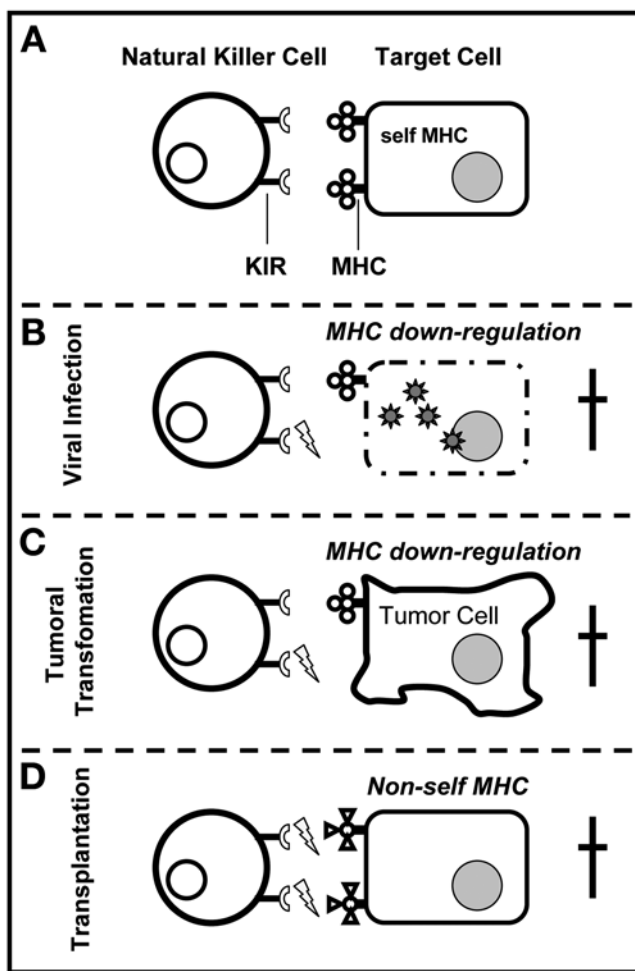
### NK CELL RECEPTORS

As described above, a distinct characteristic of NK cells is their ability to kill a target without prior sensitization. Therefore,





**Fig. 1.** Nonparenchymal cells of the liver. Twenty to 40% of all cells of the liver are not hepatocytes. One-fourth of this nonhepatocyte population is lymphocytes. NK, natural killer; TCR, T-cell receptor. Modified from ref. 126, with permission.



**Fig. 2.** NK cells and the “missing self” hypothesis. NK cells scan tissues for MHC expression. (A) In the case of normal expression of autologous (self) MHC, NK cells are inhibited via killer immunoglobulin-like receptor (KIR) and NKG2A receptors. Thus, the target cell will survive. (B, C) If MHC expression is downregulated due to viral infection (B) or tumoral transformation (C), then NK cells will be activated owing to lack of inhibition and will lyse the target cell. (D) Likewise, NK cells will be activated by heterologous (non-self) MHC. Activation leads to direct killing/cytotoxicity and to cytokine (IFN- $\gamma$ , TNF- $\alpha$ , GM-CSF) and chemokine release (MIP-1 $\alpha$ , MIP-1 $\beta$ ).

NK cells need to be kept under very tight control to prevent random killing of neighboring cells. This control mechanism was initially described as the “missing-self hypothesis” (Fig. 2). According to this dogma, inhibitory NK cell receptors recognize autologous major histocompatibility complexes (MHCs) on healthy cells and thus prevent NK cell activation (35). If, however, a cell’s MHC is downregulated as a result of a virus infection or oncogenic transformation, NK cell inhibition decreases, and the target cell can be lysed. Thus, NK cell inhibition always supersedes activation in a healthy environment.

More recently, it has been described that NK cell activation results from integration of multiple activating and inhibitory signals transmitted via a large variety of killer immunoglobulin-like receptors (KIRs), lectin-like receptors, or natural cytotoxicity receptors (36). KIRs are located on chromosome 19q13.4 and are mainly expressed by CD3<sup>-</sup>/CD16<sup>+</sup>/CD56<sup>dim</sup> NK cells. These receptors recognize MHC class I molecules such as HLA-A, HLA-B, and HLA-C and the nonclassical HLA-G. Each KIR contains two or three extracellular immunoglobulin domains (2D or 3D) and either a long or a short cytoplasmic tail. The long cytoplasmic tail is indicated by an “L” in the designated KIR name and contains two immunoreceptor tyrosine-based inhibition motifs (ITIMs), which mediate inhibitory signals. For example, the designation “KIR2DL1” describes an inhibitory KIR with two extracellular immunoglobulin domains (“2D”) and a long (“L”) cytoplasmic tail (Table 2).

There are also a number of activating KIRs (Table 3). With the exception of KIR2DL4, they usually have short cytoplasmic tails and therefore carry an “S” in their names as, for example, KIR2DS1. Short cytoplasmic tails mediate activation via DAP12 (DNAX activation protein of 12 kDa) and immunoreceptor tyrosine-based activation motifs (ITAMs) (37).

The second group of NK cell receptors, the lectin-like receptors, are encoded on chromosome 12p. This group of receptors includes the NKG2A-F receptors. NKG2A and NKG2B both form a heterodimer with CD94 and inhibit NK cells via binding to the nonclassical HLA-E molecule (Table 2). In contrast to KIR, NKG2A is highly expressed on

**Table 2**  
**Inhibitory Natural Killer Cell Receptors**

Name	CD	Expression	Ligand
<b>Immunoglobulin-like receptors</b>			
KIR2DL	CD158a	NK cell subset, memory T cells	HLA-C group 2 alleles <sup>a</sup>
KIR2DL2/3	CD158b1/b2	NK cell subset, memory T cells	HLA-C group 1 alleles <sup>b</sup>
KIR3DL1	CD158e1	NK cell subset, memory T cells	HLA-Bw4
KIR2DL5	CD158f	NK cell subset, memory T cells	Unknown
KIR3DL2	CD158k	NK cell subset, memory T cells	HLA-A3, -A11
KIR3DL3	CD158z	NK cell subset, memory T cells	Unknown
ILT-2	CD85j	NK, T, B cells, monocytes	HLA-A, -B, -C, CMV-UL18
ILT-5	CD85a	NK cells, monocytes	Unknown
LIL-8	CD85c	NK cells, monocytes	Unknown
<b>Lectin-like receptors</b>			
KLRG1		NK cell and T cell subsets, basophils	Unknown
CD94-NKG2A/B	CD159a	NK cell subset, CD8 T cells	HLA-E loaded with HLA-A, -B, -C, or -G leader peptide
<b>Other receptors</b>			
LAIR-1	CD305	NK cell subset, DCs, monocytes, T, B cells	Ep-CAM
Irp60	CD300A	NK cell subset, DCs, monocytes, T, B cells	Unknown

DCs, dendritic cells.

<sup>a</sup>HLA-C group 2 alleles encode asparagine in position 77 and lysine in position 80.

<sup>b</sup>HLA-C group 1 alleles encode serine in amino acid position 77 and asparagine in position 80.

**Table 3**  
**Activating Natural Killer Cell Receptors**

Name	CD	Expression	Ligand
<b>Immunoglobulin-like receptors</b>			
KIR2DL4	CD158d	All NK cells	HLA-G
KIR3DS1	CD158e2	NK cell subset, memory T cells	Bw4?
KIR2DS5	CD158g	NK cell subset, memory T cells	Unknown
KIR2DS1	CD158h	NK cell subset, memory T cells	HLA-C group 2 alleles <sup>a</sup>
KIR2DS4	CD158i	NK cell subset, memory T cells	HLA-Cw4?
KIR2DS2	CD158j	NK cell subset, memory T cells	HLA-C group 1 alleles <sup>b</sup>
<b>Lectin-like receptors</b>			
CD94-NKG2C	CD159c	NK cell subset, memory T cells	HLA-E with HLA-A, -B, -C, or -G leader peptide
CD94-NKG2E/H		NK cell subset, memory T cells	HLA-E with HLA-A, -B, -C, or -G leader peptide
NKG2D	CD314	NK cell subset, memory T cells	MICA, MICB, ULBP-1, -2, and -3
<b>Natural cytotoxicity receptors</b>			
NKp30	CD337	NK cells	Unknown
NKp44	CD336	Activated NK cells	Influenza hemagglutinin
NKp46	CD335	NK cells	Influenza hemagglutinin
NKp80		NK cells, some T cells	AICL (activation induced C-type lectin)
<b>Other receptors</b>			
FcγRIII	CD16	NK, some γδ T cells, NKT cells	Fc of IgG
P75/AIRM	CDw328	NK cell subset, DCs, monocytes, T, B cells	Poliovirus receptor and lectin-1

<sup>a</sup>HLA-C group 2 alleles encode asparagine in position 77 and lysine in position 80.

<sup>b</sup>HLA-C group 1 alleles encode serine in amino acid position 77 and asparagine in position 80.

**Table 4**  
**Intrahepatic T Cell Populations**

Cell surface marker	Frequency	Range
CD3+CD56+	32%	11–54%
CD3-CD56+	21%	11–51%
γδ T cells	15%	7–34%
CD8α+CD8β-	15%	4–29%
CD4-CD8-	15%	3–29%

From refs. 15 and 132.

CD16/CD56<sup>bright</sup> cells. NKG2C, -E, and -F also bind CD94 but induce NK cell activation (Table 3). NKG2D is an exception, because it does not associate with CD94 and, instead, transmits a strong activatory signal upon binding to MICA and MICB (Table 3).

The third group of NK cell receptors includes the natural cytotoxicity receptors NKp30, NKp44, NKp46 and NKp80 molecules. These receptors have the ability to activate NK cells even in the absence of additional stimuli (Table 3) (38).

## NK CELLS IN LIVER DISEASE

NK cells play a major role in the early immune response to viruses. Since a prospective analysis of intrahepatic NK cells in the early course of a virus infection cannot be performed in humans, here we will use results from mouse models to outline the role of NK cells. Although mouse NK cells and human NK cells differ with respect to surface markers and inhibitory receptors, the general mechanisms of NK cell effector functions and NK cell inhibition by self-MHCs are very similar between species.

In mice, NK cell responses have been studied in a wide variety of viral infections (reviewed in ref. 39). NK cell activation and function are detectable within the first hours of a viral infection and often precede the adaptive immune response by days to weeks. The importance of these early NK cell responses for control of viral infections is evident in experiments with NK cell-depleted or NK cell-deficient mice. In contrast to wild-type mice, these mice display an increased susceptibility to infection with mouse cytomegalovirus (MCMV) (40), herpes simplex virus (41), influenza virus (42), and coxsackievirus (43). Likewise, isolated NK cell deficiencies in humans are known to be associated with a more severe and exacerbated course of herpesvirus infections.

The NK cell response is significantly enhanced by two cytokines that are released in response to virus infections: interleukin (IL)-12 is released by activated DCs and monocytes and induces strong IFN- $\gamma$  secretion by NK cells (22). In contrast, type I interferons (IFN- $\alpha$  and IFN- $\beta$ ) are secreted by virus-infected cells and enhance NK cell cytotoxicity. Very high concentrations of IFN- $\alpha$  or IFN- $\beta$  inhibit IL-12 induction in humans (44) and mice (45) and also make splenic NK cells refractory to IL-12 stimulation (46). Therefore, NK cell effector functions can be differentially regulated. Indeed, it has been observed in MCMV infection that NK cell-mediated cytotoxicity is more important in the spleen (47), whereas NK cell-mediated IFN- $\gamma$  production dominates in the liver (46). Most of the IFN- $\gamma$ -producing NK cells in the liver appear to be recruited from the blood, because mice that lack the chemokine MIP-1 $\alpha$  cannot support high levels of IFN- $\gamma$  production in the liver and therefore are not protected from MCMV-induced death (48). In contrast, wild-type mice exhibit strong NK cell-derived IFN- $\gamma$  production in the liver and are protected from death by early inhibition of MCMV replication (49). In addition to its direct antiviral effect, IFN- $\gamma$  is also essential for the induction of chemokines that recruit activated T cells to the site of infection (48). Finally, IFN- $\gamma$  promotes polarization of antigen-specific T cells toward a Th1 type.

Mouse models have also been useful to decipher strategies by which viruses escape from NK cell responses. Many viruses block or downregulate the expression of MHC molecules on the cells they infect in order to escape from recognition by CD8 T cells. According to the missing-self hypothesis, however, downregulation of MHC molecules renders these cells more susceptible to NK cell cytotoxicity. To escape from NK cell recognition, viruses such as CMV encode MHC class homologs and/or upregulate or stabilize other NK cell-inhibiting

molecules such as HLA-E (50). Another CMV-specific strategy involves the expression of a protein called UL16, which blocks the interaction between NKG2D, an activating receptor on NK cells, and host proteins (51).

In summary, NK cells play a major role in the early phase of viral infections. Apart from direct antiviral and cytotoxic effector functions, they secrete cytokines and chemokines that help to orchestrate the innate and adaptive immune response.

## NKT CELLS

NKT cells were originally defined as cells that express a TCR along with NK cell receptors such as CD161c, CD56, CD69, and CD94. Like T cells, human NKT cells can be CD4 positive, CD8 positive, or CD4/CD8 double negative. In contrast to conventional T cells, however, they display only a limited range of TCR variable (V) region genes. A high percentage of human NKT cells present with an invariant  $V\alpha 24-J\alpha 18$  rearrangement and recognize antigens in the context of CD1d (12,52). CD1d is one of five nonpolymorphic MHC class I glycoproteins (CD1a–e) (53). It is expressed on hematopoietic APCs such as macrophages, DCs, and T and B cells and on healthy hepatocytes. Although it is now well accepted that CD1d molecules present nonprotein and glycolipid antigens, few natural CD1d-restricted antigens have been identified so far, and most are components of mycobacterial walls (54,55). Therefore, most studies employ the synthetic glycolipid  $\alpha$ GalCer to study CD1d-restricted NKT cell functions.  $\alpha$ GalCer was originally derived from marine sponge and has been shown to activate NKT subsets in mice and humans in vitro and in vivo (55,56). Because the CD1d molecule is highly conserved between species, human NKT cells recognize mice CD1d and vice versa (56).

### NKT CELL FUNCTION

NKT cells respond to TCR ligation and to DC- and Kupffer cell-derived IL-12 (57). Upon activation, NKT cells rapidly release large quantities of cytokines such as IFN- $\gamma$  and TNF- $\alpha$  (type 1 cytokines) and IL-4, IL-10, and IL-13 (type 2 cytokines) (12,58). As described above, IFN- $\gamma$  as a type 1 cytokine not only has direct antiviral functions but also contributes to the activation of other innate immune cells, such as NK cells and monocytes, as well as cells of the adaptive immune response, such as CD4 and CD8 T cells. In contrast, type 2 cytokines are involved in suppression of tissue destruction/allograft tolerance (reviewed in ref. 59). Thus, NKT cells polarize the local and systemic adaptive immune responses to either a proinflammatory type 1 (IFN- $\gamma$ , TNF- $\alpha$ ) or an antiinflammatory type 2 (IL-4, IL-10, IL-13) profile.

The second major function of NKT cells is cytotoxicity. Cytotoxicity is CD1d restricted, either Fas mediated (60,61) or perforin dependent (58,62–64) and has been shown to be important in antitumoral immune responses. In a positive feedback loop, NKT cell stimulation may also enhance activation and IL-12 production by DCs via a CD40/CD40 ligand-mediated pathway (65,66). Finally, NKT cells have been shown to release chemokines such as MIP-1 $\alpha$  and thereby attract T cells.

### NKT SUBSETS IN HUMANS

The group of NKT cells includes a variety of cells that can be distinguished by their restriction element. Invariant NKT cells (also called type I NKT cells) express NK cell markers together with an invariant  $V\alpha 24$  TCR. The percentage of  $V\alpha 24$  NKT cells in the liver is similar to that in the peripheral blood and accounts for about 0.7% of all CD3 T cells (67). In contrast to invariant NKT cells in the peripheral blood, however, invariant NKT cells in the liver express the  $V\beta 11$  chain more frequently (64.2% vs 2.9%) (67). Furthermore, most invariant NKT cells in the peripheral blood are CD4 positive (67), whereas CD8 positive (28.3%) and CD4/CD8 double-negative NKT cells (28.6%) are much more frequent in the liver. Their TCR is restricted for CD1d, and they can be activated by  $\alpha$ GalCer. Upon activation, invariant NKT cells release typical Th1 and Th2 cytokines.

Variant CD1d-restricted NKT cells express diverse TCR $\alpha$  and  $\beta$  receptors and variable NK cell markers. These cells are found in the liver and the bone marrow. Although they are restricted by CD1d and they release typical Th1 and Th2 cytokines, they cannot be activated by  $\alpha$ GalCer.

$V\delta 3$   $\gamma\delta$  T NKT cells are found mainly in the liver and display a  $V\delta 3$ -restricted TCR repertoire and variable NK cell markers. These cells also express a typical Th1 and Th2 cytokine profile, but they are not restricted by CD1d and consequently, cannot be activated by  $\alpha$ GalCer.

In addition to these relatively well-defined NKT cell subsets, other heterogeneous subgroups of NKT cells with strong expression of NK cell markers and variable TCRs and restriction elements have been found throughout the human body.

### NKT CELLS IN LIVER DISEASE

NKT cells are implicated in immune responses to bacterial, viral, and parasitic infections (reviewed in ref. 68) as well as in antitumor immune responses (69). In general, intrahepatic NKT cells appear more activated than peripheral blood NKT cells. For example, expression of the NK cell marker CD161 and the activation marker CD69 is significantly increased in  $V\alpha 24$ -positive NKT cells in the liver (67). Upon stimulation with  $\alpha$ GalCer or a combination of phorbolmyristin acetate and ionomycin,  $V\alpha 24$ -positive NKT cells of the liver predominantly produce the type 1 cytokines IFN- $\gamma$  and TNF- $\alpha$  and only a little IL-2 (67).

Since a prospective analysis of intrahepatic NKT cells in the early course of a virus infection cannot be performed in humans, we will again refer to results from mouse models. Although NKT cell functions are similar in human and mice, there are differences with respect to compartmentalization. Whereas NKT cells present only about 4% of all lymphocytes in the human liver, they represent up to 20 to 30% of lymphocytes in the mouse liver (70). A further difference between human and mouse NKT cells is that the invariant  $V\alpha$ -chain of the TCR of mouse NKT cells is the result of a  $V\alpha 14$ - $J\alpha 281$  rearrangement. Nevertheless, the mouse TCR is also restricted by CD1d, and mouse NKT cells can be activated by  $\alpha$ GalCer.

The first evidence for a role of NKT cells in infections of the liver was derived from the observation that NKT- and/or CD1d-deficient mice are more susceptible to viral (71) and bacterial infections (72,73). NKT cell activation is at least partly IL-12 dependent and results in activation-induced death, as shown by a reduction of hepatic NKT cells in wild-type mice with acute MCMV infection, compared with IL-12-deficient mice with acute MCMV-infection (22,74). Artificial activation of NKT cells by injection of  $\alpha$ GalCer has been shown to inhibit viral replication (75) and to induce protection in a mouse model of diabetogenic encephalomyocarditis virus infection (76). Likewise, activation of NKT cells with  $\alpha$ GalCer induces IFN- $\gamma$  production and downregulation of hepatitis B virus (HBV) replication in a transgenic mouse model (75). Interestingly, the influence of NKT cells in these mouse models seems at least partly owing to recruitment and activation of NK and T cells rather than to a direct effect.

In addition to the classical NKT subset, the nonclassical NKT population also seems to impact on the course of viral infections of the liver, as has been shown in a mouse model of acute hepatitis B that is initiated by transfer of innate immune cells (77). On the other hand, however, there is also a downside to therapeutic NKT cell activation, which is the induction of liver injury by activated NKT cells (78). This is evident in the concanavalin A (ConA)-induced model of hepatitis, in which liver injury is dependent on NKT cell-mediated cytotoxicity (79).

### T CELLS

Intrahepatic T cells are found scattered throughout the liver parenchyma and more concentrated in the portal tracts. Conventional T cells are either CD8 positive or CD4 positive. Both populations display a diverse TCR $\alpha\beta$  repertoire and recognize antigens in the context of MHC class I and II molecules, respectively. CD8 T cells typically outnumber CD4 T cells in the liver, and the frequency of effector/memory cells is higher than in the blood. Unconventional T cells comprise various cell types that are categorized into two major populations: T cells that express NK markers (NKT cells; see previous section) and those that do not. The latter include the major group of TCR  $\gamma\delta$  cells (15–25% of all intrahepatic T cells) and CD4/CD8 double-negative and CD4/CD8 double-positive T cells (Table 4).

#### T-CELL FUNCTIONS

Priming of T cells and elicitation of T-cell effector functions require different signals. Resting, naïve CD8 T cells require two independent signals to become fully activated. The first signal is provided by the peptide-MHC I complex through the specific TCR. The second signal (costimulation) is independent of the antigen receptor and is critical to allow full activation and differentiation of CD8 T cells (80). Thus, only few, appropriately licensed bone marrow-derived professional APCs have the ability to initiate CD8 T-cell responses (81), most likely because they express costimulatory molecules and because they carry antigens from the site of infection into lymphoid organs

(80). Because of these specific requirements, T-cell priming is thought to occur predominantly in secondary lymphoid compartments. Whether T-cell priming also occurs in the liver itself is still controversially discussed (3,81–83). Once primed and differentiated, however, CD8 T cells recognize any target cell that expresses the cognate antigen in the context of MHC class I molecules. Because peptides from intracellular pathogens are predominantly presented on MHC class I molecules, effector functions of intrahepatic CD8 T cells have received special attention. These effector mechanisms include cytolytic mechanisms and the production of cytokines, such as IFN- $\gamma$  and TNF- $\alpha$ .

Three distinct mechanisms, namely, Fas-, TNF- $\alpha$ -, and/or perforin-based cell lysis, have been implicated in CD8 T-cell-mediated hepatocyte death during inflammatory liver disease. Fas-mediated death is a rapid process that occurs within several hours and requires neither RNA nor protein synthesis. Expression of Fas (CD95), a mediator of apoptosis (84), is upregulated on hepatocytes near liver-infiltrating cells (85), especially at the advancing edges of piecemeal necrosis (84), and Fas ligand is expressed on activated, liver, infiltrating T cells (86). In fact, Fas expression levels have been shown to increase with severity of inflammation in chronic hepatitis C virus (HCV) infection (84).

TNF-mediated apoptosis can be induced by membrane-bound (87) and soluble TNF- $\alpha$  (88–90). Membrane-bound TNF- $\alpha$  is expressed on the surface of liver-infiltrating, cytotoxic CD8 T cells (87), whereas soluble TNF- $\alpha$  is predominantly produced by macrophages (91) and to a smaller extent by antigen-stimulated lymphocytes (92).

Finally, the perforin-mediated mechanism of target cell lysis may contribute to the lysis of antigen-presenting, Fas- and TNF- $\alpha$ -resistant cells (93). The pore-forming protein perforin belongs to a family of serine proteases termed granzymes (94) and is stored within cytotoxic granules of CD8 T-cells and NK cells (95). Cytotoxic granules are vectorially secreted into the intercellular space, and cell lysis is associated with the formation of membrane lesions on the target cells. Granzyme B then triggers an endogenous cell death cascade by activating intracellular caspases (96,97). Morphological changes of the target cell, such as chromatin condensation, membrane blebbing, and ultimately nuclear DNA fragmentation (apoptosis) (98) are the ultimate signals of the cell death cascade (96,97).

Apart from this lytic and cytopathic effector function, intrahepatic lymphocytes have also been shown to mediate noncytolytic control of some hepatotropic viruses. In fact, the sparse scattering of these T cells within liver lobules among a large number of hepatocytes suggests a more efficient mechanism that does not require “one-on-one” contact between effector and target cells (99). In HBV infection, cytokines such as IFN- $\alpha/\beta$ , IFN- $\gamma$ , and TNF- $\alpha$  have been shown to inhibit viral gene expression and replication (100–102) and to clear hepatocytes from most of the infecting virus without causing liver disease. HBV nucleocapsid particles, replicative viral

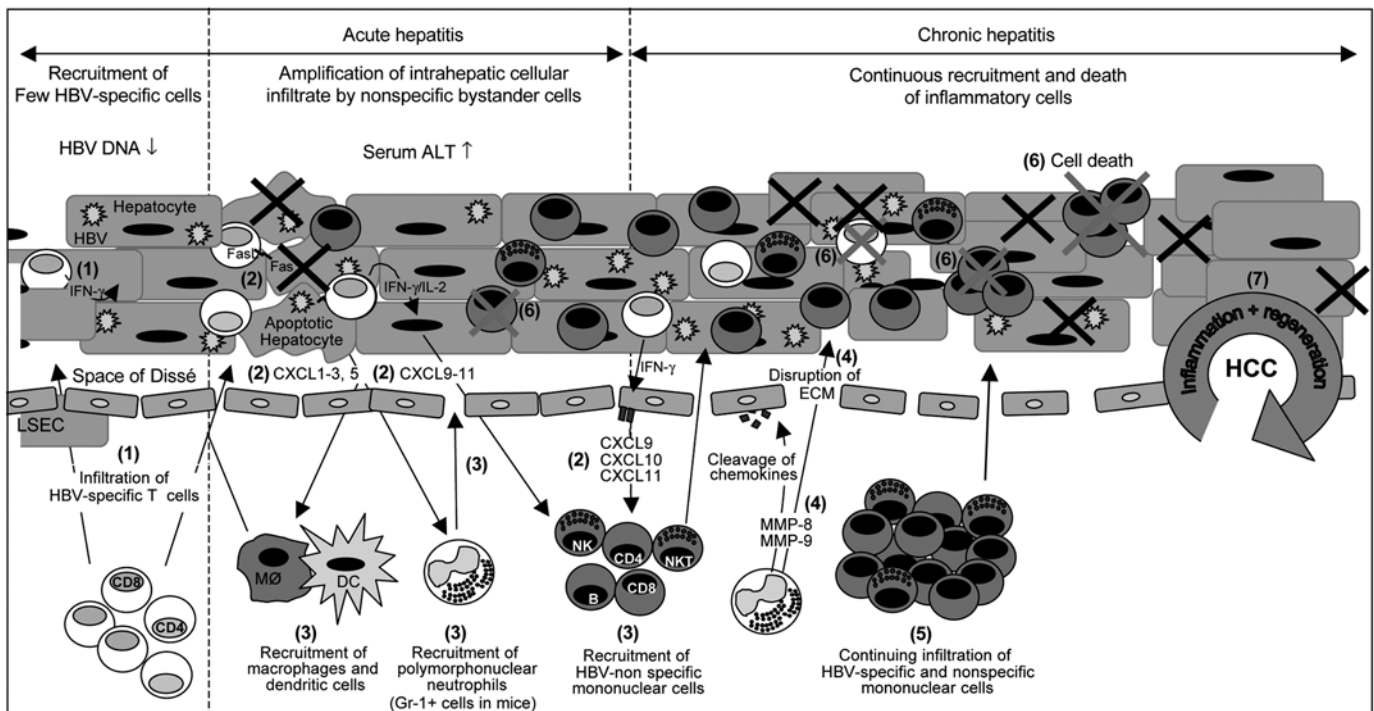
intermediates, and the episomal covalently closed circular HBV DNA, the transcriptional template of the virus, are all susceptible to these cytokine-mediated effects (103).

It is important to note that the optimal antiviral response varies from virus to virus and from organ to organ and may reflect a balance between suppressing viral replication and causing minimal tissue damage. Whereas cytopathic viruses such as vesicular poxviruses and influenza virus are mainly controlled by soluble mediators such as antibodies and interferons (104,105), control of a noncytopathic virus such as lymphocytic choriomeningitis virus (LCMV) depends critically on perforin-mediated lysis of infected cells. In the absence of perforin, persistent LCMV infection may often lead to the overproduction of cachectic cytokines, such as TNF- $\alpha$  and IFN- $\gamma$  and cell death (106). Finally, the optimal antiviral response also depends on the infected cell type: MCMV infection of the spleen, for example, is controlled by perforin-secreting NK cells, whereas MCMV infection of the liver is predominantly controlled by IFN- $\gamma$  produced by intrahepatic NK cells (47).

### T CELLS IN LIVER DISEASE

Persistent inflammatory responses in the liver owing to an ongoing T-cell response are regarded as the principal mechanism for necroinflammatory liver injury that leads to fibrosis and, ultimately, cirrhosis of the liver (107). In fact, this inflammatory process is sufficient to cause hepatocellular carcinoma, as demonstrated in a mouse model of chronic inflammation (108,109).

A detailed and sequential analysis of the factors that contribute to the immunopathogenesis of virus-induced liver disease has been performed in transgenic mice that replicate the complete HBV genome in their hepatocytes (Fig. 3). When hepatitis B surface antigen (HBsAg)-specific CD8 T cells are adoptively transferred into transgenic mice that replicate HBV in the liver, they recognize their cognate antigen, resulting in contact-dependent lysis of a small number of hepatocytes (110) and in IFN- $\gamma$ -mediated downregulation of HBV replication throughout the liver. At the same time, sinusoidal endothelial cells, macrophages, and hepatocytes produce chemokines such as CXCL9, CXCL10, and CXCL11, and vascular endothelial cells in the portal tracts produce CCL3 and CCL5. These chemokines attract additional CXCR3- and CCR5-expressing T cells as well as neutrophils, NK cells, and NKT cells (21,110–113), thereby inducing a secondary amplification of the intrahepatic infiltrate. During this process, activated peripheral blood T cells are recruited to the liver regardless of their antigen specificity (114) and rapidly outnumber the adoptively transferred HBV-specific CD8 T cells. Interestingly, recruitment of these mononuclear cells can be reduced by either inactivation of macrophages, neutralization of CXCL9 or CXCL10, or depletion of polymorphonuclear neutrophils (115). Recruitment of antigen-nonspecific mononuclear cells can also be reduced by blocking neutrophil-derived matrix metalloproteinases (MMP)-8 and MMP-9 (116). Based on these findings, it has



**Fig. 3.** Simplified schematic presentation of key factors that contribute to the pathogenesis of T-cell-mediated liver disease in hepatitis B. The model presented is based on the study of acute (93,103,115,116) and chronic (108) hepatitis in transgenic mice that replicate HBV in the liver. In this model, acute hepatitis is initiated by adoptive transfer of HBsAg-specific CD8 T cells. (1) Infiltration of HBV-specific CD8 T cells, lysis of HBV-infected hepatocytes, and interferon- $\gamma$  (IFN- $\gamma$ )-mediated downregulation of HBV replication throughout the liver occur shortly after adoptive transfer of HBsAg-specific CD8 T cells into transgenic mice that replicate HBV in the liver. Individual apoptotic hepatocytes (Councilman bodies) are detectable, but serum alanine aminotransferase, (ALT) levels remain normal. (2) IFN- $\gamma$  stimulates production of CXCL9, CXCL10, and CXCL11 by sinusoidal endothelial cells (LSEC), macrophages, and hepatocytes, production of CCL3 and CCL5 by portal tract vascular endothelium, and release of murine cytokine-induced neutrophil chemoattractant (KC), macrophage inflammatory protein-2 (MIP-2), and lipopolysaccharide-induced chemokine (The human homologs of these cytokines are CXCL1-3 and CXCL5.) (3) The released chemokines recruit NK cells and NKT cells, neutrophils, and CXCR3-positive and CCR5-positive T cells. Histological, serological, and clinical evidence of acute hepatitis results. (4) Recruitment of antigen-nonspecific mononuclear cells requires neutrophils and specific matrix metalloproteinases (MMP-8 and MMP-9), suggesting that remodeling of the extracellular matrix (ECM) by metalloproteinase facilitates leukocyte trafficking through the endothelial barrier and within the liver. (5, 6) If HBsAg-specific CD8 T cells are reconstituted, continued recruitment and death of inflammatory cells and hepatocytes contribute to the development of adenoma and eventually (7) hepatocellular carcinoma (HCC). IL-2, interleukin-2.

been suggested that the secreted, neutrophil-derived MMPs remodel the extracellular matrix of the liver and thereby facilitate intrahepatic recruitment and migration of large numbers of activated bystander cells. This bystander infiltrate is associated with significant liver injury (110) but is not required for noncytolytic downregulation of HBV replication (111,116).

Because most activated T cells are thought to undergo activation-induced cell death in the liver (114), continuous recruitment and death of antigen-specific T cells and non-specific bystander T cells and other inflammatory cells are required. In HBV- and/or HCV-infected humans, most lymphocytes infiltrate the portal tracts and reside perivascularly during the early stages of disease, and few are found intra-lobularly in contact with hepatocytes (117). As chronic liver injury progresses, the inflammatory infiltrate moves from the portal tracts toward the central veins, a feature characterized as piecemeal necrosis. Accordingly, the size of the intrahepatic inflammatory infiltrate has been used as a marker for the

severity of chronic hepatitis B and C. Ultimately, the liver lobules are surrounded and isolated from each other by newly synthesized fibrous tissue.

## CONCLUDING REMARKS AND OPEN QUESTIONS

As our understanding of innate and adaptive cellular immune responses in the liver increases, the interplay of these diverse cell populations and their roles in the outcome and pathogenesis of different types of liver diseases are increasingly recognized. Questions that remain to be answered are whether and how common inflammatory pathways can be manipulated to modify the natural history of important viral, parasitic, autoimmune, and malignant diseases of the liver.

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# 7 Cytokines in Liver Health and Disease

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## KEY POINTS

- Cytokines are soluble peptides secreted by several kinds of cells; they mediate many immune and inflammatory reactions, and regulate several biochemical processes in and around the cells that produce them. They may act on different cell types, and have overlapping effects, and their action may be local or systemic.
- Monocytes and macrophages are major cytokine sources. They are found in many tissues, but the largest number are in the liver, where they are called Kupffer cells. Nearly 80% of all macrophages in the body are Kupffer cells.
- CD4<sup>+</sup> (helper) T lymphocytes are another important source of cytokines. Two distinct subsets of CD4<sup>+</sup> helper T-cells exist, Th1 and Th2, which can be distinguished by their cytokine patterns, with Th1 cells producing mainly interleukin (IL)-2 and interferon (IFN)- $\gamma$ , and Th2 cells producing IL-4, IL-5, IL-6, IL-10, and IL-13.
- Chemokines represent a distinct cytokine subfamily with a crucial role in determining which leukocyte subsets are recruited from the circulation to injured tissue in different conditions.
- Constitutive production of cytokines is absent or minimal in most tissues, including the liver. However, as physiologic and pathologic stimuli activate cells, the production of these molecules increases, and they orchestrate the tissue's response to the stimulus. A number of inflammatory chemokines have been associated with liver diseases, and in most cases their role is clearly linked to selective recruitment of leukocyte subsets, thus playing a direct role in pathogenesis.
- Acute-phase proteins are synthesized almost exclusively in the liver, and their concentration increases rapidly after liver stimulation. During stress conditions, the hepatocytes, stimulated by cytokines produced by monocytes/macrophages at the site of injury, secrete several inducible proteins to restore homeostasis and to block the cause of injury. Acute-phase proteins have different functions: hemostatic, microbicidal, phagocytic, antiproteolytic, and antithrombotic.
- Chronic alcohol use produces adverse effects on the immune system. Several studies have demonstrated that patients with alcoholic liver disease have increased levels of the cytokines IL-1, IL-6, tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), and others, as well as the chemokine IL-8/CXCL8.
- Nonalcoholic fatty liver disease (NAFLD) may evolve into steatohepatitis (NASH), which is a metabolic liver disease in which steatosis is associated with hepatic infiltration of immune cells that leads to liver inflammation and eventually fibrosis. TNF- $\alpha$  has an important role in NASH pathogenesis.
- The first line of defence against viral infections is represented by the production of cytokines that have both antiviral and immunomodulatory actions. Cytokines play a key role in coordinating the inflammatory response against the hepatitis B (HBV) and hepatitis C (HCV) viruses, but this response may also lead to liver damage.
- A skewed immune response toward a type 1 or type 2 pattern plays a role in the pathogenesis of primary biliary cirrhosis, primary sclerosing cholangitis, and autoimmune hepatitis, the main chronic autoimmune liver diseases in adults.
- Liver mass after partial hepatectomy is replenished by replication of existing hepatocytes rather than by replication and differentiation of intrahepatic progenitor cells. The activation of multiple pathways during liver regeneration is orchestrated by cytokines like TNF- $\alpha$  and IL-1/IL-6, which interact with growth factors.
- The liver damage derived from hypoxic circumstances is commonly increased during reperfusion, a process called *ischemia-reperfusion injury*. During the ischemic phase there is activation of the endothelium with an increase in permeability and expression of adhesion molecules that are important for the recruitment of inflammatory cells in the tissue. Upon reperfusion, adherent leukocytes and activated Kupffer cells release reactive oxygen species and several cytokines, thus enhancing the inflammatory response.

## INTRODUCTION

Cytokines are soluble peptides secreted by several kinds of cells, they mediate many immune and inflammatory reactions, and regulate several biochemical processes in and around the cells that produce them. They may act on different cell types

(pleiotropic effects) and have overlapping effects (redundancy); furthermore, their action may be local or systemic. In most tissues, including the liver, constitutive production of cytokines is absent or minimal. However, as physiologic and pathologic stimuli activate cells, the production of these molecules increases, and they orchestrate the tissue's response to the stimulus. Phenotype of the immune response is a function of the repertoire of cytokines produced in the early phases (1).

Monocytes and tissue-resident macrophages are major cytokine sources. Macrophages are found in many tissues, but the largest number are in the liver, where they are called Kupffer cells (2). Nearly 80% of all macrophages in the body are Kupffer cells (3). Together with other immune cells they generate an acute inflammatory reaction, which is the body's first line of defence. Another important source of cytokines is CD4<sup>+</sup> (helper) T lymphocytes. The interaction between monocytes/macrophages and T lymphocytes activates T lymphocytes, determining their multiplication and production of cytokines. Two distinct subsets of CD4<sup>+</sup> helper T cells exist, Th1 and Th2, which can be distinguished by their cytokine patterns, with Th1 cells producing mainly interleukin (IL)-2 and interferon (IFN)- $\gamma$  (which activate CD8<sup>+</sup> cytotoxic T cells and macrophages) and Th2 cells producing IL-4, IL-5, IL-6, IL-10, and IL-13 (which activate B lymphocytes for antibody production) (4). Th1 cells and their relative cytokine products are thought to be involved in delayed-type hypersensitivity reactions and organ-specific autoimmune disorders; in contrast, Th2 cells and their cytokine products are considered to participate in allergic reactions and systemic autoimmune disorders. The signature cytokines of Th1 and Th2 subsets inhibit each other's secretions and consequently influence lymphocyte proliferation, resulting in a dynamic balance of the subsets within inflamed tissues.

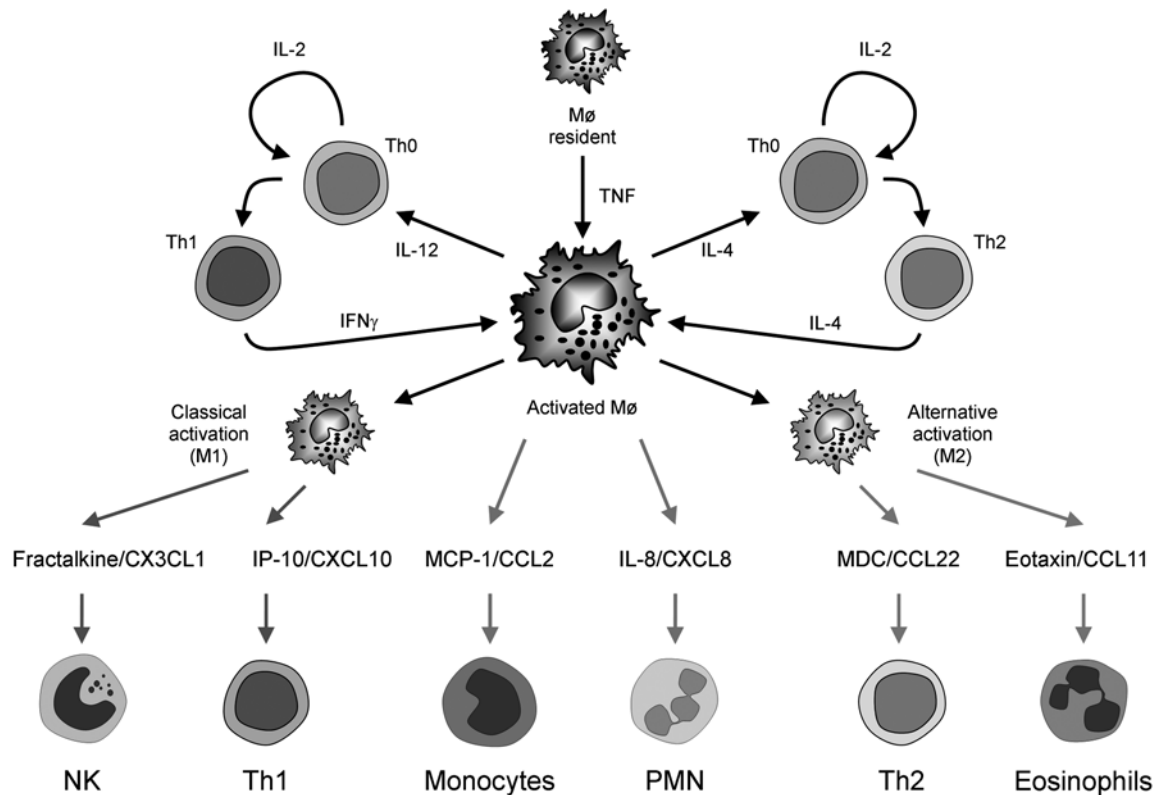
Since the original description of the Th1 and Th2 sets of cytokines, it has been recognized that cells other than CD4<sup>+</sup> lymphocytes can produce similar cytokine patterns, which has prompted a broader classification of the respective immune responses into type 1 and type 2, rather than strictly Th1 and Th2. Furthermore, a subset of cells producing both type 1 and type 2 cytokines and a subset characterized by IL-10 and transforming growth factor- $\beta$  (TGF- $\beta$ ), production have been identified and designated as Th0 and Th3 (5,6), respectively. Although a clear-cut distinction between type 1 and type 2 immune responses is more difficult in the human than in the mouse, altered Th1/Th2 balances have been demonstrated in various autoimmune diseases not only in representative animal models but also in human pathologies (7,8). Finally, a regulatory role is also played by CD4<sup>+</sup>/CD25<sup>+</sup> T lymphocytes, which mediate antigen-specific suppression of T lymphocyte responses by local secretion of IL-10 and TGF- $\beta$  (9).

The cytokine network activated in response to pathologic conditions acts through the local recruitment of distinct combinations of effector cells (Fig. 1). A distinct cytokine subfamily with a crucial role in determining which leukocyte subsets are recruited from the circulation to injured tissue in different conditions is represented by chemokines (short for

*chemotactic cytokines*), acting as chemoattractants that induce target cell migration along a gradient. The chemokine system includes about 50 members, which can be divided into four families on the basis of their molecular structure. The largest family includes 28 members mainly active on mononuclear cells (i.e., lymphocytes and monocytes), all characterized by the presence of two cysteine residues adjacent to each other in the N-terminal portion of the molecule, thus termed CC chemokines. The second family includes 16 members with one intervening amino acid separating the first two cysteine residues (10) and thus termed the CXC family. This family can be further subdivided into two groups, based on whether or not a molecule carries an ERL (glutamic-leucine-arginine) motif that immediately precedes the first cysteine residue. ERL<sup>+</sup> CXC chemokines are important in neutrophil chemotaxis and angiogenesis, whereas ERL<sup>-</sup> CXC chemokines, are angiostatic and act mainly on T lymphocytes (11). Two minor families, called C and CX3C chemokines, include a limited number of members and are mainly involved in the recruitment of selected T-lymphocyte subsets and natural killer (NK) cells. Classically, the chemokines were named according to their expression patterns or functions, but owing to the rapid discovery of new chemokines in 2000, Zlotnik and Yoshie (12) proposed a new classification system for chemokines based on the subfamily followed by a number provided by the position of the corresponding coding gene in the cluster. Thus, chemokines are now identified by a name providing information on the structural subfamily, corresponding also to the type of receptor they engage, followed by a number provided by and referring to the respective coding gene.

The biological effects of chemokines are mediated by a subfamily of G protein-coupled seven-transmembrane domain receptors. Although each chemokine receptor usually binds more than one ligand, thus having redundant activity, nonetheless they respect ligand family boundaries; therefore chemokine receptors are classified as CC chemokine receptors (CCR; 10 at present), CXC chemokine receptors (CXCR; 6 at present), C chemokine receptors (XCR; 1 at present), and CX3C receptors (CX3CR; 1 at present) (13). Some chemokines are expressed at high levels in specific tissues (tonic chemokines) and are involved in homeostatic functions such as thymocyte maturation/selection and lymphocyte recirculation (*see below*). However, most chemokines are not expressed in homeostatic conditions and are rapidly induced in pathologic conditions (fasic or inflammatory chemokines). In this case, tissue damage induces a specific cytokine milieu, which in turns defines the composition of the inflammatory response acting on the combination of chemokines present in the microenvironment (Fig. 1).

Master cytokines, which activate polarized responses differentially, regulate chemokine production. For instance, the type 2 cytokines IL-4 and IL-13 induce production of chemokines that interact with receptors preferentially expressed on polarized type 2 T cells, including MDC/CCL22 and TARC/CCL17 (agonists for CCR4), eotaxin/CCL11 (agonist for CCR3), and I-309/CCL1 (agonist for CCR8). Conversely,



**Fig. 1.** Cytokine-chemokine circuitry acting in polarized immune responses. IFN, interferon; IL, interleukin; IP-10, interferon- $\gamma$ -inducible protein; MCP, monocyte chemoattractant protein; MDC, macrophage-derived chemokine; M $\phi$ , macrophage; NK, natural killer; PMN, polymorphonuclear cells; TNF, tumor necrosis factor.

interferon (IFN)- $\gamma$  inhibits production of MDC/CCL22 in different cell types and induces expression of CXCR3 agonists, which are active on receptors expressed on type 1 T cells. Hence these chemokines supporting selective recruitment of polarized T cells and specific type 1 and type 2 effector cells expressing distinct panels of chemokine receptors are involved in the amplification of polarized responses (14).

A number of inflammatory chemokines have been associated with liver diseases (see ref. 15 and Table 2 for selected references), and in most cases their role is clearly linked to selective recruitment of leukocyte subsets; thus they play a direct (mostly negative) role in pathogenesis. Chemokine receptor inhibitors are in advanced development and might be available as therapy within the next few years. However, it is worth mentioning that although the pathognomonic biological activity of chemokines is leukocyte recruitment, some members of this large family also have other, nonchemotactic biological activities, some of which are of possible relevance in liver diseases (10). For example, CXC chemokines regulate angiogenesis (ELR<sup>+</sup> CXC chemokines being proangiogenic and ELR<sup>-</sup> CXC chemokines antiangiogenic), CC chemokines have been associated with fibrosis, and some chemokines have been demonstrated to control apoptosis and cell survival in specific cases. Thus, caution must be used in inferring a negative role for chemokine expression in the pathogenesis of liver diseases.

This is consistent with experimental data in gene-targeted animal models showing that some chemokines may play a positive role, acting as hepatocyte protectors or sustaining parenchyma regeneration (13,16).

## CYTOKINES IN THE HEALTHY LIVER

Under normal conditions liver cells produce only minimal levels of cytokines, and as a consequence only a small quantity of cytokines are detected by immunohistochemistry on liver sections. The weak staining of chemokines is confined to the vascular endothelium and to inflammatory cells around blood vessels. This observation suggests that low-level chemokine secretion occurs in normal liver and could be important for the regulation of leukocyte recruitment during physiological immune surveillance. An exception is represented by the homeostatic CC chemokine liver and activation-related chemokine (LARC/CCL20), which acts on CCR6 to regulate homeostatic recirculation in the liver of memory T cells (17).

### ACUTE-PHASE RESPONSE

An important interaction between liver and cytokines can be seen in the *acute-phase response*, an orchestrated response to tissue injury, infection, or inflammation (18). The acute-phase response is characterized by a pattern of induced hepatocyte-derived proteins and is a nonspecific first line of defence and homeostasis against a broad range of invaders. However, local

**Table 1**  
**Characteristics of Cytokines Involved in Liver Diseases**

<i>Cytokine</i>	<i>Main source</i>	<i>Effects</i>	<i>Implicated in</i>	<i>Ref.</i>
IL-1	Macrophages Antigen-presenting cells	Proinflammatory	Alcoholic disease	34,35
		Fever	Liver regeneration	33
		Acute-phase response	Ischemia-reperfusion	16
IL-6	Antigen-presenting cells Th2 cells	Proinflammatory	Alcoholic disease	34,35
		Fever	Liver regeneration	33
		Activates T lymphocytes		
		Differentiates B lymphocytes		
TNF- $\alpha$	Macrophages NK cells	Acute-phase response		
		Similar to IL-1	Alcoholic disease	34,35
			NASH	43
			Liver regeneration	33
IL-12	Activated hepatocytes	Stimulates NK cells and T lymphocytes	Ischemia-reperfusion	16
		Stimulate IFN- $\gamma$ production	Ischemia reperfusion	84
			Viral hepatitis	48,56
TGF- $\beta$	Macrophages Th3 cells	Antiinflammatory	Liver regeneration	33
		Inhibits B, T, and NK cells	Liver fibrosis	29,30
		Stimulates fibrogenesis		
IL-10	B and Th2 cells Macrophages	Antiinflammatory	Control of inflammation	4
		Inhibits IFN production		
IFN- $\alpha$	Macrophages	Stimulates B lymphocytes		
		Inhibits viral replication	Viral hepatitis	48,50
IFN- $\gamma$	Th1 cells NK cells	Stimulates NK cells		
		Modulates IL-1 and TNF- $\alpha$	Viral hepatitis	48,50
		Increases MHC expression		
		Inhibits viral replication		

Abbreviation: IFN, interferon; IL, interleukin; NASH, nonalcoholic steatohepatitis; TGF, transforming growth factor; TNF, tumor necrosis factor.

inflammation or injurious processes in the liver may also induce an acute-phase response. Acute-phase proteins are synthesized almost exclusively in the liver, and their concentration increases rapidly after liver stimulation (19). During stress conditions, the hepatocytes, stimulated by cytokines produced by monocytes/macrophages at the site of injury, secrete several inducible proteins, in order to restore homeostasis and block the cause of injury; the liver production of constitutive proteins such as albumin is therefore decreased. Acute-phase proteins have different functions: hemostatic, microbicidal, phagocytic, antiproteolytic, and antithrombotic. They can be divided into two groups, the production of which is influenced by the presence of different cytokines: type I proteins like C-reactive protein, serum amyloid A, and the C3 component of complement are released by hepatocytes in response to TNF- $\alpha$ , IL-1, and IL-6 stimulation; hepatocyte production of type II proteins, like fibrinogens,  $\alpha$ 1-antitrypsin, and ceruloplasmin is stimulated only by the IL-6 family of cytokines. These two different groups have two different types of signal transduction: IL-1-like cytokine receptors initiate the conversion of membrane sphingomyelin to ceramide via sphingomyelinase, whereas IL-6-like cytokine receptors activate Janus tyrosine kinases (20,21). Uncontrolled and prolonged action of cytokines is potentially harmful; therefore mechanisms exist that limit their activity (soluble cytokine receptors, receptor antagonists) (22).

## CYTOKINES IN LIVER DISEASE

In response to various liver injuries (viral agents, alcohol consumption, hepatotoxins, autoimmunity, ischemia), hepatocyte damage causes the recruitment of neutrophils and macrophages that produce cytokines and chemokines in hepatic tissue; the cytokines mediate the inflammatory response that leads to the regeneration of liver tissue and ultimately to the deposition of extracellular matrix by activation of hepatic stellate cells (HSCs).

Under normal conditions, the levels of these proteins that promote inflammation decrease once the infection is under control. However, if the inflammation continues for a long time, persistent production of cytokines may lead to scar tissue formation and liver cirrhosis. Thus, cytokine production can have both beneficial and harmful effects, depending on the amount and duration of cytokine release.

The main liver cells that produce cytokines are the resident macrophages, i.e., Kupffer cells, which constitute the largest reservoir of tissue macrophages in the body. Particularly important cytokines for the liver are TNF- $\alpha$ , IL-1, IL-6, IFNs, TGF- $\beta$ , and chemokines (Tables 1 and 2) (23).

The production of TNF- $\alpha$  is one of the earliest events in several types of liver injury (24). It can initiate hepatocyte apoptosis and trigger the production of other cytokines and chemokines, which together recruit inflammatory cells, kill

**Table 2**  
**Characteristics of Chemokines Involved in Liver Diseases<sup>a</sup>**

<i>Chemokine</i>	<i>Family</i>	<i>Receptor</i>	<i>Target</i>	<i>Implicated in</i>	<i>Ref.</i>
IL-8/CXCL8 (CINC)	CXC (ELR <sup>+</sup> )	CXCR1/CXCR2	Neutrophils	Alcoholic disease GVDH disease	86 87
				Bacterial hepatitis	88
				Ischemia-reperfusion	89
ENA-78/CXCL5 (MIP-2)	CXC (ELR <sup>+</sup> )	CXCR2	Neutrophils	Bacterial hepatitis	90
				Ischemia-reperfusion	89
GRO/CXCL1 (KC)	CXC (ELR <sup>+</sup> )	CXCR2	Neutrophils	Ischemia-reperfusion	91
				Bacterial hepatitis	92
IP-10/CXCL10	CXC (ELR <sup>-</sup> )	CXCR3	NK cells	Alcoholic disease	54,93
			Th1 cells	Viral hepatitis	
MIG/CXCL9	CXC (ELR <sup>-</sup> )	CXCR3	NK cells	Viral hepatitis	94
			Th1 cells	Liver cancer	95
				Graft rejection	96
SDF-1 $\alpha$ /CXCL12	CXC (ELR <sup>-</sup> )	CXCR4	Multiple	Graft rejection	96
				Liver cancer	97
MCP-1/CCL2	CC	CCR2	Monocytes	Ischemia-reperfusion	37
			Immature DCs	Alcoholic disease	98
				Liver fibrosis	99
				Bacterial hepatitis	100
MIP-1 $\alpha$ /CCL3	CC	CCR1/CCR5	Monocytes	GVDH disease	101
			Immature DCs	Bacterial hepatitis	102
			Th1 cells	Viral hepatitis	100
				Alcoholic disease	103,104
RANTES/CCL5	CC	CCR1/CCR5	Monocytes	Autoimmune diseases	105
			Immature DCs	Viral hepatitis	106
			Th1 cells	Graft rejection	107,108
Eotaxin/CCL11	CC	CCR3	Eosinophils	Fulminant hepatic failure (acetaminophen toxicity)	109
TARC/CCL17 and MDC/CCL22	CC	CCR4	Th2 cells	Fulminant hepatic failure (postinfection model)	110,111 112
LARC/CCL20	CC	CCR6	Immature DCs	Viral hepatitis	113
			Tm cells		
Fractalkine/CX3CL1	CX3C	CX3CR1	Th1 cells	Fulminant hepatic failure (acetaminophen toxicity)	114

Abbreviations: CINC, cytokine-induced neutrophil chemoattractant; MIP, macrophage inflammatory protein; MCP, monocyte chemotactic protein; IP, interferon- $\gamma$ -inducible protein; MIG, monokine induced by interferon- $\gamma$ ; SDF, Stroma-derived factor; TARC, thymus and activation-regulated chemokine; MDC, macrophage-derived chemokine; LARC, liver and activation-regulated chemokine; DCs, dendritic cells; Tm, memory T lymphocytes; GVDH, graft-versus-host disease.

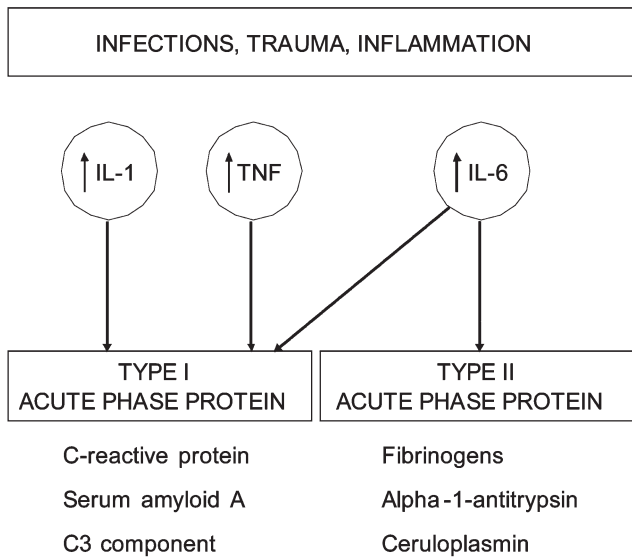
<sup>a</sup>The table reports chemokines (old/new nomenclature) associated with liver diseases, with the main target leukocytes and receptors involved. References supporting a pathogenetic role of a specific ligand/receptor in liver diseases, mostly inferred by animal models using blocking antibodies or gene-targeted animals, are provided. The names of rodent chemokines that differ from the human counterpart are provided in parentheses.

hepatocytes, and initiate a healing response that includes fibrogenesis (25) (Fig. 2). Apoptosis is a form of cell death characterized by organized nuclear and finally cellular fragmentation. It is regulated by a great number of pathways. The interaction between TNF- $\alpha$  and its cellular receptor is one of these pathways; moreover, the engulfment of apoptotic bodies by Kupffer cells induces the expression of death ligands that continue the apoptotic stimulation (26,27). TNF- $\alpha$  perpetuates inflammation through the activation of nuclear factor- $\kappa$ B (NF- $\kappa$ B), a transcriptional factor that regulates the expression of several cytokine and chemokine genes (28). Further, TGF- $\beta$  is the most potent cytokine for enhancing hepatic fibrinogenesis by stimulating the activation of HSCs (29) and is generated when apoptotic bodies are encountered

(30). Under normal conditions, HSCs are resident perisinusoidal mesenchymal cells that mainly serve to store fat and vitamin A in the liver. When activated, they assume the features of fibrogenic, contractile myofibroblasts and produce collagen, the major component of fibrotic tissue. In addition, activated HSCs mediate the inflammatory response by the production of several cytokines and chemokines (31,32). Finally, IL-1 and IL-6 are also involved in the hepatic acute-phase response (18) and in liver regeneration (33).

### ALCOHOLIC LIVER DISEASE

Chronic alcohol use produces adverse effects on the immune system; clinical studies have demonstrated that patients with alcoholic liver disease have increased levels of the cytokines



**Fig. 2.** Schematic representation of the liver acute phase. IL, interleukin; TNF, tumor necrosis factor.

IL-1, IL-6, TNF- $\alpha$ , and others, as well as the chemokine IL-8/CXCL8. Several studies have found that alcohol may increase the liver's sensitivity to these inflammatory cytokines in different ways (34). First, alcohol increases intestinal permeability, and the translocation of bacterial lipopolysaccharide (LPS) from the intestinal lumen to the portal circulation stimulates Kupffer cells to produce and release TNF- $\alpha$  into liver sinusoids. Second, alcohol enhances the sensitivity of hepatocytes to TNF- $\alpha$ . Third, elevated levels of TNF- $\alpha$  contribute to make hepatocytes susceptible to undergo apoptosis. Importantly, the levels of TNF- $\alpha$  correlate with clinical outcome (35,36).

Chemokines expressed in the sinusoids in alcoholic hepatitis would promote the recruitment of neutrophils, monocytes, and lymphocytes to the parenchyma, thus sustaining inflammation (37). The persistent presence of an inflammatory condition leads to the production of profibrogenic cytokines such as TGF- $\beta$ , which stimulate the development of liver fibrosis. TGF- $\beta$  is overproduced in the liver of patient with alcoholic cirrhosis compared with healthy subjects; it contributes to liver damage by activating HSCs (38). After an acute liver injury, parenchymal cells regenerate and replace the necrotic or apoptotic cells. This process is associated with an inflammatory response and a limited deposition of extracellular matrix (ECM). If the hepatic injury persists (i.e., persistent alcohol consumption), hepatocytes may be substituted with abundant ECM. Collagen production by HSCs is a crucial step in the development of fibrosis in patients with alcoholic steatohepatitis: the balance of production and degradation of ECM components maintains normal liver structure. The increased production with decreased degradation leads to disordered deposition of fibrillar collagen types I and III, resulting in liver fibrosis (39). Secreted collagens I and III are degraded by

members of the metalloproteinase (MMP) family (40). Some evidence indicates that initiation of degradation of fibrillar collagens I and III is made by and limited to an MMP with interstitial collagenase activity, such as MMP-1, -8, or -13. These MMPs cleave collagen at a single site a quarter of the way along the molecule. This cleavage allows the collagen to unwind partially and renders it susceptible to degradation by more promiscuous MMPs and other proteases (40). More recent evidence shows that MMP-14 and MMP-2 also have potential interstitial collagenase activity (41). It is now clear from experimental studies of liver fibrosis that progressive fibrosis is characterized not only by an exuberant secretion of collagens I and III and other matrix molecules but also by a change in the pattern of their degradation.

### NONALCOHOLIC FATTY LIVER DISEASE AND STEATOHEPATITIS

In some cases, nonalcoholic fatty liver disease (NAFLD) evolves into nonalcoholic steatohepatitis (NASH), a metabolic liver disease in which steatosis is associated with hepatic infiltration of immune cells that leads to liver inflammation and eventually fibrosis. The molecular mechanisms that lead to the development of these conditions are not clearly understood (42). TNF- $\alpha$  plays an important role in NASH pathogenesis. In addition to the effects shared with alcoholic steatohepatitis, TNF- $\alpha$  has been proposed as a factor that causes (or accentuates) and perpetuates insulin resistance. TNF- $\alpha$  seems to act like an antagonist of insulin receptors, reducing insulin sensitivity, but the mechanism is unclear and requires further investigation (43). In conditions of insulin resistance, furthermore, the liver accumulates triglycerides, thus developing steatosis (44). An increase in TNF- $\alpha$  expression has been found in patients with NASH, and levels correlate with the severity of the inflammation.

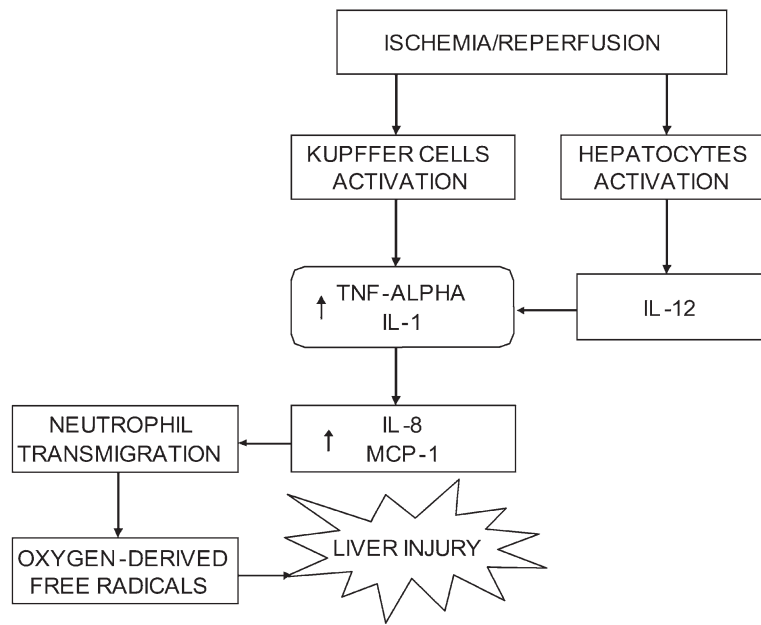
An open question relates to the "adipokine" family: these cytokines, represented by leptin and ghrelin, are secreted by adipocytes (45). Interestingly, the production of leptin is increased in patients with NASH (but also in other chronic liver diseases) (46). Leptin seems to act as a profibrogenic cytokine, directly and indirectly stimulating TGF- $\beta$  expression (47).

### VIRAL HEPATITIS

Hepatitis B (HBV) and hepatitis C (HCV) viruses are the main causes of chronic liver disease worldwide. These viruses are hepatotropic but not directly cytopathic. Importantly, the host immune response is critical in determining the resolution of the infection or the onset of a chronic form (48). An immune response too weak to clear the virus but sufficient to perpetuate the destruction of infected hepatocytes can induce chronic inflammatory disease leading to liver cirrhosis.

The first line of defence against viral infections is represented by the production of cytokines that have both antiviral and immunomodulatory actions. These share the potential to inhibit viral replication (by mediating the production of RNase and proteinase) and determine the predominant pattern of immune response. Cytokines play a key role in coordinating the inflammatory response against the virus but may also cause liver





**Fig. 3.** Schematic representation of ischemia-reperfusion liver injury. IL, interleukin; MCP, monocyte chemoattractant protein; TNF, tumor necrosis factor.

damage. Experimental evidence suggests that liver pathology in HCV-infected individuals is a direct result of the intrahepatic immune response to the virus (49). In the response to viral hepatitis, the most important cytokines are IFNs, which are activated immediately after viral infection (50). Type I IFNs (IFN- $\alpha$  and IFN- $\beta$ ) have antiproliferative and antiviral effects, and type II IFNs (IFN- $\gamma$ ) are immunomodulatory. Furthermore, IFN- $\alpha$  and IL-12 promote NK cell recruitment into the liver. Their activation occurs a few hours after HCV infection and they induce IFN- $\gamma$  and TNF- $\alpha$ , which manifest antiviral effects and stimulate the production of lymphocyte chemoattractant chemokines such as MCP-1/CCL2 and IP-10/CXCL10 (51).

Selective recruitment to the liver tissue of T cells capable of producing a Th1 response is necessary to counteract viral infections.

**HBV Infection** During acute HBV infection, several immune pathways are activated to achieve viral clearance. Self-limited HBV infection is typically characterized by an acute-phase response, followed by activation of adaptive immunity. In the presence of defects of this first line of defence, HBV infection is likely to become chronic (52). The pattern of cytokines secreted by CD4<sup>+</sup> T cells seems to be also important to resolve HBV infection: in fact, a prevalent type 1 response activates a vigorous polyclonal cellular immune response and is present in case of recovery from acute HBV infection, whereas a predominant type 2 response is less effective in resolving HBV infection and is found in chronic HBV hepatitis cases (53). Production of IFN- $\gamma$  and TNF- $\alpha$  directly inhibits HBV replication by accelerating HBV mRNA degradation and enhances cytotoxic T-lymphocyte activity (54). In addition, infected hepatocytes can undergo TNF- $\alpha$ -mediated apoptosis (55). Conversely, in chronic HBV, the

immune reaction within the liver is persistent but ineffective, and the chronic inflammation leads to persistent liver injury. The recruitment of inflammatory cells is mediated, as in the acute phase, by cytokines such as IFN- $\gamma$  (56).

**HCV Infection** Double-stranded RNA is a strong IFN- $\alpha$  inducer. However, endogenous IFN- $\alpha$ , secreted during the acute phase of the infection, is often not able to counteract the virus replication *per se* (57). The mechanisms of IFN resistance in chronic HCV infection are not clearly understood. Similar to HBV infections, a type 1 cytokine response seems to be prevalent in resolving infection, whereas a type 2 cytokine response is prevalent in chronic hepatitis. Similarly, serum levels of IL-10 correlate with more active hepatitis and a poor response to IFN therapy (58). The situation is complicated by the fact that chronic HCV hepatitis associates with a large number of T cells infiltrating the liver and producing type 1 cytokines that are not able to resolve infection while initiating a cascade of events resulting in hepatic fibrosis (59). NK cells have an important cytotoxic action during HCV infection and also secrete IFN- $\gamma$  and TNF- $\alpha$ . HCV infection results in direct suppression of NK activity and as a consequence the production of cytokines (60). Studies focused on chemokines detected an increased presence of CC chemokines in chronic HCV-related hepatitis compared with normal liver and in patients who have reached viral clearance (61).

#### AUTOIMMUNE LIVER DISEASES

It has been suggested that a skewed immune response toward a type 1 or type 2 pattern plays a role in the pathogenesis of several human autoimmune diseases such as multiple sclerosis, type 1 diabetes, and rheumatoid arthritis (8,62–64). Primary biliary cirrhosis (PBC), primary sclerosing cholangitis (PSC),

and autoimmune hepatitis are the main chronic autoimmune liver diseases in adults. Diverse cytokines have been shown to be overexpressed in the liver and serum of patients with such diseases. Despite progress in the area of lymphocyte homing, the mechanisms involved in the enrichment of T cells observed in inflammatory liver diseases are still poorly understood. Available data implicate both selective recruitment and selective retention in this process. It is also possible that, at different stages, the migration of T lymphocytes into the liver is controlled by different pathways, as indicated by evidence from cellular and cytokine studies.

**Primary Biliary Cirrhosis** There is still some controversy concerning the cytokine pattern characteristic of PBC. *In situ* hybridization revealed that the PBC liver has a significantly higher prevalence of IFN- $\gamma$  and IL-4 mRNA-positive cells compared with controls (65). However, there were considerably fewer cells with detectable levels of IL-4 mRNA than cells expressing IFN- $\gamma$  mRNA in the PBC liver, and the intensity of staining for IFN- $\gamma$  expression was highly correlated with the degree of portal inflammation. Moreover, IFN- $\gamma$  mRNA-positive cells were detected primarily in the lymphoid aggregates surrounding damaged bile ducts and in areas of piecemeal necrosis. Analysis of RNA extracted from the PBC liver has also indicated an upregulation of IFN- $\gamma$  mRNA expression (66–68). In contrast, mitogen-stimulated T lymphocytes infiltrating the PBC liver produce significantly higher levels of IL-4 and IL-10 compared with control T cells, but little IFN- $\gamma$ . Overall, these results suggest that type 1 cytokines might constitute the dominant pattern in PBC. However, we note that several reports propose an upregulation of specific type 2 cytokines, such as IL-5, IL-6, and IL-10 in PBC (66,68), although this was not an entirely consistent finding (67).

**Primary Sclerosing Cholangitis** Patients affected by PSC have a predominantly Th1 response (69,70) with high levels of TNF- $\alpha$  (71). Liver-derived T cells from PSC patients have greater intracytoplasmic TNF- $\alpha$  levels compared with those derived from patients with other autoimmune liver diseases. In addition, TNF- $\alpha$  may act synergistically with IFN- $\gamma$  to induce biliary epithelial cells to produce nitric oxide, which contributes to ductal cholestasis through the inhibition of cAMP-dependent HCO $_3^-$  secretion (72).

**Autoimmune Hepatitis** Very limited data on intrahepatic cytokine expression are available in autoimmune hepatitis (AIH). In addition to increased expression of HLA class II antigens in their hepatocytes (73), patients with AIH display a preponderant CD4 $^+$  T-lymphocyte infiltration of the portal space. These findings might indicate the involvement of T-helper cells in the pathogenesis of this disease. In response to the antigenic peptide/HLA class II complex, naive CD4 $^+$  T cells differentiate into either IFN- $\gamma$ -secreting Th1 or IL-4/IL-10-producing Th2 lymphocytes. The IL-12 produced mainly by macrophages and dendritic cells is required not only for their differentiation into Th1 cells but also to sustain the presence of memory/effector Th1 cells capable of mediating a biologic outcome. It was shown in a murine model of autoimmunity that IL-12 plays a pivotal role in Th1-dependent liver injury

(74,75). IL-12 is part of a family of cytokines that shares important functions in the regulation of both innate and adaptive immunity (76).

### LIVER REGENERATION

Several liver regeneration studies have been performed on mice after partial hepatectomy, and the results could be applied to human partial hepatectomy (77). Liver mass after partial hepatectomy is replenished by replication of existing hepatocytes rather than by replication and differentiation of intrahepatic progenitor cells. Under this condition liver regeneration requires the activation of multiple pathways that work dependently of each other. This complex system is orchestrated by cytokines like TNF- $\alpha$  and IL-1/IL-6 that interact with growth factors (33). A possible trigger for cytokine induction after hepatectomy is the increased exposure to reactive oxygen species. TNF- $\alpha$  and IL-6 levels in the blood rise greatly in the first 1 to 6 h after hepatectomy (78). TNF- $\alpha$  increases hepatocyte response to mitogenic growth factors (such as epithelial growth factor [EGF] and hepatocyte growth factor [HGF]). Under normal circumstances, hepatocytes are quiescent (i.e., in G0 phase) and are scarcely responsive to these factors, but as soon as they are exposed to both TNF- $\alpha$  and EGF, their proliferative response increases greatly. The first action of TNF- $\alpha$  is to activate MMPs, which degrade components of the ECM, starting hepatocyte replication (79). Cytokines act in the earlier phases of the hepatocyte proliferation, when quiescent hepatocytes are driven from the G0 phase to enter the G1 phase of the cell cycle; growth factors regulate the subsequent phases. The role of IL-6 is more difficult to investigate because of the complexity of its actions; a great number of genes activated in the earlier phases of liver regeneration appear to be IL-6 dependent. Probably it has an important antiapoptotic and hepatocyte survival activity (80).

### ISCHEMIA-REPERFUSION LIVER INJURY

The liver damage derived from hypoxic circumstances such as liver surgery, Budd-Chiari syndrome, or hypovolemic shock is commonly increased during reperfusion a process called *ischemia-reperfusion injury* leading to a multifactorial antigen-independent inflammatory response (81). During the ischemic phase, the endothelium is activated with an increase in permeability and expression of adhesion molecules that are important for the recruitment of inflammatory cells in the tissue. Upon reperfusion, adherent leukocytes and activated Kupffer cells release reactive oxygen species and several cytokines, thus enhancing the inflammatory response (82). In these circumstances TNF- $\alpha$  and IL-1 levels in the blood rise within minutes after oxygen delivery has been restored (16), mediate the apoptotic process, and promote the production of oxygen-derived free radicals by the secretion of chemokines with neutrophil-chemotactic activity, like IL-8/CXCL8, and monocyte-chemotactic activity, like MCP-1/CCL2. During liver ischemia-reperfusion injury, these chemokines are also present in other organs, such as the lungs, leading to damage also at these levels (83). IL-12 seems to be expressed earlier than TNF- $\alpha$  and IL-1, during the ischemic phase, and may be

responsible for the inflammatory process onset and perpetuation (84). We know little about the mechanisms that control the inflammatory response. It has been suggested that cytokines like IL-10, which are involved in the inhibition of NF- $\kappa$ B, play a key role. However, activated NF- $\kappa$ B is necessary to begin liver regeneration, and under experimental condition its inhibition leads to the extension of liver apoptosis and injury (Fig. 3) (85).

## CONCLUDING REMARKS AND OPEN QUESTIONS

Liver cytokines and chemokines represent the components of a complex scenario in liver physiology and pathology. As indicated by the large amount of data available, interaction networks appear to be more important to the final outcome of immune imbalance compared with single-mediator alterations. As a result, cytokine and chemokine response to several types of chronic and acute injury ensues, in an attempt to counteract the damage, but pathological effects are often the result, as in the case of TGF- $\beta$  and fibrosis. Importantly, cytokines are being studied as potential targets for novel treatments in several liver conditions. We note that results obtained thus far with monoclonal antibodies (such as infliximab targeting TNF- $\alpha$ ) are disappointing, yet we believe a vigorous effort is warranted in the near future to unravel new aspects of cytokine defects in liver diseases with the aim of ultimately developing new and effective treatments.

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# 8 Prevalence and Significance of Autoantibodies in Acute and Chronic Liver Diseases and Hepatocellular Carcinoma

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## KEY POINTS

- The detection of autoantibodies indicates a permanent or transient loss of self-tolerance.
- Serological autoimmunity is found in a variety of conditions including viral hepatitis and drug reactions, is usually of little clinical consequence, and does not indicate genuine autoimmune disease. Serological autoimmunity is frequent in comparison with genuine autoimmune liver disease.
- The autoantibodies most relevant for hepatological diseases include: antinuclear antibodies (ANAs), antibodies against smooth muscle actin (SMA), liver-kidney microsomal (LKM) autoantibodies, antibodies against soluble liver antigen/liver pancreas (SLA/LP), and antimitochondrial autoantibodies (AMAs).
- Autoantibodies primarily serve diagnostic purposes requiring detailed knowledge regarding specificity, methodology, and clinical background of the tested individual. They are important parameters to establish the diagnosis of genuine autoimmune disease such as autoimmune hepatitis and primary biliary cirrhosis.
- The scientific study of autoantibodies is aimed at determining the heterogeneity of autoimmune diseases and at identifying molecular targets and putative pathways involved in the loss of tolerance observed at the B-cell level.
- ANAs, AMAs, and LKM and SMA antibodies are detected by indirect immunofluorescence on rodent cryostat liver and kidney sections for screening; SLA/LP requires enzyme-linked immunosorbent assays (ELISA). Upon positivity during screening, molecular characterization is required for all autoantibodies except SLA/LP to establish disease specificity.
- Drug-metabolizing enzymes of the endoplasmic reticulum are major targets of disease-specific B-cell reactivities.

- LC-1 autoantibodies are detected by immunodiffusion and reactivity with formiminotransferase cyclodeaminase. They are not reliably detected by indirect immunofluorescence.
- Disease associations of antimicrosomal antibodies include drug-induced hepatitis, viral hepatitis, autoimmune hepatitis, and the autoimmune polyglandular syndrome type 1 (APECED, APS-1)
- A diagnostic role of autoantibody determinations in hepatocellular carcinoma is not routinely recommended.

## INTRODUCTION

Generally speaking, autoantibodies are B cell generated and also serologically detectable evidence of a loss of tolerance against cellular self structures, which can originate from different subcellular compartments (1–3). Autoantibodies have been described that target membrane-bound proteins of the cell and nuclear membranes and that reside in the cytoplasm or in other organelles such as the endoplasmic reticulum (Table 1) or mitochondria (Table 2). The proteins that are targeted include structural components such as actin or myosin and functional proteins such as metabolizing enzymes including cytochrome P450 (CYP), UDP-glucuronosyltransferases (UGTs), or pyruvate dehydrogenase (PDH). The identification of a specific epitope has stimulated research in an effort to characterize autoantibody-autoantigen reactivity as a tool to gain insight into the mechanisms of and the players involved in autoimmunity. However, the demonstration of a specific autoepitope reactivity does not preclude the possibility of crossreactivity of an exogenous antigen recognized by the immune system that displays homology with endogenous proteins of the body.

Although this is interesting in view of mimicry as a potential mechanism of autoimmunity, it can confound the disease specificity of different classes of autoantibodies and their utilization as diagnostic instruments. Disease specificity is a critical issue from the point of view of diagnostics. The recognition of ubiquitous cellular and subcellular structures present in many

**Table 1**  
**Heterogeneity of Autoantibodies Against Microsomal and Cytosolic Autoantigens: Disease Associations**

<i>Antibody</i>	<i>kDa</i>	<i>Target antigen</i>	<i>Disease</i>
Autoantigens of the endoplasmic reticulum (microsomal autoantigens)			
LKM-1	50	Cytochrome P450 2D6	AIH type 2 Hepatitis C
LKM-2	50	Cytochrome P450 2C9	Ticynafen-induced hepatitis
LKM-3	55	UGT1A	Hepatitis D-associated autoimmunity
LKM	50	Cytochrome P450 2A6	AIH type 2 Autoimmune polyendocrine syndrome type 1 (APS-1) Hepatitis C
LM	52	Cytochrome P450 1A2	Dihydralazine-induced hepatitis Hepatitis with autoimmune polyendocrine syndrome type 1 (APS-1)
	57	Disulfidisomerase	Halothane hepatitis
	59	Carboxylesterase	Halothane hepatitis
	35	?	AIH
	59	?	Chronic hepatitis C
	64	?	AIH
	70	?	Chronic hepatitis C
Autoantigens of the cytosol (soluble liver proteins)			
LC-1	58–62	Formiminotransferase cyclodeaminase	AIH type 2 AIH Hepatitis C?
SLA/LP	50	UGA repressor tRNA- associated protein	AIH type 3

Abbreviations: AIH, autoimmune hepatitis; LC-1, liver Cytosolic-1; LKM, liver-kidney microsomal; LM, liver microsomal; SLA/LP, soluble liver antigen/liver pancreas; UGT, UDP-glucuronosyltransferase.

**Table 2**  
**Heterogeneity of Mitochondrial Autoantigens**

	<i>kDa</i>	<i>Occurrence</i>	<i>Old M-classification</i>
Pyruvate dehydrogenase (PDH)			
PDH-E2 (pyruvate decarboxylase)	74	95	M2a
PDH-E1 $\alpha$ (pyruvate decarboxylase)	41	41–66	M2d
PDH-E1 $\beta$ (pyruvate decarboxylase)	36	2–7	M2e
Protein X (lipoid component of PDH)	52	95	M2c
Branched-chain ketoacid dehydrogenase (BCKD)			
BCKD-E2 (acyltransferase)	50	53–55	M2c
BCKD-E1 $\alpha$ (acyldecarboxylase)	46	?	
BCKD-E1 $\beta$ (acyldecarboxylase)	38	?	
Ketoglutarate dehydrogenase (KGD)			
KGD-E2 (succinyltransferase)	48	39–88	M2c
KGD-E1 (ketoglutarate decarboxylase)	110	Low	
E3 (lipoamide dehydrogenase)	55	38	M2c

cell types and in many organs is a principle fact surrounding autoantibody detection even in diseases that appear to affect specifically a single organ or organ system such as the liver. This requires detailed knowledge of detection methods, molecular autoepitope targets, and the clinical background of the affected individual. Only this combination can make immune serology a powerful diagnostic tool and potentially a tool for the mechanistic discovery of processes driving autoimmunity.

When immune serology is tested, it is important to realize that serological autoimmunity is not uncommon. On the one hand, autoantibodies are detectable in individuals who are clinically healthy and have an increasing autoantibody prevalence with advancing age. As an example, low-titer antinuclear antibodies (ANAs) are detectable in 20 to 50% of elderly individuals and do not necessarily indicate present disease or disease disposition in most cases (4). Serological autoimmunity is

also detected as an epiphenomenon of viral infections such as hepatitis C and hepatitis D, in alcohol abuse, as a transient phenomenon in allergic drug reactions, and even in genetically determined diseases such as the autoimmune polyglandular syndrome type 1. The example of allergic drug reactions offers one of the most plausible mechanistic explanations of the generation of autoantibodies. In these reactions a metabolizing enzyme is structurally altered by a metabolite of its substrate and subsequently immunologically recognized, leading to autoantibodies that identify the involved enzyme. These reactions are self-limiting and do not result in a permanent loss of tolerance. However, in cases of genuine autoimmune diseases, some of the same autoantigens are targeted, which demonstrates that the loss of tolerance converges on similar molecular structures.

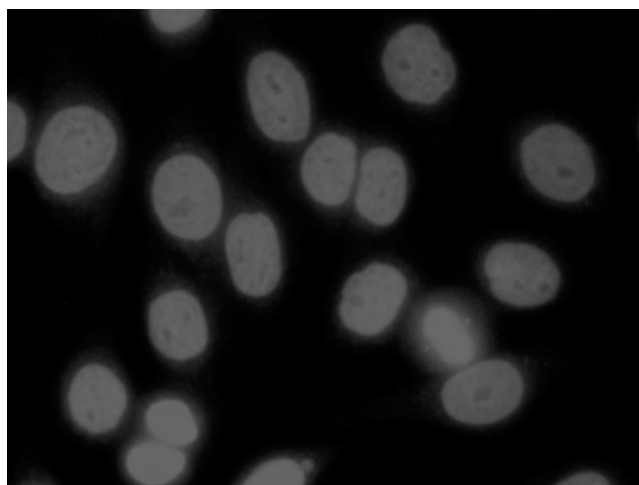
This chapter discusses the major autoantigens and autoantibodies relevant to liver diseases. Although autoimmune serology is rarely significantly involved in acute liver diseases other than drug reactions or the acute onset of a chronic autoimmune liver disease, it is a key component of the diagnosis and differential diagnosis in chronic liver diseases. These include autoimmune diseases such as autoimmune hepatitis and primary biliary cirrhosis and the serological autoimmunity found in viral infection.

### AUTOANTIBODIES IN AUTOIMMUNE HEPATITIS

Circulating autoantibodies are a classical finding in autoimmune hepatitis (AIH). Autoantibodies are the single most important finding determining diagnosis, treatment, and discrimination of autoimmune disease from chronic viral infections. The identification, molecular cloning, and recombinant expression of hepatocellular autoantigens has allowed the implementation of precise testing systems and the scientific evaluation of humoral autoimmunity associated with AIH (3,5). The autoantibodies with significance for AIH are ANAs, muscle actin (SMA) antibodies, LKM antibodies, soluble liver antigen/liver pancreas (SLA/LP) antibodies, liver cytosolic-1 (LC-1), and asialoglycoprotein receptor (ASGPR) antibodies.

#### ANTINUCLEAR ANTIBODIES

ANAs are directed against functional and structural components of the cell nucleus, nuclear membranes, or DNA. The target antigens are a heterogeneous and incompletely defined group of cellular proteins (6). To date, subtyping of the various ANA antigens offers no diagnostic or prognostic advantage. ANAs are also detected in primary biliary cirrhosis (PBC), primary sclerosing cholangitis (PSC), viral hepatitis, drug-related hepatitis, and alcoholic liver disease, and investigations have been aimed at identifying target antigens that are specific for AIH. ANAs are determined by indirect immunofluorescence on cryostat sections of rat liver and on Hep.2 cell slides. Most commonly, a homogeneous (Fig. 1) or speckled immunofluorescence pattern is encountered (7). ANAs have been found to be reactive with centromeres, ribonucleoproteins, and cyclin A (Fig. 2) (8). They represent the most common autoantibody in AIH and occur in high titers usually exceeding 1:160.



**Fig. 1.** Indirect immunofluorescence micrograph of ANAs detected on immobilized Hep.2 cells. Typical aspect of *homogeneous nuclear* staining found in a patient with autoimmune hepatitis type 1 with titers exceeding 1:160. These autoantibodies are frequently directed against dsDNA and histones and are a typical finding in type 1 autoimmune hepatitis.

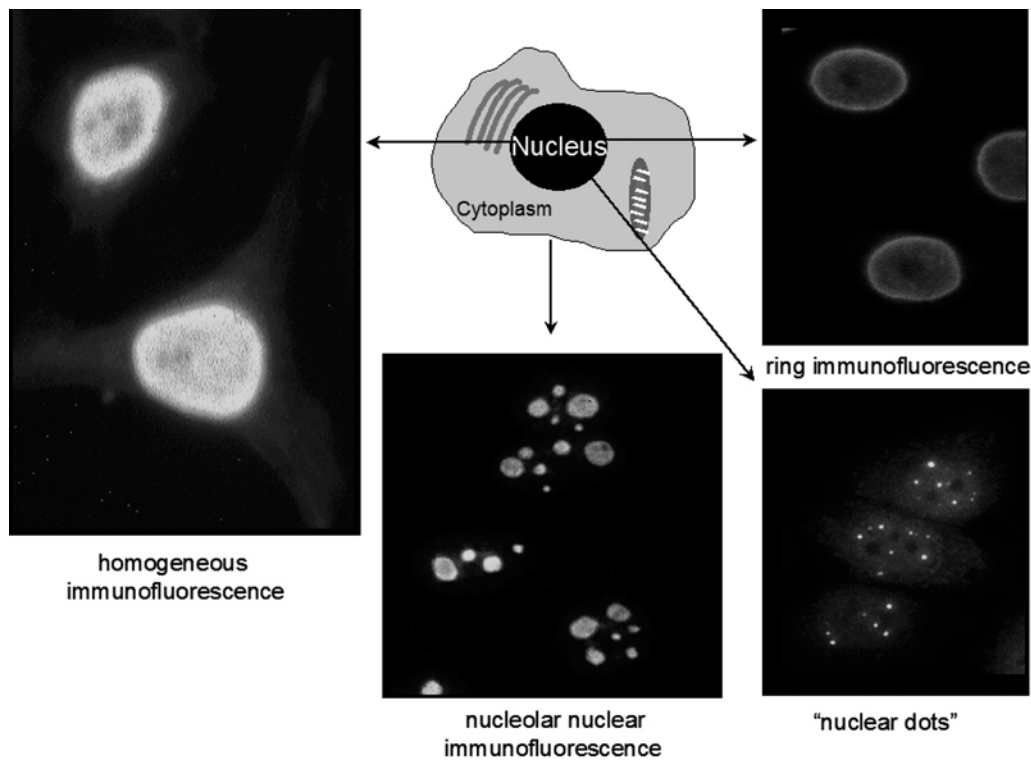
#### ANTISMOOTH MUSCLE ACTIN ANTIBODIES

SMA antibodies are directed against components of the cytoskeleton such as actin, troponin, and tropomyosin (9–11). They frequently occur in high titers in association with ANAs. However, SMA autoantibodies also occur in advanced diseases of the liver of other etiologies, in infectious diseases, and in rheumatic disorders. In these cases titers are often lower than 1:80. SMA autoantibodies are also determined by indirect immunofluorescence on cryostat sections of rat stomach (Fig. 3). SMA antibodies are associated with the HLA A1-B8-DR3 haplotype, and, probably as a reflection of this status, affected patients are reported to be younger at disease onset and to have a poorer prognosis.

#### AUTOANTIBODIES AGAINST SOLUBLE LIVER ANTIGEN

Antibodies against SLA were detected in a patient with ANA-negative AIH (12). It is now clear that the description of liver pancreas (LP) antibodies recognize the same target protein structure, leading to the designation SLA/LP autoantibodies (13,14). Anti-SLA/LP antibodies were found to be highly specific for AIH and are detectable in about 10 to 30% of all patients with AIH. In 1992, Gelpi et al. identified specific autoantibodies present in patients with a severe form of autoimmune chronic hepatitis (15). These antibodies precipitated a UGA suppressor serine tRNA-protein complex, which is probably involved in cotranslational selenocysteine incorporation in human cells. Subsequently, SLA/LP antibodies were identified as being directed against a UGA suppressor serine tRNA-protein complex and not against cytokeratins 8 and/or 18 or glutathione S-transferases, as suggested in other reports. The exact function and role of this autoantigen in autoimmunity are so far unclear. Regarding disease specificity, anti-SLA/LP antibody may be linked to the pathogenesis of the autoimmune process.





**Fig. 2.** Indirect immunofluorescence micrographs of a variety of ANAs found in autoimmune hepatitis and other autoimmune diseases and detected on immobilized Hep.2 cells. Aspect of the *nuclear membranous* (rim) immunofluorescence pattern (top right) found in a patient with autoimmune hepatitis type 1 at titers exceeding 1:160. In this pattern autoantibodies are directed against laminins (laminin B, but also laminin A and C). Membranous immunofluorescence is not a frequent finding and can indicate the existence of mixed immune syndromes including vasculitis and other features of SLE. It is clearly distinguished from a homogeneous pattern (top left). The middle panel demonstrates a *nucleolar ANA* fluorescence pattern. This pattern is *rarely* seen in autoimmune hepatitis but is common in rheumatological diseases such as scleroderma and polymyositis. If present in autoimmune hepatitis type 1, it can be indicative of overlap syndromes with rheumatological disorders. The lower right panel shows *multiple nuclear dots*. This pattern is *not typical* for autoimmune hepatitis and is mainly found in about 20% of patients with PBC. Usually AMAs are present at the same time but can also be missing in cases of ANA-positive, AMA-negative PBC. These autoantibodies are directed against the Sp100 nuclear antigen (100 kDa).

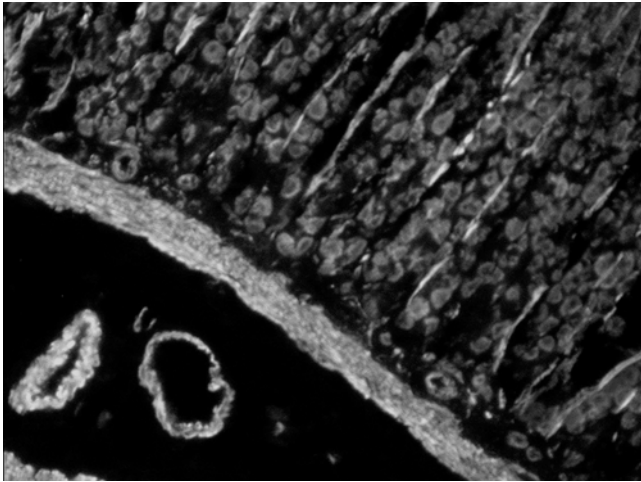
#### AUTOANTIBODIES AGAINST THE ENDOPLASMIC RETICULUM

The endoplasmatic reticulum (ER) of the cell harbors two important enzyme families, which are the main players in phase I and phase II metabolism: the CYPs and the UGTs (16). Phase I metabolism leads to oxidative modification of compounds usually by the addition of functional groups such as hydroxylation. Phase II metabolism leads to conjugation with polar prosthetic groups such as glucuronic acid (glucuronidation). In the case of the UGTs, glucuronidation leads to a water-soluble glucuronide, which is targeted for renal or biliary elimination. Both enzyme families are preferred targets of a B-cell response in autoimmune liver diseases (Fig. 4 and Table 1). In 1973, Rizzetto discovered autoantibodies reactive with the proximal renal tubulus (Fig. 5A) and the hepatocellular cytoplasm by indirect immunofluorescence (Fig. 5B) (17). These autoantibodies, termed LKM-1, were associated with a second form of ANA-negative AIH (18). Between 1988 and 1991, the 50-kDa antigen of LKM-1 autoantibodies was identified as cytochrome P4502D6 (CYP2D6) (19–21). A second type of LKM autoantibodies, LKM-2, was found to be directed

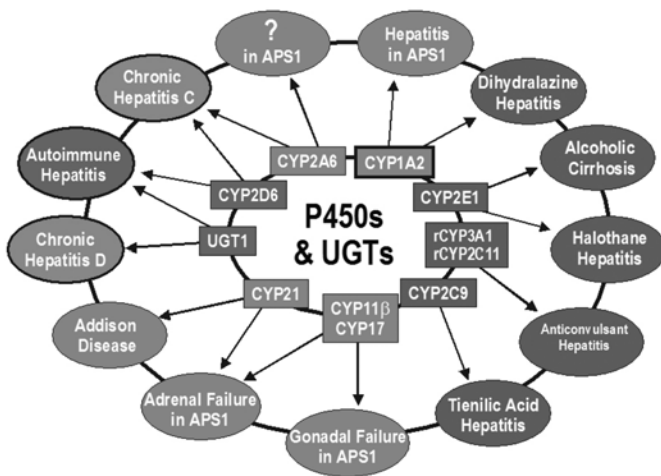
against a different target, CYP2C9, and is induced by drug exposure in susceptible individuals (22). A third group of LKM autoantibodies, LKM-3, was identified in 6 to 10% of patients with chronic hepatitis D virus infection (HDV) by Crivelli 1983 (23). These autoantibodies are directed against family 1 UDP-glucuronosyltransferases (UGT1A) and also occur in autoimmune hepatitis (24,25).

#### AUTOANTIGEN AND AUTOANTIBODY DEFINITIONS OF LKM/LM AUTOANTIBODIES

One of the prominent features of autoimmune diseases, but not restricted to these, is high titers of autoantibodies. The refined analysis of serological findings in patients with serological autoimmunity and genuine autoimmune disease is ongoing, since not only reliable diagnostic tests are required but also clues are sought to unravel the pathophysiology of the obvious loss of tolerance associated with the detection of autoantibodies. Autoantibodies binding liver and kidney tissue (LKM) are directed against microsomal targets (expressed in the ER of these two organs) and exhibit a remarkable heterogeneity of targets, with a high degree of specificity for different disease conditions (summarized in Table 1 and Fig. 4). The



**Fig. 3.** Typical immunofluorescence pattern of SMA autoantibodies detected on rat stomach cryostat sections. This serum shows immunoreactivity with the muscularis mucosae and muscularis propria layers of rat stomach. Note that the mucosa is excluded from reactivity. This autoantibody is often detected in conjunction with ANA in autoimmune hepatitis type 1.



**Fig. 4.** Diversity of autoantibodies against endoplasmic reticulum (microsomal) targets in autoimmune hepatitis, drug-induced hepatitis, viral hepatitis, and genetic disease (autoimmune polyglandular syndrome type 1; APECED/APS-1). CYP, cytochrome P450; UGT, uridine diphosphate glucuronosyltransferase.

most progress has been achieved by the molecular identification of specific microsomal protein targets and their recombinant expression, leading to specific testing systems as well as the possibility of studying epitope recognition patterns.

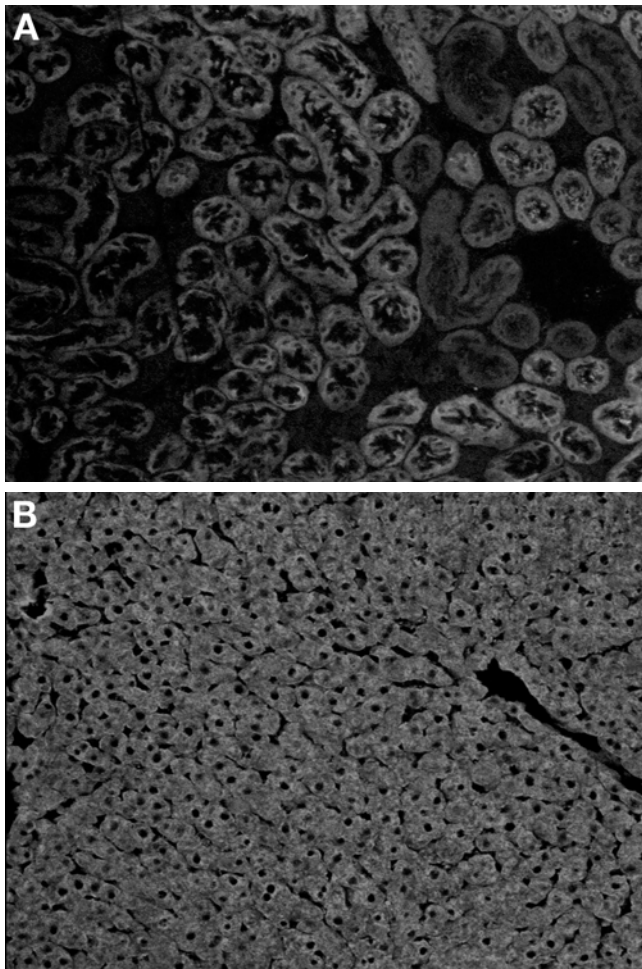
LKM-1 autoantibodies recognize a major linear epitope between amino acids 263 and 270 of the CYP2D6 protein (Fig. 6) (20). These autoantibodies inhibit CYP2D6 activity in vitro and are capable of activating liver-infiltrating T lymphocytes, indicating a combination of B- and T-cell activity in the autoimmune process involved. In addition to linear epitopes,

LKM-1 autoantibodies have also been shown to recognize conformation-dependent epitopes (26). CYP2D6 has been found to be expressed on the hepatocellular surface, and its expression appears to be regulated by cytokines. LKM-2 autoantibodies are directed against CYP2C9, which is involved in the metabolism of ticrynafen, a diuretic no longer in use. This association explains a mechanism of possible (transient) loss of tolerance in autoimmunity. Upon exposure and metabolism of specific drugs, the involved drug-metabolizing enzyme is biochemically altered and subsequently immunologically recognized, leading to autoantibody formation (27). This is how the specificity of drug-induced serological autoimmunity for precise autoantigen targets is explained. LKM-3 autoantibodies recognize UDP-glucuronosyltransferase, an enzyme system expressed in the inner membrane of the ER. LKM-3 autoantibodies have not been found to be inhibitory. Drug-associated LKM-3 autoantibodies have not been described.

Liver microsomal (LM) autoantibodies, which are characterized by an immunofluorescence pattern selectively staining the hepatocellular but not renal cell cytoplasm have been found to be directed against CYP1A2. These are found in patients treated with dihydralazine (28) but also in a genetically determined disease (autoimmune polyglandular syndrome [APECED]) (29). For screening purposes LKM and LM autoantibodies are first visualized by indirect immunofluorescence (Fig. 5A and B) on rodent cryostat sections of liver and kidney tissue. Subclassification is achieved by enzyme-linked immunosorbent assay (ELISA) and Western blot, preferably using recombinant antigens. As outlined in the introduction, antimicrosomal autoantibodies exhibit a broad range of associations. The clinically most relevant ones are discussed in the following sections.

**Autoimmune Hepatitis Type 2-Associated Microsomal Autoantibodies** AIH-2 is characterized by the presence of LKM-1 autoantibodies against CYP2D6 (30,31). In 10%, LKM-3 autoantibodies against UDP-glucuronosyltransferases are also present (25). In contrast to AIH type 1, additional organ-specific autoantibodies are present such as antithyroid, anti-parietal cell, and anti-Langerhans cell autoantibodies. The number of extrahepatic immune syndromes such as diabetes, vitiligo, and autoimmune thyroid disease has been reported to be more prevalent. Serum immunoglobulin levels are moderately elevated, with a reduction in IgA. AIH type 2 is a rare disorder that affects 20% of AIH patients in Europe but only 4% in the United States, possibly because of genetic variability or differences in testing strategies.

LKM autoantibodies have been extensively studied for their role as markers not only of AIH type 2 but also for differential diagnostic purposes in order to offset other hepatic diseases, to gain insight into the immunological mechanisms involved in AIH, and to evaluate their prognostic role. Testing of 26 LKM-positive sera was carried out using Western blotting with partial sequences of recombinant CYP2D6. Eleven of these sera recognized a short minimal epitope of eight amino acids with the sequence DPAQPPRD (20). Twelve other clones recognized a larger epitope containing this eight-amino-acid



**Fig. 5.** Indirect immunofluorescence showing LKM-1 autoantibodies on rat kidney and liver cryostat sections. Serum of a patient with autoimmune hepatitis type 2. (A) Typical indirect immunofluorescence pattern of LKM-1 autoantibodies detecting the proximal (cortical) renal tubules but excluding the distal tubules located in the renal medulla, which corresponds to the tissue expression pattern of the autoantigen CYP2D6. (B) Using rat hepatic cryostat sections, a homogeneous cellular immunofluorescence staining is visualized excluding the hepatocellular nuclei (LKM-1).

core sequence. A search of electronic data bases revealed an interesting match of the minimal epitope with the primary structure of the immediate early protein IE 175 of Herpes Simplex Virus-1 (HSV-1) (Fig. 6). Sequence identity was present for the sequence PAQPPR. Therefore affinity-purified LKM-1 (anti-CYP2D6) autoantibodies were used in Western blots with lysates of BHK-cells infected with HSV. The autoantibody specifically detected a band at 175 kDa that demonstrated crossreactivity with an HSV-specific protein of 175 kDa. The hypothesis that molecular mimicry may underlie this form of autoimmunity was further suggested by a case study (32). In a pair of identical twins, one sister suffered from AIH type 2, and the other one was healthy. Interestingly, only the sister suffering from AIH was HSV positive, and her serum recognized the viral 175-kDa protein in lysates of

HSV-infected cells. Molecular mimicry may contribute to the development of AIH-2 by weakening self-tolerance to certain protein targets. So far evidence for the mimicry hypothesis in AIH is not convincing.

Further work on epitope mapping was performed resulting in the identification of three minor epitopes on CYP2D6. Most patients with AIH-2 recognize the epitope of amino acids 257 to 269, including the core sequence of DPAQP-PRD. With lower frequencies, another epitope of amino acids 373 to 389 was detected and two infrequent epitopes consisting of amino acids 373 to 389 or 410 to 429 (33). Since linear peptides were unable to absorb the inhibitory activity of LKM-1 autoantibodies on CYP2D6 activity, the presence of conformational autoantibodies in LKM-1 sera was suggested. Another major epitope located at amino acids 321 to 373 has been characterized that appears to be three dimensional and is no longer reactive when cut into overlapping pieces (26) (Fig. 7). The recognition of epitopes located between amino acids 257 and 269 appears to be a specific autoimmune reaction of AIH and discriminatory against LKM-1 autoantibodies associated with chronic HCV infection (Fig. 8) (34).

#### Hepatitis C-Associated Microsomal Autoantibodies

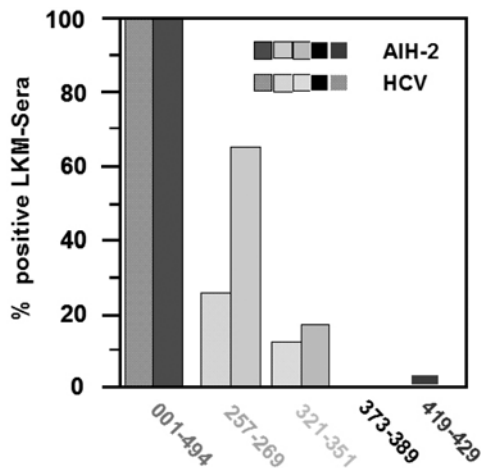
Hepatitis C is associated with an array of extrahepatic manifestations, including mixed cryoglobulinemia, membranoproliferative glomerulonephritis, polyarthritis, porphyria cutanea tarda, Sjögren's syndrome, and autoimmune thyroid disease (35). Not surprisingly, numerous autoantibodies are found to be associated with chronic hepatitis C. Similar to AIH, antinuclear, SMA, LKM, and antithyroid antibodies are found with a high prevalence.

The examination of LKM autoantibodies in HCV patients revealed that although anti-CYP2D6 titers are similar to titers in AIH-2, differences exist regarding the epitopes recognized by LKM autoantibodies. In patients with AIH-2, the epitope of amino acids 257 to 269 is recognized with a significantly higher prevalence than in chronic hepatitis C (Fig. 8). In addition, the immune reaction appears to be more heterogeneous than in AIH, as indicated by recognized protein targets of 59 and 70 kDa (36).

LKM autoantibodies in chronic hepatitis C may indicate an increased risk of disease exacerbation. A patient with a high LKM-1 titer and autoantibodies directed against an epitope of amino acids 257 to 269, which is preferentially recognized by patients with AIH-2, showed exacerbation of the disease under interferon treatment (34). In contrast to other patients with HCV infection, this patient further recognized a rarely detected epitope on the C-terminal third of the protein. These results suggest that epitope mapping may contribute to the identification of patients at risk of exacerbating their disease.

Another autoantibody was detected in patients infected with HCV and HGV. About 2% of HCV-positive sera in general and 7.5% of LKM-1-positive HCV sera recognize CYP2A6 (37). This autoantibody appears to occur more frequently in HCV-infected patients with LKM-1 autoantibodies. Interestingly anti-CYP2A6 autoantibodies are not detected in patients with





**Fig. 8.** Liver-kidney microsomal (LKM)-1 autoantibodies directed against CYP2D6 display differences in autoepitope recognition in genuine autoimmune hepatitis (AIH) type 2 and LKM-1 autoantibodies found in hepatitis C virus (HCV) infection. The greatest differences are seen in an epitope found between amino acids 257 and 269 (26).

#### Microsomal Autoantibodies in Autoimmune Hepatitis Associated With the Autoimmune Polyglandular Syndrome

The APS-1 syndrome is characterized by a number of autoimmune disorders involving endocrine and nonendocrine organs including mucocutaneous candidiasis, hypoparathyroidism, and adrenal insufficiency (establishing the diagnosis when two of the latter are present) (41). In 10% of patients, autoimmune hepatitis is present. APS-1 has greatly increased our understanding of autoimmune diseases since it has a monogenic association with mutations in the *autoimmune regulator (AIRE)* gene. AIRE is expressed in medullary epithelial cells of the thymus, accounting for less than 0.1% of thymic cells (42). The transcription factor encoded by the *AIRE* gene regulates the expression of a multitude of antigens required for the negative selection of autoreactive T cells in the thymus (43). In AIRE-deficient mice, less autoantigen is expressed in thymic medullary epithelial cells, resulting in a higher number of higher reactive T cells in the periphery, which contributes to the establishment of autoimmune disease. AIH in APS-1 syndrome leads to the formation of autoantibodies against CYP1A2 and CYP2A6 (44). AIH can be the first clinically apparent component of this syndrome, in particular in children (45). However, retrospective analysis of adult patients with AIH has not detected an increased frequency of variant AIRE alleles (46).

**Microsomal Autoantibodies and Drug Reactions** A small percentage of patients treated with therapeutic drugs can develop severe hepatitis, which is characterized by lymphocytic liver infiltrations and autoantibodies directed against hepatic proteins (27). It is believed that drug-metabolizing enzymes, mainly CYPs, create reactive metabolites, which in turn modify either the metabolizing CYP enzyme itself and/or other hepatic proteins (Fig. 4). In susceptible patients these modified proteins induce an immune response, resulting in severe “drug-induced

hepatitis.” Modified proteins preferentially include CYPs, which themselves are then the target for autoantibodies. As typical examples, tienilic acid-induced hepatitis, dihydralazine hepatitis, halothane hepatitis, and anticonvulsant-induced hepatitis have been characterized. It is debated whether alcoholic liver disease is caused in part by an autoimmune reaction against hepatic proteins, directed against both acetaldehyde- and hydroxyethyl-modified hepatic proteins (47). It is suggested that metabolism of ethanol by CYP2E1 generates hydroxyethyl radicals, which can represent targets of autoimmunity.

#### Microsomal Autoantibodies of Unknown Relevance

LKM autoantibodies have also been identified to react with yet unidentified proteins. These include antigens with molecular weights of 35, 57, 59, and 70 kDa. These autoantibodies are predominantly found in AIH, HCV infection, and halothane hepatitis (36).

#### General Role of Antimicrosomal Autoantibodies

Although detailed molecular analyses can provide a high degree of specificity and possible disease associations with LKM/LM autoantibodies, the diagnosis of the disease association is usually reached by the exclusion of other causes of liver disease. It is interesting that the autoepitopes spanning different associations (Fig. 4) lie on a relatively small homologous portion of the CYP molecule (Fig. 9) across isoforms. Immunofluorescence is only a screening option because a positive finding — although suggestive of autoimmune liver disease — may just reflect serological autoimmunity associated with viral infection (31). LKM immunofluorescence therefore does not indicate disease or organ specificity of the underlying pathology. In these cases a refined analysis with molecular antigen-based methods such as ELISA and Western blot is required; in very rare cases an attempt at identifying the epitope recognition pattern may be of value.

#### LIVER CYTOSOLIC AUTOANTIBODIES

LC-1 autoantibodies were detected in the 1990s and are best found by immunodiffusion rather than indirect immunofluorescence (48). Immunofluorescence is often confounded by the bright presence of LKM patterns, which obscure LC immunofluorescence. Therefore, LC-1 autoantibodies are most likely overlooked when immunofluorescence is employed as the only method of screening or detection. The corresponding autoantigen was described 1999 (49). The antigen recognized by anti-LC1 was identified as formiminotransferase cyclo-deaminase (FTCD). FTCD is a metabolic enzyme involved in the conversion of histidine to glutamic acid and is most highly expressed in the liver. It is bifunctional and composed of distinct FT and CD domains connected by a short linker. Anti-LC-1 sera recognize distinct epitopes on FTCD preferentially localized to the FT domain of FTCD. Antibodies against LC-1 were found in up to 50% of patients with AIH-2 (50,51). Less frequently, anti-LC-1 may be associated with SMA and ANA in sera from patients with AIH type 1 and chronic hepatitis C infection. In addition, anti-LC-1 has been shown in studies to represent the only serological marker in 10% of patients with AIH. Contrary to most other autoantibodies in AIH, anti-LC-1 seems

	$\beta$ 5-2	helix I				
P450bm3 - 243	P - E T - - G E P L D D E N I R Y Q I I T F L I A G H E T T S G L L S F A L Y F					
CYP1A2 - 294	G P R A S G - N L I P Q E K I V N L V N D I F G A G F D T V T T A I S W S L M Y					
CYP2D6 - 282	A K G N P E - S S F N D E N L R I V V A D L F S A G M V T T S T T L A W G L L L					
CYP2C9 - 274	E K H N Q P - S E F T I E S L E N T A V D L F G A G T E T T S T T L R Y A L L L					
CYP3A1 - 282	S K D K E S H T A L S D M E I T A Q S I I F I F A G Y E P T S S T L S F V L H S					
CYP21B - 267	P S M E E <b>G S G Q L L E G H V H M A A V D L L I G G T E T T A N T L S W A V V F</b>					
	helix J	helix J'				
P450bm3 - 280	L V K N P H V L Q K A A E E A A R V L V D P - V P S Y K - - - Q V K Q L K Y V G					
CYP1A2 - 333	<b>L V T K P E I Q R K I Q K E L D T V I G R E R R R P R L S - - - D R P Q L P Y L E</b>					
CYP2D6 - 321	<b>M I L H P E V Q R R V Q Q E I D D V I G Q V R R P E M G - - - D Q A H M P Y T</b>					
CYP2C9 - 313	<b>L L K H P E V T A K V Q E E I E R V I G R N R S P C M Q - - - D R S H M P Y T D</b>					
CYP3A1 - 322	L A T H P D T Q K K L Q E E I D R A L P <b>N K A P P T Y D - - - T V M E M E Y L D</b>					
CYP21B - 307	<b>L L H H P E I Q Q R L Q E E L D H E L G P G A S S S R V P Y K D R A R L P L L-N</b>					
	helix K	$\beta$ 6-1	$\beta$ 1-4	$\beta$ 2-1	$\beta$ 2-2	$\beta$ 1-3
P450bm3 - 316	M V L N E A L R L W P T A P A - F S L Y A K E D T V L G G E Y P L E K G D E L M					
CYP1A2 - 370	<b>A F I L E T F R H S S F L P F T I P H S T T R D T T L N G F Y I P K K C C V F V</b>					
CYP2D6 - 358	<b>A V I H E V Q R F G D I V P L G V T H M T S R D I E V Q G - F R I P K G T T L I</b>					
CYP2C9 - 350	<b>A V V H E V Q R Y I D L L P T S L P H A V T C D I K F R N - Y L I P K G T T I L</b>					
CYP3A1 - 359	<b>M V L N E T L R L Y P I G N R L E R V C K K - D V E I N G - V F M P K G S V V M</b>					
CYP21B - 347	<b>A T I A E V L R L R P V V P L A L P H R T T R P S S I S G - Y D I P E G T V I I</b>					
	helix K'	helix K''	Meander			
P450bm3 - 365	V L I P Q L H R D K T I W G D D V E E F R P E R F E - - N P S A I P Q H A F K -					
CYP1A2 - 410	<b>N Q W Q V N - H D P E L W E D P S E F R P E R F L T A D G T A I N K P L S E K M</b>					
CYP2D6 - 397	T N L S S V L K D E A V W E K - P F R F H P E H F L D A Q G H F V K P E A F L -					
CYP2C9 - 389	I S L T S V L H D N K E F P N - P E M F D P H H F L D E G G N F K K S K Y F M -					
CYP3A4 - 397	I P S Y A L H R D P Q H W P E - P E E F R P E R F S K E N K G S I D P Y V Y L -					
CYP21B - 386	P N L Q G A H L D E T V W E R - P H E F W P D R F L E P G K N S R A L A F - - -					
	Cys-Pocket	helix L				
P450bm3 - 401	- P F G N G Q R A C I G Q Q F A L H E A T L V L G M M L K H F D F - - E D H T N					
CYP1A2 - 449	<b>M L F G M G K R R C I G E V L A K W E I F L F L A I L L Q Q L E F F S V P P G V</b>					
CYP2D6 - 435	- P F S A G R R A C L G E P L A R M E L F L F F T S L L Q Q H F S F S V P T G Q P					
CYP2C9 - 427	- P F S A G K R I C V G E <b>A L A G M E L F L F L T S I L Q N F N L K S L V D P K</b>					
CYP3A1 - 433	- P F G N G P R N C I G M R F A L M N M K L A L T K V L Q N F S F Q P C K E T Q					
CYP21B - 422	- - - G C G A R V C L G E P L A R L E L F V V L T R L L Q A F T L L P S G D A L					

Fig. 9. Alignment of autoepitopes on different CYP proteins in autoimmune hepatitis and drug-induced hepatitis as well as adrenal autoimmunity.

to correlate with disease activity and may be useful as a marker of residual hepatocellular inflammation in AIH.

#### ANTINEUTROPHIL CYTOPLASMATIC AUTOANTIBODIES

Antibodies to neutrophil cytoplasmic antigens ANCA were detected in 65 to 95% of sera from patients with AIH type 1 and additionally in sera from patients with PSC (Fig. 10). ANCA are detected by immunofluorescence, which distinguishes two patterns: cANCA, with a diffuse cytoplasmic staining of neutrophils and pANCA, which exhibits a rim-like staining of the perinuclear cytoplasm. In AIH, atypical pANCA (also termed xANCA) are usually found that display a pANCA immunofluorescence pattern but do not show reactivity with myeloperoxidase, one of the major autoantigens of classical ANCA. Recent data have shown reactivity with a nuclear envelope protein (52). The discrimination of ANCA is difficult, because ANAs frequently also stain ethanol-fixed neutrophils. The target antigen in AIH is unknown, but, apart from myeloperoxidase, proteinase 3 and elastase have been ruled out as candidates. The role of ANCA in AIH is unclear,

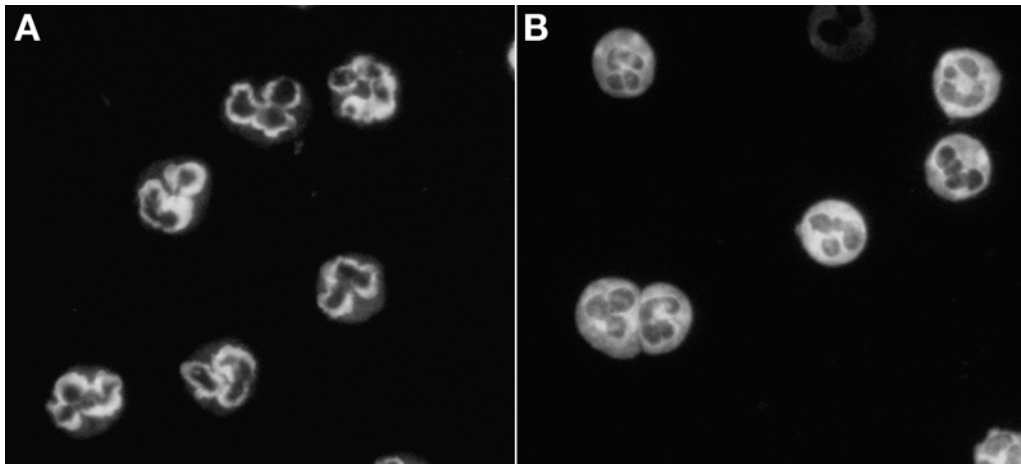
but routine determination may be useful to identify patients formerly classified as having cryptogenic hepatitis (53).

#### ANTIASIALOGLYCOPROTEIN RECEPTOR ANTIBODIES

Antibodies against ASGPRs (54) were observed in up to 90% of all patients with AIH and can coexist with ANA, SMA, and anti-LKM-1. However, they are not disease specific and can also be found in viral hepatitis, drug-induced hepatitis, and PBC. Levels of antiasialoglycoprotein antibodies correlate with inflammatory disease activity and might be used as additional marker to monitor treatment efficacy.

#### PRIMARY BILIARY CIRRHOSIS AND THE CHARACTERIZATION OF AUTOANTIGENS

It is generally believed that the autoimmune attack on the small intrahepatic bile ducts in PBC is mediated by cellular mechanisms and that this process is the main contributor to the pathophysiology of PBC (55–57). Although cellular autoimmunity is the defining process of patient survival and hepatic function, humoral autoimmunity is the main diagnostic feature



**Fig. 10.** Immunofluorescence study showing antineutrophil cytoplasmic antibodies (ANCA) with a typical pANCA (**A**) and cANCA (**B**) distinction. These autoantibodies are found in autoimmune hepatitis type 1 (ANA and SMA positive) in up to 95% but are not considered to be a specific diagnostic finding in AIH. When further analyzed, they frequently do not exhibit reactivity with myeloperoxidase (pANCA) or proteinase 3 (cANCA) in AIH.

of this disease. High-titer antimitochondrial antibodies (AMAs) were first described by Mackay in 1958 (58). In 1967 the target antigen of AMA was localized within the inner mitochondrial membrane and termed M2 (59). In 1985, the further analysis of M2 antigens led to their subdivision into individual antigen fractions between 36 and 74 kDa molecular weight (60–64) (Table 2). In 1987, molecular cloning of the 74-kDa antigen led to the identification of the ketoacid dehydrogenase multiprotein complex (OADC) as the major autoantigen of PBC-associated AMA (65). Autoantibodies directed against members of the OADC represent those previously defined as anti-M2 autoantibodies. These AMAs are PBC specific and can be separated from nonspecific AMAs using molecularly defined seroimmunological methods (5).

The OADC consists of three major antigens: pyruvate dehydrogenase (PDH), branched chain ketoacid dehydrogenase (BCKD), and ketoglutarate dehydrogenase (OGD) (66,67) (Table 2). Every enzyme in itself consists of three subunits with individual enzymatic activities: E1 (decarboxylase), E2 (dihydro lipoamide acyltransferase), and E3 (lipoamide dehydrogenase).

In 95% of all North American and European, and 65% of all Japanese PBC sera, AMAs are directed against the E2 subunit of PDH (PDH-E2). PDH-E2 represents the 74-kDa autoantigen identified first as part of the M2 antigen fraction. AMAs mainly belong to the IgM class of immunoglobulins, but IgA, IgG1, and IgG3 class autoantibodies are also regularly detected. The further analysis of PBC sera has demonstrated that 53 to 55% are reactive with the E2 subunit of BCKD (BCKD-E2), which corresponds to the earlier identified 52-kDa antigen of M2. In addition, 39 to 88% of PBC sera display autoantibodies directed against the E2 unit of OGD (OGD-E2), corresponding to the 48-kDa component of M2. Reactivity of these three major subspecies of PBC-specific AMAs has a number of common features: immunoreactivity favors epitopes

on the E2 subunit in all three cases, the recognized epitopes are of considerable sizes and are conformation dependent, and they are localized within the lipoyl domain of the molecules. Epitopes have been characterized for PDH-E2 (93 amino acids) (66,68), BCKD-E2 (227 amino acids) (69), and OGD-E2 (81 amino acids) (70). Autoantibodies against PDH-E2 occur together with anti-BCKD-E2 in 60% of cases. In about 10 to 20% of PBC patients, anti-BCKD-E2 autoantibodies are detected alone, the significance of which is not clear.

Autoantibodies directed against the other components of the OADC are of minor diagnostic importance. Anti-PDH-E1 $\alpha$  autoantibodies have been detected in 41 to 66% of PBC patients and have been implicated as a serological indicator of coexisting systemic sclerosis (71). However, this test is not routinely employed. Autoantibodies against protein X, an autoantigen of 56 kDa, have been described and found to be completely crossreactive with PDH-E2 antibodies (72,73).

In 89% of PBC patients, AMAs have also been detected in the bile. These were directed against PDH-E2 (79%), BCKD-E2 (32%), and OGD-E2 (5%) and were always found when AMAs of the same reactivity were also present in the serum (74). Almost half of these biliary AMAs were of the IgA subtype, which were directed against the same autoepitopes as serum AMAs. Interestingly, the presence of PDH-E2, BCKD-E2, and OGD-E2 antigen was detected in bile of PBC patients, indicating that the humoral response in these patients may be antigen driven by OADC antigen or proteins crossreactive with this antigen. AMAs of the IgA subtype, the expression of PDH-E2 antigen (or a crossreactive antigen) on biliary epithelial cells (72,75) in PBC patients, may indicate that PBC could represent a mucosal disease entity. AMAs and PDH-E2 or crossreactive antigens are also detected in the saliva of PBC patients, which may represent additional evidence for this hypothesis (76). AMAs in saliva and bile are not part of the routine determination of AMAs in PBC patients, and their diagnostic significance is unknown.

## B-CELL EPITOPES AND T-CELL REACTIVITY IN PBC

When PBC biopsies are assessed, it is obvious that an intense cellular reaction is present in the portal tracts and is focused on the bile ducts. To establish a relationship with humoral autoimmunity, peptide specificities of the PDH-E2 antigen were studied, leading to the identification of autoreactive CD4<sup>+</sup> clones proliferating in response to an amino acid motif located between 163 and 176 as well as 36 and 49 (77,78). With respect to the OGDH-E2 molecule, a CD4 cell motif was identified between amino acids 100 and 113. When these motifs are aligned, a common amino acid sequence of ExETDK is found. The analysis of T-cell precursor frequencies showed a 100-fold higher incidence in the liver and regional lymph nodes of PBC patients compared with peripheral blood. They were also lower in more advanced stages of PBC and absent in PSC. Interestingly, the B-cell epitopes on PDH-E2 map to a similar region between amino acids 164 and 183 and 38 and 57, demonstrating an overlap of B-cell and CD4<sup>+</sup> T-cell epitopes.

Similarly, CD8<sup>+</sup> cells are a prominent feature of the cellular infiltrate observed in the liver biopsies of PBC patients. A recent analysis identified a CD8<sup>+</sup> cell epitope between amino acids 159 and 167 (KLSEGDLLA) (79). These cell clones responded to stimulation with full-length PDH-E2, and PDH-E2 complexed with purified AMAs from PBC patients as well as with monoclonal antibody (80). As seen for CD4<sup>+</sup> cells, 159 to 167 precursor frequencies were 10-fold higher in PBC livers and in early stages of PBC. Combined, these data show that antibody epitopes align with both CD4<sup>+</sup> and CD8<sup>+</sup> cell epitopes and share a common peptide motif, ExETDK, which is also shared to some extent with PDH-E2 of *E. coli* (ExDK). This points to a favored hypothesis of mimicry in the pathogenesis of PBC.

Taken together, epitope analyses show a defined B-cell response and a PDH-E2-driven cellular response in the liver involving presentation by antigen-presenting cells and dendritic cells aimed at the biliary epithelium (Fig. 11). The antigen recognition shows a remarkable overlap in this process.

## CHARACTERIZATION OF ANTINUCLEAR ANTIBODIES IN PBC

ANAs, are routinely used as a diagnostic marker in a large number of immune-mediated diseases including autoimmune liver diseases and rheumatological diseases (6). ANAs have also been identified as a serological parameter in up to 52% of patients with PBC. The question is whether these antibodies can be employed to contribute to the diagnosis of PBC by identifying AMA-negative cases of PBC. Antigens of the nuclear pore complex have emerged as secondary antigens in the serological diagnosis of PBC (81,82) (Table 3). Autoantibodies against a 210-kDa glycoprotein of the nuclear membrane (gp 210) (83,84) are well characterized. They are highly PBC specific and occur in 10 to 47% of patients. Although these autoantibodies have been found to exhibit a high specificity for PBC, they persist after orthotopic liver transplantation and do not appear to indicate disease recurrence in this situation (85–87). The epitope has been mapped to the

carboxy terminus of the protein and is recognized by all gp210-positive sera (88).

Nucleoporin p62 is targeted in 32% of PBC sera and also appears to be disease specific (89). In about 20% autoantibodies are detected against Sp100, a nucleoprotein of 100 kDa molecular weight (90). Sp100 appears to exhibit a high specificity for PBC and has also been found to persist after orthotopic liver transplantation for PBC (86). The prognostic significance of these autoantibodies is most likely as low as that found for PBC-specific AMAs (91). Molecular analyses have identified linear Sp100 epitopes in PBC sera (92). One study identified cyclin A as a human autoantigen in hepatic and extrahepatic diseases (8). Anti-cyclin A autoantibodies were detected in 7% of patients with PBC and more frequently in AIH type 1. Other antinuclear autoantibodies with specificity for PBC include the lamin B receptor (93) and the promyelocytic leukemia-associated protein PML (94).

When ANAs are detected in PBC, they frequently display unique immunofluorescence patterns such as nuclear dots (i.e. Sp100) or a nuclear ring-like pattern (laminins, gp210) (Fig. 2). Although in AIH the predominant ANA pattern is a homogeneous or speckled immunofluorescence appearance, ANAs in PBC or AMA-negative PBC are frequently distinguishable during screening by immunofluorescence for nuclear dots or ring patterns. Cases of these autoantibodies in patients with the clinical presentation of PBC and the absence of AMAs are rare but may be the only seroimmunological clue to establishing the diagnosis of PBC in a selected number of patients.

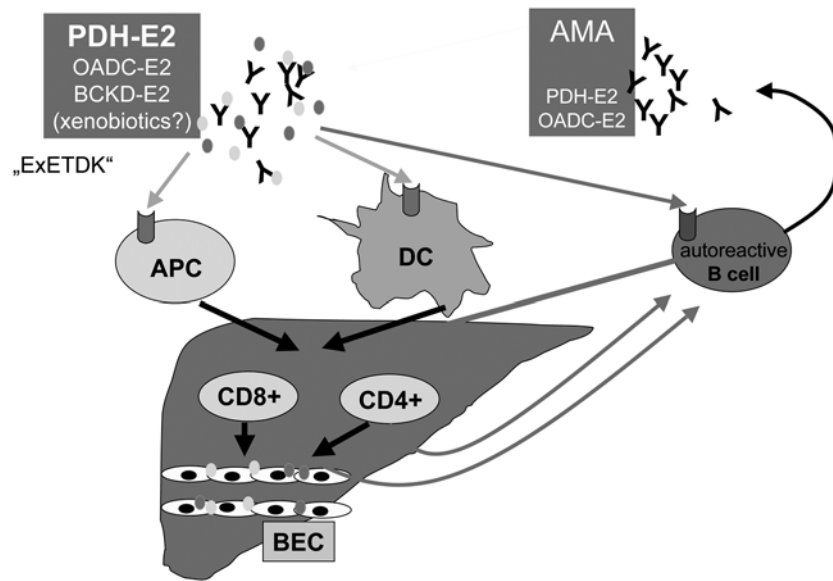
## CHARACTERIZATION OF AUTOANTIGENS IN OVERLAPPING AUTOIMMUNE SYNDROMES

It is an interesting and clinically significant observation that overlap syndromes between different autoimmune diseases of the liver are present in about 18% (95,96). In about 5% of patients with a primary diagnosis of AIH, signs and symptoms of PBC (bilirubin and alkaline phosphatase elevation, liver biopsy) exist. On the other hand, 19% of patients with a primary diagnosis of PBC also have markers or signs of AIH (95,96).

The overlap of PBC and AIH is characterized by the presence of ANAs in 67% and antibodies against SMA in 67% (Fig. 12). Since it has been reported that patients with an overlap of PBC and AIH can respond to corticosteroid treatment equally well as patients with primary AIH, the identification of this variant group by autoantibody characterization is required and contributes to the establishment of a safe and efficacious therapeutic strategy. Overlap syndromes share a number of common features including hypergammaglobulinemia, the presence of ANAs, and interface hepatitis in the histological examination. A specific test to identify and classify overlap syndromes has not yet been established; however, the autoantibody profile allows for a subclassification, in particular the presence or absence of PBC-specific AMAs (97).

The definition of another overlapping syndrome, autoimmune cholangiopathy, is diagnostically and clinically not precisely established. It is a matter of perspective whether autoimmune cholangiopathy is viewed as a subentity of AIH type 1 (98), or





**Fig. 11.** Graphic representation of a model of the immune attack on the biliary epithelium in primary biliary cirrhosis based on B-cell and T-cell data discussed in the text. In this model B- and T-cells act synergistically to produce biliary damage. A role of the diagnostic antimitochondrial antibodies (AMAs) found in PBC patients, specifically directed against PDH-E2 or crossreactive antigens, is also suggested for the pathogenesis of the disease. APC, antigen-presenting cells; DC, dendritic cells; BEC, biliary epithelial cells.

**Table 3**  
**PBC-Associated Antinuclear Antibodies**

Anti-gp210
Anti-nucleoporin p62
Anti-Sp100
Anti-laminin B receptor
Anti-cyclin A
Anti-promyelocytic leukemia protein (PML)

as an AMA-negative form of PBC (99). One case report (among others) has illustrated the diagnostic dilemma: in this report, a 56 yr old Caucasian woman was treated for AMA-positive disease with ursodeoxycholic acid, which led to the normalization of her elevated serum alkaline phosphatase (100). After 18 mo of treatment, alkaline phosphatase and aspartate aminotransferase levels increased, AMA titers disappeared, and previously negative ANA titers were detectable. All parameters normalized after treatment with corticosteroids. This case not only demonstrates a switch of serological markers (AMA to ANA) but also a switch of required treatment regimen. Based on these reports, AIH and PBC may coexist or be subject to disease progression from PBC to AIH. Treatment based on the autoantibody profile proved to be effective and demonstrates the validity of autoantibody testing in overlapping syndromes of autoimmune liver diseases.

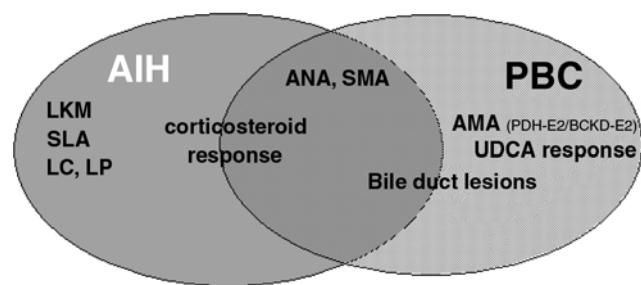
### AUTOANTIBODIES IN HEPATOCELLULAR CARCINOMA

The presence of autoantibodies in patients with hepatocellular carcinoma (HCC) and other malignancies was reported in the 1970s and 1980s and even earlier (101,102). The prevalence

of autoantibodies in HCC is not surprising, and two potential explanations may contribute to this. First, many chronic liver diseases, which have a high prevalence of serological autoimmunity, represent a predisposition for the development of HCC; among them is chronic viral hepatitis (HCV, HBV, HDV). Moreover, rheumatological symptoms are a frequent clinical observation in patients with malignant diseases and may reflect a predisposition for serological autoimmunity (103). Second, mechanisms leading to the deregulation of death pathways and nuclear cycling, as well as other processes, lead to profound changes in the nuclear protein repertoire, which, in addition to cell death and exposure of cell protein from degraded cells to the immune system, may lead to a loss of tolerance (104). In terms of this hypothesis, autoantibodies such as ANA (105) but also proteins against p53 (106), human telomerase reverse transcriptase (hTERT) (107), and cyclin B1 (108) have been detected. The development of ANA as well as titer elevations has been reported to coincide with neoplastic transformation, which appears to substantiate this hypothesis (105). The prevalence varies between 9 and 31%. A predictive or diagnostic role in the absence of elevated  $\alpha$ -fetoprotein has been suggested but remains to be conclusively shown.

### CONCLUDING REMARKS

Autoantibodies represent a powerful diagnostic tool and also serve as a scientific window for the study of mechanisms involved in autoimmunity and the loss of tolerance. Today it is almost inconceivable that an efficiently treatable chronic liver disease characterized by autoantibodies such as AIH was once debated and its existence challenged. The multitude of different autoantibodies reported to date require an increasing awareness



**Fig. 12.** Features of overlapping autoimmune diseases (“overlap syndrome”) in autoimmune hepatitis (AIH) and primary biliary cirrhosis (PBC) (3). The establishment of a true overlap is reached by the documentation of hepatitis and cholestasis biochemically, a histology compatible with both diseases (presence of biliary lesions in otherwise typical features of AIH), as well as the presence of anti-mitochondrial autoantibodies (AMAs) and autoantibodies typical of AIH (i.e., antinuclear antibody [ANA]). For antibody nomenclature, see Table 1. PDH-E2, E2 subunit of pyruvate dehydrogenase; BCKD-E2, E2 subunit of branched chain ketoacid dehydrogenase; LC, liver cytosolic; LKM, Liver-kidney microsomal; LP, liver pancreas; SLA, soluble liver antigen; SMA, smooth muscle actin.

not only of their specificities but also of the methodology employed in their detection, the clinical circumstances surrounding the patient, and also the historic development of the individual markers. The most significant challenge for the practising hepatologist is the discrimination between serological autoimmunity (present in many disease entities and even in otherwise healthy appearing individuals) and genuine autoimmune disease, which is rare and requires treatment. Although AMAs are highly disease specific for PBC and SLA/LP antibodies appear to have a high predictive value for AIH, most autoantibodies can only be of value after careful assessment and adequate testing methodology. This is becoming increasingly difficult in view of the increasing numbers and specificities of autoantibodies detected and detectable in humans. From a scientific perspective, autoantibodies confront us with the realization that autoimmune diseases lead to serological heterogeneity. AIH can be characterized by ANA, SMA, SLA/LP, and LKM autoantibodies. Although the loss of self-tolerance is indicated by all these autoantibodies, the exact mechanisms remain elusive, and a defined antigen-based process that is convincingly reproducible in animal models is still lacking. Autoantibodies define candidate proteins for such processes. The example of HCC illustrates that proteins involved in cell cycling and cell death can become targets of an immune response and appear to reflect steps occurring in carcinogenesis. Their diagnostic value nevertheless remains controversial. At present autoantibody testing is a valuable diagnostic tool and an inherent component of every hepatological workup.

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# 9 The Role of Inflammation and Immunity in the Pathogenesis of Liver Fibrosis

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## KEY POINTS

- There has been continued clarification of the cellular source of extracellular matrix (ECM) in hepatic fibrosis, major advances in understanding signaling and transcriptional events, and exciting insights into the biology of fibrosis progression and resolution.
- Both fibrosis and cirrhosis are the consequences of a sustained wound-healing response to chronic liver injury, and they are determined by the nature and severity of the underlying liver disease as well as the extent of hepatic fibrosis.
- Even cirrhosis may regress, although the inflammatory and immunologic determinants of reversibility are uncertain.
- The hepatic lymphocyte populations are very diverse and are dominated by cells that are rare in other parts of the body including natural killer (NK), natural killer cells with a T-cell receptor (NKT), T cells with the standard  $\alpha\beta$  T-cell receptor (TCR $\alpha\beta$ ), T cells with the  $\gamma\delta$  receptor (TCR $\gamma\delta$ ), and B cells.
- The sinusoidal structure, low flow rates and resident Kupffer cell population all contribute to retention of activated T cells in the liver.
- The identification of pattern recognition receptors including Toll-like receptors (TLRs) has been a crucial advance, whose impact on fibrosis progression and resolution is not yet clearly understood.
- The activated hepatic stellate cell (HSC) is the primary source of fibrosis in liver disease; however, related mesenchymal cell types from a variety of sources may also make measurable contributions.
- Degradation of interstitial, or scar, matrix is required for fibrosis regression, and Kupffer cells, or liver macrophages, may regulate this response.
- Stellate cells can amplify the inflammatory response by inducing infiltration of mono- and polymorphonuclear leukocytes.
- The two aspects of immunomodulation of liver fibrosis that are best understood are the interactions between HSCs and NK cells and the impact of the Th1/Th2 dichotomy of CD4<sup>+</sup> T cells on fibrogenic activity.

## INTRODUCTION

Hepatic fibrosis represents a ubiquitous response of the liver to acute or chronic injury. Tremendous progress in understanding the pathophysiology of this wound-healing response has led to realistic expectations for treating fibrosis in patients with chronic liver disease owing to either viral hepatitis or metabolic or autoimmune diseases, among others. There has been continued clarification of the cellular source of extracellular matrix (ECM) in hepatic fibrosis, major advances in understanding signaling and transcriptional events, and exciting insights into the biology of fibrosis progression and resolution (*see refs. 1–4 and references therein for more general reviews*).

The clarification of interactions between the immune system and fibrogenic response has been among the most exciting developments in fibrosis research during the past 5 yrs (5). In the liver, these advances include evidence of direct interactions between immune cell subsets and fibrogenic cells in liver, the emergence of natural killer (NK) cells as determinants of hepatic stellate apoptosis and thus fibrosis resolution, the establishment of hepatocellular apoptosis as an inflammatory and fibrogenic stimulus, and the growing recognition that hepatic stellate cells (HSCs) contribute to the innate immune response. These and other observations underscore the prospect for eventually manipulating these interactions therapeutically.

Whereas fibrosis accompanies progressive liver injury and may vary from mild to extensive, cirrhosis is the end stage of fibrosis of the hepatic parenchyma, resulting in nodule formation that can lead to altered hepatic function and blood flow. Both fibrosis and cirrhosis are the consequences of a sustained wound-healing response to chronic liver injury, with variable clinical manifestations that are determined by the nature and severity of the underlying liver disease as well as the extent of hepatic fibrosis. Recent studies suggest that cirrhosis is a slowly progressive disease whose risk of complications accrues over time, with an

annual mortality rate of 4% in patients infected with chronic hepatitis C virus (HCV) (6). Among patients with cirrhosis, approx 70% of deaths are directly attributable to liver disease (7), the largest fraction of which is due to hepatocellular carcinoma (HCC) (6). The overall burden of liver disease in the United States—the vast majority of which is caused by chronic disease with fibrosis—continues to expand, and it has a growing economic and social impact (8).

Remarkably, recent studies suggest that not only is fibrosis reversible, but in selected patients even cirrhosis may regress, although the determinants of reversibility and its likelihood in patients with chronic liver disease are not completely understood (9). Moreover, the relative contribution of immune interactions to reversibility is unknown. Still, the continued clarification of how the immune system regulates both fibrosis progression and regression, combined with basic science advances in understanding of both acquired and innate immunity, augur well for significant progress in exploiting this knowledge to the benefit of patients.

This chapter will review the immune cellular components and general pathophysiology of hepatic fibrosis and then emphasize our growing knowledge of the immune and molecular mediators of fibrosis, which establish the basis for how these advances might lead to immunomodulation of liver fibrosis.

## IMMUNE CELLULAR COMPONENTS IN LIVER

The unique and important role of resident immune cells in liver has only recently been appreciated. The healthy liver has a very large and diverse number of immune cell populations, as demonstrated by analysis of isolated cell populations following enzymatic digestion. Healthy rodent and human livers contain approximately  $1$  to  $3 \times 10^6$  cells per gram of tissue, the composition of which is unique. The hepatic lymphocyte populations are very diverse and are dominated by cells that are rare in other parts of the body including NK, natural killer cells with a T-cell receptor (NKT), T-cells with the standard  $\alpha\beta$  T-cell receptor (TCR $\alpha\beta$ ), T cells with the  $\gamma\delta$  receptor (TCR $\gamma\delta$ ), and B cells. In several liver diseases histological analysis has identified large populations of immune cells (*see below*).

Each of these cellular populations has a distinct origin, regulatory pathway, and effector function that may influence liver fibrosis. The cells of the innate immune system (NK and NKT) are phylogenetically older and provide the first response to pathogens. NK cells are relatively abundant in the liver and comprise 25 to 30% in humans and 10 to 20% of the intrahepatic lymphocyte (IHL) population in mice. Morphologically, they are large granular lymphocytes (Pit cells), with the majority found in the sinusoidal lumen in contact with Kupffer and endothelial cells (10). Their predominant function is cytotoxicity toward a range of targets including tumor cells and virally infected cells. This activity is mediated by a number of effector mechanisms, including CD95-L, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), and perforin/granzyme. In addition, they augment the immune response by stimulating T cells and macrophages through the production of cytokines, the most important of which is interferon- $\gamma$  (IFN- $\gamma$ ). The full maturation

and survival of NK cells is also dependent on IFN- $\gamma$  as NK cells from IFN- $\gamma$ -deficient mice do not express TRAIL and have poor cytotoxic function. In addition to IFN- $\gamma$ , NK cells secrete a number of other cytokines including tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin-5 (IL-5), IL-10, and IL-13 (11). Their direct cytotoxicity is augmented by their ability to stimulate the adaptive immune response (12).

An important mechanism of NK cell regulation is via membrane-bound receptors that provide inhibitory signals. Class I molecules are a well-characterized set of ligands for these inhibitory receptors, and they minimize NK cell cytotoxicity toward cells with normal or high levels of class I expression. The inhibitory receptors have a range of structures including the immunoglobulin superfamily and C-type lectin-like family that result in phosphorylation of immunoreceptor tyrosine-based inhibitory motifs (13). NK cells also possess membrane-bound activating receptors including CD16 and NKG2D. Ligands for NK cell-activating receptors have a similar structure to class I molecules and have been found on several HCCs, but not normal hepatocytes. The signal from many of the stimulatory receptors is strong enough to overcome the inhibition from the presence of conventional class I molecules. The potential targets for NK cell cytotoxicity are broader than just tumor and virus-infected cells, because NK cells can induce apoptosis of Purkinje cells as well as HSCs.

NKT cells display many of the features of NK cells including inhibitory and activating receptors, potent cytotoxic function, and the production of IFN- $\gamma$  and IL-4. NKT cells also possess TCRs. Most NKT cells in the liver have an  $\alpha\beta$  TCR with invariant TCR V $\alpha$ -J $\alpha$  combination, with V $\alpha$ 14 and J $\alpha$ 281 in the mouse, and the homologous V $\alpha$ 24 and J $\alpha$ Q in humans. These are termed classical NKT cells, and their development is dependent on nonpolymorphic class I molecules (CD1). In addition to the classical NKT cell population, a smaller CD8-expressing NKT cell population has been identified. This population is CD1 independent and does not use the invariant TCR. CD8-expressing NKT cells display cytotoxic ability but have a more restricted cytokine production, with predominant production of IFN- $\gamma$ . TCR $\gamma\delta$ -expressing NKT cells are also present and mostly lack CD4 and CD8, although some are CD8<sup>+</sup>. The development of  $\gamma\delta$ NKT cells is MHC independent, and their cytokine profile is dominated by IFN- $\gamma$  production. NKT cells are involved in immune responses including tumor rejection, immune surveillance, protection against microbial infection, and control of autoimmune diseases. They are also important in experimental models of liver injury including concanavalin A-induced hepatitis and endotoxin-induced liver injury.

T cells in the liver with the  $\alpha\beta$  TCR have many important differences from  $\alpha\beta$  T cells in lymph nodes and the spleen. The majority display markers of activation and are undergoing the cell cycle. A significant percentage (5–10%) are undergoing apoptosis, which increases significantly in the presence of large quantities of high-affinity peptide. Most hepatic T cells are thought to undergo activation in the spleen and lymph nodes and are subsequently retained in the liver via an intracellular adhesion molecule-1 (ICAM-1) and vascular adhesion protein-1

(VAP-1)-mediated process. The sinusoidal structure, low flow rates, and resident Kupffer cell population all contribute to this retention. Activated T cells retained by the liver have clear functions, which include classical cytotoxicity toward hepatocytes as well as potentially regulatory function via IFN- $\gamma$ , IL-4, IL-10 and IL-13. The liver is relatively enriched for CD8<sup>+</sup> T cells compared with the lymph node and spleen. In addition, there is a significant population of T cells that do not express CD4 or CD8 (double negative [DN]), and many of these are thought to be preapoptotic. CD4<sup>+</sup> T cells with well-defined Th1 and Th2 profiles have been identified in the liver, and adoptive transfer of each of these populations demonstrates that these cells can survive for weeks. Interestingly, the transferred CD4<sup>+</sup> Th1 cells become nonfunctional, but the CD4<sup>+</sup> Th2 cells retain their functionality (14,15). These data and the bias toward Th2 by antigen presentation within the liver suggest that CD4<sup>+</sup> T-cell development and survival in the liver is biased toward a Th2 phenotype.

A subgroup of T cells with significant regulatory activity toward other components of the immune system has been identified. These cells, which are functionally defined as regulatory T cells (T-reg), consist of a heterogeneous population. Phenotypically, the best characterized population expresses CD4<sup>+</sup>/CD25<sup>+</sup>, as well as the forkhead transcription factor foxp3 (16). This is a key factor in murine regulatory cell development and confers a regulatory phenotype upon forced expression (17). Such T-regs require initial activation via the TCR and then express their suppressive function in an antigen-nonspecific manner. The mechanism of the immunosuppression is not fully understood with evidence for the requirement of cytotoxic T-lymphocyte antigen-4 (CTLA-4), IL-10, and transforming growth factor- $\beta$  (TGF- $\beta$ ) (18). The immunoregulatory nature of the T-reg effect is underscored by its ability to be overcome by IL-2 or CD28 costimulation. Other less well-defined populations of T-regs secrete predominantly IL-10 (Tr1) or TGF- $\beta$  (Th3). Increased numbers of T-regs have been identified in HCC tissue and also in the peripheral blood of patients with chronic HBV infection.

### **PATTERN RECOGNITION RECEPTORS: GENERAL FEATURES**

The mechanisms by which complex organisms detect the presence of infectious agents have been one of the most intriguing in immunology. The identification of the germline encoded molecules including Toll-like receptors (TLRs) has been a crucial advance. These receptors are members of an expanding group of molecules known as pattern recognition receptors (19–21). TLRs recognize relatively invariant structures called pathogen-associated molecular patterns (PAMPs) that are shared by many pathogens but not expressed by the host. Examples of PAMPs include lipopolysaccharide (LPS), lipoteichoic acid (LTA), and unmethylated CPG DNA of bacteria lipoarabinomannan (LAM) of mycobacteria. These PAMPs are recognized by specific TLRs and result in a cascade of signaling molecules with upregulation of effector molecules (22). One group of effector molecules consists of reactive oxygen intermediates and

antimicrobial peptides. A second group consists of costimulatory molecules that are upregulated and increase the efficiency of activation of the adaptive immune response. A third group includes cytokines, chemokines, and adhesion molecules. As can be surmised from this activation TLRs has far-reaching consequences on immune activation and provides a rapid response to pathogens.

The TLRs are, however, only a subgroup of pattern recognition receptors, with a non-TLR group termed the caterpillar protein family. This includes the two molecules NOD1 and NOD2 as well as a group of 14 NALP proteins (23). There has been great interest in NOD2 based on its association with susceptibility to Crohn's disease, and mutations of members of the NALP family have been shown to be responsible for rare, mostly autosomal recessive, periodic fever syndromes (24). The role of NALPs in the immune response is currently poorly understood.

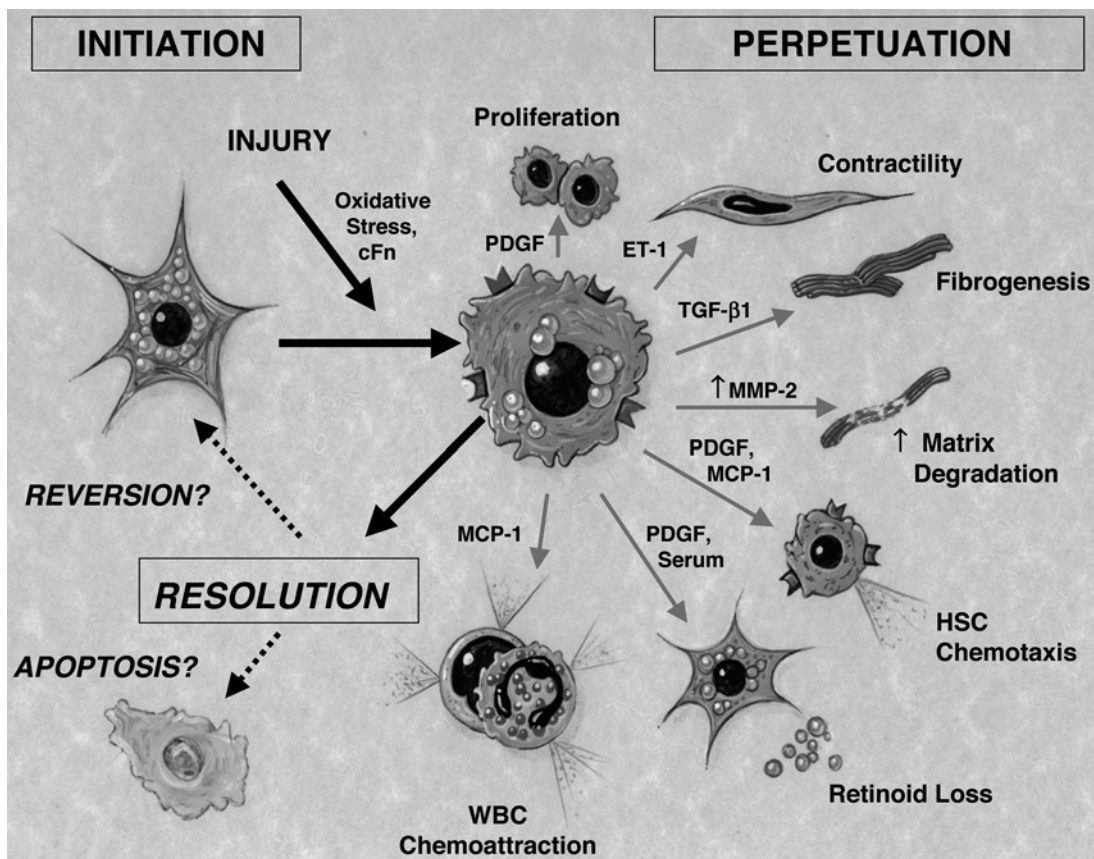
### **CELLULAR PATHOPHYSIOLOGY OF HEPATIC FIBROSIS AND THE ROLE OF HEPATIC STELLATE CELLS**

The identification of the cellular sources of ECM in hepatic fibrosis has laid the groundwork for defining mechanisms of fibrosis and potential therapies. The HSC (previously called the lipocyte, Ito, fat-storing, or perisinusoidal cell) is the primary source in normal and injured liver. In addition, related mesenchymal cell types from a variety of sources may also contribute measurably to total matrix accumulation, including classical portal fibroblasts (25–27) (especially in biliary fibrosis), bone marrow-derived cells (28,29), and possibly mesenchyme through epithelial-mesenchymal cell transition into hepatocytes (EMT) (30). Although EMT is well established in the kidney (30–32), its importance in liver fibrosis is less certain.

HSCs are resident perisinusoidal cells in the subendothelial space between hepatocytes and sinusoidal endothelial cells (*see* refs. 4 and 33 for reviews). They are the primary site for storing retinoids within the body. Stellate cells can be recognized by their vitamin A autofluorescence, perisinusoidal orientation, and variable expression of a number of the cytoskeletal proteins including desmin, glial acidic fibrillary protein, vimentin, and nestin, among others (34,35). In strict terms, “stellate cells” may represent a heterogeneous population of mesenchymal cells with respect to cytoskeletal phenotype, vitamin A content, and localization (34,35), but collectively they are the key fibrogenic cell type in the liver. Moreover, a remarkable plasticity of the stellate cell phenotype has been documented *in vivo* and *in culture*, precluding a strict definition based only on cytoskeletal phenotype (36,37). Stellate cells with fibrogenic potential are not confined to the liver and have been identified in the pancreas, for example, where they contribute to desmoplasia in chronic pancreatitis (38) and carcinoma (39).

Studies *in situ* in both animals and humans with progressive injury have defined a gradient of changes within stellate cells that collectively are termed *activation* (Fig. 1). Stellate cell activation refers to the transition from a quiescent vitamin A-rich cell to a highly fibrogenic cell characterized morphologically





**Fig. 1.** Role of stellate cell activation in hepatic fibrosis. Following liver injury, hepatic stellate cells undergo *activation*, during which they are transformed from quiescent vitamin A-rich cells into proliferative, fibrogenic, and contractile myofibroblasts. The major phenotypic changes after activation include proliferation, contractility, fibrogenesis, matrix degradation, chemotaxis, retinoid loss, and white blood cell (WBC) chemoattraction. Key mediators underlying these effects are shown. The fate of activated stellate cells during the resolution of liver injury is uncertain but may include reversion to a quiescent phenotype or selective clearance by apoptosis. ECM, extracellular matrix; cFn, cellular fibronectin; PDGF, platelet-derived growth factor; ET-1, endothelin 1; TGF-β1, transforming growth factor β1; MMP-2, matrix metalloproteinase-2; MCP-1, monocyte chemoattractant protein-1; HSC, hepatic stellate cell. (From ref. 75, with permission.)

by enlargement of rough endoplasmic reticulum, diminution of vitamin A droplets, ruffled nuclear membrane, appearance of contractile filaments, and proliferation. Cells with features of both quiescent and activated cells are often called transitional cells. As noted above, proliferation of stellate cells occurs in regions of greatest injury, which is typically preceded by an influx of inflammatory cells and is associated with subsequent extracellular matrix accumulation.

Conceptually, activation occurs in two phases, *initiation* and *perpetuation* followed by *resolution* when liver injury has subsided. Initiation refers to the earliest events that render cells responsive to cytokines, and perpetuation connotes those responses to cytokines that collectively enhance scar formation (*see below*). Resolution refers to the fate of activated stellate cells when the primary insult is withdrawn or attenuated (4).

Once stellate cells are “primed” by initiating factors, perpetuation occurs, which can be subdivided into at least six distinct events that can occur simultaneously. Features of the perpetuated phenotype are detailed in the following section.

### PROLIFERATION

Platelet-derived growth factor (PDGF) is a key stellate cell mitogen (40), whose signaling pathways have been well characterized in this cell type (41). In addition to proliferation, PDGF stimulates  $\text{Na}^+/\text{H}^+$  exchange, providing a potential site for therapeutic intervention by blocking ion transport (42). Other compounds with mitogenic activity toward stellate cells include vascular endothelial cell growth factor (43), thrombin (44,45), epidermal growth factor (EGF), TGF-α, keratinocyte growth factor (46), and basic fibroblast growth factor (bFGF) (47). Signaling pathways for these and other mitogens have been greatly clarified in stellate cells (41).

### CHEMOTAXIS

Stellate cells can migrate toward cytokine chemoattractants (41,48) mediated by a number of transmembrane receptors (41,49,50).

### FIBROGENESIS

Increased matrix production is the most direct way that stellate cell activation generates hepatic fibrosis. TGF-β1 is the

most potent fibrogenic factor identified to date; it stimulates the production of matrix components including collagen, cellular fibronectin and proteoglycans (51). Signals downstream of TGF- $\beta$  converge a family of bifunctional molecules known as Smads, which refine or enhance TGF- $\beta$ 's effects downstream of its receptors (52–54). Smads 2 and 3 elicit distinct signaling responses that favor stellate cell activation and fibrogenesis (41), whereas Smad 7 is inhibitory via activity of Id protein (55), making it an attractive molecule to utilize in antifibrotic therapies (56). The response of Smads in stellate cells differs between acute and chronic injury to further favor matrix production (55,57,58).

It is important to emphasize that although most analyses of TGF- $\beta$  in hepatic fibrosis have focused on its potent fibrogenic activity, it is also a highly immunoregulatory molecule (59). However, the potential importance of TGF- $\beta$ 's immunomodulatory activity—via effects mediated through T-cell subsets or fibrogenic cells—in mediating hepatic fibrosis has been largely overlooked.

### CONTRACTILITY

Contractility of stellate cells may be a major determinant of early and late increases in portal resistance during liver fibrosis. Activated stellate cells impede portal blood flow both by constricting individual sinusoids and by contracting the cirrhotic liver, since the collagenous bands typical of end-stage cirrhosis contain large numbers of activated stellate cells (*see ref. 1 for review*).

The major contractile stimulus toward stellate cells is endothelin-1, whose receptors are expressed on both quiescent and activated stellate cells but whose subunit composition may vary (1). Increased endothelin levels result from increased endothelin-converting enzyme (ECE) activity due to stabilization of the ECE mRNA (60).

Another key contractile mediator in activated stellate cells is angiotensin II, which is synthesized by activated stellate cells in an NADPH-dependent pathway (61–63).

Locally produced vasodilator substances may oppose the constrictive effects of endothelin-1 (64,65). Nitric oxide, which is also produced by stellate cells, is a well-characterized endogenous antagonist to endothelin.

### MATRIX DEGRADATION

Quantitative and qualitative changes in matrix protease activity play an important role in ECM remodeling accompanying fibrosing liver injury. An enlarging family of matrix metalloproteinases (also known as matrixins) has been identified, which are calcium-dependent enzymes that specifically degrade collagens and noncollagenous substrates (*see refs. 66 and 67 for reviews*). In liver, “pathological” matrix degradation refers to the early disruption of the normal subendothelial matrix, which occurs through the actions of at least four enzymes: *matrix metalloproteinase 2 (MMP-2)* (also called gelatinase A or 72-kDa type IV collagenase) and *MMP-9* (gelatinase B or 92-kDa type IV collagenase), which degrade type IV collagen, *membrane-type metalloproteinase-1 or -2*, which activate latent MMP-2, and *stromelysin-1*, which degrades

proteoglycans and glycoproteins and also activates latent collagenases. Stellate cells are a key source of MMP-2 (68), MMP-13 in rodents (69), and stromelysin (68).

Failure to degrade the increased interstitial, or scar, matrix is a major determinant of progressive fibrosis, and Kupffer cells, or liver macrophages, have emerged as key determinants of this response. An elegant genetic model in mice recently demonstrated that macrophage depletion during fibrosis progression attenuates fibrosis, whereas depletion during fibrosis regression augments fibrosis (70). It is unknown whether these divergent responses reflect different subpopulations of macrophages or different functions of the same macrophage population (Fig. 2) (71). Regardless, the findings reemphasize the potentially important role of macrophages—a key component of the hepatic immune system—in regulating fibrogenesis and point to the need for further studies of this cell type.

Progressive fibrosis is associated with marked increases in tissue inhibitor of metalloproteinases (TIMP-1) (72,73) and TIMP-2 (74), leading to a net decrease in protease activity and therefore more unopposed matrix accumulation. Stellate cells are the major source of these inhibitors (66). Sustained TIMP-1 expression is emerging as a key reason for progressive fibrosis, and its diminution is an important prerequisite to allow for reversal of fibrosis. It is unclear whether the activity of macrophages in fibrosis regression is related to interactions with or modulation of TIMP-1.

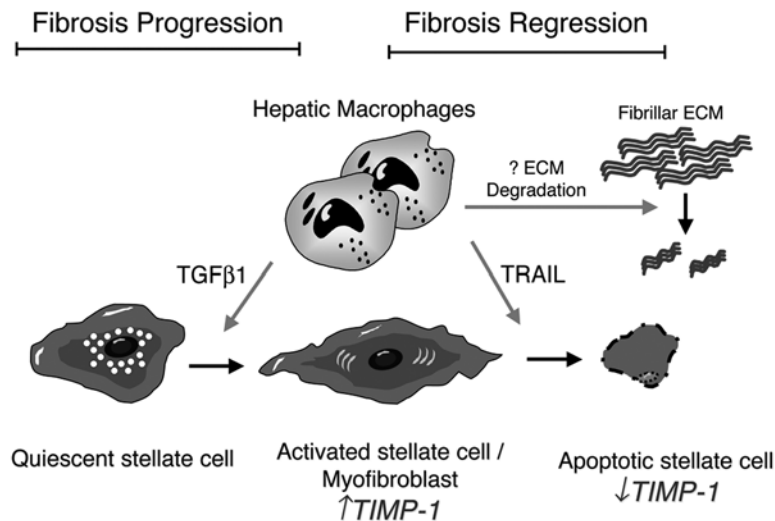
### RETINOID LOSS

As stellate cells activate, they lose their characteristic perinuclear retinoid (vitamin A) droplets and acquire a more fibroblastic appearance. In culture, retinoid is stored as retinyl esters, whereas stellate cells activate the retinoid released outside the cell as retinol, suggesting that there is intracellular hydrolysis of esters prior to export (75). However, it is unknown whether retinoid loss is required for stellate cells to activate and which retinoids might accelerate or prevent activation *in vivo*.

### ROLE OF STELLATE CELLS IN INFLAMMATORY SIGNALING AND INNATE IMMUNITY

Stellate cells are assuming an increasingly central role in our understanding of hepatic inflammation. They can amplify the inflammatory response by inducing infiltration of mono- and polymorphonuclear leukocytes. Activated stellate cells produce chemokines that include monocyte chemoattractant protein-1 (MCP-1) (64), CCL21 (76), regulated on activation, T-cell expressed and secreted (RANTES), and CCR5 (77). They also express TLRs (78), indicating a capacity to interact with bacterial LPS, which in turn stimulates stellate cells (79). Stellate cells can also function as antigen-presenting cells (80) that can stimulate lymphocyte proliferation or apoptosis (81). In addition to mononuclear cell chemoattractants, stellate cells produce neutrophil chemoattractants, which could contribute to the neutrophil accumulation characteristic of alcoholic liver disease.

In addition to regulating leukocyte behavior, stellate cells may in turn be affected by specific lymphocyte populations.



**Fig. 2.** Role of macrophages in the progression and regression of hepatic fibrosis. Hepatic macrophages may elicit divergent effects on liver fibrosis by promoting stellate cell activation in the face of continued injury and fibrosis and stellate cell apoptosis during fibrosis regression during recovery, once injury has subsided. Evidence from other studies implicates transforming growth factor- $\beta$  (TGF- $\beta$ 1) as one potential paracrine stimulator of stellate cell activation by macrophages, whereas tumor necrosis factor-related apoptosis ligand (TRAIL) may mediate stellate cell apoptosis during fibrosis regression associated with recovery. Apoptosis associated with loss of tissue inhibitor of metalloproteinase-1 (TIMP-1) may unmask latent matrix protease activity released by either macrophages, stellate cells, or other cell types. It is not certain whether the same macrophages account for the divergent activities of this cell type or whether different macrophage subsets mediate these opposing pathways. ECM, extracellular matrix. (Modified from ref. 71 based on findings in ref. 70).

For example, CD8 cells harbor more fibrogenic activity toward stellate cells than CD4 cells (82), which may explain in part the increased hepatic fibrosis seen in patients with HCV/HIV coinfection, in which CD4/CD8 ratios are reduced, compared with in patients monoinfected with HCV alone.

The role of pattern recognition receptors in HSCs is also being uncovered. Activated human HSCs express TLR4 and the other two molecules (CD14 and MD2) that together form the LPS receptor complex (78). In activated human HSC; low concentrations of LPS induced activation and NF- $\kappa$ B and JNK, leading to expression of chemokines and adhesion molecules in activated human HSCs. Mouse HSCs express TLR4 and TLR2 and respond to a range of PAMPs including LPS, LTA, and *N*-acetyl muramyl peptide with secretion of IL-6, TGF- $\beta$  and MCP-1 (79). These *in vitro* results suggest that bacterial wall products produce an inflammatory phenotype in HSCs but notably do not induce matrix deposition, since fibronectin and collagen transcripts were not increased. Signaling to HSCs via TLR4 may function to enhance an adaptive immune response against bacterial pathogens, and HSCs would facilitate this response by helping with the recruitment of immune cells and amplifying the initial signal. It is also possible that ligation of TLR4 is just the initial step in a series of signals required for differentiation of HSCs into a fully fibrogenic phenotype. This may be by recruitment of Th2-type Kupffer cells or other immune cells, which provide additional signals such as IL-13.

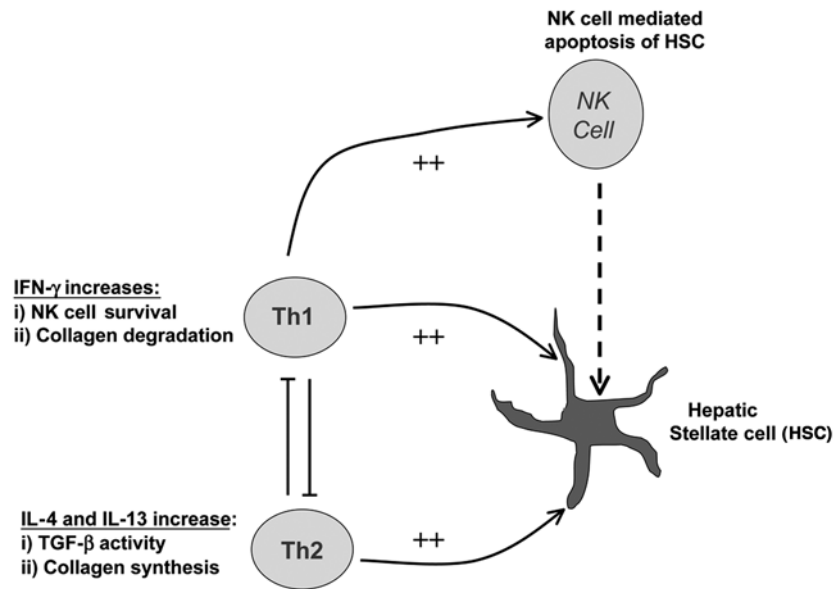
Although it has been determined that TLRs and members of the caterpillar family recognize molecular patterns in pathogens, there is no theoretical constraint limiting recognition of

self-molecular patterns, which are usually hidden inside cells. In fact, there is increasing evidence that self-molecules may activate some of these receptors. The best evidence is presentation of apoptotic mammalian DNA that is relatively CpG rich and can activate TLR9 (83). This pathway is important in autoactivation of B cells and may play a role in the activation of HSCs by apoptotic cells (84). A further example is the activation of immune cells by uric acid, which is dependent on the presence of NALP3 and the adaptor molecule apoptosis-associated speck-like protein containing a caspase-recruitment domain (85). It will be important to identify the molecules from apoptotic bodies that provoke HSCs, as none have been identified; pattern recognition receptors may play an important role in this process. Of equal importance, the identification of apoptotic fragments from damaged hepatocytes as fibrogenic stimuli is an important conceptual advance, which has led to new approaches to antifibrotic, hepatoprotective therapies using caspase inhibitors in patients with chronic liver disease (86).

## IMMUNOMODULATION OF LIVER FIBROSIS

The elucidation of novel pathways of immune regulation and effector subsets in normal and diseased liver has provoked exploration of how these cells affect liver fibrosis, particularly HSCs. Currently the two aspects of immunomodulation of fibrosis that are best understood include the interactions between HSCs and NK cells and the impact of the Th1/Th2 dichotomy of CD4<sup>+</sup> T cells on fibrogenic activity (Fig. 3).

During resolution of liver fibrosis, a significant proportion of activated stellate cells are undergoing apoptosis, but the underlying mechanism has not been clarified (66). In culture, HSCs



**Fig. 3.** Model of immune regulation of liver fibrosis. Th1 and Th2 cells inhibit each other's development and also have opposing effects on liver fibrosis. Th1 cytokines (predominantly interferon- $\gamma$  [IFN- $\gamma$ ]) stimulate natural killer (NK) cell function, and also stimulate enzymes active in collagen degradation. This has the effect of increasing hepatic stellate cell (HSC) apoptosis, thus limiting new matrix deposition and increasing breakdown of established matrix. Th2 cells via interleukin-4 (IL-4) and IL-13 increase transforming growth factor- $\beta$  (TGF- $\beta$ ) activity and collagen synthesis of HSCs.

are sensitive to CD95-L and TRAIL-mediated apoptosis, and NK cells can induce apoptosis of HSCs by a TRAIL-mediated mechanism (87). In a recent study (87), an antifibrotic effect of NK cells is indicated by the presence of increased fibrosis in mice depleted of NK cells by anti-asialo-GM1 antibody and by decreased fibrosis after NK cell activation by a TLR3 ligand poly I:C. The NK cell-induced HSC apoptosis was specific for activated HSCs that expressed the NK cell-activating receptor NKG2D. The activated NK cells deliver a lethal blow to HSCs by inducing apoptosis with TRAIL. In this study NK cell function was dependent on IFN- $\gamma$ , and provided an explanation for earlier experiments demonstrating an important antifibrotic role for IFN- $\gamma$  (88). The antifibrotic role of NK cells was further supported by evidence of direct adhesion to HSCs in mouse livers and the development of greater fibrosis in mice genetically deficient in NK cells (89). Most recently, these findings have been reinforced by studies in humans with HCV (90).

NK cells can induce apoptosis of virally infected cells and tumor cells with low expression of class I, but induction of apoptosis of normal cells by NK cells is a relatively new concept and has been demonstrated for NK cells, immature dendritic cells, and neurons in the dorsal root ganglion (91,92). The roles of NK cells in HSC apoptosis and NK cell activation by TLR3 in reducing liver fibrosis raise important questions about how these functions are altered during chronic viral infections with HCV and HIV, as well as by therapeutic immunosuppression. HCV is expected to activate TLR3 and, based on the above paradigm, would activate NK cells and decrease liver fibrosis. This is contrary to a large amount of clinical data on the role of HCV in the progression of liver fibrosis. However, the interaction of HCV with the TLR3 pathway is

much more complex than simple activation, and a number of viral mechanisms actually decrease signaling through this pathway. These include proteolysis by HIV NS3/4A serine protease of the adaptor proteins, which link TLR3 to kinases responsible for activating a number of antiviral responses (93). Ligation of CD81 on NK cells by HCV E2 also inhibits NK cell function. This inhibition of NK cell function by HCV would be expected to decrease HSC apoptosis and increase liver fibrosis. Therefore, adaptation by HCV to limit the innate immune response may result in increased liver fibrosis. Decreased NK cell function has also been demonstrated in HBV infection, but the underlying mechanisms have not been identified.

The antifibrotic role of NK cells is also consistent with the clinical data of increased liver fibrosis in the setting of therapeutic immunosuppression. The effect of single immunosuppressive agents on NK cell function is minimal, but the combination of cyclosporine and corticosteroids results in significant loss of NK cell cytotoxicity (94). In addition, cyclosporine renders some cells resistant to NK cell-mediated cytotoxicity. The effect of HIV infection on NK cell number and function is more complex. Some NK cell subsets coexpress CD4 and HIV coreceptors and are targets for infection with HIV. NK cells from HIV-infected patients have reduced cytolytic activity and decreased production of cytokines (95). The hypothesis that NK cells limit liver fibrosis by inducing HSC apoptosis can serve as a model for explaining the above clinical observations. This model predicts that NK cell function will be relatively impaired in individuals with rapid progression of fibrosis and compared with those in whom liver fibrosis progresses slowly.

The role of NKT cells in liver fibrosis is less well understood. The observation that CCl<sub>4</sub>-induced fibrosis is not diminished in

**Table 1**  
**Impact of Genetic Background Fibrosis Susceptibility in Different Mouse Strains<sup>a</sup>**

<i>Manipulation</i>	<i>Deficiency</i>	<i>Strain/sex</i>	<i>Injury</i>	<i>Fibrosis</i>	<i>Ref.</i>
RAG2 <sup>-/-</sup>	T, B, NKT	BALB/M	CCl <sub>4</sub>	Reduced	104
SCID	T, B, NKT	BALB/M	CCl <sub>4</sub>	Reduced	96
SCID	T, B, NKT	B6/M	CCl <sub>4</sub>	Increased	96
SCID	T, B, NKT	BALB/M	CCl <sub>4</sub>	Reduced	89
SCID-Beige	T, B, NKT, NK	BALB/M	CCl <sub>4</sub>	No difference	89
IFN-γ <sup>-/-</sup>	IFN-γ	BALB/M	CCl <sub>4</sub>	Increased	96
IFN-γ <sup>-/-</sup>	IFN-γ	B6/M	CCl <sub>4</sub>	Increased	96
B2m <sup>-/-</sup>	CD8 <sup>+</sup> T, NKT	B6/M	CCl <sub>4</sub>	No difference	104
MHCII <sup>-/-</sup>	CD4 <sup>+</sup> T	B6/M	CCl <sub>4</sub>	No difference	104
Jh <sup>-/-</sup>	B cells	BALB/M	CCl <sub>4</sub>	Reduced	104
uMT <sup>-/-</sup>	B cells	B6/M&F	Schistosoma	Increase	104
TCRδ <sup>-/-</sup>	αδT	B6/?	CCl <sub>4</sub>	No difference	104
CD1 <sup>-/-</sup>	Conventional NKT	??	CCl <sub>4</sub>	No difference	87
mIgM-Tg	Immunoglobulin	BALB/?	CCl <sub>4</sub>	No difference	104
LPM2a	Immunoglobulin	BALB/?	CCl <sub>4</sub>	No difference	104
Anti-ASGM-1	NK (some NKT)	B6/M	DDC diet	Increased	87

<sup>a</sup>The fibrosis is compared with the wild-type mouse of the same strain.

CD1-deficient mice indicates that this population is not essential for the development of liver fibrosis, but such experiments can easily mask more complicated biological functions. In particular, NKT cells can activate NK cells and may reduce liver fibrosis by enhancing NK-mediated HSC apoptosis. In addition not all NKT cell development is dependent on CD1, and these nonclassical NKT cells are known to produce IFN-γ which has potent antifibrotic activity.

A vital role of the adaptive immune system in modulating fibrosis is evident by the significant difference in liver fibrosis in mice of the C57BL/6 and the BALB/c mouse strains. C57BL/6 mice have significantly less fibrosis in response to CCl<sub>4</sub> compared with BALB/c mice, and these differences are negated in the absence of B, T and NKT cells (96). This finding suggests that the strain-dependent differences in C57BL/6 and BALB/c mice are predominantly owing to the adaptive immune system. In C57BL/6 mice the CD4<sup>+</sup> T-cell response is predominantly skewed toward Th1, in contrast to that of BALB/c mice, which is skewed toward Th2. The important role of the Th1 cytokines was confirmed by increased fibrosis in C57BL/6 and the BALB/c mice lacking IFN-γ and limiting fibrosis by injection of IFN-γ (96).

The regulation of fibrosis by the Th1/Th2 dichotomy in the immune response of liver is consistent with the activity of Th1/Th2 in fibrosis in general and has been explored in a number of genetic mouse models of liver fibrosis (Table 1). In a number of models of fibrosis induction, the use of different cytokine-deficient mice has shown that fibrogenesis is strongly linked to the development of a Th2 response involving IL-4, IL-5, and IL-13. In the presence of a strong Th1 inflammatory response, the development of fibrosis is very limited (97,98). For example, in a rodent model of schistosomiasis-induced liver fibrosis, treatment with IFN-γ or IL-12 had no effect on infection, but collagen deposition was greatly reduced (98). Th1 and Th2 cytokines activate very different gene transcription programs,

In tissues with a Th1 immune response, the transcription of IFN-γ-dependent genes is upregulated, with little activation of genes involved in fibrosis. In a Th2-dominated response, genes known to be important in fibrosis are upregulated, including *procollagen-1*, *MMP2*, *MMP9*, and *TIMP1* (99,100). Since IL-4 and IL-13 share a pathway involving IL-1 receptor antagonist (IL-4Ra) and Signal transducer and activator of infection 6 (STAT6) signaling, there has been great interest in the relative roles of these two cytokines in liver fibrosis. Experiments in which IL-4 and IL-13 were inhibited independently identified IL-13 as the dominant fibrotic cytokine. In schistosomiasis infection, inhibiting IL-13 resulted in an 85% decrease in collagen deposition (101). The greater role of IL-13 relative to IL-4 may reflect the relatively greater amount of IL-13 in most inflammatory conditions. An additional reason for the potency of IL-13 may be through a positive effect on increasing TGF-β activity by inducing the production of latent TGF-β and activating TGF-β through upregulation of MMPs that cleave the latent TGF-β complex.

The interactions between the immune system and HSCs are not unidirectional; instead, there is significant evidence that HSCs also modulate the hepatic immune response. This is best demonstrated by their expression of the costimulatory molecule B7-H1 (programmed death ligand -1 [PDL-1]) on activated but not resting HSCs (102). B7-H1 binds to PD1, which is an Ig superfamily member related to CD28 and CTLA-4, but which lacks the membrane proximal cysteine that allows these molecules to homodimerize (103). PD1 is expressed on a range of immune cells including CD4<sup>+</sup> T cells, and at very low levels PD1 activation are sufficient to inhibit the earliest stages of T-cell activation. PD1 also inhibits expression of the cell survival gene *bcl-xl* and limits activation of Akt. The final effect of PD1 may be very context dependent and influenced by the stage of T-cell differentiation and the degree of stimulation via the TCR. HSCs induced apoptosis of T cells activated in an alloassay

but did not inhibit proliferation or cytokine production. This suggests that activated HSCs have a mechanism for inhibiting T-cell-mediated cytotoxicity and, conversely, can induce T-cell apoptosis. These findings may have implications for survival of HSCs during a T-cell-mediated immune response but the result may also be induction of T-cell tolerance against antigens expressed on HSCs.

## CONCLUDING REMARKS AND OPEN QUESTIONS

The field of inflammation and immunity in the pathogenesis of liver fibrosis is so new that there are far more questions than answers. Still, some major insights have emerged in the past 5 yr, including the importance of hepatocyte apoptosis as a fibrogenic stimulus, the early evidence of differential activity of specific lymphocyte subsets on fibrogenesis and HSC apoptosis, the participation of HSCs in innate immunity, the central regulatory role of macrophages in fibrosis progression and regression, and dysregulation of hepatic immunity in chronic liver diseases, in particular HCV. A coherent, integrated picture of these intersecting pathways is not yet possible; however, clear directions for the future have become evident. First, the molecular basis for how different lymphocyte populations interact with HSCs and other fibrogenic cells should be characterized, in particular the role of adhesion and cell-surface molecules expressed on HSCs. Second, the full spectrum of pattern recognition receptors and their cognate ligands in HSCs remains unknown. Third, the finely tuned responses of macrophages to injury and their interactions with fibrogenic cells must be elucidated. Finally, the genetic control of immune interactions in fibrosis must be explored, as insights in this area could greatly illuminate our understanding of hepatic inflammation, disease susceptibility and progression, and response to specific therapies. Accelerating progress in our understanding of normal immune regulation will lead to advances in elucidating parallel pathways in the liver affecting normal function and disease. Thus, the area of immunity and hepatic fibrosis is likely to remain one of the most exciting and fruitful areas of inquiry for the foreseeable future.

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# 10 Clinical Use of Immunopathology Techniques in Liver Diseases

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## KEY POINTS

- The liver is anatomically structured for both innate and adaptive immunity. The innate immune system includes dendritic cells, Kupffer cells, NK cells, and NKT cells. The adaptive immune system includes T cells and B cells, both resident and received.
- Liver diseases are caused by many etiologies, including metabolic abnormalities, infections, autoimmunity, drug and chemical toxicity, mechanical, and genetic abnormalities.
- Liver diseases are often expressed as inflammation (hepatitis) during the course of the disease. This is usually caused by influx of immune cells and production of cytokines.
- Hepatic injury in many liver diseases is directly or indirectly caused by immune mechanisms.
- Immunological techniques in combination with molecular techniques are increasingly used in clinical diagnosis and management.
- Immunoassays are used to detect antigens or antibodies. These techniques play a key role in the establishment of an infectious etiology of liver diseases, such as HBV and HCV infection.
- Detection of autoantibodies is important in the diagnosis of autoimmune liver diseases. Good examples are anti-LKM antibody for type II autoimmune hepatitis and antimitochondrial antibody for primary biliary cirrhosis.
- Liver biopsy plays a critical role in the diagnosis and management of liver diseases. It is essential to assess the severity of liver injury and to provide insight into potential causes (including evaluating simultaneous liver injury from multiple etiologies).
- Routine histological examination combined with selected histochemical stains is still the most commonly used approach for morphological diagnosis and evaluation of liver diseases.

- Immunohistochemical staining is gaining increasing application in liver tissue evaluation. It can specifically identify resident cell types in the midst of ongoing liver injury, infectious pathogens, abnormal molecules, and cancer cell types.

## INTRODUCTION

The liver is the largest organ in the human body. It synthesizes and processes essential circulating proteins, detoxifies endogenous and exogenous substances, engages in bile formation for the elimination of amphiphilic and water-insoluble molecules from the body, and constitutes a unique immunological site. Its location astride the splanchnic and systemic circulation creates a critical role for immunological processing of antigens in the splanchnic circulation. The liver anatomic structure is well suited for these biological functions, as it contains 80% of the resident macrophages in the body, Kupffer cells, and has a substantial resident population of lymphocytes and dendritic cells. The liver also has a unique ability to be subject to simultaneous damage from multiple sources, owing in part to the propensity of humans to expose themselves to infectious agents. Hepatic injury may arise from the following general causes: infectious; intrinsically immune-mediated; drug-induced (including alcohol); metabolic (including nonalcoholic fatty liver disease); mechanical (especially vascular); and environmental. The immune response, directly or indirectly, plays a crucial role in hepatocellular damage. Clinical determination of the causes and severity of liver disease requires synthesis of clinical information, laboratory data, and morphological assessment of the liver tissue status. This chapter focuses on the laboratory and morphological assessment of liver disease. Particular focus is given to immunological techniques.

To utilize adequately the immunological techniques in liver disease management, it is necessary to understand the immunological basis of liver diseases. Moreover, understanding immunological mechanisms of the liver damage forms the basis for developing enhanced immunological tests for clinical use. Therefore, we first summarize the basic facts on the immunological basis of liver diseases.

**Table 1**  
**Inflammatory Cells in Hepatitis**

<i>Cell type</i>	<i>Cell marker</i>	<i>Function</i>
Innate immune system		
Kupffer cells (KC)	CD68	Antigen presentation Phagocytosis
Dendritic cells (DC)	CD1	Antigen presentation
Natural killer cells (NK)		Antitumor
Natural killer T cells (NKT)		Immunoregulation
Adaptive immune system		
B lymphocytes	CD20	Antibody response
T lymphocytes	CD3	
T-helper cells	CD4	Immunoregulation
Cytotoxic T cells	CD8	Target cell apoptosis
Regulatory T cells (T-reg)	CD4 <sup>+</sup> /CD25 <sup>+</sup>	Immunoregulation

## IMMUNOLOGICAL BASIS OF COMMON LIVER DISEASES

Inflammation is an important part in the immunological process. On one hand, pathogens can be eradicated or confined by inflammation; on the other hand, inflammation causes tissue damage, sometimes irreversible. Hepatitis—inflammation of liver tissue—is the predominant form of clinical liver disease. Inflammation is a sophisticated process tightly controlled by the host immune system and modulated by pathogenic factors. A well-balanced tissue inflammation usually favors the host eliminating the underlying pathogenic factors, especially viruses. However, uncontrolled or persistent inflammation will cause significant tissue damage. The key players in the inflammatory process are immune cells. These cells are involved in different stages of tissue inflammation. The abundance of these cells is used to evaluate the timing of inflammation and the underlying pathogenic factors. Detection and characterization of these cells are important for laboratory diagnosis of liver disease. A summary of the inflammatory cells that accumulate during hepatic injury (and their abbreviations) is given in Table 1.

Hepatitis occurs in almost all liver diseases, but the onset, progression, and outcome of the hepatitis vary significantly depending on the etiology. Major causes of hepatitis in clinical practice are hepatotropic viral infection, alcoholic and non-alcoholic steatohepatitis, autoimmune hepatitis, and drug-induced liver injury. Viral hepatitis is more frequently accompanied by lymphocyte-predominant inflammation; alcoholic hepatitis is often exemplified with numerous neutrophils; drug-related hepatitis tends to have more eosinophils; and autoimmune hepatitis is characterized by the presence of a large number of plasma cells. The detailed mechanisms of the inflammatory cell responses to different pathogens are not well defined. It is believed that the immune system plays a key role in hepatitis.

### THE INNATE IMMUNE SYSTEM

The liver is a multifunctional organ. Besides its well-known role in body metabolism, its role in immune regulation must also be recognized (1). In the first instance, hepatocytes constitute 80% of the cells in the liver. Of the remaining 20%, bile duct epithelial cells comprise only 1%, sinusoidal

endothelial cells 10%, Kupffer cells (the resident macrophages of the liver) 4%, and lymphocytes 5% (including T cells, B cells, natural killer [NK] cells, and natural killer T [NKT] cells). Of these nonparenchymal cells, endothelial cells, Kupffer cells, NK cells, and NKT cells are all part of the innate immune system. With its average mass of 1800 g in an adult, the liver is thus particularly enriched with cells of the innate immune system, compared with other parenchymal organs. Although this has immediate value for dealing with foreign antigens released from the gut into the splanchnic circulation, it also means that the liver is well equipped for an immune response to neoantigens expressed within its substance.

One of the key functions of the innate immune system is to process antigens for adaptive immune cells. The cells that have an antigen presentation ability are called antigen-presenting cells (APCs). The endothelial cells and Kupffer cells in the liver along with the circulating dendritic cells have the properties of APCs (2). These cells are among the first groups of cells to encounter antigens circulated in the liver. The outcome of the antigen presentation by these cells can be dramatically different according to cell type. For instance, Kupffer cells may help to initiate a robust immune response, whereas endothelial cells may give immune tolerance to the antigen (3,4). Although the mechanisms by which the Kupffer cells and endothelial cells interact with adaptive immune cells are not known, cytokines are presumably the key factors in regulating this process. The cytokines are usually induced by Toll-like receptors (TLR) signaling pathways initiated by TLR recognition of pathogen components (5).

Kupffer cells play a role in all forms of hepatitis, as an obligate anatomical companion. Indeed, they comprise 80% of the systemic host mononuclear phagocytic system (6). They reside normally on the luminal aspect of the sinusoidal endothelium, so as to engulf particulate material and microorganisms that arrive via the splanchnic circulation from the gut. Kupffer cells are potent scavengers for systemic and gut-derived inflammatory mediators and cytokines (7). Hepatocellular death is rapidly followed by Kupffer cell phagocytosis of the residual debris. For example, when an isolated hepatocyte undergoes apoptosis, it is routinely engulfed by a nearby Kupffer cell within 2 to 4 h (8). With smoldering hepatocellular apoptosis, clumps of macrophages can accumulate in the parenchyma. Such macrophages can persist in the parenchyma for an extended period, most likely weeks to months, serving as sentinels of prior hepatocellular injury and death. This feature has served as a guide for the pathologist to assess liver histopathology. Hepatic damage more extensive than just apoptosis of isolated hepatocytes engenders recruitment of circulating macrophages. The most dramatic example is massive hepatic necrosis, in which the vast expanse of the hepatocellular parenchyma undergoes cell death. With survival of the patient over the ensuing hours and days, the hepatic parenchyma becomes a sea of macrophages amid the cellular debris. Their phagocytic and migratory action facilitates removal of the nonviable material, clearing the way for regeneration and recovery of the liver tissue.

NK and NKT cells are most abundant in the liver. The retention mechanisms of these cells in the liver are not known (9). NK and NKT cells can participate in the immune response without prior antigenic stimulation (9). NK cells—and potentially NKT cells as well—appear morphologically as *pit cells*, large granular lymphocytes that reside in the sub-endothelial interstices of the space of Disse (10). Cell-surface markers have been used to identify these cells specifically. These cells can produce high levels of proinflammatory (Th1) and antiinflammatory (Th2) cytokines (11). NK cells are major producers of interferon (IFN)- $\gamma$  (a proinflammatory cytokine); NKT cells produce IFN- $\gamma$ , or interleukin-4 (IL-4); (an anti-inflammatory cytokine). IFN- $\gamma$  enhances the dendritic cell expression of proteins involved in cellular antigen processing and presentation, including proteasome subunits and MHC molecules. In addition, IFN- $\gamma$  induces additional chemokines or cytokines that affect T and B cells. The immunological roles of NK and NKT cells are implicated in tumor surveillance, viral infection, and transplantation rejection (12,13). Hepatitis B virus (HBV) and hepatitis C virus (HCV) can activate NK and NKT cells, which leads to secretion of IFN- $\gamma$  and IFN- $\beta$  (14). These cytokines exhibit antiviral activity through noncytopathic mechanism.

#### THE ADAPTIVE IMMUNE SYSTEM

Adaptive immunity, mainly performed by T and B cells, plays a critical role in hepatitis. One key feature of adaptive immunity is the antigen specificity. Robust and specific adaptive immune responses are paramount for clearance of viral infection. Antigen-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cells are involved in eradication of HBV and HCV infection, during both acute and chronic infection (15). CD4<sup>+</sup> helper T cells recognize short antigenic peptides displayed in the antigen-binding groove of HLA class II molecules; these peptides are derived from intracellular proteolytic cleavage of exogenous antigens such as viruses (16). CD4<sup>+</sup> T cells secrete lymphokines that modulate the activity of antigen-specific B cells and CD8<sup>+</sup> T cells (17). A CD4<sup>+</sup> T-helper type 1 secretion profile (Th1) consists of antigen-dependent production of IL-2 and IFN- $\gamma$ . A T-helper type 2 secretion profile (Th2) consists of IL-4 and IL-10 secretion. It is the Th1 cytokine profile that enhances CD8<sup>+</sup> T-cell cytolytic activity (18).

Cellular immunity against intracellular viral pathogens involves CD8<sup>+</sup> cytotoxic T cells (cytotoxic T lymphocytes [CTLs]) as the effector arm. CTLs respond to viral peptides presented by infected cells in the antigen-binding groove of HLA class I molecules (19). CTL-mediated lysis of virus-infected host cells by Fas/FasL or perforin can lead to viral clearance. However, hepatocyte cell death may have an impact on clinical liver function if new hepatocytes are not regenerated in time. Fortunately, hepatocellular death is not an obligatory outcome of viral infection, as CTLs can secrete antiviral cytokines to induce noncytolytic inhibition of viral gene expression and replication (20,21). Therefore, balancing the cytolytic and noncytolytic antiviral systems is fundamentally important in viral pathogenesis. In chronic viral infections, such as HBV and HCV, a major hypothesis is that inadequate

CTLs permit persistent viral infection and hence forms the substrate for chronic necroinflammatory hepatic injury.

It is well established that adaptive immunity is tightly regulated to achieve a critical balance between robust responses against pathogens and immune tolerance to self. Understanding this regulation is the “holy grail” of immunology, for which the molecular details remain to be defined. Recently, a population of lymphocytes has garnered particular attention, CD4<sup>+</sup> T cells constitutively expressing the IL-2-receptor  $\alpha$ -chain (CD25): CD4<sup>+</sup>/CD25<sup>+</sup> T cells [T-regs]. T-regs represent about 5 to 10% of peripheral CD4 T cells (22). T-regs regulate the activation of CD4<sup>+</sup> and CD8<sup>+</sup> T cells by suppressing their proliferation and effector function. This suppressive action is thought to be critical in preventing the activation of autoreactive T cells (23). Suppression occurs both through cell-cell contact and possibly through release of inhibitory cytokines (24). Interestingly, patients with autoimmune hepatitis have a reduced number of circulating T-regs at the time of diagnosis, whereas HCV patients have an increased number of T-regs (25). These observations led to the hypothesis that manipulation of the numbers of T-regs may have an implication in the treatment of various liver diseases, usually caused by either suppressive adaptive immunity or overreactive autoimmunity. Hence, testing this hypothesis will further elucidate the role of T-regs in liver pathobiology.

#### RECRUITMENT AND INFLUX OF INFLAMMATORY CELLS

The key event in inflammatory cell recruitment and influx is margination and egress. Leukocyte extravasation involves expression of vascular adhesion molecules by activated endothelial cells, margination and rolling of leukocytes expressing the cognate ligands, adhesion of the leukocytes to the endothelium, transmigration across the endothelium, and migration within the extravascular space toward a chemotactic stimulus. The vast circulation of the liver, with both splanchnic influx of venous blood and direct arterial perfusion, facilitates the retention of inflammatory cells in the liver in response to inflammatory stimuli. Recruitment of lymphocytes, in particular, may be driven by expression of powerful chemoattractants, not only by the sinusoidal endothelium but also by parenchymal hepatocytes (26). In the case of macrophage recruitment, the chemokine macrophage inflammatory protein 1 $\alpha$  (MIP-1 $\alpha$ ) mediates the recruitment of inflammatory NK cells (27). Intrahepatic production of MIP-1 $\alpha$  is accomplished through IFN- $\alpha$  and IFN- $\beta$  stimulation of the innate immune system in the liver to generate monocyte chemoattractant protein-1 (MCP-1) (28), which in turn recruits MIP-1 $\alpha$ -producing inflammatory macrophages to the liver (29).

Unlike vascular leakage accompanied by inflammation elsewhere in the body, the fenestrated sinusoidal endothelium ensures that there is free exchange of plasma fluid with the extravascular space within the hepatic parenchyma. Hence, the liver is not subject to interstitial edema in the same sense as in other body tissues. Swelling of the liver occurs during hepatitis owing to swelling of hepatocytes themselves.

In the case of viral infection, inflammatory cell recruitment in the liver is relatively well defined. In the acute phase, lymphocytes first suffuse the hepatic parenchyma (hepatic lobules) and target virus-infected cells by recognizing peptides presented on the cell surface. As the infection settles into a chronic phase, portal tracts characteristically become populated with a mixed inflammatory cell population dominated by lymphocytes, with admixed macrophages and scattered granulocytes. It is still not well understood how the inflammatory cells accumulate in the portal tracts. If viral clearance does not occur, the portal tract lymphocytes are capable of attacking the surrounding hepatocytes, resulting in hepatocyte apoptosis (piecemeal necrosis), which is also referred to as *interface hepatitis*. This is a characteristic feature of progressive chronic hepatitis.

**Hepatocyte Apoptosis** A critical consequence of inflammation is hepatocellular death. Hepatocellular death takes two broad forms: apoptosis and necrosis (30). Necrosis is usually caused by mechanical injury or tissue ischemia. Apoptosis is an active form of cell death (suicide) in which cells exhibit cytoplasmic shrinkage, cell membrane blebbing, chromatin condensation, and cellular fragmentation into small membrane-bound *apoptotic bodies* (31). There are at least two mechanisms by which a cell initiates apoptosis: the extracellular “death receptor” pathway, whereby extracellular ligands binding to “death receptors” activate the apoptosis pathway, and the intracellular mitochondrion cytochrome c pathway (32). Regardless of the entry point stimulating apoptosis, the effector arm of the apoptotic pathway is activation of caspases and endonucleases, which induce the cleavage of structural proteins and DNA, respectively. In the liver, apoptotic bodies have long been referred to as acidophilic bodies or Councilman bodies (33). Identification of apoptotic bodies indicates current and ongoing hepatocellular apoptosis, since apoptotic hepatocytes are engulfed within a matter of hours by Kupffer cells or other macrophages (34). Apoptosis is an essential physiological process. It plays a critical role in hepatic development and remodeling. It is also an important host defense system against infected cells, controlling infection.

### LABORATORY APPROACHES FOR LIVER DISEASE DIAGNOSIS

The role of laboratory tests in the diagnosis and management of clinical liver diseases cannot be overemphasized. Since its first utilization in 1913, when a phthalein dye was used to investigate liver function, laboratory tests have been widely used for liver disease diagnosis and management. The ultimate goal of any laboratory test is to gain information on etiology and severity of liver injury, functional status of the liver, and therapeutic responses to a given therapy. Because of the complexity of liver function and disease expression, a panel of tests is commonly performed. Serum or plasma-based laboratory tests are still the most commonly used tests in clinical practice, largely because of their noninvasive nature, readily availability, and relative specificity in reflecting liver injury.

**Table 2**  
**Serum-Based Tests for Autoimmune Liver Diseases**

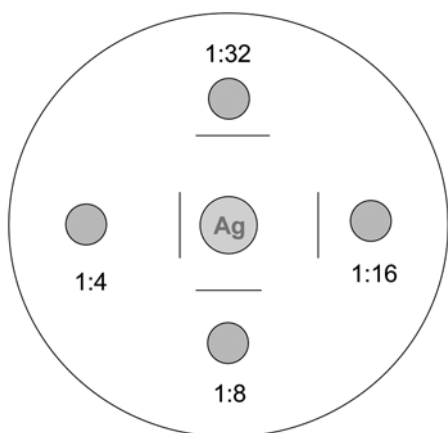
<i>Test</i>	<i>Clinical utility</i>
Aminotransferases	Hepatocytic injury
Alkaline phosphatase	Hepatobiliary disorders
$\gamma$ -Glutamyl transpeptidase	Hepatobiliary disorders
Bilirubin	Liver injury and function
Prothrombin time	Liver function
Albumin	Liver function
Anti-smooth muscle antibody (SMA)	PBC or autoimmune
$\gamma$ -Immunoglobulin	Autoimmune
Antinuclear antibody (ANA)	Autoimmune
Antimitochondrial antibody (AMA)	Autoimmune
Anti-liver-kidney microsomal (LKM) antibody	Autoimmune hepatitis, type II
Hepatitis A Virus (HAV) serology	Hepatitis A infection
Hepatitis B Virus (HBV) serology and antigens	Hepatitis B infection
Hepatitis C Virus (HCV) serology and viral RNA	Hepatitis C infection
Ceruloplasmin	Wilson's disease
Ferritin	Hemochromatosis
$\alpha$ 1-antitrypsin	$\alpha$ 1-antitrypsin deficiency

### NONINVASIVE SERUM-BASED DIAGNOSTIC APPROACHES

Serum-based laboratory tests are still the most commonly used tests in clinical hepatology practice. These tests are summarized in Table 2. The classical examples are chemistry-based aminotransferases, bilirubin, alkaline phosphatase, and  $\gamma$ -glutamyl transpeptidase. These markers are generally elevated in serum when there is active hepatocellular or biliary tract injury. Determination of serum albumin, prothrombin (PT), and coagulation factor levels is used to evaluate synthetic function of the liver, since the liver is almost the only source for these proteins. Serological tests are employed to determine the presence of antibodies against a specific microbial pathogen or autoantibodies. Serum is also the common source for identification of antigens from microbial pathogens that infect the liver. Although detection of viral pathogens by itself is not directly related to autoimmune diseases, exclusion of viral infections is almost always needed during a clinical workup. Clinical presentation is often similar in autoimmune disorders and in viral hepatitis. Therefore, viral testing is usually done for patients who have clinical hepatitis. The major techniques used in the tests listed in Table 2 are chemical assays, immunoassay, and nuclear acid-based molecular assays, such as polymerase chain reaction (PCR). These techniques have dramatically advanced over the past several decades. Automation of these tests is common in a modern clinical laboratory.

### PRINCIPLES OF IMMUNOLOGICAL TECHNIQUES USED IN SERUM-BASED TESTS

It is beyond the scope of this chapter to review detailed methodology for each immunoassay. Instead, we will summarize the important principles for some major categories of immunological techniques. Almost all the immunological



**Fig. 1.** Schematics of the immunodiffusion method. A solution containing a known specific antigen (Ag) is loaded in the central well of an agarose plate. Different dilutions of patient serum are loaded in the peripheral wells. The black lines represent the precipitation lines formed by antigen and antibody complex. The higher the antibody concentration in the patient serum sample, the closer the precipitation line is to the antigen well.

assays are based on the property of antigen and antibody binding. The interactions of antigen and antibody can be classified as primary (antigen-antibody complex), secondary (interaction of immune complexes), and tertiary (interaction of immune complexes with immune cells). A variety of different assays have been designed to detect the final antigen and antibody complexes qualitatively and quantitatively (35).

**Immunodiffusion** This is a technique that is based on the formation of antigen and antibody complexes precipitated in gel matrix, which is a visible precipitin line. The formation of the complex is dependent on the molecular size of the antigen, the concentration of the antigen and antibody, and the structure of the supporting gel matrix. Fig. 1 shows the basic principle of this technique. The antigen solution is loaded in the center. Patient samples with different dilutions will be loaded in the peripheral wells. After incubation, precipitin lines are visible, indicating the presence of corresponding antibody in the patient sample. The line tends to be closer to the wells with lower concentration of antibodies because the lower the concentration, the slower the diffusion rate. This procedure is used for identification of an antigen or an antibody (36). It is simple and inexpensive but lacks the speed and the sensitivity for quantification. This technique is currently less frequently used alone in clinical laboratories, but as a component of immunoelectrophoresis, the same principle is still applicable.

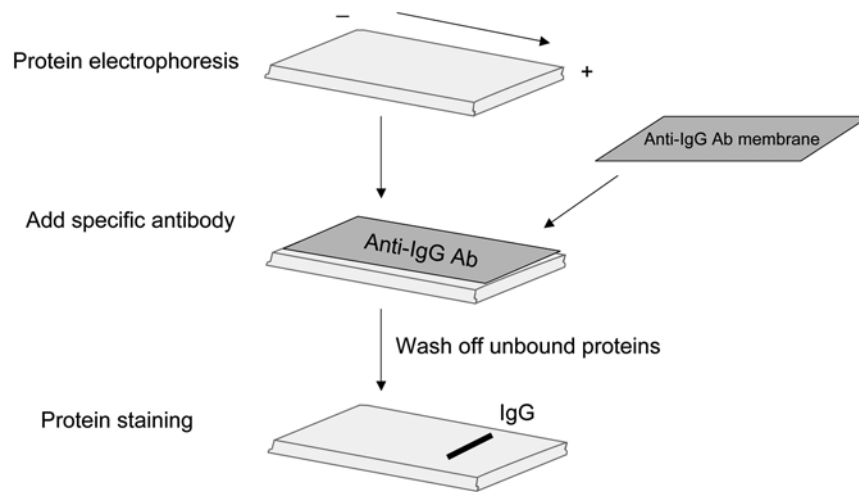
**Immunolectrophoresis and Immunofixation Electrophoresis** Immunolectrophoresis is used to detect proteins or immunoglobulins (mainly IgM, IgG, and IgA) in patient serum. This method permits the differentiation between monoclonal or polyclonal immunoglobulin reactivity (37). The best example of using this test is monitoring for a monoclonal immunoglobulin spike in patients with suspected multiple myeloma. This is a two-stage procedure. The serum sample is

first separated in an agarose gel through electrophoresis. Then corresponding antibodies are used for immunodiffusion assay, as discussed in the previous section. Precipitin arcs will form if there is a specific antigen (specific type of immunoglobulin) in the patient serum. Because the final result is entirely based on the presence or absence of a precipitin line, it is not a quantitative assay. Moreover, the complexity of the patient serum may present a great challenge in interpreting the result.

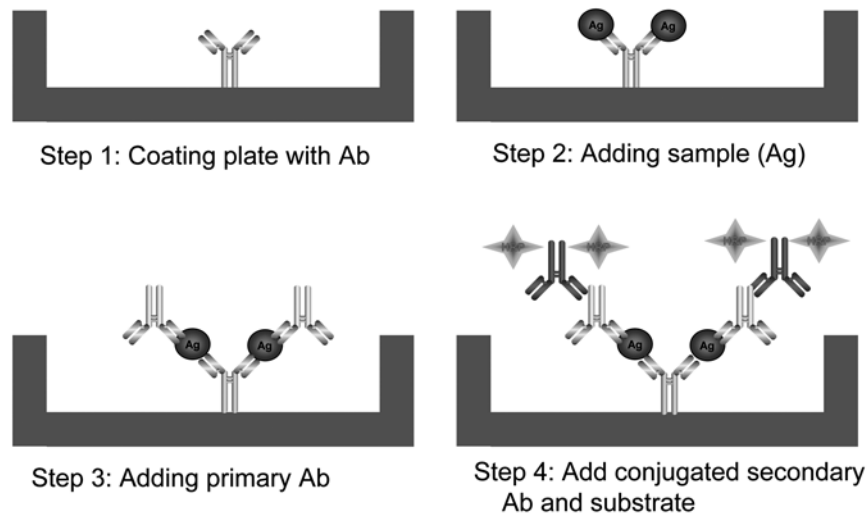
Immunofixation electrophoresis is a modified method based on a principle similar to that of immunoelectrophoresis (38,39). Like isoelectric focusing (IEF), this is a two-step procedure (Fig. 2). First the samples are separated in agarose gel by electrophoresis. Then the antibody-soaked filter paper or cellulose acetate strips are applied over the gel. The antibody will diffuse into the gel (immunodiffusion). Immunoprecipitation will occur in the gel if there is specific antigen-antibody complex formation. The precipitated complex can be detected by standard protein staining. This method is easier to perform, reproducible, and quantitative. It has been widely used in clinical laboratories, particularly for determination of the presence of monoclonal immunoglobulins (monoclonal gammopathy) and the type-specific light chain or heavy chain.

**Radioimmunoassay** The principle of radioimmunoassay (RIA) is based on the proportional binding of radioisotope-labeled antigen and antibody. The use of radiation would allow detection of trace amount of molecules. A radioisotope-labeled antigen (such as hepatitis B surface antigen [HBsAg]) binds to its specific antibody forming an immunocomplex (40). The known amount of HBsAg or a test sample containing HBV will proportionally displace the radioisotope-labeled HBsAg antigen in the immunocomplex. The immunocomplex is then separated from the soluble phase, and the intensity of the radiation in the complex will be detected. The radiation intensity is inversely related to the amount of antigen in the test samples. This test requires the antibody to have a high specificity and a high purity. Many radioisotopes have been used for this assay, but iodine ( $^{125}\text{I}$ ) is the most commonly used radioisotope because of its easy incorporation into the amino acid structure. RIA is an extremely sensitive immunoassay. This method is exclusively used in detection of trace amount of drugs or hormones. It has also been used to detect HBV antigens or antibody, as well as HCV antibodies. Although this is an extremely sensitive test, the technical facility requirements prevent its broad use in all clinical laboratories.

**Enzyme Immunoassay** Enzyme immunoassay (EIA) is one of the most commonly employed immunological techniques in clinical laboratories. The assay uses an enzyme-labeled antibody to bind a specific antigen, followed by exposure of the enzyme-specific substrate, resulting in a colorimetric product that can be detected and quantified. There are many variants of tests based on the principle of EIA. One of the most commonly used EIA tests is enzyme-linked immunosorbent assay (ELISA) (41). ELISA specifically refers to a solid-phase type, in which solid material (a 96-well plate) adsorbs protein (antibody or antigen) to its surface. The purpose of solid surface adsorbing protein is to separate the bound enzyme immunocomplex from



**Fig. 2.** Schematics of immunofixation electrophoresis. Antigens in the sample are first resolved in an agarose gel (protein electrophoresis). A membrane soaked with a specific antibody (IgG) is then placed on the agarose gel, followed by incubation to allow antibody (Ab) diffusion into the gel. The specific antigen-antibody complex will form and be retained in the gel, whereas unbound antibody elsewhere in the gel can be rinsed away. The retained band of antigen-antibody complex can then be visualized by protein staining.



**Fig. 3.** Schematics of the enzyme-linked immunosorbent assay (ELISA). The coating antibody (Ab) is fixed on the surface of a microtiter plate (Step 1). Test samples containing antigen (Ag) are then added to the plate to allow antigen capture by the coating antibody (Step 2). A soluble primary antibody is then added to the medium (Step 3); this primary antibody usually binds to antigen epitopes that are different than those bound by the coating antibody on the plate. This step is then followed by addition of an enzyme-labeled secondary antibody (Step 4). The most commonly used enzyme is horseradish peroxidase (HRP). Finally, a color-generating substrate (e.g., diaminobenzidine) is added to the medium; the colorimetric changes reflect the presence of specific antigen in the sample. A critical advantage of ELISA is the amplification of detectable signal through the primary–secondary antibody binding steps.

the free enzyme. The commonly used “sandwich” method is a representative example of ELISA (Fig. 3). In this procedure, a specific antibody is fixed to the microtiter plate by incubation in an appropriate buffered solution. The testing sample is incubated with the fixed antibody, followed by washing off the unbound antigens. The second primary antibody is then incubated with the antigen–antibody complex fixed on the surface of the microtiter plate. This antibody must be able to recognize

a different epitope compared with the antibody fixed to the plate. Secondary antibody conjugated with an enzyme is then added. After the unbound secondary antibody is washed off, substrate is added. The colorimetric solution will be analyzed. This technique is simple, reliable, and inexpensive. Thus, it has been widely used for qualitative and quantitative detection of antigens from pathogens and antibodies that initiate in the human serum. The principle of the test is outlined in Fig. 3.

EIA is now used for detection of pathogen antigens (such as HBV, HCV, influenza A virus, adenovirus, *Giardia* organisms, *Clostridium difficile* toxin, and *E. coli* Shiga toxin) (42). It is also used to detect antibodies (such as anti-double-stranded DNA antibodies). EIA has been used for initial HCV screening (43). ELISA can be used to determine the presence of anti-HCV antibodies. A confirmatory test is the immunoblot assay (RIBA), which is a strip immunoblot assay including NS5 and c33c recombinant HCV proteins as well as c100p, 5-1-1p, and c22p synthetic peptides to detect antibodies to HCV in human serum or plasma (44). In this procedure, a patient serum sample is incubated with the membrane, which is pre-coated with HCV peptides. After the unbound serum proteins are washed off, peroxidase-conjugated goat antihuman IgG antibody is incubated with the blot, followed by addition of a substrate, 4-chloro-1-naphthol. The positive result exhibits color bands on the blot.

To improve the sensitivity of the EIA test further, based on a similar principle, fluorescent dyes and chemiluminescence agents are increasingly used for antibody labeling (45,46). The antigen-antibody complexes are then detected and quantified by special instruments. These new techniques have the advantages of high throughput and easier automation; hence their clinical applications are expected to expand rapidly in the near future.

### CELL-BASED IMMUNOASSAYS

As we discussed earlier, in the immunological basis of common liver disorders, immune cells play a critical role in liver diseases. Therefore, characterization of these cells should be of value in understanding the disease process. To achieve this end, many immunological techniques have been developed to gain information on immune cells (47). Up to now, most of these cell-based immunological methods, such as phenotyping and functional analysis of lymphocytes, have been used in research laboratories but not routinely in clinical laboratories for liver diseases. HLA typing is commonly used to determine potential high-frequency genotypes. The test can be performed using HLA-specific antibodies incubated with patient white blood cells. Recently, DNA-based HLA genotyping has become more popular.

Flow cytometry has been widely used in modern clinical laboratories (48,49). This technology allows one to examine multiple characteristics at the single cell level. Cell size and granularity can be readily analyzed with the instrument. With numerous available antibodies, immunophenotyping of cells is routinely performed in laboratories. One of the key advantages of flow cytometric analysis is its ability to detect several labeled markers (by different fluorescence dyes, such as fluorescein isothiocyanate, phycoerythrin, Cy3, Cy5, and so on) on a single cell. For liver diseases, flow cytometric analysis is generally used to define infiltrative hematological disorders in the liver, largely because of the availability of well-characterized antibodies and their associations with biological phenotypes. For example, to investigate a liver tumor composed of small blue cells, immunophenotyping by flow cytometry using

antibodies against B cells (CD20) and T cells (CD3) has a remarkable diagnostic value. Flow cytometry can also be used to determine the content of DNA in tumor cells (DNA ploidy). Although it is feasible to characterize lymphocytes from liver tissue and peripheral blood by flow cytometry (21), their clinical implications are yet to be defined.

Other immune cell-based techniques, such as mixed lymphocyte culture, EliSpot assay, cytotoxicity assay (CTL assay), assays for phagocytosis, and complement assays are widely used in research laboratories (50). We will not discuss these techniques here because of their currently limited application in clinical practice. There are several good references for readers (51–53).

### LIVER TISSUE-BASED DIAGNOSTIC APPROACHES

Tissue diagnosis, mainly liver biopsy, plays an essential role in the management of liver diseases in clinical practice. Over the past several decades, many techniques have been used for gaining the maximal information from liver biopsy tissue. Among these techniques, immunohistochemical staining is one of the most important and widely used. Liver biopsy is routinely used for evaluating liver diseases. It plays an essential role in clinical hepatology practice. It is the required technique for visualizing disease processes in the liver at the microscopic level. Liver biopsy may involve *cutting*, whereby intact pieces of tissue are obtained either by a cutting needle or by a scalpel for histological processing. Liver biopsy may also involve *aspiration*, in which a thin needle is inserted into the liver substance, and cellular material is aspirated under suction; this variant yields dispersed specimens for cytologic analysis and/or clumps of tissue for histological examination.

The first cutting needle biopsy device was introduced by Vim and Silverman in 1938 and was used in procedures requiring several minutes for percutaneous placement of the needle and withdrawal of tissue specimens. A key refinement was the introduction of the Menghini cutting needle in 1958; the use of this needle in percutaneous needle biopsy procedures requires only a second or two of penetration and withdrawal (54). The resultant substantial decrease in bleeding complications enabled percutaneous liver biopsy to become a routine procedure in the evaluation and management of patients with suspected liver disease. More recently introduced cutting needle biopsy devices such as the Tru-cut biopsy and biopsy guns provide for semiautomation of the percutaneous procedure. Transjugular liver biopsy is also used in some clinical situations (55). Immunological and molecular biological techniques have been increasingly used to gain more information using liver biopsy tissues. Before describing the immunological techniques, we first briefly summarize the routine approaches toward examination of liver biopsy specimens.

To interpret a liver biopsy accurately, it is essential to handle and prepare the tissue specimen correctly. Depending on the nature of the suspected diseases, a variety of laboratory techniques have been used to enhance diagnostic sensitivity and specificity. In most instances, liver biopsy tissue is routinely fixed in buffered formalin and processed for microscopy, but

**Table 3**  
Commonly Used Stains for Liver Tissue Diagnosis

Stain	Usage
Hematoxylin & eosin	General tissue stain
Periodic acid-Schiff	Glycogen and glycoproteins
Periodic acid-Schiff with diastase	Glycoproteins
Masson Trichrome	Connective tissue
Reticulin	Delicate Collagen fibers
Prussian Blue	Hemosiderin
Oil Red O	Lipid droplets
Rubeanic acid	Copper
Hall	Bilirubin
Congo Red	Amyloid
Shikata	Elastin fiber

this is not always the best choice. In the setting of possible hematological disorders affecting the liver, it is essential to have some fresh (not formalin-fixed) tissue submitted to a laboratory for flow cytometric analysis. When inherited metabolic disorders are suspected, it is necessary to preserve a portion of the liver tissue by immediate immersion in liquid nitrogen for biochemical, enzymatic, and/or molecular analysis; a small portion also should be placed in electron microscopy fixative for potential ultrastructural analysis. If an infectious etiology other than hepatotropic viral infection is suspected, a portion of fresh tissue may be submitted for microbiology cultures. In some special circumstances, if lymphocytes need to be isolated for liver tissue, preservation of the biopsy tissue for up to several hours with cell culture medium is required.

To visualize the histopathological changes in a liver biopsy, the tissue sections need to be stained. The most common tissue stain is hematoxylin & eosin (H&E), which stains the nucleus blue and the cytoplasm red. Other commonly used stains, as listed in Table 3, are routinely used for highlighting specific components of cellular and connective tissue. Examples of these stains in liver biopsy are shown in Fig. 4. Trichrome stain is used for assessing the degree of liver fibrosis that almost invariably accompanies all liver diseases. This stain is particularly useful for differentiating acute hepatic architectural collapse from advanced liver fibrosis. In the former, there is no significant dense collagen deposit. It is also useful for evaluating fibrosis reversal, which has been recently demonstrated in many studies (56,57). Reticulin stain highlights the thin fibers around the hepatic sinusoidal structure (hepatic cords). This stain can aid in differentiation of regenerated nodules (less than three cell layers thick) and well-differentiated hepatocellular carcinoma (more than three cell layers thick). In the setting of nodular regenerative hyperplasia, a condition accompanying many autoimmune disorders, reticulin stain is particularly helpful because it shows the "compressing" hepatic plates by the regenerative hepatic nodules comprised of two-cell-layer hepatic plates. Periodic acid Schiff (PAS) stains are useful for highlighting the basement membranes of bile duct epithelium, detection of glycogen storage, and detection of glycoprotein accumulation. One of the best examples

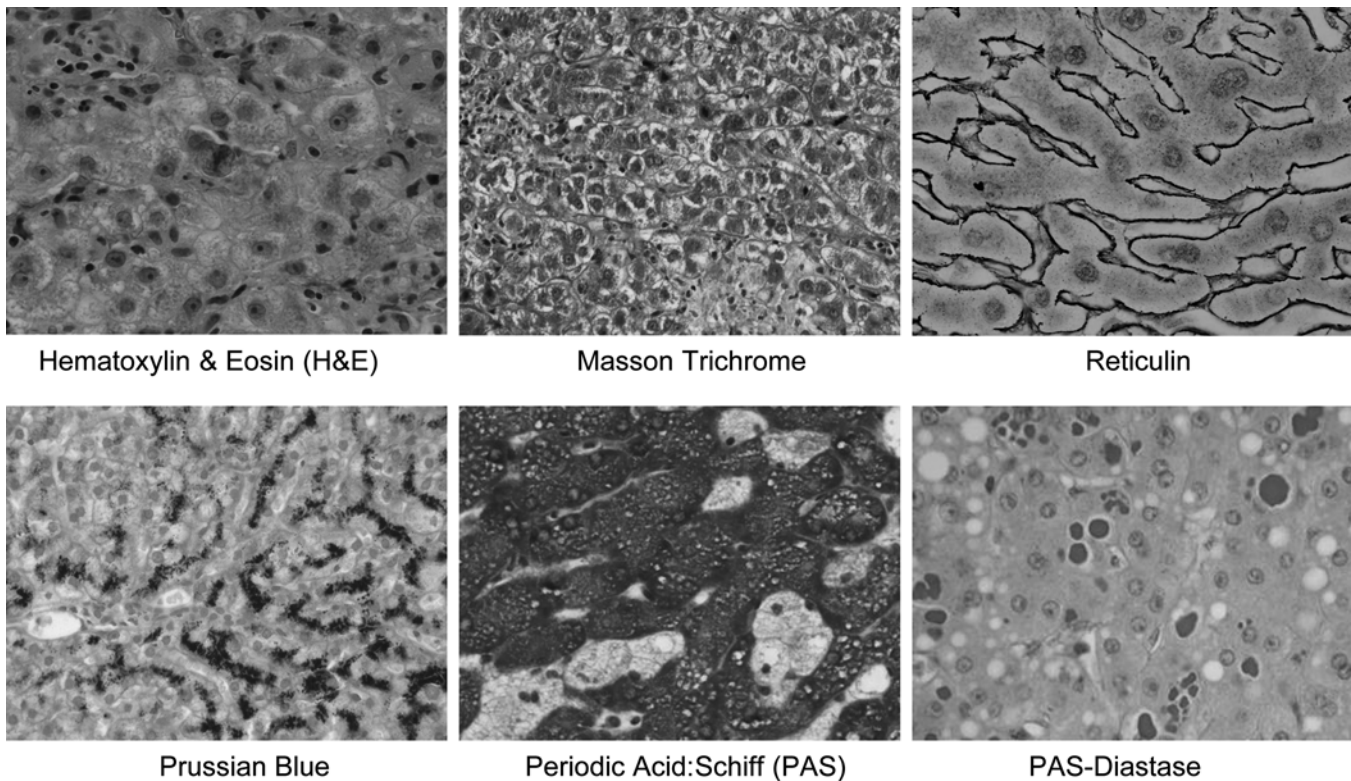
**Table 4**  
Antibodies Used in Liver Tissue Diagnosis

Antibody	Clinical utility
Hep Par 1	Hepatocyte-specific antigen
Cytokeratin 19	Bile duct epithelium
Cytokeratin 8	Hepatocytes
Cytokeratin 18	Hepatocytes
Cytokeratin 7	Hepatocytes and bile duct
Cytokeratin 20	Colon carcinoma
$\alpha$ -Fetoprotein (AFP)	Cancer markers
Carcinoembryonic antigen (CEA) polyclonal	Bile canaliculi
Epidermal growth factor receptor (EGFR)	Overexpressed in hepatocellular carcinoma (HCC)
Hepatitis B surface antigen (HBsAg)	HBV surface infection
Hepatitis B core antigen (HBcAg)	HBV core infection
Adenoviral antigen	Adenoviral infection
Cytomegalovirus antigen (CMV)	CMV infection
Herpes viral antigen	Herpes viral infection
Albumin	Hepatocytes
$\alpha$ 1-antitrypsin	Hepatocytes
S-100	Melanoma
Human melanoma black (HMB)-45	Melanoma
Chromogranin	Neuroendocrine tumor
Synaptophysin	Neuroendocrine tumor
Anti- $\lambda$ -chain	Plasmacytoma

is the correlation of PAS-diacetate-resistant globules with the  $\alpha$ -1-antitrypsin deficiency Z genotype. A number of chemical stains are used for detection of mineral accumulation in hepatocytes or other resident cells. For example, Prussian Blue is used for hemosiderin detection, and Rubeanic acid is used for copper accumulation in hepatocytes.

The most commonly used immunological technique for liver biopsy is immunohistochemistry. Immunostaining of liver tissue was first reported in 1963 (immunofluorescence) and in 1970 (immunoperoxidase). Up to 2005, more than 300 commercially available antibodies have been used for tissue diagnosis in pathology laboratories, and more than 11,000 citations in the literature have mentioned liver immunostaining. The commonly used antibodies in liver pathology are listed in Table 4. The principle of this technique is similar to that of EIA (discussed above in enzyme immunoassay), except the antigens are in the tissue section. The labeled antibody binds a specific antigen on the tissue section. The labeling marker is usually an enzyme (e.g., peroxidase, alkaline phosphatase). The presence of this enzyme will generate colorimetric precipitate in the tissue *in situ* after reaction with a corresponding specific substrate. To increase the sensitivity of this technique, a signal amplification step is often used. The most common is the biotin and avidin system, referred to as the *ABC method*. The major steps of this technique





**Fig. 4.** Commonly used stains in liver histology. In addition to routine hematoxylin & eosin staining, the chemical stains most commonly used to evaluate liver histopathology include the following. Masson trichrome stain highlights fibrous tissue blue. Reticulin stain highlights the fine fibers around hepatic plates. Prussian blue stain shows blue hemosiderin pigment in a liver biopsy with increased iron storage. PAS stain shows glycogen in hepatocytes, and PAS-D detects the presence of glycoprotein granules in hepatocytes with  $\alpha$ 1-antitrypsin deficiency.

are illustrated in Fig. 5. Most of the chromogens use DAB, which forms a dark-brown reaction product.

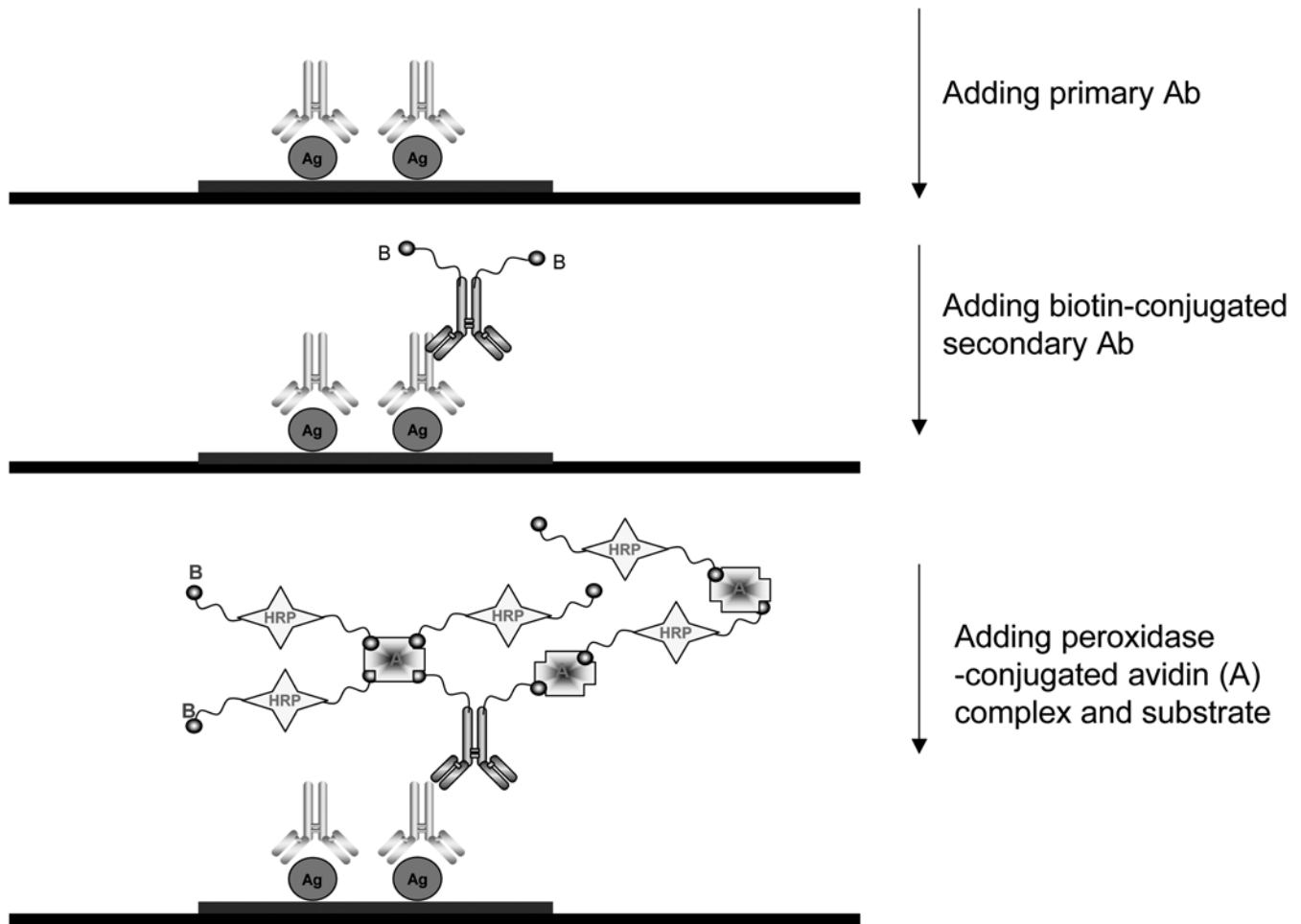
The general applications of immunostaining in liver tissue are as follows:

1. Identification of native cell types in the liver, such as hepatocytes, bile duct epithelial cells, stellate cells, lymphocytes, and vascular cells (Fig. 6).
2. Molecular characterization of liver diseases, such as viral antigen detection (HBV, adenovirus, herpesvirus, and so on (Fig. 7).
3. Detection of abnormal molecules, such as amyloid,  $\alpha$ -1-antitrypsin polymers, Mallory bodies, or fibrinogen deposit (Fig. 8).
4. Identification of tumor markers, such as  $\alpha$ -fetoprotein,  $\beta$ -catenin, and epithelial growth factor receptor (Fig. 9).
5. Characterization and classification of tumors, such as hepatocellular carcinoma, cholangiocarcinoma, and various metastatic cancers.

Although most resident cells in the liver are readily identifiable in routine H&E stains, immunostains are extremely valuable for characterization of these cells in pathologic conditions. Sometimes it is difficult to differentiate hepatocellular carcinoma (HCC) from cholangiocarcinoma. In this setting, immunostains using hepatocyte-specific antibody, Hep Par 1,

and bile duct epithelium-specific antibody, CK19, are important. More than 90% of HCCs are Hep Par1 positive and CK19 negative, whereas almost all cholangiocarcinomas are positive for CK19 (58). The antigenic nature of Hep Par 1 has not yet been identified (59). Using a CK19 marker to identify bile ducts is valuable for better characterization of the intrahepatic biliary anatomy and confirmation of the absence of bile ducts in the setting of chronic rejection. Stellate cells are resident cells in the liver but are difficult to identify in routine H&E stain. It is well documented that stellate cell activation is involved in hepatic fibrosis. When these cells are activated, smooth muscle actin (SMA) will be overexpressed. Based on this characteristic, immunostain for SMA has been used to detect these cells in liver fibrosis (60,61). Other cell markers are used for characterization of tumor cells in the liver. For example, the endothelial markers CD31 and factor VIII are positive for vascular tumors; chromogranin and synaptophysin are positive for neuroendocrine tumors; and S-100 and HMB45 are positive for metastatic melanomas.

Immunohistochemistry is particularly important for detection of viral pathogens, because it often offers a highly specific etiological diagnosis. Immunostains for cytomegalovirus (CMV), adenovirus, herpesvirus, epstein-barr virus (EBV), and HBV are readily available in pathology laboratories. Immunostain for HCV is not routinely used because the current antibodies



**Fig. 5.** Illustration of ABC immunohistochemistry. A tissue section is incubated with a primary antibody (Ab), followed by addition of a biotin-labeled (B) secondary antibody. Enzyme (horseradish peroxidase [HRP])-labeled avidin (A) is then added, which binds to the biotin to form an enzyme-avidin-biotin-antibody complex. The colorimetric substrate for the enzyme is then added, so the complex can then be visualized by a localized colored reaction product, placed in the context of tissue organization. Similar to ELISA, there is substantial amplification of detectable signal through use of the biotin-avidin binding sequence. Ag, antigen.

are not sensitive enough to detect the presumably low amount of viral antigens. Specific detection of CMV and EBV is critical in the liver transplantation setting, when differentiating cellular rejection vs viral infection is critical for choosing correct therapies.

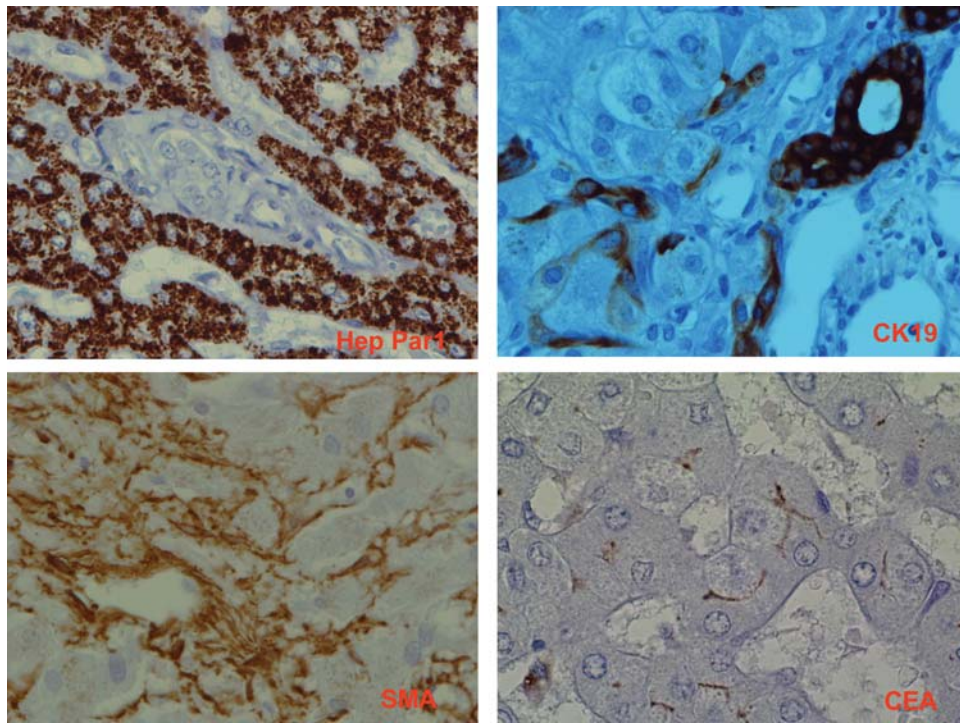
Identification of abnormal molecules by immunohistochemistry is useful for histological diagnosis. Although accumulated  $\alpha$ -1-antitrypsin (AAT) molecules are detectable by PAS-diastrase stain, immunostain using anti- $\alpha$ -1-antitrypsin offers a specific diagnosis. Amyloidosis can involve the liver. When this occurs, immunohistochemical stain will help to determine the nature of the proteins deposited.

Detection of tumor markers helps not only in making a correct diagnosis but also in selecting a specific therapy. In HCC,  $\alpha$ -fetoprotein is elevated in approx 50% of the cases. Evaluating  $\alpha$ -fetoprotein status will aid in monitoring tumor progression and response after therapy. Epithelial growth factor receptor (EGFR) has been found to be elevated in many

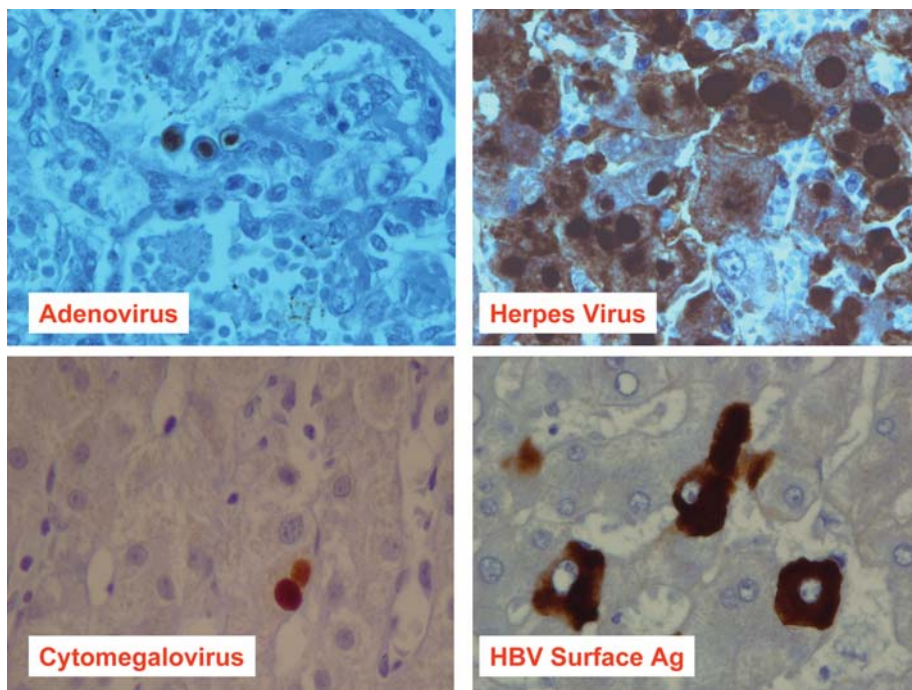
carcinomas including HCC. Several EGFR inhibitors are available (62). Therefore, detection of EGFR status in HCC may be useful for guiding therapy, although clinical application of EGFR in HCC has not been firmly established (63).

## CONCLUDING REMARKS AND OPEN QUESTIONS

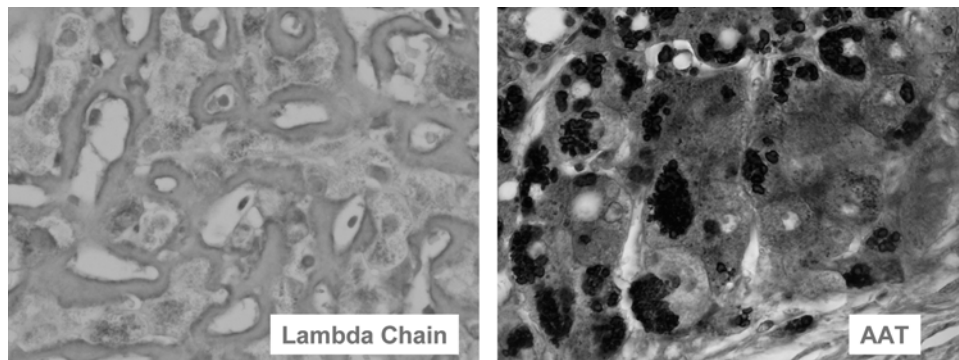
Liver diseases are invariably affected, either directly or indirectly, by both the innate and the adaptive immune system. Laboratory approaches aiming at immunological aspects of diseases are critical in clinical diagnosis and therapy. Immunological techniques have been widely used for liver disease research and clinical practice. Most of the techniques involved in evaluation of the function of immune cells, antigen mapping, and immunophenotyping are mainly used in research laboratories. Techniques used in clinical settings are predominantly serum-based immunoassays and tissue-based immunohistochemistry. These techniques are used for identification of specific antigens or infectious pathogens, characterization



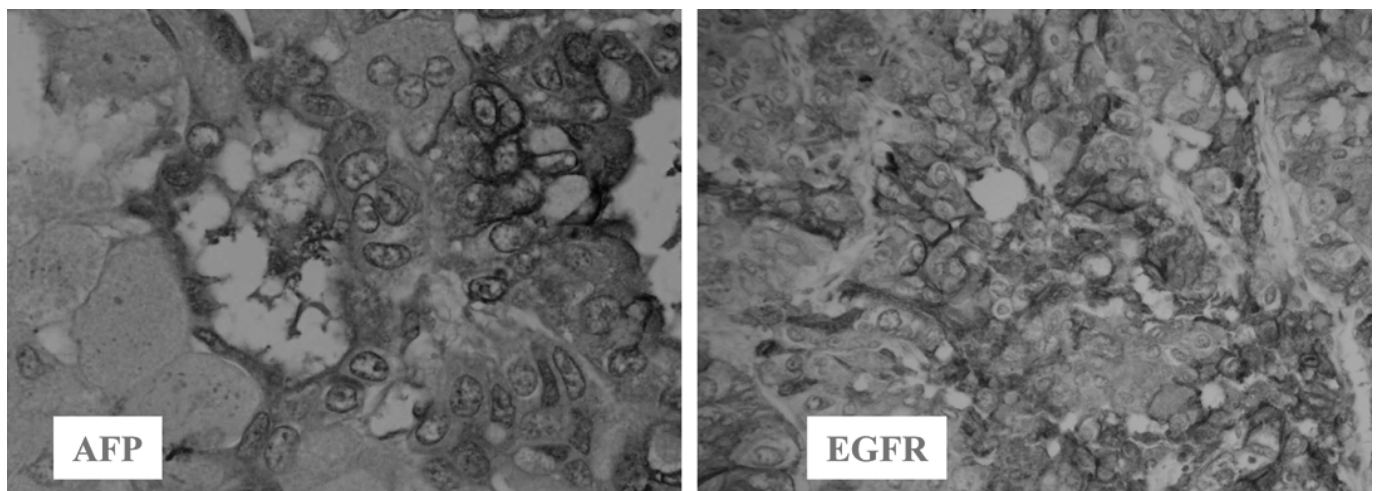
**Fig. 6.** Immunohistochemistry of resident cells in liver tissue. Hep Par1 antibody detects an unidentified hepatocyte-specific antigen in human hepatocytes. Virtually all human hepatocytes are positive for this stain. Anti-cytokeratin 19 (CK19) antibody specifically detects bile duct epithelial structures in the liver: bile ducts, bile ductules, and the bile duct epithelia of canals of Hering. Anti-smooth muscle actin (SMA) shows the presence of activated stellate cells in the setting of liver fibrosis. Polyclonal carcinoembryonic antigen (CEA) highlights the bile canalicular structure.



**Fig. 7.** Immunohistochemistry for pathogens. Virus-specific antibodies are used to detect the presence of viral proteins in liver tissue. Anti-adenoviral antibody or anti-herpes viral antibody shows the characteristic viral nuclear inclusions. Anti-CMV antibody detects both nuclear and cytoplasmic viral inclusions. Anti-hepatitis B viral (HBV) antibody against surface antigen (HBsAg) shows intense cytoplasmic staining in the infected hepatocytes.



**Fig. 8.** Immunohistochemistry for molecular abnormalities. Antibody for  $\lambda$ -chain shows the presence of amyloid deposit in the space of Disse. Anti- $\alpha$ 1-antitrypsin (AAT) antibody reacts with the protein aggregates (polymers) in a patient with AAT deficiency.



**Fig. 9.** Immunohistochemistry for tumor markers. Two clinically used tumor markers are shown by immunohistochemical staining: AFP ( $\alpha$ -fetoprotein) and EGFR (epithelial growth factor receptor). Both sections are of hepatocellular carcinoma.

of tissue markers, detection of tumor markers, and classification of cancers. The immunological techniques are rapidly evolving. Better designed antibodies, more sensitive detection technologies, and the availability of automated systems will further enhance the ability and capacity of clinical laboratories to diagnose liver disease.

With more understanding of the immunological basis of liver diseases, more techniques are expected to be available for clinical applications. Advances in genomics and proteomics will significantly change clinical laboratory practice. For instance, HBV and HCV viral load tests constitute large portion of clinical laboratory practice. We expect that more liver disease markers will be identified and applied in the clinical setting.

Although numerous studies have indicated that lymphocytes in liver tissue are key players in liver immunology, the clinical applications of this knowledge have not been well defined. Technically, it is feasible to detect various lymphocytes within

liver tissue and peripheral blood, but the main problem is that we do not know how the information is to be used in clinical practice. It is apparent that the outcome of this type of translational research will have a great impact on the management of immunological liver diseases. Immunological techniques will definitely play a critical role in this effort.

The challenging questions are:

1. What is the impact of nucleic acid-based tests on the utilization of immunoassays?
2. How are we going to take advantage of proteomic discoveries to design and apply more diagnostic tests using immunological techniques?
3. Numerous immunological techniques are available to evaluate immune cells functionally. How are we going to apply these techniques to clinical practice?
4. Predicting disease progression is increasingly important in modern medicine. What is the role of immunoassays in liver disease prognosis?

5. Hematology has been revolutionized by the characterization of cell markers on hematopoietic cells. Can we utilize similar techniques to immunophenotype hepatocytes and inflammatory cells within the liver?
6. How are we going to balance the economic issues of utilizing more advanced laboratory tests in clinical hepatology practice?

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# 11

## Tumor Immunology

### *Hepatocellular Carcinoma, Cholangiocarcinoma, and Metastatic Neoplasms*

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CHRISTOPHER L. BOWLUS

#### KEY POINTS

- Primary liver cancers including hepatocellular carcinoma (HCC) and cholangiocarcinoma (CCA) are increasing in prevalence among Western populations, and few effective therapies are available.
- NK cells play a central role in the innate immune response to tumors. Inhibitory (KIR) and stimulatory receptors (NKG2D) on NK cells are involved in the recognition of tumor cells, which often decrease KIR ligands (MHC class I) or increase NKG2D ligands (MIC, H6O, Rae1).
- The expression of mutated proteins or the overexpression of normal proteins can lead to an adaptive immune response against tumors. In order to illicit an immune response, tumor antigen must be processed and presented from MHC class I and/or II on antigen-presenting cells (macrophages and dendritic cells).
- Several of the cancer-testis tumor antigens, which are normally restricted to male germinal cells, are frequently expressed by HCC but not CCA or metastatic colon cancer.  $\alpha$ -Fetoprotein (AFP) is also frequently expressed by HCC, whereas CA19-9 is associated with CCA. Carcinoembryonic antigen is often expressed by CCA and metastatic colon cancer.
- Based on the phenotype and reactivity of tumor-infiltrating lymphocytes, CD8 T cells are the main effector cells in the antitumor immune response. CD8 T-cell responses against cancer-testis antigens and AFP have been found in healthy controls and HCC patients. Whether they play a significant role in clinical outcomes is unclear.
- Inflammatory responses to HCC and CCA in general are uncommon, suggesting that most tumors avoid immune surveillance. Several potential mechanisms have been identified including downregulation of MHC class I, expression of FasL, inhibition of Fas signaling, and immunosuppressive effects of AFP.
- The goal of immunotherapy is to illicit specific immune response against tumor antigens. Obstacles to achieving

this goal include identification of tumor antigens that are expressed in most tumors and can be processed and presented by most MHC class I alleles. In addition, tolerance to cell antigens must be broken.

- Strategies of immunotherapy in HCC have included the use of cytokines to induce MHC class I expression on tumors and stimulate antigen presentation, adoptive transfer of various effector cells, loading of dendritic cells with tumor lysates, and immunization with tumor antigens.
- Success of immunotherapy has been limited to adjuvant treatment of patients undergoing HCC resection. Two studies, one with infusion of stimulated, autologous peripheral blood lymphocytes and the other with tumor lysate pulse dendritic cells, have shown significant improvements in tumor-free and overall survival.
- Future investigations to optimize immunotherapy protocols will likely lead to practical therapies that stimulate tumor-specific immune responses and improve the currently dismal outcomes of liver neoplasms.

#### INTRODUCTION

Most tumors of the liver arise from hepatocytes giving rise to hepatocellular carcinoma (HCC), or biliary epithelial cells giving rise to cholangiocarcinoma (CCA), or the tumors are metastatic, often from colon cancer. HCC and CCA are associated with other diseases of the liver, which often incite chronic inflammation. In the case of HCC, chronic viral hepatitis is often present, although other non-inflammatory liver diseases such as hemochromatosis and  $\alpha$ 1-antitrypsin deficiency also increase the risk of HCC. Inflammatory conditions of the biliary tract, including liver fluke infestation and primary sclerosing cholangitis, are associated with CCA. These findings suggest that the immune response plays an important role in the development of many of these tumors. However, the immune response to tumors may be equally important in preventing the development or progression of liver tumors. Furthermore, directing an immune response against tumor cells has been a goal of many cancer vaccine trials, with some promising results.

### TUMOR IMMUNE SURVEILLANCE

The recognition that the immune system may play a role in the natural history of cancer arose from observations that some cancer patients with bacterial infections experienced tumor regression. In the 1960s and 1970s, as the cellular and molecular basis of immunity was defined, the theory of cancer immunosurveillance was developed. Lewis Thomas and MacFarlane Burnet postulated that a normal function of lymphocytes is to protect against tumor development (1,2). Initial studies in athymic nude mice failed to support this theory. However, later experiments have identified important roles for interferon (IFN)- $\gamma$  and perforin (3–5).

In particular, mice deficient in IFN- $\gamma$  signaling have a high rate of spontaneous and carcinogen-induced tumors. In addition, mice deficient in the  $\alpha$ -chain of the IFN- $\gamma$  receptor or mice unable to signal through the IFN- $\gamma$  receptor because of a deficiency in signal transducer and activator of infection-1 (STAT-1) have an increased rate of tumor formation compared with wild-type mice deficient in the p53 tumor suppressor (3). Additional studies in mice deficient in recombination activating gene 2 (RAG2), INF- $\gamma$  receptor 1 (R1), or STAT-1 have shown similar increases in susceptibility to carcinogen-induced tumors as well as epithelial tumors (6). Furthermore, mice deficient in both lymphocytes and IFN- $\gamma$  signaling, i.e., RAG2/STAT1 double-knockout mice, are even more prone to tumor development than RAG2 knockouts. These studies and others have established the presence of immunosurveillance in these experimental models and the role of lymphocytes and IFN- $\gamma$  acting through IFN- $\gamma$  signaling in tumor cells.

The importance of immunosurveillance in human cancer outside of virally mediated tumors is debatable, but evidence in support of the theory is accumulating. Antitumor T cells and antibodies have been detected in numerous cancer types. However, a strong correlation between the presence of these markers and clinical outcome is primarily limited to melanoma, colorectal cancer, and renal cell carcinoma. In the absence of treatment, vitiligo of melanoma, a sign of an antitumor immune response, has long been associated with an improved prognosis (7–9). In addition, IFN- $\alpha$ 2b treatment, which induces an autoimmune response manifested by the production of autoantibodies, is strongly associated with a better prognosis than treatment without an autoimmune response (10). In colon cancer, infiltration of effector memory CD8 T cells has recently been associated with a lower rate of metastases and better survival (11).

Recently, the interplay between innate and adaptive immunity in cancer immunity has been recognized, most notably in the use of attenuated bacillus Calmette-Guerin (BCG) for the treatment of bladder cancer. Microbial DNA, which is the active antitumor agent of BCG, is a key stimulant of innate immunity (12). Bacterial but not vertebrate unmethylated CpG motifs can activate the innate immune system by binding of the Toll-like receptor (TLR)-9. However, it is also important to note that inflammation can also promote tumor progression. This is particularly true of the innate immune system (13,14).

### IMMUNE RESPONSE TO TUMOR

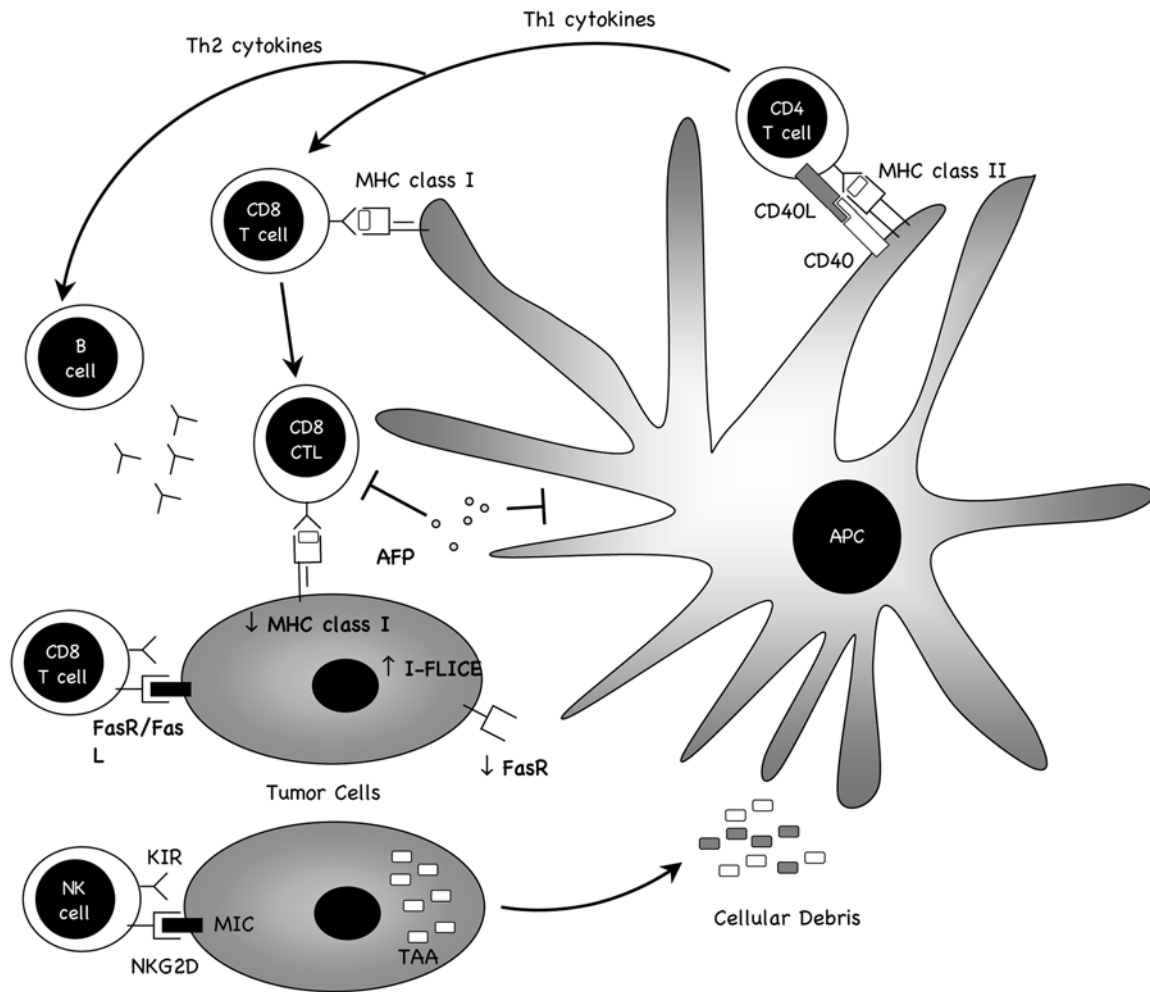
Cytotoxic T cells, natural killer (NK) cells, and antibodies all show activity against tumor cells in vitro (Fig. 1). In mouse models, tumor immunogenicity is mediated by CD8<sup>+</sup> and CD4<sup>+</sup> T cells as well as NK cells. In human cancers, tumor-infiltrating CD4<sup>+</sup> and CD8<sup>+</sup> T cells have been cloned that recognize tumor antigens presented on the major histocompatibility complex (MHC). These peptides can be either tumor specific or tumor associated. *Tumor-specific antigens* are found only in tumor cells and are derived from mutated proteins or proteins derived from recombinant genes as a result of chromosomal translocation. *Tumor-associated antigens* are derived from normal cellular proteins that are aberrantly or overexpressed by tumor cells and to which the immune system is not tolerant. Tumor-associated antigens are frequently proteins that are normally expressed in immunologically privileged sites or at very low levels.

Activation of tumor-specific T cells requires the presentation of tumor antigens by MHC class I or II molecules. In the case of CD8<sup>+</sup> T cells, 8- to 11-amino-acid peptides are produced from cytosolic proteins by the proteasome complex. Peptides are transferred into the endoplasmic reticulum by transporter associated with antigen presentation (TAP) transporters, where they are bound to MHC class I molecules and subsequently expressed on the surface of tumor cells.

Tumor antigens can also be taken up by dendritic cells (DCs), which present exogenous peptides on MHC class II molecules to CD4<sup>+</sup> T cells, further enhancing the proliferation and effector mechanisms of CD8<sup>+</sup> T cells via Th1 cytokines. In addition, costimulatory signals such as CD40/CD40L promote dendritic cell maturation and cross-presentation of antigen on MHC class I molecules to CD8<sup>+</sup> T cells. Furthermore, Th2 cytokines can activate B cells, producing antibodies against tumor antigens, which may elicit an antibody-dependent cellular cytotoxicity.

In addition to the adaptive immune response to tumor cells, the innate immune system can play an important role in the immune response to tumors (15). NK cells have long been known to recognize many different tumor cells but not normal self-cells. Initial studies showed that reduced or abolished expression of MHC class I, frequently found in tumor cells, was central to the tumor killing effects of NK cells. Subsequently, inhibitory receptors on NK cells were identified that prevent the class-I specific killing. In humans these receptors include killer immunoglobulin-like receptors (KIRs) and CD94/NKG2A. KIRs are immunoglobulin-like and bind directly to MHC class I molecules. In contrast, CD94/NKG2A binds to a peptide derived from the signal sequence of MHC class I, which is presented on the nonclassical class I molecule HLA-E. Recently, stimulatory signals have also been identified that are induced by tumor cells and are recognized by NK cells. NKG2D is a stimulatory receptor expressed on NK cells, T cells, and macrophages that can recognize MHC class I chain-related protein (MIC), H6O, retinoic acid early inducible protein 1 (Rae 1) and UL16 binding protein (ULBP). These





**Fig. 1.** Mechanisms of tumor immune response and evasion. Tumor cells lacking MHC do not inhibit natural killer (NK) cells through inhibitory receptors such as killer immunoglobulin-like receptors (KIRs). Stimulatory NK receptors such as NKG2D bind ligands including MHC class I chain-related proteins (MIC, which are upregulated). Tumor-associated antigens (TAAs) are taken up by antigen-presenting cells (APCs), especially dendritic cells, which present peptides on MHC class II to CD4 T cells. Costimulatory signals (CD40/CD40L) increase antigen presentation and cross-presentation of tumor antigens to CD8 T cells. Th1 and Th2 cytokines activate CD8 T cells and B cells, leading to a tumor-specific immune response. Evasion of the immune response may involve the downregulation of MHC class I to reduce the presentation of TAA. In hepatocellular carcinoma,  $\alpha$ -fetoprotein (AFP) secretion from tumor cells may inhibit APC function through several mechanisms and may also inhibit T- and B-cell functions. Cholangiocarcinomas and metastatic colon cancers express FasL, which can induce apoptosis of CD8 T cells. Although cholangiocarcinoma also expresses FasR, apoptosis is inhibited by the inhibitor of Fas-associated death domain-like IL- $1\beta$ -converting enzyme (I-FLICE).

proteins are increased on tumor cells and virally infected cells and can lead to tumor rejection *in vivo*.

With this background in the immune response to tumor, we will discuss the specific tumor antigens associated with HCC, CCA, and metastatic colon cancer and the attempts at manipulating the immune response for therapeutic benefit. When data are available, we will also discuss clinical trials, which to date have had only modest success.

## HEPATOCELLULAR CARCINOMA

### IMMUNE RESPONSE TO HCC

Inflammatory infiltrates within HCC tumors are uncommon, but several studies have suggested that they are clinically

relevant (16–19). A study of 163 HCC specimens found a marked inflammatory cell infiltrate in only 11 cases (16). Infiltrates consisted primarily of CD8<sup>+</sup> T cells and were associated with necrosis of cancer cells. The 11 cases with inflammatory infiltrates had a remarkably lower recurrence rate (9.1%) compared with controls (47.7%) and a better 5-yr survival (100% vs 65.1%, respectively). Infiltration with DCs along with lymphocytes has also been associated with lower recurrence rates and better survival (18).

The lack of significant immune response does not appear to be caused by a lack of MHC class I expression or defects in antigen processing (20), rather, it may be owing to tumor-infiltrating CD4<sup>+</sup>/CD25<sup>+</sup> regulatory T cells (19). Compared

with liver-infiltrating lymphocytes, CD4<sup>+</sup>/CD25<sup>+</sup> T cells are increased in the tumor-infiltrating lymphocyte population (2.4% vs 8.7%, respectively) and although the numbers of peripheral CD4<sup>+</sup>/CD25<sup>+</sup> T cells do not differ between patients with HCC and healthy controls, a significantly greater proportion express the immunosuppressive cytokine transforming growth factor- $\beta$  (TGF- $\beta$ ; 55.5% vs 2.9%, respectively).

### HCC TUMOR ANTIGENS

**$\alpha$ -Fetoprotein** Serum  $\alpha$ -fetoprotein (AFP) is well established as a tumor marker for HCC. AFP is normally expressed by the fetus and appears in the serum, where it reaches peak levels of 3 mg/mL at 10 to 13 wks of gestation. At birth, serum levels drop to 30 to 100  $\mu$ g/mL, and in normal adults levels are normally 1 to 3 ng/mL. Of HCC tumors, 50 to 70% secrete AFP and can reach serum levels over 1 mg/mL. However, elevated levels up to 200 ng/mL are also found in patients with viral and autoimmune hepatitis without HCC (21,22).

A naturally occurring immune response to AFP was first suggested by the identification of antibodies against AFP in the serum of patients with HCC (23). During the same period AFP was shown to be processed and a specific peptide presented by MHC class I molecules (24). Subsequently, several MHC-AFP peptide complexes have been shown to elicit cytotoxic T lymphocytes (CTL) responses and cytokine release in both healthy controls and patients with HCC, suggesting that anti-AFP T cells are not deleted during ontological development of the immune system (25,26). Interestingly, the frequency of CTL responses appears to increase with advanced disease, suggesting that the CTL response is not a significant factor in preventing tumor progression. However, treatment of HCC (usually with local ablative therapy) was associated with an increase in anti-AFP CTL responses. These treatments may enhance the presentation of AFP and activation of specific AFP-responsive T cells.

Akeel et al. have also found CD4<sup>+</sup> T cells responsive to AFP epitopes in patients with HCC (27). CD4<sup>+</sup> T cells were identified in peripheral blood lymphocytes (PBLs) cultured in the presence of an AFP peptide predicted to be bound by HLA-DR13. The CD4<sup>+</sup> T cells were characterized as having a Th1 phenotype and recognized the peptide in the context of HLA-DR but not HLA-DQ or class I. In contrast to the anti-AFP CD8<sup>+</sup> T cells, no CD4<sup>+</sup> T cell response could be generated from healthy controls or from patients with chronic liver disease without HCC. In addition, CD4<sup>+</sup> T-cell responses were strongly associated with early-stage disease and low levels of serum AFP, suggesting that the CD4<sup>+</sup> T-cell response may be more important than the CTL response in inhibiting the progression of HCC.

Alternatively, the lower CD4<sup>+</sup> T-cell response in HCC patients with high AFP levels may be directly related to the immunosuppressive effects of AFP. Multiple effects of AFP on immune response have been reported including downregulation of MHC class II molecules on monocytes (28,29) and inhibition of T- and B-cell responses (30–34). In addition, AFP impairs dendritic function and induces their apoptosis (35). Specific

effects of AFP on DCs include the downregulation of CD40 and CD86 as well as decreased production of interleukin-12 (IL-12) and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ). Moreover, DCs from HCC patients with high AFP levels produce lower levels of TNF- $\alpha$  ex vivo compared with healthy controls. These effects may be mediated by increases in leukotriene synthesis induced in monocytes by specific AFP receptors (36,37).

**Cancer-Testes Antigens** In the early 1990s, the first tumor antigen was cloned from a melanoma and shown to elicit a cytotoxic response from autologous lymphocytes (38). Designated melanoma antigen 1 or MAGE-1 (and subsequently renamed MAGE-A1), it was found to be restricted to testes among normal tissues but expressed in a number of tumors. Subsequent identification of antigens with similar features lead to the concept of cancer-testes antigens as a group of proteins that are normally restricted in expression to male germ cells and are occasionally found in ovary tissue and trophoblasts (39). They include MAGE-A genes, NY-ESO-1, and SSX. These genes are encoded on the X-chromosome and are often induced in tumors by promoter hypomethylation. Genetic, serologic, and bioinformatics approaches have been used to identify a large number of cancer-testes genes, many of which spontaneously produce cellular and/or humoral immune responses in cancer patients.

A number of cancer-testes genes have been shown to be expressed in some HCCs, although the frequencies vary considerably (Table 1). In 1996, Yamashita and colleagues first reported the presence of MAGE-1 mRNA in 16 of 20 resected HCC tumors but none in nontumor liver tissue (40). Subsequent studies have found a wide range of frequencies (0.19–0.78) in MAGE-1 expression as well as a number of other cancer-testis antigens in HCC. It is unclear whether this variability reflects technical differences between studies or true differences in the biology of HCC in different regions. However, a recent study comparing 40 HCC specimens from Beijing and 33 from Guangxi province in China found a significant difference in the frequency in MAGE-3 expression between HCC from the two locations (32.6% vs 70.0%, respectively) (41). Inconsistencies have been found in the associations between the expression of cancer-testis antigens and clinical outcomes. Suzuki et al. reported that patients with MAGE-1 expressing HCC had lower AFP levels and a better recurrence-free survival (42). However, others have failed to find associations of cancer-testis antigens with HCC stage or AFP levels (43,44).

Spontaneous CTL responses to cancer-testis antigens have been documented in patients with HCC (45–49). Dong et al. used a computer-based epitope prediction algorithm to design potential antigens from the MAGE sequences that would be expected to be bound by the HLA-A2.1 allele present in 50% of the Chinese population (45,50). They found that the sequence QLVFGIEVV, corresponding to residues 159 to 167, is bound by HLA-A2.1 and induces a MAGE-specific CTL response against HCC cell lines expressing MAGE.

Zhou and colleagues took this a step further and screened an HCC tumor expressing MAGE-1 and MAGE-3 for MAGE peptides spontaneously presented on HLA-A2 (49). In contrast

**Table 1**  
**Frequency of Cancer-Testis Antigens in Hepatocellular Carcinoma**

Total no. of cases	BAGE											Ref.					
	MAGE-1	MAGE-2	MAGE-3	MAGE-4	GAGE	GAGE1-6	GAGE1-2	No. (frequency)	SSX-1	SSX-2	SSX-4		SSX-5	SCP-1	NY-ESO-1	CTp11	HCA587
20	16 (0.80)																40
50	23 (0.46)	17 (0.34)	21 (0.42)	8 (0.16)													115
33	26 (0.78)		14 (0.42)														43
45	27 (0.60)																116
60	18 (0.30)	9 (0.15)	15 (0.25)														42
22	15 (0.68)		10 (0.46)														117
33	22 (0.67)		13 (0.39)			12 (0.36)	10 (0.30)	7 (0.21)									44
30									24 (0.80)	14 (0.47)	22 (0.73)	10 (0.33)	2 (0.07)	11 (0.37)			118
21	4 (0.19)		5 (0.24)	1 (0.05)	8 (0.38)				8 (0.38)	2 (0.09)	2 (0.09)		6 (0.29)	0 (0.00)			119
30	21 (0.70)		16 (0.53)														120
105	79 (0.75)								76 (0.72)						66 (0.63)	59 (0.56)	121
73	51 (0.70)		35 (0.48)							26 (0.36)				31 (0.42)			41
20	16 (0.80)		9 (0.45)														47

to the sequence predicted by Dong et al., they identified a MAGE-1 peptide (FPSLREAAL) corresponding to residues 294 to 302 as well as a MAGE-3 peptide (MAGE-3<sub>271-279</sub>, FLWGPRALV). However, specific CD8<sup>+</sup> T cells detected by tetramer staining and CTL responses to the latter antigen could only be detected at low frequency after tumor recurrence.

Interestingly, the MAGE-1 and MAGE-3 peptides identified by Dong et al. are different from those found to be highly immunogenic in melanoma, namely, MAGE-1<sub>161-169</sub> and MAGE-3<sub>271-279</sub> (38). Zerbin and colleagues were able to detect tetramer-positive CD8<sup>+</sup> T cells to MAGE-1<sub>161-169</sub>/HLA-A\*0101 and MAGE3<sub>271-279</sub>/HLA-A\*0201 among tumor-infiltrating lymphocytes but only in 1 patient each among 10 patients with HCC expressing MAGE-1 and MAGE-3 mRNA (47). Expansion of MAGE-specific T cells after 10 d of culture with peptide resulted in the detection by tetramer staining of MAGE-3 in only one additional patient. The MAGE-1<sub>161-169</sub>-specific CD8<sup>+</sup> T cells were oligoclonal, based on the limited number of T-cell receptor (TCR) V $\beta$  chains expressed. They were functional and capable of killing target cells. Phenotypically, they were characterized as CD45RA<sup>-</sup>/CCR7<sup>-</sup>/CD62L<sup>-</sup> and CCR5<sup>+</sup>, and on mitogenic stimulation only 41.8% expressed INF- $\gamma$  compared with 78.4% of nonantigen-specific CD8<sup>+</sup> T cells.

More frequent antigen-specific CD8<sup>+</sup> T-cell responses have been found to NY-ESO-1b 157–165 (SLLMWITQC), presented by HLA-A2 (46). Peptide stimulation of peripheral blood mononuclear cells (PBMCs) elicited antigen-specific CD8<sup>+</sup> T-cell responses as measured by INF- $\gamma$  ELISPOT in 5 of 16 HLA-A2 HCC patients with NY-ESO-1b-expressing tumors. In addition, 6 of 12 subjects had detectable CD8<sup>+</sup> T cells with antigen-specific tetramers. No significant correlation was found between CD8<sup>+</sup> T-cell responses and tumor stage. Similar studies have also identified CD8<sup>+</sup> T cells in tumor-infiltrating lymphocytes directed against antigens derived from MAGE-A10 and SSX-2 (48).

In summary, spontaneous immune responses to HCC are uncommon, perhaps due owing the immunosuppressive effects of AFP and regulatory T cells. When an immune response occurs, it may have significant effects on the progression of tumor. The cancer-testis antigens are frequently expressed in HCC, but no single family member is universally expressed. Although spontaneous CD8<sup>+</sup> T cells against cancer-testis antigens are infrequent, these proteins remain promising targets for immunotherapy against HCC as will be discussed later in Immunotherapy Trials.

## CHOLANGIOCARCINOMA

CCA is an uncommon primary liver tumor that arises from the biliary epithelium. However, the incidence has been noted to be rising in Western countries, including the United States (51). Only a few select cases of CCA are candidates for curative therapy by surgical resection or liver transplantation. Long-term survival for surgical resection is only 20% at 2 yr. Because this tumor is rare, investigations into tumor-associated antigens and immune responses have been limited.

## IMMUNE RESPONSE

The immune response to cholangiocarcinoma appears to be less intense compared with that of HCC. In studying the effects of cytokines on tumor-infiltrating lymphocytes, Shimizu et al. recovered tumor cells and infiltrating lymphocytes at ratios of 7.6:6.4, 8.0:2.1, and 4.8:4.7 from HCC, CCA, and metastatic liver tumors, respectively, suggesting a much less intense inflammatory response to CCA compared with either HCC or metastatic liver tumors (52). Most of these lymphocytes, whether from HCC, CCA, or metastatic liver tumors are T cells with a memory phenotype expressing CD45RO (53). Notably, expansion of tumor-infiltrating lymphocytes from CCA proliferated *in vitro* much more slowly than those from HCC.

Evasion of CCA from immune surveillance has been suggested to involve Fas-mediated pathways of apoptosis. CCAs express Fas ligand (FasL) and disable Fas receptor (FasR), two key players in the regulation of apoptosis in immune tolerance and carcinogenesis (54,55). FasL is normally expressed by cells at immunologically privileged sites, where it induces apoptosis of activated T cells expressing FasR. Although low levels of FasL are expressed in cultured normal cholangiocytes, expression at both the protein and mRNA level are greater in cell lines derived from CCA (54). In addition, FasL is not detected by immunohistochemistry or *in situ* hybridization on normal bile duct epithelium but is present in dysplastic and well-differentiated cholangiocarcinoma (55). FasL on cholangiocarcinoma cells is able to induce apoptosis of Fas-sensitive T cells, and apoptotic lymphocytes are more frequently observed surrounding CCA tumors than in surrounding tissue, suggesting a possible mechanism of immune evasion.

In addition to FasL, CCA cells express FasR, particularly in early stages. However, poorly differentiated CCA is characterized by a decrease in FasR expression, which would be expected to make such tumors insensitive to FasL-bearing T cells. Furthermore, CCA cells express high levels of Fas-associated death domain-like IL-1 $\beta$ -converting enzyme (FLICE) inhibitor (I-FLICE), a competitive inhibitor of caspase-8 that is part of the Fas-mediated apoptosis pathway. Thus, CCA inhibits immune surveillance by inducing cell death of activated T cells through FasL but is protected from autoapoptosis and T-cell-mediated apoptosis. Other mechanisms that may inhibit activated T cells include replication competent avian splice1 (RCAS1) and mucin1 (MUC1), which are frequently expressed by CCAs and have been associated with induction of apoptosis in activated T cells (56–62).

## TUMOR-ASSOCIATED ANTIGENS

Investigations into the expression and immune responses to tumor-associated antigens in CCA have been limited. Gene microarray studies have identified several genes that are upregulated in CCA, but nothing is known about the immune response to their proteins (63,64).

**Carbohydrate Antigen 19-9** Carbohydrate antigen 19-9 (CA19-9) was originally identified by a monoclonal antibody

raised against a human colorectal carcinoma cell line. The epitope was later identified as a sialylated lacto-*N*-fucopentaose II carbohydrate related to the Lewis blood group antigens and is found on high-molecular-weight mucin. The biologic function of CA19-9 is unknown, but it may be a marker of mucins that can induce apoptosis in activated T cells, contributing to the evasion of immune surveillance (56).

CA19-9 has subsequently been identified on many adenocarcinomas, notably pancreatic cancer, ovarian cancer, and CCA. Serum CA19-9 is frequently elevated in patients with CCA but is also elevated in benign conditions including biliary obstruction. Its usefulness as a screening or diagnostic test for CCA is controversial (65–70). CA19-9 can be detected by immunohistochemistry on normal bile duct cells, but it is greatly increased in 80 to 91% of CCAs (71,72). Whether a humoral or T-cell-mediated response to CA19-9 develops in cholangiocarcinoma or other CA19-9-expressing tumors has not been investigated.

**Carcinoembryonic Antigen** More than 4 decades ago, Gold and Freedman first identified the tumor-associated antigen carcinoembryonic antigen (CEA) in human colon cancer (73). CEA was originally thought to be restricted to fetal and cancer tissue, but it now appears to be normally expressed in a number of adult tissues (74). The structure of CEA is related to the immunoglobulin superfamily. Several related genes have been identified and included in the CEA gene family. Although CEA is normally expressed by colon epithelium, serum levels are frequently elevated in patients with colon cancer. This has led to its use as a tool for colon cancer surveillance.

Nonomura et al. found CEA expression in 42 of 44 CCAs, with more prominent expression in poorly differentiated tumors (75). Serum levels of CEA are also often elevated in patients with CCA and may add diagnostic benefit to CA19-9 alone (76,77).

Despite early reports of anti-CEA antibodies in serum from patients with CEA-producing tumors, it is more likely that very few patients spontaneously develop anti-CEA immune responses (78–85). Initial reports in the 1970s may have identified antibodies crossreacting with related antigens. T-cell-mediated responses to CEA have not been detected in healthy controls or patients with CEA-producing tumors (86).

**Cancer-Testis Antigens** A limited number of studies have identified the expression of cancer-testis antigens in CCA. Okami and colleagues found MAGE-1 and MAGE-3 mRNA to be expressed in a minority of CCAs, 5 and 7 of 32, respectively (87). In contrast, CEA was present in 26 of the 32 specimens. Tsuneyama et al. identified MAGE-3 by immunohistochemistry in 32 of 68 (47%) invasive cholangiocarcinomas (88). Finally, Utsunomiya et al. reported on the expression of NY-ESO-1, SCP-1, and SSX-4 in addition to MAGE-1 and MAGE-3 in 2, 6, 3, 1, and 4 of 20 CCAs (89). Expression of at least one of the genes was present in only 50%. Thus, CCA appears to express cancer-testis antigens less frequently than HCC but still at rates that may make them suitable targets for tumor immunotherapy.

## METASTATIC LIVER NEOPLASM

Colorectal cancer is the most common metastatic neoplasm in the liver. As discussed above in cholangiocarcinoma, the frequency and phenotype of tumor-infiltrating lymphocytes is similar in metastatic liver tumors and HCC (52,53). However, metastasis is associated with a lower frequency of tumor-infiltrating CD8<sup>+</sup> memory T cells in the primary lesion, suggesting evasion of immune surveillance (11). Several mechanisms have been identified that may allow neoplastic cells to avoid immunosurveillance. Unlike early-stage colon cancer, in which FasL expression is found in approximately half of the cases, hepatic metastases almost always express FasL (90). As in CCA, early studies supported the tumor counterattack hypothesis of inducing apoptosis of activated T cells and hepatocytes, which may facilitate tumor invasion; recently these findings have been called into question (91–93). In contrast, HLA expression in metastatic and poorly differentiated colon cancer is lower compared with primary lesions, suggesting a decrease in presentation of tumor-associated antigens as a mechanism to evade tumor surveillance (94).

As mentioned in carcinoembryonic antigen above, CEA is frequently increased in metastatic colon cancer and is clinically useful for cancer surveillance following resection. However, there does not appear to be a significant humoral or cellular immune response to CEA. Several studies have assessed the expression of cancer-testis antigens in colon cancer and found that they are infrequently expressed (39). Nevertheless, the expression of MAGE-3 has been associated with liver metastases, and humoral responses to cancer-testes antigens as well as other novel colon cancer antigens are more frequent in metastatic disease (95,96).

## IMMUNOTHERAPY TRIALS

Tumors of the liver, whether they be primary (HCC and CCA) or metastatic, typically have a poor prognosis and limited therapeutic options. Specifically targeting the immune response to tumor tissue through various strategies has been a lofty goal, which has recently demonstrated some promising clinical results (Table 2). Targeting an immune response to a mutated protein such as p53 or  $\beta$ -catenin would in theory be tumor specific. However, in order to generate an immune response, the mutation would need to be processed and presented by APCs on MHC molecules. In addition, despite common “hot spots” for mutations in these genes, designing therapeutic vaccines to these proteins would require numerous combinations to be applicable to most patients. Similarly, targeting tumor-associated antigens requires the protein to be processed and presented and tolerance to be broken. As discussed above, several tumor-associated antigens are frequently expressed in these tumors, (presented by MHC molecules) and at times spontaneously break tolerance. Immunization with tumor antigens or lysates and stimulation with cytokines either to increase tumor immunogenicity or decrease the immunosuppressive tumor microenvironment are approaches that have been employed in attempts to overcome these barriers. The remainder of this chapter will

**Table 2**  
**Clinical Trials of Immunotherapy in Hepatocellular Carcinoma**

Treatment	No.	Control group	Stage	End points	Response	Ref.
<i>Cytokines</i>						
INF- $\gamma$ /IL-2 + chemotherapy i.a.	20	None	Unresectable III/IV	Tumor size AFP	↓ Size in 14/20 Normalization in 12/20	98
INF- $\gamma$ /GM-CSF s.c.	15	Historical	Unresectable III/IV	Survival Tumor size	No difference 1 partial response 14 responses	100
OK-432 i.m + IL-2/ cyclophosphamide/Adriamycin i.a.	24	None	Unresectable III/IV	Tumor size	7 stable disease 3 progressive disease	97
INF- $\gamma$ /IL-2 +chemotherapy i.a.	20 20	Resection alone		Survival Intrahepatic recurrence	No difference 8 vs 0	99
INF- $\alpha$ 2a + cisplatin/doxorubicin/5-FU	94 94	Doxirubicin	Unresectable III/IV	Survival Tumor size	No difference	122
<i>Adoptive immunotherapy</i>						
IL-2 + LAKs + Adriamycin i.a.	12	Adriamycin i.a.		Survival	No difference	101
IL-2 + LAKs + Adriamycin i.a.	12	Adriamycin i.a.		Recurrence	No difference	102
IL-2 + LAKs + Adriamycin i.a.	12	Adriamycin i.a.		Recurrence	↓ Recurrence (8.3% vs 50%)	102
IL-2-stimulated TILs i.a.	10	Historical		Recurrence	↓ Recurrence (19.4% vs 41.6%)	104
IL-2/CD3-stimulated PBLs	76 74	Resection alone		Overall survival Disease-free survival	No difference	106
<i>Dendritic cells (DCs)</i>						
GM-CSF/IL-4 DCs + tumor	2	None	IV	Survival	1 alive at 3 yr	109
GM-CSF/IL-4 DCs + (tumor + KLH + TNF- $\alpha$ )	10	None	Unresectable	DTH Tumor size	7 of 10 1 partial response	108
GM-CSF/IL-4 DC + (tumor + TNF- $\alpha$ ) + IL-2 s.c.	2	None	IV	Tumor size	No response	111
GM-CSF/IL-4 DCs intratumor	14	None	Unresectable	Tumor size	2 partial responses	107
GM-CSF/IL-4 DCs + (tumor + IL-1 $\beta$ /IL-6/TNF- $\alpha$ /PGE <sub>2</sub> )	31	Historical	IV	Survival	↑ 1- and 2-yr survival	110
<i>Tumor vaccines</i>						
AFP peptides	6	None	IV	Tumor size	No response	113
Formalin-fixed tumor + GM-CSF/IL-2/BCG	19 22	Resection alone	I/II/III/A	T-cell response Recurrence-free survival Overall survival	↑ AFP T-cell response ↑ 2-yr recurrence-free and overall survival	114

Abbreviations: i.a., intraarterial (hepatic); s.c., subcutaneous; LAKs, lymphokine-activated killer cells; TILs, tumor-infiltrating lymphocytes; PBLs, peripheral blood lymphocytes; KLH, keyhole limpet hemocyanin; BCG, bacillus Calmette-Guérin; PGE<sub>2</sub>, prostaglandin E<sub>2</sub>; AFP,  $\alpha$ -fetoprotein; DTH, delayed-type hypersensitivity; 5-FU, 5-fluorouracil; GM-CSF, granulocyte-macrophage colony-stimulating factor; IC, interleukin; INF, interferon; TNF, tumor necrosis factor; i.m., intramuscular.

summarize the human clinical trials involving immunotherapies for the treatment of liver neoplasms.

## HCC

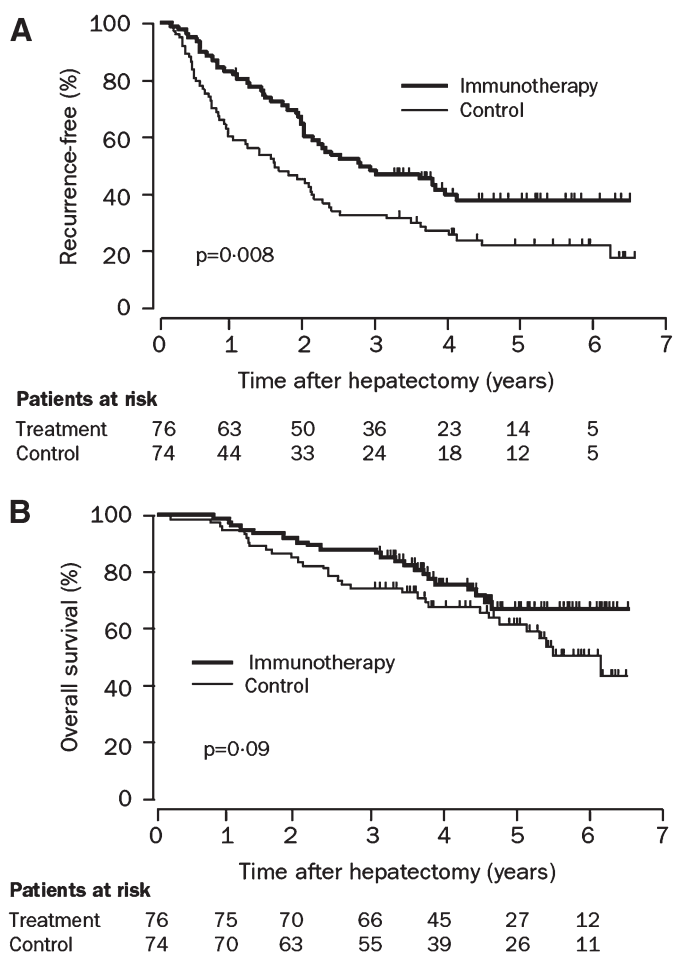
**Cytokines** Several small studies have been performed to assess the efficacy and safety of IFN- $\gamma$  and IL-2 targeted to tumor by administration through the hepatic artery. Two studies involved unresectable HCC and the infusion of IL-2 (97,98). Oka et al. treated 24 patients who had unresectable HCC with hepatic artery infusion of recombinant IL-2, Adriamycin, and cyclophosphamide. OK-432, a streptococcal preparation that is reported to induce innate immunity through TLR-4 and induce maturation of DCs, was given intramuscularly. By imaging criteria, responses were complete (CR) in four, partial (PR) in three, minor (MR) in seven, no change (NC) in seven, and progressive disease (PD) in three. The 2-yr survival rate of the responders (CR+PR+MR) was 80% but 0% in the nonresponders (NC+PD).

In a similar study, Lygidakis et al. treated 20 patients who had unresectable HCC with transarterial chemotherapy along with INF- $\gamma$  and IL-2 emulsified in a Lipiodol-Urografin mixture targeted to the spleen and the liver tumor (99). A decrease in tumor size occurred in 14 of the 20 patients, and serum AFP levels declined in 14 patients, reaching normal levels in 12. The same group performed a randomized study in patients with resectable HCC. The treatment group received the INF- $\gamma$  and IL-2 emulsion pre- and postoperatively. Eight patients developed intrahepatic tumor recurrence in the control group within 3 to 26 mo of follow-up. In contrast, none of the patients receiving INF- $\gamma$  and IL-2 developed recurrence after 4 to 27 mo of follow-up.

More recently, a phase I study of the safety and tolerability of IFN- $\gamma$  and granulocyte-macrophage colony stimulating factor (GM-CSF) given subcutaneously was performed in patients with unresectable stage III or IV HCC (100). A partial response was observed in only one patient, and 6-mo and 1-yr survival was not better than expected (40 and 20%, respectively).

**Adoptive Immunotherapy** Several approaches have been taken in an attempt to expand effector cells *in vivo* and subsequently reinfuse them. Two early randomized studies investigated the prevention of recurrence in resectable HCC by lymphokine-activated killer (LAK) cells isolated from spleens taken at the time of surgery in combination with Adriamycin (101,102). Only 12 patients were allocated to each arm in these studies. The first suggested a decrease in the rate of recurrence, but no significant differences in survival or tumor recurrence were noted in the second study.

Unlike LAK cells, which are not tumor specific, tumor-infiltrating lymphocytes (TILs) are primarily T cells that contain tumor-antigen-specific reactivity. Indium<sup>111</sup>-labeled TILs infused into the hepatic artery have been shown to traffic to tumors preferentially (103). Wang et al. reported on 10 patients treated with TILs isolated from resected tumors and infused via the hepatic artery (104). The recurrence rate of the treated patients was lower than that of historical controls (19.4 and



**Fig. 2.** Time to first recurrence (A) and overall survival (B) after resection of hepatocellular carcinoma in patients treated with autologous peripheral blood lymphocytes activated *in vitro* with IL-2 and CD3 vs controls. (Reprinted from ref. 106.)

41.6%, respectively). In addition, a direct comparison of TILs vs LAK cells in stage IV HCC demonstrated a superior response with the cytotoxic TILs in terms of tumor regression (105).

The largest adoptive transfer study involved the use of autologous PBLs, stimulated with IL-2 and anti-CD3 antibody and infused intravenously (106). In this study, 150 patients with HCC undergoing resection were randomly assigned to adoptive immunotherapy ( $n = 76$ ) or no adjuvant therapy ( $n = 74$ ). After a median follow-up of 4.4 yr, immunotherapy reduced the frequency of recurrence (59% vs 77%,  $p = 0.01$ ) and improved recurrence-free survival ( $p = 0.008$ ; Fig. 2). A nonstatistically significant improvement in overall survival was also observed.

**Antigen-Presenting Cells** Five studies have been published on the use of DCs pulsed with tumor or tumor lysates *ex vivo* and reinfused to stimulate a tumor-specific immune response (107–111). The first study involved the treatment of two patients with advanced HCC, one of whom appeared to

improve. Another study treated 20 patients with various tumor types, 2 of which had HCC, but no signs of a clinical response were found. Iwashita et al. treated 10 unresectable HCC patients with DCs loaded with tumor lysate and found that 7 developed a delayed-type hypersensitivity response, indicating successful vaccination. However, only one patient had a minor tumor response.

More recent studies have injected DCs directly into the tumor with radiotherapy (107). Of 12 patients completing treatment, there were two partial responses and four minor responses. AFP decreased by more than 50% in three patients, and AFP-specific immune responses were identified in eight patients. In contrast to direct injection of untreated DCs, Lee et al. reinfused DCs pulsed with autologous tumor lysates weekly for 5 wk (112). After the first 14 patients were treated, they treated 17 more patients but added monthly infusions after the first pulse therapy. The latter group had a significantly better 1-yr survival (63.3% vs 10.7%,  $p < 0.001$ ), suggesting that ongoing refinements in the scheduling of immunotherapy treatments may lead to significant improvements in efficacy.

**Tumor Vaccines** Despite the high frequency of AFP production in HCC and the ability to isolate AFP-specific T cells, AFP peptide vaccination has not shown clinical success (113). In a phase I clinical trial, six HLA-A2 patients with HCC were immunized with immunodominant AFP peptides. All six generated T-cell responses to some or all of the peptides, but no clinical responses were identified. DC-based therapies using AFP peptides are currently ongoing.

Vaccination with autologous tumor has also been reported. A randomized study of formalin-fixed autologous tumor mixed with IL-2, GM-CSF, and BCG has shown promising results in preventing recurrence in patients undergoing HCC resection (114). In this studies 41 patients were randomized to receive vaccine treatment ( $n = 19$ ) or no adjuvant therapy ( $n = 22$ ). At a median follow-up of 15 mo, the risk of recurrence in vaccinated patients was reduced by 81% (95% confidence interval, 33–95%;  $p = 0.003$ ). In addition, vaccination improved recurrence-free survival ( $p = 0.003$ ) and overall survival rates ( $p = 0.01$ ).

## CONCLUDING REMARKS AND OPEN QUESTIONS

Anecdotal experiences have suggested that in a small number of patients with liver cancers, either primary or secondary, immune responses could lead to significant clinical improvements. The identification of specific immune responses to tumor-associated antigens has lent further support to the theory of immunosurveillance. Promising results with immunotherapy as an adjuvant therapy for resectable HCC suggests a potential role for these therapies. Genetic and immunologic approaches to characterize tumor-associated antigens further and the immune responses they illicit should lead to new treatment approaches. Future studies incorporating multiple strategies aimed at inducing or enhancing tumor-specific immune responses hold a promise of improved outcomes for these tumors, which otherwise have a dismal prognosis.

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**BACTERIAL, PARASITIC,  
AND VIRAL INFECTIONS  
OF THE LIVER**

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**II**

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# 12 Innate and Adaptive Immune Responses to Bacterial and Parasite Infections

## *Clinicopathological Consequences*

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### KEY POINTS

- **The innate immune system** is comprised of hereditary components that provide an immediate first line of defence to ward off pathogens continuously. Many effectors contribute to its action: physical and chemical barriers (skin, stomach acid, mucous coating of gut and airways, cough), phagocytic cells (macrophages and neutrophil granulocytes), and other components such as lysozyme, the complement system, and acute-phase proteins (i.e., C-reactive protein).
- **The adaptive (acquired) immune system** is based on the humoral and cellular immune systems (cytotoxic T cells and T-helper cells).
- Every immune response represents a **host defence strategy** to contain spread of infection, but it is also responsible for the tissue pathological damage and for the clinical manifestations.
- **Mycobacterial infections:** liver involvement during *Mycobacterium tuberculosis* infection varies with the stage of pulmonary or systemic infection, being common in case of miliary disease. Up to two-thirds of patients with primary pulmonary TB have some kind of liver involvement. The ability to form granulomas is critical to control the diffusion of the infection; the granulomatous inflammation represents in fact a specialized tissue mechanism of host defence, circumscribing the infected macrophages within a limited area and inducing a potent antimicrobial activity. The two cells most responsible for the immune response are the macrophages and CD4<sup>+</sup> T lymphocytes.
- **Brucellosis**, like tuberculosis, is a chronic granulomatous infection caused by *Brucella*, which is a facultative intracellular pathogen. The primary pathology during this infection is the noncaseating granuloma. Cell-mediated immunity is crucial in limiting the infection.
- **Pyogenic liver abscesses** are principally caused by malignant biliary obstruction, but the hematogenous diffusion, from intestinal inflammatory process (diverticulitis, appendicitis, colon cancer) is also possible. Abscess development is a host defence strategy, mainly determined by the local cellular immune response, to contain the spread of infection.
- **Malaria** is an intracellular protozoan parasite whose life cycle is determined by its ability to evade the innate and/or the adaptive immune response. The liver is affected during malaria infection in different degrees, and the malarial hepatopathy is a heterogeneous syndrome, ranging from mild elevation of liver function tests to fulminant liver failure.
- **Schistosomiasis** is a helminthic infection causing a wide spectrum of disease. The balance between Th1- and Th2-type cytokines influences the extent of the pathology and the development of the fibrosis, one of the typical features of hepatic schistosomiasis. As the granulomas enlarge, there is a preferential development of the Th2 response.
- **Amebiasis** is caused by *Entamoeba histolytica*. Liver abscess is the typical extraintestinal manifestation, and its development depends on both parasite and immune system host factors. The amoeba has to be capable of causing alterations in intestinal permeability, to secrete a specific proteinase pattern, to induce apoptosis, and to resist complement-mediated lysis. Indirect evidences suggests that cellular immunity is an important factor in the protection against *E. histolytica*.
- **Visceral leishmaniasis** is an intracellular protozoal infection that primarily targets the macrophages of the liver. An ineffective cell-mediated immune response is associated with active disease progression, clinically characterized by hepatomegaly, splenomegaly, and pancytopenia. If untreated, the disease could be rapidly fatal. Most immunocompetent individuals develop a successful T-cell-mediated defence that is able to prevent clinical disease but may not eliminate the parasite. This immune reaction is mainly based on the formation of granulomas.

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- **Echinococcosis** results in humans when they become accidental hosts for a cystic intermediate stage of one of the two major species of canine tapeworms belonging to the genus *Echinococcus*. A combined Th1 and Th2 cytokine profile appears to be crucial for prolonged parasitic growth and survival. Th1 cytokines promote the initial cell recruitment around the parasite vesicles, inducing a chronic cell infiltrate and the formation of the typical periparasitic granuloma.

## MYCOBACTERIAL INFECTIONS

A vast spectrum of illness can result from infection owing to *M. tuberculosis*, *Mycobacterium avium*, and *Mycobacterium leprae*, but the representative organism of this genus is *M. tuberculosis*. *M. tuberculosis* infection is estimated to infect 1.6 billion people worldwide or approximately one-third of the world's population, killing about 3 million people each year (1,2). More than 90% of tuberculosis (TB)-related deaths occur in developing countries, and the disease has huge social and economic costs. Nations with a high prevalence of HIV have witnessed the greatest increase in the number of TB cases (3). Diffusion of the infection depends on inhalation of aerosols from individuals with pulmonary infection. The development of the disease occurs in less than 10% of infected persons and is significantly increased by impaired cell-mediated immunity. Liver involvement varies with the stage of pulmonary or systemic infection, being common in the case of miliary disease. However, it had been demonstrated that up to two-third of patients with primary pulmonary TB have some kind of liver involvement (4).

### IMMUNE RESPONSE

*M. tuberculosis* is characterized by a complex cell wall rich in mycolic acids, peptidoglycan, and arabinogalactan, surrounding the cell membrane. Many of these components are responsible for immune system stimulation, whereas the phagocytosing macrophages initiate the host immune response (5). Macrophages initially secrete proinflammatory cytokines, such as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin-1 (IL-1), and IL-6, which leads to an influx of cells to the site of infection (6,7). T cells, particularly Th1, are critical in the immune response; in fact, by secretion of interferon- $\gamma$  (IFN- $\gamma$ ) and IL-2, they contribute to the control of infection. Individuals defective in IFN- $\gamma$  or IFN- $\gamma$  receptors are prone to more severe disease (8). The contribution of both macrophages and T cells leads to the formation of the typical tissue immune response of TB, which are the granulomas (9). Specific antibodies against *M. tuberculosis* have not been found to be of primary importance in host defence against the infection.

### CLINICAL MANIFESTATIONS AND PATHOLOGY

The liver may be involved in several ways during the course of TB, ranging from the hepatic granulomas (the most common) and the tuberculomas to TB of the biliary tract and miliary TB (10).

**Granulomatous Disease** Symptoms and signs of liver disease are usually occasional findings, and most patients present nonspecific features like general malaise, fatigue, weight

loss, anorexia, and fever (11). Physical examination may reveal hepatomegaly or splenomegaly. Alkaline phosphatase and  $\gamma$ -glutamyl transpeptidase elevation may be present (12). The typical features of the tubercular granulomas are represented by caseation necrosis within the granuloma and irregularity of the contour, with a very dense rim of lymphocytes surrounding the lesion. Multinucleated giant cells, fibroblasts, eosinophils, mast cells, and basophils may surround the granuloma, but the epithelioid cell is the essential element. Usually these lesions are relatively few, generally 1 to 2 mm in diameter, and they are frequently found periportal. The regular liver architecture and its function are not usually affected (13).

**Tuberculoma** When multiple large caseating granulomas coalesce, they form the tuberculoma, which is typically larger than 2 mm in diameter. The patients in this case usually present ascites, splenomegaly, and lymphadenopathy (14). At ultrasound (US), detectable tuberculomas usually manifest as round, hypoechoic masses (15).

**Miliary Tuberculosis** This entity follows blood-borne dissemination of *M. tuberculosis*. The clinical presentation is varied, but the presence of multiple granulomas in the liver is characteristic. It is usually rapidly fatal: the first signs, such as general malaise and weight loss, are nonspecific, and the following course, if the disease is left untreated, is rapid. The US appearance consists of a homogeneously enlarged liver or a diffuse hyperechogenicity (15).

**Biliary Tuberculosis** This is observed in case of direct involvement of the biliary tree by the granulomas. Cholangitis, as a consequence of rupture of a caseating granuloma into the bile duct, is a very uncommon event. Clinical features may vary, and abdominal pain, general malaise, and obstructive jaundice can occur (10).

## BACTERIAL INFECTIONS OF THE LIVER

The liver is frequently involved during systemic and intestinal bacterial infections, thanks to the dual blood supply from the hepatic artery and the portal vein. The patient may present with signs of severe liver dysfunction, especially in case of immunodeficiency, or subclinically with mild biochemical or histological abnormalities, in the case of immunocompetent patients.

### BRUCELLOSIS

Brucellosis is a chronic granulomatous infection caused by several species of *Brucella*, a small Gram-negative coccobacillus. Brucellosis is the commonest zoonotic infection worldwide. Its epidemiology has drastically changed during the last few years because of sanitary and socioeconomic reasons, together with the evolution of international travel. Several areas, such as Latin America, have achieved control of the infection, but new foci of human brucellosis are emerging, particularly in central Asia (16). *Brucella* is now considered a monospecific genus, the *Brucella melitensis*; all the other species, such as *Brucella suis* and *Brucella abortus*, are subtypes (17). Humans are infected through direct contact with contaminated animal parts or indirectly through unpasteurized milk or dairy products.

**Table 1**  
**Bacterial Infections of the Liver**

<i>Organism</i>	<i>Immune response</i>	<i>Clinical features</i>	<i>Pattern of liver injury</i>
<i>Salmonella typhi</i>	Initial control of infection by the reticuloendothelial system, followed by adaptive immune response, based on release of TNF- $\alpha$ , IFN- $\gamma$ , IL-12, IL-18, and IL-15	Acute hepatitis with hepatomegaly, splenomegaly, jaundice, and fever with rigors	Nonspecific hepatitis, steatosis, minimal portal infiltration, and hepatocyte cloudy swelling
<i>Neisseria gonorrhoeae</i>	The immune response is mainly anticorporal, and inflammatory cytokines are poorly represented	Perihepatitis owing to gonorrhea (Fitz-Hugh-Curtis syndrome): fever, right upper quadrant pleuritic pain, and lower abdomen tenderness	Perihepatitis
<i>Francisella tularensis</i> (tularemia)	Initial control depends on IFN- $\gamma$ and TNF- $\alpha$ ; this response allows the specific immune response dominated by T cells	Hepatitis-like syndrome, elevation of aminotransferases, and rare hepatomegaly	Rare hepatic abscess and granulomas
<i>Yersinia pseudotuberculosis</i> , <i>Y. enterocolitica</i> , and <i>Y. pestis</i>	Innate immune response executed by macrophages, which recognize <i>Yersinia</i> cell envelope components through Toll-like receptor 4; <i>Yersinia</i> is able to survive by inducing apoptosis in the infected macrophages	Jaundice and hepatomegaly (in case of septicemia)	Multifocal liver abscesses and granulomas
<i>Treponema pallidum</i>	The innate response is activated by the treponemal lipoproteins, recognized by macrophages via Toll-like receptor 2; the consequent cells recruited are mainly Th1 type, producing IL-2, INF- $\gamma$ , and IL-12	Congenital: hepatomegaly, ascites, and portal hypertension Acquired: mild elevation of liver function tests or acute hepatitis with hepatomegaly and splenomegaly, rare jaundice Tertiary: jaundice (rare) and Budd-Chiari syndrome	Congenital: small epithelioid granulomas, and severe portal and interstitial fibrosis Acquired: granulomas, focal necrosis, cholestasis, pericholangiolar inflammation, and vasculitis Tertiary: bile duct obstruction owing to hepatic gummae

Abbreviations: IFN, interferon; IL, interleukin; TNF, tumor necrosis factor.

After entering the human body, brucellae are taken up by local tissue lymphocytes and consequently spread hematogenously throughout the body, thanks to a particular tropism for the reticuloendothelial system. The infection can localize in a variety of organs, including the liver, which is routinely affected (18).

**Immune Response** Brucellae are facultative intracellular pathogens. Although most brucellae are rapidly eliminated by phagolysosome fusion inside the macrophages, 15 to 30% survive within the cells of the reticuloendothelial system, where they persist and replicate for long time, in gradually evolving compartments. Brucellae reside inside the acidified phagosome, which also limits the antibiotic action (19). Cell-mediated immunity is crucial in limiting the infection. *Brucella* activates natural killer (NK) cells by release of IL-2 by macrophages. IFN- $\gamma$  is in turn released by NK cells, and it plays a central role in the pathogenesis of brucellosis, by activating other macrophages, by inducing apoptosis and cytokine production, and by increasing the expression of antigen-presenting

molecules (20,21). Antibody response plays a limited part in the overall host response.

**Pathology** The liver shows a granulomatous hepatitis with a marked inflammatory infiltrate and occasionally fibrosis. Noncaseating granulomas can develop, and frequently multiple microgranulomas can be scattered throughout the parenchyma. These lesions are typically composed of a small number of histiocytes expanding and producing compression atrophy of the surrounding hepatocytes. Less commonly, the infection can produce different type of abscesses: small multifocal abscesses are frequently observed, and a form of “pseudotumoral hepatic brucella caseous necrosis” or brucelloma is also described (22).

**Clinical Manifestations** Brucellosis has an insidious onset, characterized by recurrent fever with headache, weakness, night sweats, backache, and joint pain. Hepatomegaly, splenomegaly, and lymphadenopathy are often present. Jaundice is rare. These symptoms and signs are secondary to a granulomatous or nonspecific hepatitis (18–22). Ascites may be observed, either as a temporary exacerbation of preexisting

hepatic disease or as a frank peritonitis (23). Blood tests may reveal mild leukopenia and relative lymphocytosis, along with mild anemia and thrombocytopenia, mainly attributable to hypersplenism and bone marrow involvement, with mild increases in transaminases and alkaline phosphatase. In the case of hepatic abscess, US shows an iso- or hypoechoic lesion, containing some hyperanechoic areas and calcifications (24,25).

### PYOGENIC LIVER ABSCESS

The epidemiology of pyogenic abscess has significantly changed owing to the increasingly invasive management of biliary and pancreatic disease (26). The incidence rate in the Western countries is reported to be 7 to 22 per 100,000 hospital admissions (27). Pyogenic abscesses, especially when multiple, may be caused by hematogenous dissemination (from gastrointestinal sources, such as diverticulitis, appendicitis, colonic cancer, or adenoma), ascending cholangitis, or superinfection of necrotic tissue. However, the most common present cause of hepatic abscesses is malignant biliary obstruction. Diabetes mellitus is one of the most common associated diseases. This entity is a potentially life-threatening disease, with significant mortality ranging from 6.5 to 40% previously reported in literature (28). More than 50% of liver abscesses are polymicrobial, but *Escherichia coli* is the most common bacterium.

**Immune Response** Abscess development is a host defence strategy to contain the spread of infection, but it is also responsible for the clinical manifestations. When the bacteria arrive in the liver, the first immune response is determined by phagocytes, such as macrophages and polymorphonuclear leukocytes. These cells are attracted by many components of the bacterial cell walls, which create a chemoattractant gradient, followed by the phagocytes. These cells are responsible for the release of proinflammatory cytokines, such as IL-1, IL-6, and TNF- $\alpha$ , which increase the immune response leading to local control of the infection (29).

**Pathology** Pyogenic abscesses at macroscopic examination are solitary or multiple lesions, with a diameter ranging from millimeters to centimeters. At histopathological analysis, the cavity may reveal the presence of multiple locules, usually filled with dense, purulent material and lined by fibrous tissue. The fibrous capsule is typically very thick and could extend inside the surrounding liver parenchyma. The edges of the lesions are composed of epithelioid macrophages, lymphocytes, eosinophils, and neutrophils (30).

**Clinical Manifestations** The clinical features of pyogenic liver abscess are not specific; they include fever, abdominal pain, typically localized in the upper right quadrant, and vomiting. The abscesses may be clinically occult ("cold"), manifesting only as weight loss and vague abdominal pain. Hepatic biochemical abnormalities are non-specific, including slightly elevated bilirubin and transaminases, together with hypoalbuminemia and leukocytosis (31,32). At US, pyogenic abscesses may manifest as discrete hypoechoic nodules or undefined areas of altered hepatic echogenicity, mainly located in the right lobe. In the case of large abscesses, the US appearance could vary from hypo- to hyperechoic with various

internal echoes and debris. Gas may be evidenced within the lesion (33).

### PARASITIC INFECTIONS OF THE LIVER

Liver parasites span a wide range of complexity, and different species mature and reproduce within hepatocytes, reticulo-endothelial cells, the portal venous system, and the bile ducts. Well-adapted parasites cause minimal acute injury to the host organ as they generate enormous numbers of progeny that pass into the blood or bile with the potential to infect other hosts, but when a parasite enters a poorly adapted species or organ, acute or severe injury could happen. Successful parasites have evolved to accommodate the defences and immunologic responses of normal hosts; hosts with abnormal or compromised responses are at risk of severe disease manifestations.

#### MALARIA

Malaria is the most important parasitic infection in humans, with an estimated 500 million people affected each year worldwide and a total of approx 2 million deaths, mostly children, each year (34). It is caused in humans by intracellular protozoa of 4 species (*Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale*, and *Plasmodium malariae*), but *P. falciparum* causes most infections and is responsible for the most severe disease. All are transmitted by mosquito bite, the female *Anopheles*, and all involve the uptake of the sporozoites, the invasive form of the mosquito, by hepatocytes (35). Malaria sporozoites actively cross the sinusoidal cell layer and pass through Kupffer cells prior to hepatocyte invasion. The liver is the site of this initial preerythrocytic cycle, during which the sporozoite undergoes schizogony to form a schizont, which divides to produce a large number of merozoites. The process of schizogony happens in the liver without involving or hampering its function. Merozoites are released by rupture of hepatocytes into circulation, where they invade erythrocytes. *P. falciparum* and *P. malariae* are not associated with any residual liver stage after release of merozoites, whereas *P. vivax* and *P. ovale* are associated with a persistent exoerythrocytic stage, the hypnozoite, which persists in the liver and eventually matures into schizonts. Some of the released cells develop into gametocytes, which are ingested again by mosquitos during bites, allowing resumption of the cycle.

**Immune Response** Following repeated infections, the gradual acquisition of mechanisms that limit the inflammatory response to the parasite is observed together with development of the antibody repertoire. Infection with a parasite variant that is not recognized by the exiting antibodies or infection in children who have not yet developed a fully protective immune system brings a greater risk of developing severe disease and death; conversely, humans with intact host defences usually recover from acute episodes of malaria. The hypothesis is that the parasite is able to inhibit the innate and/or the adaptive inflammatory cytokine response. *P. falciparum* would act by abrogating IL-12 secretion (responsible for NK cell activation), switching to IL-10 production, with a subsequent reduction in T-cell proliferative response. The failure of NK cells to produce a strong response, including an adequate release of



INF- $\gamma$  and TNF- $\alpha$ , could correlate with a worse parasite replication containment (36–38). In vitro and in vivo studies have implicated antibodies, CD8<sup>+</sup> and CD4<sup>+</sup> T cells, cytokines (TNF- $\alpha$ , INF- $\gamma$ , and IL-12), and nitric oxide (NO) as critical effectors in protection against hepatic malaria, but the whole mechanism has not been fully elucidated (39,40).

**Pathology** During the erythrocytic stage of the infection, Kupffer cells take up released hemoglobin degradation products, known as malarial pigment (hemozoin), which appears as dark cytoplasmic granules in liver specimens. Histopathological examination of the liver shows evidence of a wide spectrum of changes: swollen hepatocytes, inflammatory portal infiltrates with lymphocytes, parasitized red blood cells, and steatosis. Centrizonal necrosis has been reported with a different prevalence, being described as characteristic of malarial hepatitis (41) or rarely associated (42). A more recent paper described centrizonal necrosis in 25% of *P. falciparum* malaria cases with jaundice (43). Cholestasis is rarely described.

**Clinical Manifestations** The liver is affected during malaria infection in different degrees, and the malarial hepatopathy is a heterogeneous syndrome with at least two different clinical patterns: the patients categorized as group A present a fulminant clinical illness, acute renal failure, purpura, asterixis, or impaired sensorium. Group B is characterized by fever, headache, vomiting, and only a modest elevation of conjugated bilirubin, aspartate aminotransferase, or alkaline phosphatase; this variant is quite common during *P. falciparum* infection (up to 60% of cases) (44). The term “malarial hepatitis” has often been used to describe hepatocellular jaundice in patients with malarial infection, but the clinical significance of this entity has not been completely elucidated. However, the diagnosis of this disease could be based on the following criteria (45): (1) demonstration of *P. falciparum* infection; (2) a threefold rise in ALT, with or without conjugated hyperbilirubinemia; (3) absence of clinical serological evidence of drug or viral hepatitis; and (4) clinical response to antimalarial drugs or autopsy evidence of disseminated falciparum infection. Clinically, the patients exhibit hepatomegaly and splenomegaly, and jaundice could be present. The incidence of jaundice in malaria is reported as widely variable, from 3% (46) of cases up to 62% (47).

### SCHISTOSOMIASIS

Schistosomiasis is a trematode infection affecting more than 200 million persons worldwide: 120 million of them have symptoms, and 20 million have severe illness. Five species of schistosome are known to infect humans, but *Schistosoma mansoni*, *Schistosoma japonicum*, *Schistosoma mekongi*, or *Schistosoma intercalatum* are the species associated with chronic hepatitis and intestinal fibrosis. The free-swimming larval forms of the parasite, known as cercariae, enter the body by penetration of the skin and transform into immature worms. Larvae migrate first to the lungs through the venous circulation; then they reach the left heart and consequently the systemic circulation. After several days, the worms migrate to the portal venous system; sexual reproduction occurs in the portal vein

where adult worms reside and eggs are laid. Eggs production starts 4 to 6 wk after the infection and continues for the whole life of the worm (up to 5 yr). Eggs pass from blood vessels into tissues, including intestinal or bladder mucosa, from where they are shed in the feces or urine (48).

Hepatic schistosomiasis occurs when the eggs are not excreted but are trapped by the portal venules corresponding to the egg size, about 50  $\mu$ m, and the disease results from the host's immune response to the eggs themselves.

**Immune Response** The eggs in the liver remain viable for about 3 wk and determine a first immune response that is primarily Th1 in type, with increased production of INF- $\gamma$ , NO, and TNF- $\alpha$  and the recruitment of eosinophils and granuloma formation (49). In particular, the balance between Th1- and Th2-type cytokines influence the extent of the pathology and the development of the fibrosis. As the granulomas enlarge, there is a preferential development of the Th2 response: granulomas that surround schistosome eggs in the liver are dependent on CD4 cells largely of the Th2 phenotype (50). The Th2-type response is probably determined partly by an initial innate immune response. It had been shown, in fact, that soluble egg molecules react with Toll-like receptors, activating dendritic cells and, ultimately, Th2-type responses (51). The intensity and duration of infection determine the amount of antigen released and the severity of the chronic reaction: most granulomas grow at the site of maximal egg concentration (liver, intestine, and genitourinary tract).

**Pathology** The final result of hepatic schistosomiasis with heavy parasitic infection is severe portal fibrosis and greatly enlarged fibrotic portal tracts, resembling clay pipestems thrust through the liver (termed Symmers' pipestem fibrosis) (52). Normal liver architecture is preserved, lobular architecture is retained, and nodular regenerative hyperplasia is not observed. This fibrosis is reversible, at least in part. In an animal model of schistosomal hepatic fibrosis, the liver tissue response after *S. japonicum* was evaluated at different time points after infection, showing that the degree of hepatic fibrosis was correlated with the density of eggs and granulomas in the liver tissue, but lesions regressed spontaneously, even in the higher dose infected group, as the pigs underwent a self-cure (53).

In a well-studied murine model of hepatic schistosomiasis, collagenolysis predominated over continuous collagen synthesis and deposition after the cure of infection and cessation of egg deposition. It is not clear to what extent this phenomenon is involved in human fibrosis (54).

The association between hepatitis B virus (HBV) chronic hepatitis and *S. mansoni* infection was found to increase the risk of hepatocellular carcinoma in an Egyptian study (55).

**Clinical Manifestations** Up to 1 wk after skin penetration by the cercarial form of the parasite, a maculopapular eruption may arise at that site. A potentially fatal acute illness, Katayama fever, is a form of acute schistosomiasis, common in areas of high transmission rate. It is a serum sickness-like syndrome triggered by the onset of deposition of an egg into host tissues. Clinical features of this entity are not specific and include respiratory and/or abdominal symptoms (right upper quadrant

pain and bloody diarrhea) together with fever, headache, and myalgias. Tender hepatomegaly and splenomegaly could be present (56). Advanced hepatic schistosomiasis is characterized by signs and symptoms related to the portal fibrosis and to the presinusoidal portal hypertension. Patients can present with esophageal and gastric variceal bleeding and important splenomegaly. Hepatocellular synthetic function is usually preserved until the last stage of the disease, and patients have normal or nearly normal liver function tests for a long course. When present, laboratory evidence may include blood eosinophilia, anemia, hypoalbuminemia, increased urea and creatinine, and hypergammaglobulinemia. In addition, important reductions in erythrocytes, leukocytes, and platelets could be present owing to splenic sequestration.

Symptomatic splenomegaly may persist after infection resolution, and splenectomy is very common in endemic areas (57). US findings that characterize chronic schistosomiasis owing to *S. mansoni* consist of wall thickening of the portal vein, determining the typical "bull's-eye" appearance, which represents an anechoic portal vein surrounded by an echogenic mantle of fibrous tissue (58). Growth retardation and late development is specifically associated with schistosomiasis in heavily infected children. Coinfection with viral hepatitis, either HBV or HCV, is also possible considering that the regions with a high prevalence of schistosomiasis usually have a high endemicity of chronic viral hepatitis. The association between the two infections determines faster deterioration of the liver, and severe illness is very common. Most people hospitalized for severe bleeding, ascites, or decompensated liver failure have both schistosomiasis and chronic viral hepatitis (59–61).

### AMEBIASIS

*E. histolytica* is considered the second or third leading cause of death among the parasitic diseases, with an estimated 40,000 to 100,000 people dying yearly from amebiasis (61). It is distributed throughout the world, in almost all countries where the barriers between human feces and food or water are insufficient: Africa, Central and South America, and India have the highest morbidity and mortality (62). Two genetically distinct species of *Entamoeba* are described: the commensal, *Entamoeba dispar*, and the pathogen, *E. histolytica* (63).

A great number of patients infected with *E. dispar* or some strains of *E. histolytica*, which remain in the luminal surface of the bowel, are asymptomatic. Amebiasis in its invasive form is responsible for amoebic colitis, which involves only a relatively small proportion of infected individuals. Once through the bowel wall, trophozoites invade the portal circulation and disseminate systemically, reaching the liver to cause hepatic amebiasis and its distinctive lesion, the amoebic abscess.

**Immune Response** Intestinal invasion depends on parasite and host factors: first, the parasite needs to have a specific genetic (64,65) and immunoenzymatic profile, making it capable of causing alterations in intestinal permeability (66) and has to secrete a specific proteinase pattern (67), induce apoptosis (68), and resist complement-mediated lysis. It is not clear whether

protective immunity to amebiasis exists: indirect evidence suggests that cellular immunity is an important factor in protection against *E. histolytica*. Splenectomy (69) or the use of steroids (70) accelerates liver abscess formation. In animal models of amoebic abscess, an acute inflammatory reaction, dominated by neutrophils, is observed in the early stages at the edge of the lesion. Neutrophils release mediators that cause hepatocyte death and extend the damage to distant cells; as *E. histolytica* can kill cells without direct contact, most hepatocytes die from apoptosis (71). The increasing numbers of lesions will coalesce to form a larger lesion, the abscess itself.

**Pathology** Histological features of amoebic liver abscesses include a scant inflammatory reaction at the edge and a rim of connective tissue, which surrounds a well-circumscribed region. The content of the central cavity is a thick exudate, containing liquefied cells, and cellular debris; it can be creamy and white in color or dirty brown and pasty, known as "anchovy paste." This material is nearly sterile, and the amoeba is rarely found in the cavity itself; the abscesses can become purulent in case of a secondary bacterial infection. The adjacent liver parenchyma is often completely unaffected (72).

**Clinical Manifestations** In cases of amoebic colitis, patients develop bloody diarrhea and abdominal pain. These symptoms could last several weeks; fever is uncommon (less than 40% of patients), but weight loss and anorexia can be observed. Some days or months after the onset of diarrhea, or even without a history of intestinal amebiasis, the clinical manifestations of hepatic abscess can appear. The abscesses mainly affect 18- to 50-yr-old men (73). The hepatic lesion is usually solitary and frequently at the right lobe, close to the capsule. Consequently, the typical physical sign is hepatomegaly accompanied by symptoms such as fever, right upper quadrant pain, and hepatic tenderness. The pain may radiate to the shoulder or to the right side of the neck. In the unusual case of an abscess of the left lobe, the patient suffers epigastric pain, radiating to the left back (71). If the abscess compresses the diaphragm, cough and dyspnea may be present, with dullness and rales in the right lung base (74). In case of ruptured abscess in the peritoneum, abdominal pain with guarding and rigidity is observed (75). Jaundice is very uncommon, with a reported prevalence of 5% of cases. However, if present, jaundice is associated with a worse prognosis. Laboratory findings include moderate leukocytosis, without eosinophilia, mild anemia, either normochromic or hypochromic, and increased levels of alkaline phosphatase and erythrocyte sedimentation. In patients with multiple abscesses, the leukocytosis may be severe, with a prevalent neutrophilic component. At US, an amoebic abscess is typically located near the liver capsule; it appears as oval or round, and it is hypoechoic, with low-level internal echoes and no relevant wall echoes. The central abscess cavity may show multiple septa and sometimes air bubbles (76).

### LEISHMANIASIS

Visceral leishmaniasis, or kala-azar, is a potentially fatal vector-borne disease, caused by the infection of the reticulo-endothelial cells of the liver, spleen, bone marrow, and other

organs, such as the dermis and nasooropharyngeal mucosa, by an intracellular protozoal parasite, *Leishmania*. In the Indian subcontinent, where nearly half of the new symptomatic world's cases are observed, the incidence is 250,000 new cases per year (78). A total of about 21 species of *Leishmania*, which are transmitted by different species of phlebotomine sandflies, can cause visceral, cutaneous, and mucocutaneous pattern: the visceral variant usually involves the liver, and it is determined by *Leishmania donovani* (79). Human beings are incidental hosts of infection, and other mammals (such as rodents and canids) are reservoir hosts. Besides infection from the bite of sandflies in endemic areas, *Leishmania* can be transmitted by blood transfusion, shared needles, sexual contact, or transplantation of infected organs (80).

**Immune Response** The fundamental principle of the immunoregulation of leishmaniasis is that the parasite, which replicates in the quiescent macrophages, is killed by activated macrophages and that the outcome of the disease is conditioned by the nature and effectiveness of the T-cell and cytokine responses (mainly IFN, IL-2, and IL-12), early in infection (79,81). It seems clear that the pattern of the initial innate immune response in the initial phase of infection is determinant for switching to the Th1 or Th2 response (82). The Th1 response would be responsible for INF- $\gamma$  production and parasite resistance, whereas the Th2 reaction and the secretion of IL-4 would confer susceptibility. If the cell-mediated immune system or other defence mechanisms are defective (i.e., in case of malnutrition or HIV), full clinical expression or reactivation can occur (83,84).

**Pathology** The pathological findings correlate with the predominant host response: in case of minimal disease and few parasites visible in liver specimens, epithelioid granulomas may be present. The granulomatous inflammation represents a specialized tissue mechanism of host defence, circumscribing the infected macrophages within a limited area and inducing a potent antimicrobial activity. The complete elimination of the parasite seems a rare event, whereas more often parasite quiescence is observed. In case of ineffective immune response, overt disease is observed, accompanied by numerous parasites multiplying within activated Kupffer cells and macrophages, the appearance of myofibroblasts, the deposition of intralobular collagen, and effacement of the space of Disse with connective tissue (85). Visceral leishmaniasis is also associated with severe intralobular fibrosis, which appears to be fully reversible after treatment (86).

**Clinical Manifestations** Infection remains asymptomatic or subclinical in many cases, or it can follow an acute, sub-acute, or chronic course. When symptomatic, the disease becomes life threatening after an incubation period of weeks to months. The major clinical manifestations of visceral leishmaniasis include fever, severe cachexia, hepatomegaly, splenomegaly, lymphadenopathy, pancytopenia (anemia, thrombocytopenia, and leukopenia with neutropenia, marked eosinopenia, and a relative lymphocytosis and monocytosis), hypergammaglobulinemia (mainly the IgG form with polyclonal B-cell activation), and hypoalbuminemia. All organs with reticuloendothelial cells may be involved, including the entire gastrointestinal tract. Although pronounced liver

fibrosis may be common, ascites is a rare finding. When signs and symptoms of leishmaniasis become clinically evident, treatment is mandatory, as the disease could be rapidly fatal: most patients experience an improvement of fever during the first week of treatment, but hepatomegaly, splenomegaly, and pancytopenia usually do not resolve until weeks or, sometimes months, after treatment (79,87). The best indicator of treatment success is represented by the freedom from clinical relapse for at least 6 mo (88).

## ECHINOCOCCOSIS

Echinococcosis, or hydatid disease, is an endemic infection in many countries, including the Middle East, the areas bordering the Mediterranean Sea, South Africa, Northern Canada, Australia, and New Zealand. However, with immigration and widespread traveling, it can also be observed in many other countries. Mortality rates associated with hydatid disease are low, but the morbidity is relevant, especially in relation to the common requirement of multiple surgical interventions (89). Echinococcosis results in humans when they become accidental hosts for a cystic intermediate stage of one of the two major species of canine tapeworms belonging to the genus *Echinococcus*. These two main species (*Echinococcus granulosus* and *Echinococcus multilocularis*) are of primary medical and public health importance; two other species (*Echinococcus vogeli* and *Echinococcus oligathrus*) have been rarely described in humans. *E. granulosus* is responsible for cystic echinococcosis, whereas the *E. multilocularis* is the cause of the alveolar form, which is relatively uncommon. Humans become infected by ingestion of eggs of the tapeworm, either by eating food contaminated with eggs excreted by domestic (often sheep-herding) or wild dogs, or other canines (wolves or foxes). The ingested embryos invade the intestinal mucosa and proceed up to the liver, through the portal venous system (90).

**Immune Response** A combined Th1 and Th2 cytokine profile appears crucial for prolonged parasitic growth and survival. It may be hypothesized that Th1 cytokines promote the initial cell recruitment around the parasite vesicles, inducing a chronic cell infiltrate and the formation of the organized periparasitic granuloma, fibrosis, and necrosis. The Th2 cytokines, and most of all IL-10, with its anti-inflammatory action, if prevalent, could be responsible for the ineffective immune response (91). On the other hand, parasites may avoid the immune system of the host by their low immunogenicity, by interfering with the mechanisms of antigen presentation, and by inhibiting T cells or macrophages (92). Antibody production is often impressive and is used for the diagnosis, but it does not correlate with protection against the parasite.

**Pathology** At histopathological analysis, a hydatid cyst is a fluid-filled structure delimited by three layers: the outer pericyst, which corresponds to the compressed and fibrosed liver tissue, derived by the chronic immune response of the host; the endocyst, made up of a varying number of concentric layers of hyaline placed on top of each other; and the germinative layer, which covers the inside of the cyst and consists of a monolayer of viable pluripotent cells.

The cysts formed in *E. multilocularis* infection are less well limited, since there are no sharp limits between the parasitic tissue and the liver parenchyma: alveolar echinococcosis is characterized by a multivesicular structure surrounded by an extensive fibroinflammatory host reaction. The lesion behaves like a slow-growing cancer, with frequent invasion of biliary and vascular walls (93). The poor vascularization of the parasitic mass often leads to necrosis in the central part of the lesion. Liver abscess owing to superimposed bacterial infection of the necrotic area may occur in this disease.

**Clinical Manifestations** During cystic echinococcosis, the initial phase of primary infection is always asymptomatic, and it may remain asymptomatic for many years. Most hydatid cysts come to clinical attention because of their enlargement with a consequent mass effect or because of their rupture. The primary organ affected is the liver (70% of patients), mainly the right lobe. About 90% of cysts are limited to the liver, lung, or both; however, ectopic cysts (2–3% of cases) in the kidney, spleen, brain, heart, and bone may produce unusual findings. Common complications include rupture into the biliary tree with secondary cholangitis, biliary obstruction or extrinsic compression, subphrenic abscess formation, and intraperitoneal rupture, with eventual anaphylaxis (94).

Alveolar echinococcosis typically presents later than the cystic form, as it could have an incubation period of 15 yr. If untreated, the alveolar form could be fatal. More than 30% of cases become clinically evident, with cholestatic jaundice, epigastric pain, anorexia, and fatigue, and patients have hepatomegaly. Extrahepatic primary disease is described in only 1% of cases.

Laboratory tests are usually characterized by eosinophilia in case of complicated cysts, whereas routine laboratory findings are not of diagnostic relevance (95).

US findings are variable and range from purely cystic to solid-appearing pseudotumors. Wavy bands of delaminated endocyst may be noted internally. Cyst wall calcifications, from tiny to massive, are often described peripherally, together with compression and fibrous reaction of the surrounding liver parenchyma (96).

## CONCLUDING REMARKS

The function of the immune system is to defend the body from external agents, including bacteria and parasites. Central to this function is the ability to react and to kill the pathogens. Many cells take part in the immune response, including macrophages and neutrophil granulocytes (innate response) and cytotoxic T cells and T-helper cells (adaptive response). Tissue injury determined by pathogens is one of main sources of information that launches inflammation, which in turn launches immunity. Injured host cells release alarm signals that activate antigen-presenting cells; in addition, “microbial nonself cells” induce an innate immune response, which in turn triggers an adaptive immune response. The immune reaction itself, both acute and chronic, is mainly responsible for the clinical features of the infection, but many infections are clinically silent, reflecting the ability of adaptive immune mechanisms to prevent disease.

In the nonimmune-compromised individuals, infections are more clinically overt and can become severe or life threatening. Overall patterns of disease are strongly influenced by the previous immunological experiences of the host. Understanding the circuits that confer and control the immune response holds important therapeutic promise.

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# 13 Immune Response to Hepatitis A and E Viruses

## *Role in Disease Pathogenesis and Viral Elimination*

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JOHANNES HADEM AND MICHAEL P. MANNS

### KEY POINTS

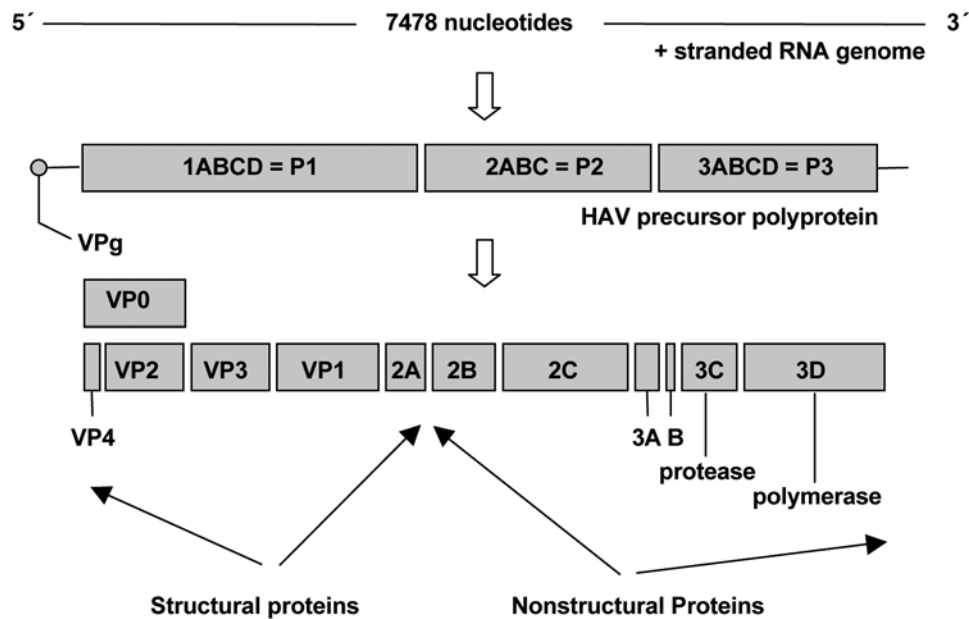
- Hepatitis A virus (HAV) is a small, plus-strand RNA virus in the hepatovirus genus of the picornavirus family and is the most common defined cause of viral hepatitis worldwide. Following receptor-mediated entry into the cytoplasm of the hepatocyte, the HAV genome is transcribed into a 250-kDa polyprotein, whose cleavage products involve structural and nonstructural proteins.
- HAV infection is usually acquired via the fecal-oral route and is associated only with acute (self-limiting) forms of viral hepatitis. An age over 40 yr and the presence of pre-existing liver disease define risk factors that predispose individuals to a symptomatic (icteric), and potentially fatal course of disease.
- Acute hepatitis A is diagnosed by detection of anti-HAV-IgM. These antibodies persist for about 6 mo and are probably of heterogenous antigenic specificity. A number of major antigenic domains have been demonstrated on structural and to a lesser extent also on nonstructural proteins. Some of them are likely to be discontinuous in nature and arise during the assembly of the viral capsid.
- Liver injury during acute HAV infection is probably not a direct cytopathic effect of the virus but mediated by HLA-restricted T lymphocytes during an immunopathologic response to antigens expressed within hepatocytes.
- Possible host mechanisms to clear the virus may include: (1) recruitment of cytotoxic T cells/NK cells from the periphery to the liver, (2) HLA-restricted killing of virus-specific CD8+ T lymphocytes (CTL), and (3) secretion of interferon ( $\gamma$ ) by CTL, which may facilitate chemotaxis and have direct antiviral properties.
- Worldwide, the incidence of acute hepatitis A is decreasing, and the prevalence of preexisting immunity among adults is declining in parallel.
- Monovalent and combination vaccines are presently available to prevent hepatitis A. They contain formalin-inactivated viral particles. All currently licenced vaccines

have a high protective efficacy and proven safety when administered to children 2 yr or older or to adults, with low rates of adverse events.

- According to current CDC recommendations, vaccination should be administered to all children at 1 yr of age, and members of certain risk groups (i.e., men having sex with men, travellers, illegal drug users, and patients with preexisting liver disease, who have an increased risk of developing a fatal course of disease when superinfected by HAV).
- Hepatitis E virus (HEV) is a plus strand RNA virus of approx 7.5 kb. Four genotypes have been recognized so far. Genotypes 3 and 4 appear to circulate in animals. HEV causes epidemics in regions with poor sanitary conditions.
- Acute hepatitis E is diagnosed by detection of anti-HEV-IgM or fecal HEV-RNA. Not much is known about immune responses in acute hepatitis E.
- A recombinant HEV vaccine has recently been shown to prevent clinical hepatitis E in male Nepalese volunteers.

### INTRODUCTION

Hepatitis A virus (HAV) is a nonenveloped small RNA virus in the hepatovirus genus of the picornavirus family (1) and is the most common defined cause of viral hepatitis worldwide. In the United States, the number of notified cases annually is around 23,000, but estimates of the real number of cases of clinical disease range up to 75,000 per year. The infection is usually transmitted via a fecal-oral route and is associated only with acute forms of viral hepatitis. Much higher virus titers are found in bile and in stool than in blood. Whereas infection in children and the very young is most often unrecognized, most infections in adults are symptomatic and associated with acute icteric hepatitis. Risk factors for a fulminant clinical course include an age greater than 40 yr and some forms of preexisting liver disease. As the incidence of HAV infection among children and adolescents has declined in many countries owing to improved socioeconomic status, these individuals are at increased risk of disease later in life because of the lower prevalence of immunity (2).



**Fig. 1.** Organization of the hepatitis A virus (HAV) genome, the HAV polyprotein, and its cleavage products. The 5' and 3' noncoding regions flank the open reading frame (ORF), which encodes for structural and nonstructural proteins. VPg, genome-linked protein.

Diagnosis is made on the medical history, clinical features, and a positive anti-HAV-IgM antibody. A number of changes in the humoral and cellular arm of the immune system have been reported during acute HAV infection. These changes are likely to be responsible for the pathological lesion in acute hepatitis A since HAV does not induce any visible cytopathic effects and probably does not interfere with the macromolecular synthesis of its host cell (3).

Hepatitis E virus (HEV) is a small, nonenveloped RNA virus that is presently classified into a separate genotype of hepatitis E-like viruses. In contrast to HAV, HEV is more restricted to tropical and subtropical developing countries (4). Originally identified as a principal cause of acute hepatitis in India and China (5), HEV is now commanding attention in regions of Sudan and Iraq where civil conflicts have led to unsanitary conditions (6). HEV is spread by fecally contaminated water in such areas but is not transmitted from person to person. High attack rates are found in adults between 15 and 40 yr of age. Although the mortality associated with HEV is similar to that of hepatitis A, a mortality rate of 20% has been reported for pregnant woman during outbreaks in developing countries (4). The pathogenesis of hepatitis E is poorly understood, but humoral and cellular immune responses play a major role (7). The diagnosis of acute hepatitis E is based on detection of HEV-IgM antibodies in serum or HEV-RNA in serum or feces (8).

This chapter reviews the available data on the immunopathogenesis, prophylaxis, and treatment of HAV and HEV infections, giving an overview on the virology, immune responses to viral antigens expressed by the infected hepatocyte and epithelium of the gastrointestinal tract, important clinical features

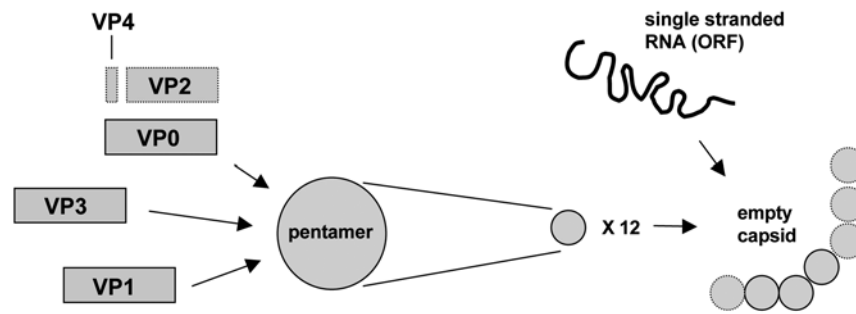
of the acute hepatitis caused by these viral pathogens, and recent advances in vaccination strategies.

## GENOMIC STRUCTURE AND REPLICATION CYCLE OF HAV

The identification of HAV dates back to 1973, when a virus-like antigen was discovered by immune electron microscopy as the probable causative pathogen of an acute form of infectious hepatitis (9). Formerly defined as enterovirus type 72, HAV is now classified as the only species of the genus *Hepatovirus* of the picornavirus family. It is an icosahedral, nonenveloped particle, 27 nm in diameter, with its RNA genome being single strand, positive sense, and approximately 7.5 kb in length (1). The genome organization includes a 5' nontranslated segment of approx 734 bases in length, followed by a single long open reading frame (ORF) encoding a polyprotein of approx 2227 amino acids, and a short 3' noncoding region that terminates in a 3' polyadenylic acid tract. A small, genome-linked protein (VPg) is covalently attached to the 5' end of virion RNA (Fig. 1).

The extended basilar surface of the hepatocyte is exposed to the space of Disse and through it to the venous sinusoids, via which HAV is likely to reach the liver during early stages of the infection (10). Attachment to the cellular receptor HAV-CR-1, a mucin-like glycoprotein, might facilitate viral entry into the cell (2). A recent study on the mechanisms underlying the hepatotropism of HAV demonstrated that HAV-specific immunoglobulin A (IgA) mediates infection of hepatocytes with HAV via the asialoglycoprotein receptor, which binds and internalizes IgA molecules. This was shown for mouse as well as human hepatocytes (11).





**Fig. 2.** Assembly of the HAV virion. VP1, VP3, and VP0 (and possibly its cleavage products VP2 and VP4) form pentamers, 12 of which are then united to build the empty capsid. Infectious HAV virions additionally contain genomic RNA and VP<sub>g</sub> (genome-linked protein). ORF, open reading frame.

As with other picornaviridae, the virus next penetrates the cellular membrane by endocytosis, followed by the release of the viral RNA (uncoating). Replication of the genome occurs in the cytoplasm of the infected cell, with synthesis of a complementary negative strand, which then serves as template for the positive strands. The process of transcription proceeds asymmetrically, with an excess of plus-strand molecules synthesized under direction of the virus-specified 3D pol RNA-dependent RNA polymerase (2).

The polyprotein encoded by the ORF has a molecular mass of about 250 kDa. Proteolytic cleavage of the viral polyprotein P1-P2-P3 is central in the viral life cycle and leads to liberation of the capsid proteins (VP0, VP3, VP1, or VP1-2A) from the P1 or P1-2A domain and of the nonstructural proteins from the P2 and P3 domains. It has been proposed that P1-2A is the functional precursor of the structural proteins (12). Possibly to enlarge the array of viral proteins, picornaviral polyprotein processing results in intermediate and mature products that apparently have distinct functions within the viral life cycle.

Common to all picornaviruses is the major proteinase 3C pro, which excises itself from the P3 domain of the polyprotein (13). It was shown that HAV-3C pro is able to liberate all structural and nonstructural proteins from the primary translation product (14). An additional proteinase, 2A pro, or an unusual nonenzymatic step, specifically catalyzes the liberation of the structural proteins' precursor. Polypeptide 3AB, known as a precursor of the genome-linked protein VP<sub>g</sub> in poliovirus, has been shown to interact with membranes in HAV, and proteins 2C and 2BC also have the potential to rearrange intracellular membranes. Other stable P3-processing intermediates have been detected, but their roles within the life cycle have not yet been directly assessed.

Efficient liberation of structural proteins from P1-2A seems to be necessary but not sufficient for productive HAV capsid formation, a step that is probably promoted by polypeptides flanking the proteinase 3C pro (13). Although the specific study of HAV assembly has been hampered by its slow growth and relatively low yield in tissue culture, HAV morphogenesis is thought to be similar to that of poliovirus, the prototype picornavirus. Poliovirus capsids are assembled from 12 subunits called

pentamers (15). These subunits contain five copies of a protomer that consists of one molecule of each of the capsid proteins 1AB (VP0), 1C (VP3), and 1D (VP1), with a fourth polypeptide possibly also being involved (16) (Fig. 2). HAV pentamers have a sedimentation coefficient of 14S; in addition, HAV 70S (empty capsid) and 135S RNA-containing particles have been described (17,18). Whether HAV-VP4 does participate in capsid formation is not clear. Considerable controversy has also surrounded the 2A segment of the HAV genome, which codes for the 2A protein and is necessary for RNA replication in poliovirus. In HAV, however, the nonstructural 2A protein segment is not required for RNA synthesis (19), but might play a role in capsid assembly (20). Although there is only one serotype of the hepatitis A virus, distinct genotypes have been described in human infections (21), with a nucleotide sequence variation ranging from 15 to 25%. However, all the human strains are very closely related antigenically. Even in comparison with HAV strains unique to nonhuman primate species, there is strict conservation of antigenic function despite substantial genetic divergence (22).

The viral assembly is followed by vesicular packaging of the viral particles and finally, the release of those vesicles at the apical surface of the hepatocyte. This part of the cellular membrane forms a well-demarcated groove that encircles the cell and provides access to the biliary canaliculi through which components of bile (including HAV during acute hepatitis A) are secreted from the liver into the feces (23). It is tempting to speculate that this vectorial secretion of progeny virus may involve either the normal vesicular cellular protein sorting system or perhaps specialized hepatocellular transporter proteins involved in secretion of biliary lipids and bile salts at the canalicular membrane (10,24).

## EVIDENCE FOR INFECTION OF THE GASTROINTESTINAL EPITHELIUM

The transmission of HAV is generally caused by the ingestion of material contaminated with feces containing HAV. However, the pathological sequence of events that begins with entry of the virus via the gastrointestinal tract and ultimately results in hepatitis is not well understood (10).

Resistance to acid pH and detergents accounts for the ability of HAV to transit through the stomach (20). Virus replicated in the hepatocyte is secreted across the apical canalicular surface of the hepatocyte into the bile, a process that may involve vesicular transport mechanisms (2). However, as relatively large amounts of virus are present in feces from 1 to 4 wk after exposure, a primary, extrahepatic site of replication for this highly hepatotropic agent has long been postulated. Early experiments involving immunohistological evaluation of intestinal tissue from infected nonhuman primates provided no evidence for the presence of virus within the gastrointestinal mucosa (10). However, more recent data demonstrated the presence of specific HAV antigen within the cytoplasm of epithelial cells from the small intestine of tamarins and New World owl monkeys (25).

Recent studies suggest that the infection of polarized cultures of Caco-2 cells with hepatitis A virus results in an extensive release of progeny virions through apical cellular membranes (10). Caco-2 cells most closely resemble epithelial cells of the small intestinal villi and crypts. The uptake of HAV was at least 30- to 40-fold more efficient via the apical surface, which could imply a greater abundance of the HAV receptor in this area. Similarly, release of progeny HAV virions occurred almost exclusively via the apical cellular membrane via a mechanism not dependent on cellular lysis. This release of newly replicated virus would result in an increase in the amount of virus present within the lumen of the gastrointestinal tract and an amplification of the inoculum (10). Viral antigen may be detected in the feces as late as 2 wk after the onset of symptoms, and viral RNA can be detected in feces by reverse-transcription polymerase chain reaction (PCR) for up to 2 mo after the peak elevation of enzymes (2). However, the infectivity of feces is dramatically reduced following resolution of the acute liver injury, and long-term fecal shedding of infectious virus has not been documented.

As shown for Caco-2 cells, the infection of intestinal epithelial cells is unlikely to play a role as the primary infection site for HAV in respect to the restricted basolateral release of viral particles (10). Instead, transcytosis by specialized M cells overlying Peyer's patches in the distal ileum, which is a mechanism of poliovirus entry into the organism, might be relevant. There is a significant viremia that parallels fecal shedding of HAV and typically persists for several weeks during the prodromal and early clinical phase of the illness. This viremia is likely to be the source of virus spread among illicit drug users of injection drugs and has led to contamination of some lots of high-purity, solvent-detergent inactivated clotting factors (2).

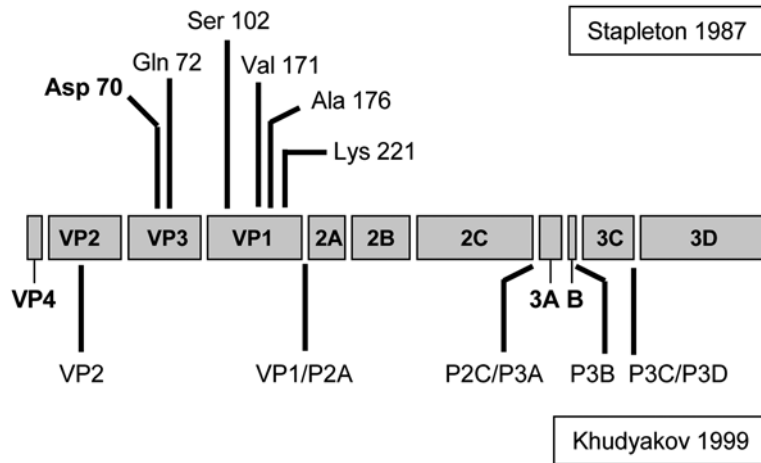
### ANTIGENIC EPITOPES OF THE HEPATITIS A VIRUS POLYPROTEIN AND ITS CLEAVAGE PRODUCTS

HAV contains a single-stranded, plus-sense RNA genome with a single long ORF encoding the HAV polyprotein (rV-ORF) with a molecular weight of about 250 kDa. Structural and non-structural proteins are generated by posttranslational proteolytic processing. Sucrose density gradients of rV-ORF-infected cell lysates contain peaks of HAV antigen with sedimentation

coefficients of about 15S (pentamers) and 70S (empty capsids), suggesting that major epitopes are located on structural proteins of HAV. Studies with monoclonal antibodies could demonstrate several antigenic epitopes within an immunodominant neutralization antigenic site on 14S subunits. In contrast, other epitopes within this site were formed upon assembly of 14S subunits into capsids. Thus, these epitopes were probably built either by a conformational change in the antigenic site or by the juxtaposition of epitope fragments present on different 14S subunits during assembly of 14S into 70S particles (15). This view is supported by observations that polyclonal or monoclonal antibodies obtained against native HAV demonstrate only marginal reactivity with denatured capsid proteins. Similarly, antibodies raised to purified capsid proteins did not neutralize HAV efficiently (26).

X-ray crystallographic determinations of virus structures have contributed substantially to our current understanding of the structural organization and function of picornaviruses. However, in the case of HAV, the production of quantities of purified virus sufficient for crystallographic studies represents a daunting task (27,28). An important approach toward mapping the HAV neutralizing epitopes was to identify mutations within the HAV capsid proteins that result in resistance to neutralization with monoclonal antibodies (28) (Fig. 3). In one study, neutralization escape mutants selected from a rapidly replicating HM175 strain of HAV were identified at the Asp-70 and Gln-72 residues of the capsid protein VP3, as well as at Ser-102, Val-171, Ala-176, and Lys-221 of VP1. The data support the existence of an immunodominant neutralization site involving residues of VP3 and VP1 and a second, potentially independent site involving residue 221 of VP1. As some of the monoclonal antibodies compete effectively with polyclonal human postreconvalescent antibody for attachment to the virus, it is likely that the immunodominance of the epitopes recognized also extends to humans (29). Others have suggested a continuous epitope at amino acid residues 110 to 121 (VP3) (30) and found neutralization escape mutations at residues Pro-65, Asp-70, Ser71 (VP3), Asn-104, Lys-105, Gln-232 (VP1) for the HAS 15 strain of HAV (31). Since most escape mutants demonstrate a change at amino acid residue Asp-70 (VP3), this residue is likely to be of primary importance for antibody binding. It has been suggested that two sites on VP1 interact with a single VP3 site to form the immunodominant epitope, although the antigenic sites on these capsid proteins might be located too far from each other to fit under a single immunoglobulin footprint (31,32). Interestingly, the highly restricted number of residues identified as sites of mutation (key amino acid residues) could reflect quite stringent structural constraints imposed by the need to retain biological activity of the capsid (27). Although it is likely that the sites of mutation detected are located within the antigenic region, this is not necessarily the case, as neutralization resistance can be conferred by amino acid substitutions outside a neutralization epitope.

If the B-cell epitopes of HAV are linear protein epitopes formed directly from the primary amino acid sequence, then



**Fig. 3.** Antigenic reactivity of different domains of the HAV polyprotein. Stapleton et al. (29) suggested an immunodominant neutralization site by examining the development of neutralization escape mutants detected by monoclonal antibodies and involving VP1 and VP3. Khudyakov et al. (28) tested synthetic peptides spanning the HAV polyprotein using human serum from acutely infected individuals. Interestingly, significant antigen recognition was found on nonstructural proteins.

binding to synthetic individual overlapping peptides should identify them. Unfortunately, most epitopes on globular proteins recognized by antibody are discontinuous, and this makes characterization rather demanding, since one cannot predict which residues are likely to be brought together in space to form the epitope. Accordingly, the search for immunoreactive HAV peptides has been frustrating so far. Data describing a VP1 peptide that induced anti-HAV neutralizing antibodies in an animal model could not be reproduced by others (33).

The difficulties in modeling HAV peptides imposed by the conformational nature of the HAV capsid antigenic sites led to the application of synthetic peptide combinatorial libraries. The strategy is to screen a large library of (chemically) synthesized (hexa)peptides capable of mimicking the main antigenic structure of HAV with a defined monoclonal antibody. By using the divide-couple-recombine approach, a recent study was able to identify a peptide that reacted specifically with monoclonal and polyclonal anti-HAV antibodies and, in mice, induced a specific antiviral immune response. Furthermore, the peptide could also be used in an enzyme-linked immunosorbent assay (ELISA) for revealing a primary immunoglobulin M immune response in sera of acutely infected human patients. However, no sequence homology was found between the identified peptide and the HAV capsid proteins VP1 and VP3. Although it seems possible that the identified peptide behaves as mimotope (i.e., a small linear amino acid sequence that contributes to a discontinuous epitope), the structural relationship to the HAV epitope must remain unclear in the absence of elucidation of the X-ray crystallographic structure of the antibody-antigen complex (34).

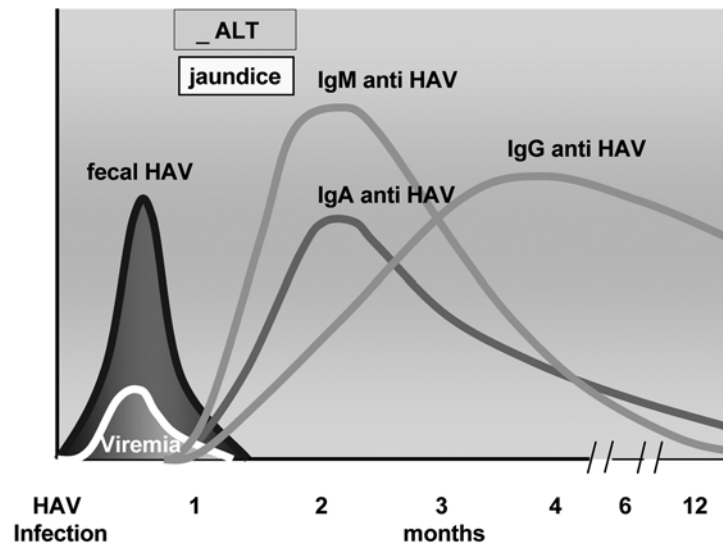
A recent study examined 237 overlapping 20-mer synthetic peptides spanning the entire HAV polyprotein by using a panel of serum samples from acutely HAV-infected patients. Forty-two antigenic domains were identified, 19 of which were found within the structural proteins; 22 were located

within the nonstructural proteins, with one domain spanning the junction of VP1 and P2A proteins. Five of these domains were considered immunodominant, as judged by the breadth and strength of their linear immunoreactivity, and were located within VP2, VP1/P2A, P2C/P3A, P3B, and P3C/P3D, respectively. Interestingly, four of the five most immunoreactive domains are derived from small HAV proteins and/or encompass protein cleavage sites separating different HAV proteins. Additionally, nonstructural proteins (P2A, P3A, and P3B) could be shown to be of particular antigenicity. An analysis of the immunoreactivity of synthetic peptides with HAV seroconversion panels (obtained from humans and chimpanzees) demonstrated that both IgM and IgG antibodies can be detected with these peptides for a short time around the acute phase of HAV (28).

Despite enormous efforts, not much is known to date about the conformational structure of the immunodominant neutralization binding site of HAV. However, it is interesting to note that all of the five afore-mentioned strong antigenic peptides are hydrophilic and folded  $\alpha$ -helices separated by strong  $\beta$ -turns, as predicted by a computer-assisted analysis of the secondary structure (28). However, statements about linear antigenic sequences are of restricted value, for most of the relevant epitopes might be discontinuous in nature.

## HUMORAL IMMUNE RESPONSES IN HAV INFECTION

The diagnosis of hepatitis A is made during acute illness by demonstrating anti-HAV of the IgM class (Fig. 4). These antibodies can be detected when serum aminotransferase activity is elevated and fecal HAV shedding is still occurring. The IgM anti-HAV levels reach their peak during the acute and early convalescent phases and become undetectable in 75% of patients 6 mo after onset of infection. It is likely that the initial antibody response to HAV also involves IgG and



**Fig. 4.** Scheme of humoral antibody responses during the acute convalescent phase of hepatitis A. Viral shedding in the stool usually stops within about 30 d of the onset of infection. IgM anti-HAV levels reach their peak during the acute and early convalescent phases and become undetectable in 75% of patients after the onset of the infection, whereas IgG anti-HAV remains detectable for many years.

IgA antibodies, since 7S antibodies may be present as early as 2 d after onset of illness (35). However, significant levels of neutralizing antibodies cannot be detected in either saliva or fecal suspensions from most experimentally infected primates or naturally infected humans, suggesting that the secretory antibody response to the virus is quite limited. Although secretory antibodies have an important role in natural immunity to polio, for example, they are not likely to be important for protection against hepatitis A (36). Serum IgG anti-HAV peaks during the convalescent period and remains detectable for many years. However, surveys of populations generally infected at early ages suggest that antibody may decline in some persons to levels no longer detectable by currently available immunoassays. Such persons are probably still protected from symptomatic reinfection but may have a resurgent anti-HAV response devoid of an IgM component upon reexposure to the virus. Serum neutralizing activity against the virus appears in parallel fashion with antibody detected by immunoassay and may be present 3 to 5 d before the onset of symptoms (37).

Several assays are available to measure IgG and IgM anti-HAV. The most widely used procedures are competitive inhibition (blocking) immunoassays, which measure the ability of a test serum to block the binding of labeled antibody to virus that has been captured onto a solid-phase support (e.g., the HAVAB by Abbott Laboratories). Thus both IgM and IgG anti-HAV are detected in these assays (38). When a World Health Organization anti-HAV reference reagent is tested in parallel, these readily available and well-standardized tests are also able to quantify the anti-HAV antibody titer (39). Another substantially more sensitive method for detection of viral neutralizing antibodies is the radioimmunofocus inhibition test (RIFIT). In this case, HAV replication foci developing

underneath agarose overlays are detected by the staining of acetone-fixed cell sheets with radiolabeled antibodies to the virus, followed by autoradiography. In neutralization assays, however, sensitivity and specificity are strongly determined by several test parameters, such as cutoff values and sera dilutions, making this highly labor-intensive assay available for larger research laboratories only (38).

Currently, the only available source of immunoreactive proteins for the development for competitive inhibition immunoassays is inactivated HAV derived from cell culture, which is currently used by all commercial companies that manufacture HAV tests. In addition to the inconvenience and cost associated with the production, purification, and standardization of cell culture-derived HAV antigen, current commercially available assays are unable to discriminate between natural infections and vaccine-induced immunity (28). The immune system of the infected individual can produce antibodies to both the structural and the nonstructural proteins during a natural infection, whereas an inactivated vaccine induces antibodies only to the structural proteins (40). Synthetic nonstructural proteins might therefore be useful in differentiating inactivated vaccine-induced immunity from natural infection, although HAV recombinant proteins are apparently poorly antigenic (28). Recently, antibodies to the nonstructural 3C proteinase of HAV could be specifically detected by ELISA in the serum of chimpanzees experimentally infected with virulent HAV and in the serum of naturally infected humans. In contrast, these antibodies were not detected by this assay in serum from HAVAB-seropositive chimpanzees that had been immunized with inactivated HAV (41). Further improvements in the expression of recombinant HAV proteins might therefore be of particular interest for the future development of HAV serodiagnostic tests.

## CELLULAR IMMUNE RESPONSES AND CONCEPTS FOR THE PATHOGENESIS OF LIVER DAMAGE DURING HAV INFECTION

The immunological response to infection with wild-type hepatitis A virus is complex and likely to involve both the cellular and humoral limbs of the immune system (42). There seems little question that induction of T-cell immunity, including the appearance of CD8<sup>+</sup> human leukocyte antigen-restricted cytotoxic T cells, is important in the pathogenesis of hepatitis A (1,43,44). However, in comparison with hepatitis B and hepatitis C, not much is known about the cellular immune response in hepatitis A, which is the most common liver disease in developing countries. Most of the work on this topic was done several years ago, before vaccination became available. Since then, new concepts concerning the control of viral infections by cytokine-mediated noncytolytic mechanisms have broadened our knowledge of the virus–host interaction, and much work needs to be done to clarify the role of these concepts, for HAV infection in particular.

Cell necrosis in several viral infections in humans can be mediated by cytotoxic T lymphocytes (CTLs) (45) that recognize viral antigens on the surface of infected cells in the context of HLA class I (43). Cytotoxic peripheral blood lymphocytes capable of lysing autologous HAV-infected skin fibroblasts can be detected in patients with acute hepatitis A but not in controls without antibodies against HAV. Interestingly, the cytotoxicity of peripheral blood lymphocytes is relatively low during viremia but peaks 2 to 3 wk after onset of icterus, i.e., after normalization of laboratory findings (46). This might be owing to recruitment of CTLs from the periphery to the liver during the very acute phase of hepatitis A, or it might suggest that virus-specific CTLs are generated too late to be of any significance for recovery from HAV infection (47).

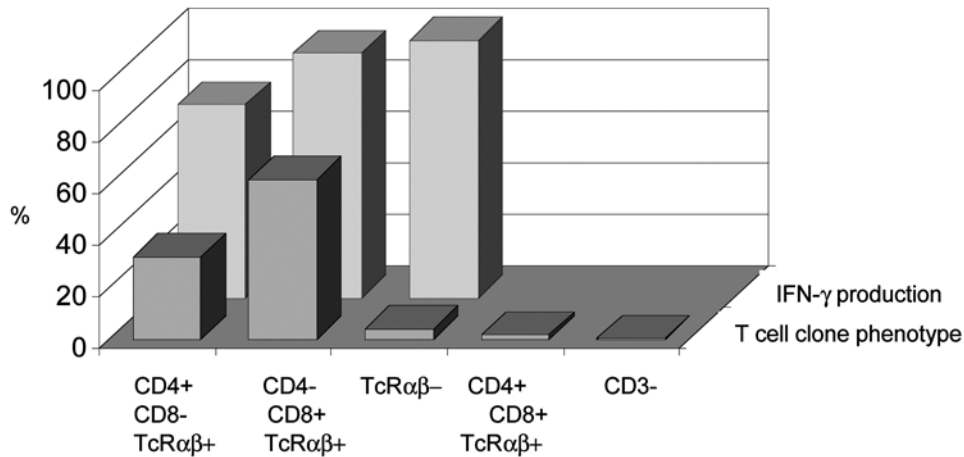
Examination of CTLs derived from the liver during the acute phase of hepatitis A demonstrated that about 50% of liver-infiltrating CD8<sup>+</sup> clones are HAV specific and can kill HAV-infected skin fibroblasts in an HLA-restricted manner (44). Since the virus used for infection of target cells was a virus adapted to growth in fibroblasts and had therefore possibly experienced changes in antigenicity during the process of adaptation, the actual fraction of virus-specific CTLs was possibly even higher (47). Electron microscopic studies showed that the interaction between an HAV-specific liver-derived CD8<sup>+</sup> clone with noninjured autologous HAV-infected skin fibroblasts eventually resulted in total necrosis, in which numerous elongated filopodia of the attacking lymphocyte infiltrated the HAV-infected skin fibroblasts (44). CD8<sup>+</sup> T lymphocytes dominate in the infiltrate over CD4<sup>+</sup> cells during the acute phase of the disease, whereas after recovery the CD8/CD4 ratio is back to normal value (47).

The production of interferon- $\gamma$  (IFN- $\gamma$ ) by T lymphocytes has been recognized to be an important step in the control of viral replication, as in murine cytomegalovirus infection. Various investigations that tried to clarify the role of IFNs in hepatitis A showed that HAV was not capable of inducing

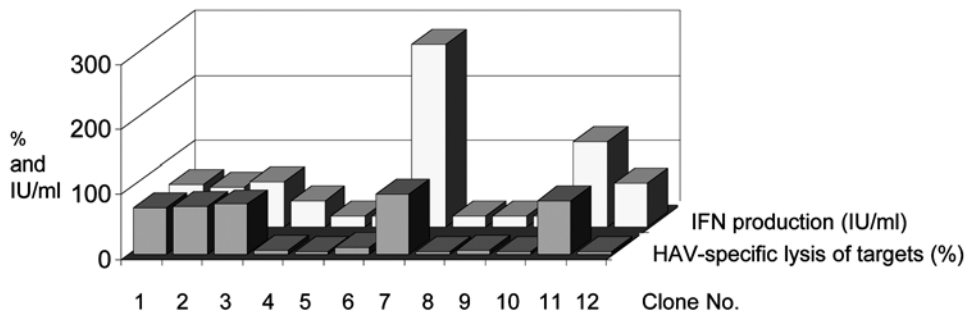
measurable IFN- $\alpha$  levels in lymphocytes or IFN- $\beta$  levels in fibroblasts (48). Similarly, several reports also indicated that patients with HAV infection do not produce IFN in the acute or convalescent stage of the disease (49). There is, however, evidence that IFN- $\gamma$  has the capacity to terminate persistent infection of fibroblasts by HAV (43). As shown for other viral infections (50), sensitized CTLs have been recognized as a prime source of this immune IFN. The HAV-specific production of IFN- $\gamma$  correlates temporally with the development of HAV-specific cytotoxicity and is to a great extent mediated by CD8<sup>+</sup> lymphocytes (43). A clonal analysis of infiltrating T lymphocytes in liver tissue in viral hepatitis A demonstrated specific cytotoxicity against autologous infected fibroblasts in about 50% and variable IFN- $\gamma$  production among the T-cell clones (Figs. 5 and 6). Interestingly, in one patient with a second exacerbation of the disease, more than 20% of all clones had a natural killer (NK) cell-like phenotype (47). NK cells are large granular lymphocytes with a characteristic morphology that bind to high molecular weight glycoproteins on the surface of virally infected cells. Their potential importance during an infection with hepatotropic viruses is underlined by the fact that NK cells account for 20 to 30% of intrahepatic lymphocytes. NK cells have been shown to mediate cytotoxicity and produce IFN- $\gamma$  during the initial period of primary infections in mice. Their role in HAV, however, has not been examined so far.

Some of the various modulating influences of IFNs on the immune system might be of special interest in the context of viral hepatitis: First, the expression of IFN- $\alpha/\beta$  was able to clear replicative intermediates from the hepatocyte in a murine model of HBV infection (51), indicating an attractive mechanism of noncytolytic viral purging. Second, there is evidence that IFN- $\gamma$  contributes to the recruitment of non-antigen-specific CD8<sup>+</sup> lymphocytes, which might exert harmful effects during viral hepatitis. Third, IFN- $\gamma$  induces the expression of cell-surface proteins, including major histocompatibility complex antigens on many different cell types including hepatocytes (43,52). It is tempting to speculate about the potential relevance of these mechanisms in hepatitis A. However, the events in the cascade of immunologic changes during acute HAV infection have not been uncovered so far.

Recent studies might have provided an explanation for the prolonged period of clinical quiescence in the face of mounting viral replication. Interferon regulatory factor 3 (IRF-3) is a cytoplasmic transcription factor that is phosphorylated after viral infection of the cell and induces IFN- $\beta$  synthesis, thereby promoting viral clearance. This activation of IRF-3 is initiated by double-stranded RNA (dsRNA) and mediated via Toll-like receptor 3 or the retinoic acid-inducible gene I pathway (53). Interestingly, HAV has been shown to inhibit dsRNA-induced IFN- $\beta$  gene expression as well as dsRNA-induced apoptosis (54). This inhibition of IFN- $\beta$  is likely to be mediated by blockage of IRF-3 activation through the retinoic acid-inducible gene I pathway (54a). This might be one mode of action by which HAV disrupts cellular mechanisms of viral clearance and evades the host immune response (20). In contrast to the



**Fig. 5.** Phenotype distribution of 257 liver-derived T-cell clones from a patient with acute hepatitis A and percentage of clones producing interferon- $\gamma$  (IFN- $\gamma$ ). Since the antigen specificity of the CD4<sup>+</sup> and CD4<sup>-</sup>/CD8<sup>-</sup> TcR $\gamma^+$  clones could not be tested, mitogen was used to test for the capacity to produce IFN- $\gamma$ . (Modified from ref. 47.)



**Fig. 6.** Hepatitis A Virus (HAV)-specific cytotoxicity and interferon- $\gamma$  (IFN- $\gamma$ ) production by liver-derived CD8<sup>+</sup> clones from a patient with acute hepatitis A. CD8<sup>+</sup> clones were incubated on autologous HAV-infected fibroblasts. <sup>51</sup>C release and IFN- $\gamma$  concentration were determined from the supernatants. Cytotoxicity to uninfected fibroblasts and associated IFN- $\gamma$  production was less than 8% and less than 16 IU/mL, respectively. (Modified from ref. 47.)

hepatitis C virus, HAV does not appear to interfere with activation of the transcription factor nuclear factor- $\kappa$ B (54).

Hepatocytes in hepatitis A have been shown to express HLA class I antigens, whereas these molecules are not or only weakly expressed on the surface of normal human hepatocytes (43). IFN- $\gamma$  is thought to be one of the major mediators for this effect, resulting in enhancement of an efficient T-cell-mediated immune attack (43). Additionally, the fact that there is only one HAV serotype might give further evidence for the hypothesis that the cellular pathway of the immune system is responsible for eliminating HAV. RNA-dependent RNA polymerase of single-strand plus-sense viruses generally has an ineffective proofreading function, causing about 1/100,000 mistakes during production of the RNA chain. The irregular nucleotide within the newly transcribed minus strand is then causing an amplified mistake during the synthesis of plus-sense copies. If the picornavirus is controlled by the humoral arm of the immune system, these mutations, which may lead to changes in the antigenic property, will be

responsible for the development of antigenic drift. In contrast, in the case of HAV, in which T cells instead of antibodies are likely to be responsible for viral elimination, there is no positive selection of the antigenic drift variants; a single serotype is maintained.

An exciting field is the study of viral coinfections and its implications for immune response. Recently, it has been shown that an acute HAV infection can have the potential to suppress markedly the replication of an underlying chronic HBV infection. This is probably mediated by the induction of cytokines. At the time of HAV infection a sharp peak in the IFN- $\gamma$  level occurred just before HBV DNA and hepatitis B early antigen (HBeAg) began to decrease below the limit of detection. The HBV-specific T-cell response was not modified, and HBV replication relapsed after resolution of hepatitis A (55).

In conclusion, the mechanisms of liver injury in hepatitis A are poorly characterized so far. In contrast to other picornaviruses, HAV generally causes an inapparent and persistent rather than a cytolytic infection in cell cultures *in vitro* (44).

The roles of different immune mechanisms in the elimination of the virus and in the inflammatory reaction are still unclear (43). Earlier studies had shown that hepatocyte destruction in HAV infection is unlikely to be mediated by complement-dependent cytolytic antibodies to HAV (56). Therefore HLA-restricted, virus-specific, CLTs could play an important role, possibly via secretion of inflammatory cytokines like IFN- $\gamma$ , which might then exert direct antiviral mechanisms to clear HAV noncytolytically (2,51). Additionally, the introduction of HLA class I tetramers offers new possibilities to characterize a certain subset of T cells with defined specificity.

## CLINICAL AND HISTOLOGICAL FEATURES OF ACUTE HAV INFECTION

HAV is transmitted almost exclusively via the fecal–oral route. Person-to-person spread is enhanced by poor personal hygiene and overcrowding, and large outbreaks as well as sporadic cases have been traced to contaminated food. In addition, certain groups appear to be at risk for parenterally transmitted HAV. In support of this argument, outbreaks of hepatitis A have been increasingly recognized in users of illicit injection drugs (2,57). Infection with HAV results in a broad spectrum of sequelae, ranging from subclinical infection, to clinical infections with or without jaundice, to acute liver failure and possible death. The risk of infection associated with jaundice increases with age.

After an incubation period of 15 to 45 d, a variable pattern of prodromal symptoms develops. Anorexia, nausea and vomiting, abdominal pain, fatigue, malaise, cough, and arthralgias may precede the onset of jaundice by 1 to 2 wk. The development of dark urine and jaundice marks the beginning of the icteric phase, which is often accompanied by mild-to-moderate tender hepatomegaly on physical examination. Complete clinical and biochemical recovery is to be expected 1 to 2 mo after all cases. HAV infection does not have a chronic phase and does not cause chronic hepatitis. However, relapsing hepatitis A has been described in 6 to 10% of patients, and some individuals develop a cholestatic form of the disease.

A serum bilirubin of around 40  $\mu\text{mol/L}$  (2.5 mg/dL) is the threshold for differentiating nonicteric from icteric hepatitis. The absolute measures of coagulation factors such as prothrombin time, prothrombin levels, International Normalized Ratio (INR), and factor V levels are good parameters to identify those at risk of developing acute liver failure.

The characteristic histological features of acute HAV infection are random areas of lobular hepatitis with spotty necrosis associated with a mononuclear portal and periportal cell infiltrate. This comprises predominantly lymphocytes and histiocytes but also includes neutrophils and eosinophils.

## HAV INFECTION IN PREEXISTING LIVER DISEASE

The occurrence of acute hepatitis A in the setting of pre-existing chronic liver disease theoretically puts such patients at increased risk of morbidity and mortality compared with previously healthy individuals experiencing acute hepatitis. This is likely to be true for hepatitis A superimposed on

chronic hepatitis B, chronic hepatitis C, and other chronic liver diseases (58).

Between 1983 and 1988, 115,551 cases of hepatitis A were reported to the Centers for Disease Control and Prevention (CDC), with an overall case fatality rate of 0.33%. Fatalities occurred predominantly in the older population, with 72.4% of deaths occurring in patients over the age of 49 yr. The risk of death was estimated to be increased by 59-fold in chronic hepatitis B surface antigen (HbsAg) carriers and by 23-fold in chronic liver disease (58).

In a 7-yr prospective study from Italy, 163 patients with chronic hepatitis B and 432 patients with chronic hepatitis C were followed and monitored for a superinfection with HAV. In contrast to the relatively good outcome of the HBV patients superinfected with HAV, there was a substantial risk of fulminant hepatitis and death associated with HAV superinfection in the HCV group: 17 of those 432 patients (3.9%) experienced hepatitis A, 7 patients of whom (41%) developed fulminant hepatitis resulting in death in 6 patients (35%) (59).

These data support the CDC recommendation that patients with preexisting liver disease should receive hepatitis A vaccination (60).

## HAV VACCINATION: STRATEGIES AND SAFETY

Within the United States, roughly 50,000 cases of acute hepatitis were reported annually between 1984 and 1993, but many more went unreported (61). HAV was responsible for most (47%) of those cases. Whereas immunity to hepatitis A approaches 100% in developing countries, only about one-third of the population in the United States shows detectable anti-HAV indicating immunity.

Thus, there is a growing subpopulation at risk for HAV infection that might be associated with a severe course of disease in older individuals and those with preexisting liver disease. Acute hepatitis may be a serious, even fatal illness and is often associated with a prolonged convalescence, thus representing a considerable disease burden (16). Once HAV infection occurs, there is no specific antiviral therapy (apart from the administration of HAV immunoglobulin, which will be discussed later in this section). Supportive care can include a high-calorie diet, intravenous feeding, and cholestyramine (in case of severe pruritus). In cases of fulminant hepatitis, maintenance of fluid balance and vital parameters, correction of hypoglycemia, and control of bleeding and hepatic encephalopathy are the main goals. Meticulous intensive care is the one factor that does appear to improve survival. Orthotopic liver transplantation is resorted to with increasing frequency and excellent results in patients with fulminant hepatitis.

In 1973, Feinstone and colleagues at the National Institutes of Health were the first to identify HAV as the causative agent of acute hepatitis (9). The growth of HAV in culture together with the development of sensitive and specific serological techniques then allowed further studies in the field of HAV vaccination. The first vaccine to be introduced worldwide was Havrix<sup>®</sup> (SmithKline Beecham, Philadelphia, PA) in 1992, which was licensed in the United States in 1995. A second

**Table 1**  
**Recommendations for Use of Hepatitis A Vaccine**

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**Routine immunization**

All children at 1 yr of age (i.e., 12–23 mo); children who are not vaccinated by 2 yr of age can be vaccinated at subsequent visits

**Increased risk of hepatitis A**

Persons traveling to or working in countries with high or intermediate hepatitis A virus (HAV) endemicity, such as Mexico, the Caribbean, Southeast Asia, South and Central America, and Africa

Men who have sex with men

Illegal drug users

Individuals who work with HAV-infected primates or with HAV in research laboratories

Persons with clotting factor disorders

Outbreaks in communities with high or intermediate rates of hepatitis A

**Increased risk of more severe disease**

Persons with chronic liver disease

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Modified from refs. 62 and 69.

vaccine, VAQTA® (Merck, West Point, PA), was licensed in the United States in 1996 (62). These are notable more for their similarities than their differences. Both contain formalin-inactivated viral particles (HM175 and CR326F strains, respectively) produced in infected human diploid fibroblasts. It might be important to know that whereas VAQTA is formulated without a preservative, Havrix contains 2-phenoxyethanol. Both inactivated vaccines are adsorbed to aluminum hydroxide and thus should not be frozen (16).

Both Havrix and VAQTA appear to be of similar immunogenicity (62,64). Two controlled field trials have confirmed the high protective efficacy of these vaccines. A single 25-unit dose of VAQTA was given to 1,037 children at high risk for hepatitis A in New York and provided complete protection (65). The level of protection was similarly high among Thai children who completed a primary immunization series with two doses of 360 ELISA U each of Havrix (16,66). More than 95% of healthy adults develop anti-HAV antibodies within 1 mo after receiving a single dose of vaccine. Therefore postvaccination testing is not necessary, although anti-HAV antibodies are often undetectable by ELISA (62). The recommended schedules for Havrix and VAQTA include a single primary immunization followed by a booster dose after 6 mo. This probably provides protective antibody levels for more than 20 yr. Neither vaccine should be given to persons with a history of allergy to any vaccine component or children under the age of 2 (60). The safety of hepatitis A vaccine in pregnant women needs to be further evaluated, although risk for the fetus is likely to be low (62).

Both vaccines are well tolerated, and no serious adverse events in post marketing monitoring have been unequivocally attributed to either vaccine (16,62). Among the adverse effects are: mild local reactions, soreness at the site of intramuscular injection in up to 56%, and fever in up to 4%. A few potentially life-threatening adverse events have been reported whose causal links to HAV vaccine remain unclear.

Earlier recommendations for broader immunization of children in regions of the United States with high HAV incidence have led to an impressive 88% decline in reported HAV cases in those states (67). A similarly sharp decrease in disease rates could also be observed after the implementation of a

hepatitis A immunization program in Israel (68). In October 2005, the Advisory Committee for Immunization Practices (ACIP) of the CDC has therefore added a provisional statement to the recommendations for the use of hepatitis A vaccines in the United States (Table 1) (60,69). Routine immunization is now recommended for all children at 1 yr of age (i.e., 12–23 mo). Children who are not vaccinated by 2 yr of age can be vaccinated at subsequent visits. Children under 3 yr of age who attend preschool day-care centers have an important role in the transmission of HAV in some communities, even though they are rarely symptomatic when infected.

According to the ACIP guidelines, vaccination should also be administered to persons at increased risk for hepatitis A, i.e., persons traveling or working in countries with increased HAV endemicity, men who have sex with men, illegal drug users, individuals in research laboratories, and persons with clotting factor disorders. Furthermore, persons with preexisting chronic liver disease should receive HAV vaccination (60). Pre vaccination testing for anti-HAV might be particularly cost-saving in patients with chronic liver disease, owing to the relatively high anti-HAV prevalence in these individuals (62).

Several studies have evaluated the immunogenicity of HAV vaccines in patients with chronic liver disease. In general, vaccination in this patient group has been shown to be safe and efficacious. In an open multicenter study, comparing the efficacy of Havrix in patients with compensated liver disease (among them chronic hepatitis B and C) with healthy subjects, there was a higher seroconversion rate among the healthy individuals (93%) compared with those who had chronic hepatitis C (74%) or nonviral chronic liver disease (83%) after administration of a single dose. However, there was no significant difference in the seropositivity rates among these groups after completion of the vaccination schedule. Nevertheless, this study demonstrated that postvaccination anti-HAV titers in patients with preexisting liver disease, although seroprotective (more than 10 mIU/mL), are significantly lower than those in healthy individuals (70).

Data on the efficacy of HAV vaccines in end-stage liver disease and liver transplant recipients have been inconsistent (71). HAV vaccination seems to be safe in liver transplant



recipients (72), and seroconversion rates following a booster dose have been reported to be as high as 97% in this patient group. However, seroconversion after complete HAV vaccination is significantly less common in decompensated liver disease, with the Child-Pugh Score predicting the vaccination response (73). Possibly owing to the concomitant immunosuppressive therapy, antibody titers decline much more rapidly, leading to a significantly lowered proportion of HAV-protected patients 2 yr after complete immunization (74). These findings indicate that patients with chronic liver disease should receive vaccination before the development of hepatic decompensation.

In 2001, the Food and Drug Administration (FDA) licensed a combined hepatitis A and B vaccine (Twinrix<sup>®</sup>) for use in persons aged 18 yr older. Any person in this age group having an indication for both hepatitis A and B vaccination can be administered Twinrix, including patients with chronic liver disease, users of illicit injectable drugs, men who have sex with men, and persons with clotting factor disorders. Primary vaccination consists of three doses, given on a 0-, 1-, and 6-mo schedule, the same schedule as that used for a single antigen hepatitis B vaccine (75). A prospective, randomized, comparative U.S. trial of Twinrix with corresponding monovalent vaccines suggested that this new combination vaccine is of comparable safety and immunogenicity (76). Furthermore, the persistence of anti-HAV and anti-HBs following Twinrix administration is similar to that following single-antigen hepatitis A and B vaccine administration at 4 yr of follow-up (75).

Candidate live, attenuated HAV vaccines have been developed using viruses that have been adapted to grow in cell culture (20). Such a vaccine has received relatively wide use in China and appears capable of inducing protective antibody levels (95). One study suggested, however, that a single dose of this attenuated hepatitis A vaccine lacked efficacy in preventing asymptomatic HAV infection (78).

Despite their importance in preventing morbidity associated with acute hepatitis A, vaccines have little to offer after a person has been exposed. Thus, when hepatitis A is recognized in a patient, close family member, or household contact, immune globulin should be given for prophylaxis, optimally within 2 wk after exposure (16). Immunoglobulin is a sterile preparation of concentrated antibodies (immunoglobulins) made from pooled human plasma. Immunoglobulin provides protection against hepatitis A through passive transfer of antibody. When used for preexposure prophylaxis, a dose of 0.02 mL/kg of immunoglobulin administered intramuscularly (i.m.) confers protection for less than 3 mo, and a dose of 0.06 mL/kg immunoglobulin administered i.m. confers protection for 5 mo or less. When administered within 2 wk following exposure to HAV (0.02 mL/kg i.m.), immunoglobulin is greater than 85% effective in preventing hepatitis A. Persons who have been administered one dose of hepatitis A vaccine at least 1 mo before exposure to HAV do not need immunoglobulin (60).

The ACIP's call for immunization of all children in the United States will further lower the morbidity associated with HAV in the United States. However, to achieve the goal

of eliminating HAV transmission, world-wide hepatitis A vaccination programs have to be implemented.

## GENOMIC STRUCTURE AND REPLICATION CYCLE OF HEV

The existence of HEV was first suspected in 1980, when cases of water-borne hepatitis in India were recognized not to be due to hepatitis A. In 1983, the virus was visualized by immune electron microscopy, but it took another 6 yr before the viral genome was cloned by Tam et al. and the virus was named hepatitis E virus (4). Since HEV has not been grown efficiently in cell culture, information about its molecular biology has been obtained mainly from recombinant technologies (79). The HEV genome consists of a linear, single-stranded, positive sense RNA of approximately 7.5 kb containing a 3' poly (A) tail and 3' noncoding regions; it encodes for three ORFs. ORF1 (5079 nt) encodes a polyprotein of about 1690 amino acids, which can be cleaved to non-structural proteins that are involved in viral genome replication and viral protein processing. Additionally, ORF1 contains the Y and X domains with unknown function (77). ORF3 (369 nt) encodes for a 123 amino acid protein that partitions with the cytoskeleton in cell fractionation studies after expression in eukaryotic cells. Although the function of ORF3 protein is unknown, the aforementioned observations prompted the hypothesis that ORF3 protein might serve as a cytoskeletal anchor site, where ORF2 and HEV-RNA could bind and subsequently begin viral nucleocapsid assembly (80). ORF2 (1980 nt) is translated to a 660 amino acid protein that represents the major, if not the only, protein in the virion. ORF2 expression in insect cells is followed by nonviral proteolytic processing into smaller proteins, which are likely to represent structural proteins and can participate in the formation of virus-like particles. However, the size or modifications of the ORF2 protein in infectious virions have not been characterized so far. HEV replicates in the cytoplasm of hepatocytes and is shed in the feces. It is not known whether there are extrahepatic sites of replication or how ingested virus reaches the liver (79).

The genomes of several HEV strains from different parts of the world can be grouped into at least four major genotypes (77). Two human strains from Asia (genotype 1) and Mexico (genotype 2) can be distinguished from genotype 3 virus, which was shown to circulate naturally in swine and to possess the capability of interspecies transmission (81). In 1999, a fourth HEV strain was discovered in Taiwanese swine (82). It is likely that HEVs infecting swine are attenuated and can cause subclinical infections that might explain the relatively high seroprevalence of anti-HEV even in developed countries. The four HEV genotypes apparently comprise a single serotype (79).

## HISTOLOGICAL FEATURES OF HEV INFECTION AND THE POSSIBLE PATHOGENETIC ROLE OF HUMORAL AND CELLULAR IMMUNE RESPONSES

Acute hepatitis E is morphologically characterized by focal necrosis, ballooned hepatocytes, and acidophilic degeneration

of hepatocytes. Cholestatic forms may present with bile stasis and glandular transformation of hepatocytes. No data are available on the exact mechanism of hepatocellular injury in HEV infection. Although it is unlikely that HEV mediates a direct cytopathic effect, this has been difficult to confirm because a cell culture system is presently lacking.

Two studies from India have examined immunological alterations in acute hepatitis E. The first study demonstrated that the proportion of positive results in a lymphocyte proliferation assay using seven peptides of ORF2 and ORF3 was higher in patients with hepatitis E (11/21) than in controls (5/22,  $p < 0.05$ ) (83). The same group recently published data on immune responses to phytohemagglutinin (PHA) and HEV peptides as well as cytokine production by peripheral blood mononuclear cells (PBMCs) in pregnant women with acute hepatitis E. This study has several limitations. First, the number of patients and controls was low, and second, only a few patients with acute hepatitis E had a significant lymphoproliferative response to HEV peptides (i.e., stimulations indices were less than 2), which contrasts to the results of the former study. There was, however, slightly increased IFN- $\gamma$  production in nonpregnant patients with hepatitis E compared with the control group. The various modulating influences of IFNs in viral hepatitis have been discussed above. As in hepatitis A, IFN- $\gamma$  might be secreted by CTLs or NK cells in acute HEV infection, thereby supporting viral clearance, but this has not been investigated so far. Another interesting result was that pregnant women with acute hepatitis E had the lowest lymphoproliferative responses to PHA, with lower production of IFN- $\gamma$  and higher production of interleukin-4 (IL-4). Although pregnancy itself is believed to skew the cytokine responses toward the Th2 type, the aforementioned findings were not demonstrated in pregnant or nonpregnant controls (7). Whether this Th2 bias in pregnant patients with acute hepatitis E is associated with the severe course of hepatitis E in pregnancy needs to be clarified in the future.

ORF2-derived antigens expressed from baculovirus in insect cells and, to a lesser extent, antigens expressed in *E. coli* have yielded the best serologic tests to diagnose present or past HEV infection (4). One of these assays with a high specificity for anti-HEV was developed at the U.S. National Institutes of Health (NIH), (84). Such tests demonstrated approximately 20 to 90% seropositivity in areas with HEV endemicity and 1 to 20% seropositivity in populations in which hepatitis E is seldom diagnosed. Interestingly, there is an unexplained difference in the pattern of antibody acquisition between India and Egypt: anti-HEV seropositivity in India is found in 30 to 40% of Indian adults, but exceeds 60% among 10-yr-old children from Egypt. Further puzzling is the question of why the age-stratified seroprevalence of anti-HAV antibodies in India is so dissimilar from that of anti-HEV antibodies (6). Because anti-HEV antibodies following infection of rhesus monkeys with one of the four mammalian HEV genotypes are broadly crossreactive (85), the available serological tests are likely to detect acute hepatitis E regardless of the underlying HEV strain. IgM anti-HEV antibodies indicate an acute hepatitis E infection and are present for up to 4 mo. The IgG class of

anti-HEV rises to variable titers during early convalescence and can be detected up to several years (Fig. 7).

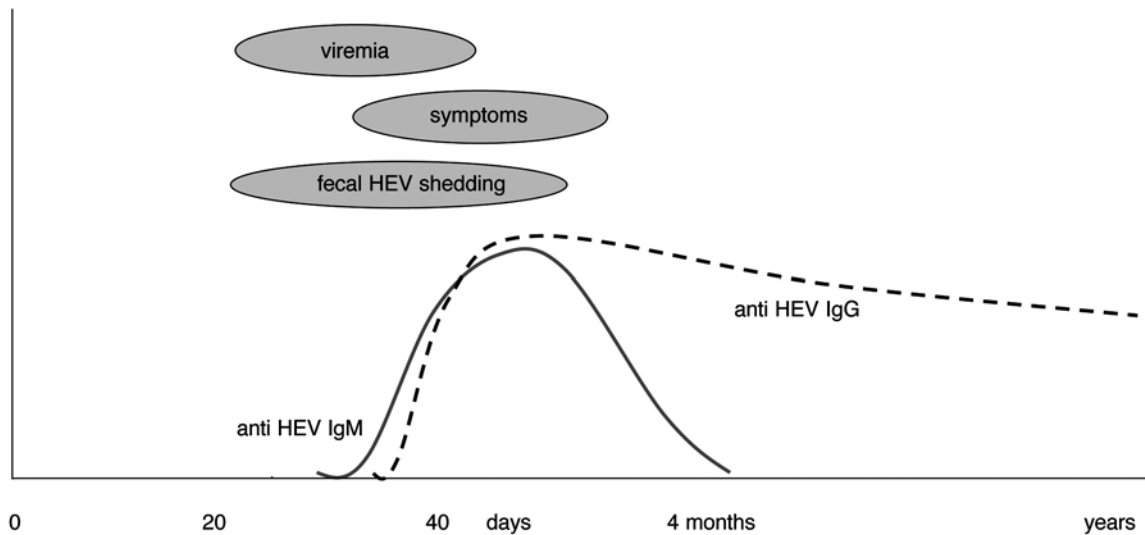
## CLINICAL FEATURES OF ACUTE HEV INFECTION

Much about the epidemiology of hepatitis E is unknown (6). HEV is spread by fecally contaminated water in endemic areas. Since person-to-person transmission is uncommon (rate of secondary attack rates after household contacts, 0.7–2.2%), the hypothesis of animal-to-human spread has attracted attention. The fact that anti-HEV antibodies can be found in swine, numerous rodents, and even domesticated animals like sheep, cattle, and chickens has led some authors to suggest that hepatitis E might represent a zoonosis (79). Indeed, recent clusters of hepatitis E cases in Japan have been traced to the ingestion of undercooked deer meat and pig liver (86).

The incubation period of HEV infection ranges from 15 to 60 d (87). HEV infection is self-limiting and never progresses to chronicity. The clinical signs and symptoms of acute hepatitis E resemble those of acute hepatitis A. The overall case fatality rate is 0.5 to 3%, but mortality rates of up to 20% have been reported for pregnant women who were infected during outbreaks of hepatitis E in developing countries (4).

## RECENT ADVANCES IN HEV VACCINATION

Earlier studies have shown that people previously infected with HEV are protected during epidemics of the disease (88). Vaccination of rhesus monkeys with a recombinant ORF2 protein from genotype 1 resulted in almost complete protection against HEV infection for at least 6 mo (89). Just recently, the broad cross-genotype neutralization of HEV was demonstrated in cell culture assay (85). Monolayers of Hep G2/C3A cells were inoculated with genotype 1 HEV mixed with either anti-HEV or an appropriate control. As determined by immunofluorescence microscopy, anti-HEV from vaccinated or infected rhesus monkeys neutralized the virus and showed broad crossreactivity between the four genotypes. Among several other candidate peptides derived from ORF2 and ORF3, a recombinant truncated ORF2 protein (amino acids 112–607) has attracted attention because of its high immunogenicity and induction of neutralizing antibodies (90). So far, ORF2 gene or its fragments have been found in prokaryote cells, insect cells, yeast cells, animal cells, and tomatoes (77). The only HEV vaccine candidate that has progressed to the stage of clinical trials is the recombinant ORF2 protein spanning amino acids 112 to 607 expressed in insect cells via a baculovirus vector, which was developed at the NIH. This vaccine was shown to be safe and immunogenic in a phase I trial including 88 American volunteers and another phase I trial in 22 Nepalese volunteers in whom vaccination at 0, 1, and 6 mo resulted in a 100% anti-HEV seroconversion after 7 mo (91). Purcell et al. observed that the HEV vaccine in rhesus monkeys lead to vaccine-induced protection against HEV disease but only incomplete protection against HEV infection. In March 2007, Shresta et al. published a large phase II trial of a similar or identical HEV vaccine administered almost exclusively to 2000 male Nepalese volunteers (96). HEV vaccine effectively



**Fig. 7.** Scheme of humoral antibody responses during the acute convalescent phase of hepatitis E. Viral shedding in the stool usually stops within about 50 d after hepatitis E virus (HEV) exposure. IgM anti-HEV levels reach their peak during the acute phase of infection and are detectable for 3 to 4 mo, whereas IgG anti-HEV can remain detectable for several years.

prevented clinically overt HEV infection. Although the use of vaccine to prevent asymptomatic HEV infection was not investigated (97), this trial certainly represents a milestone study on the way towards a commercially available protective vaccine against hepatitis E.

Novel vaccination technologies include cDNA vaccination and immunization with recombinant HEV-like particles. Intramuscular injection of HEV ORF3 cDNA that had been expressed in prokaryote cells resulted in anti-HEV-IgG seroconversion in 12 of 16 mice (92). Recombinant virus-like particles (rVLPs) spontaneously assemble after the expression of a 111 amino acid N-terminal fragment of the capsid protein in the baculovirus system (93). HEV rVLPs given orally to cynomolgus monkeys protected these animals against HEV infection (94). These results suggest that HEV rVLP could be a candidate for an oral hepatitis E vaccine (77).

### CONCLUDING REMARKS AND OPEN QUESTIONS

The availability of safe and extremely effective inactivated hepatitis A virus vaccines since the early 1990s has contributed to a declining interest in the molecular virology of HAV and the pathogenesis of hepatitis A. Only a few data exist on the interaction between the host's immune response and HAV, and further research is definitely necessary to clarify why HAV does not persist in the infected host, whereas HCV does (20). Although the CDC has just recently called for universal HAV vaccination of all children at 1 yr of age in the United States, this will hardly influence the 1.5 million cases of acute hepatitis A reported worldwide annually. To reduce the morbidity of hepatitis A, further improvements in sanitary conditions and vaccination programs on a worldwide basis are necessary.

The eruption of new hepatitis E cases in regions with poor sanitary conditions demonstrates that HEV has the potential

to cause explosive epidemics when the infrastructure breaks down owing to civil conflicts (6). Much of the research on HEV and hepatitis E vaccine development has been done in the laboratory of Robert H. Purcell and Suzanne U. Emerson, who have also developed a vaccine that has recently been tested in Nepal. Further investigation is necessary on the host-HEV interaction, the long-term protection against hepatitis E provided by the first available vaccine, and the development of potential oral vaccinations with HEV recombinant virus-like particles.

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# 14 Role of the Immune Response in Hepatitis B

## *Determinants of Severity, Chronicity, and Response to Antiviral Therapy*

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### KEY POINTS

- HBV is a noncytopathic, hepatotropic DNA virus. A central feature of the life cycle of a virus is the synthesis of viral DNA from an RNA template. HBV can cause chronic hepatitis, leading to liver cirrhosis and hepatocellular carcinoma.
- Variable outcome of disease depends on the balance between viral parameters and the immune system; dose of inoculum, kinetics of viral replication, and tissue tropism are balanced by specificity, kinetics, and the strength of innate and adaptive immune responses.
- HBV does not enter a logarithmic phase of replication until 4 to 5 wk after infection, and activation of innate immunity is not detectable during the early phases of HBV infection.
- The differences in the adaptive immune response to HBV, which characterize chronic and resolved patients, are heavily influenced by the immunological events occurring during the initial phase of HBV replication.
- The ability to mount an efficient, virus-specific helper and cytotoxic T-cell response is essential for control of HBV infection.
- The establishment of HBV chronicity leads to a state of collapse of virus-specific adaptive immunity that is not absolute but appears to be mainly regulated by the quantity of HBV replication present in chronic hepatitis B patients.
- The control of HBV infection was thought to be dependent on the destruction of infected cells by the immune system. However, virus-infected hepatocytes could be controlled by cytokine-dependent curative mechanisms that do not require hepatocyte destruction.
- HBV-specific cytotoxic T cells (CTLs) mediate protection but can also be the principal effector of liver damage. An efficient HBV-specific CD8 response can promote viral

control without persistent liver pathology, whereas an inadequate CTL response may contribute to liver pathology not only directly but also via the recruitment of non-antigen-specific T cells into the infected liver.

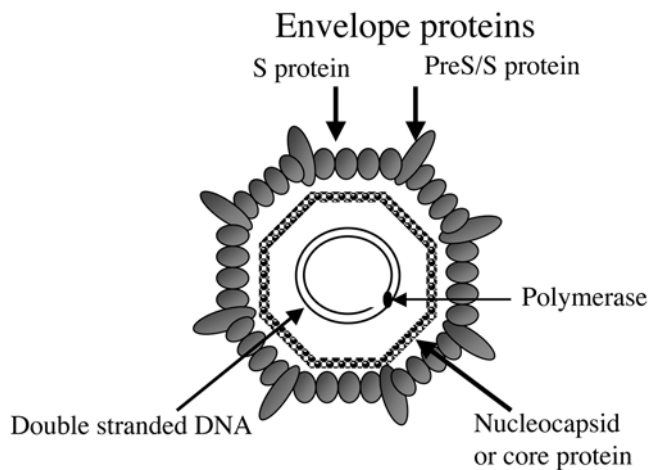
- Inhibition of viral replication with antiviral drugs can restore HBV-specific T-cell responses, but the restoration is often transient.

### INTRODUCTION

The hepatitis B virus (HBV), a member of the Hepadnaviridae family, is a hepatotropic noncytopathic DNA virus that, despite the presence of an effective prophylactic vaccine, is estimated to infect 300 million people, with a particularly high prevalence in Asia and Africa (1).

HBV causes liver diseases that vary greatly in severity from person to person. Some subjects control infection efficiently and clear the virus from the bloodstream either without clinically evident liver disease or with an acute inflammation of the liver (acute hepatitis) that can resolve without long-term clinical sequelae. Other patients fail to clear the virus and develop chronic infection. Most chronically infected patients remain largely asymptomatic without life-threatening liver disease, but 10 to 30% develop liver cirrhosis with possible progression to liver cancer. The rate of HBV chronicity is low in adult infections (5% or lower), but age and route of infection influence the outcome, with exposure in neonatal life leading to a high rate of HBV persistence (1). Outcome of infection and the pathogenesis of liver disease are determined by virus and host factors, which have been difficult to elucidate fully because the host range of HBV is limited to humans and chimpanzees.

The study of animal models of related *Hepadnavirus* infections and transgenic mouse models able to express individual HBV genes or replicate the entire viral genome have clarified several aspects of HBV infection. Furthermore, the ability to analyze many immunological phenomena *ex vivo* through direct quantification of antigen-specific T cells in humans and chimpanzees has considerably increased our knowledge of HBV pathogenesis.



**Fig. 1.** The structure of HBV particle.

This chapter reviews the major recent concepts in the immunopathogenesis of HBV infection. After describing parameters that can influence the outcome of infection, we focus our attention on the distinctions of HBV immunity between resolved and persistently infected patients. We next examine how the demonstration of noncytopathic mechanisms of HBV clearance has changed our current understanding of the pathogenesis of liver damage during chronic infection with HBV. In light of the importance of coordinate expansion of cellular immune responses in the successful control of HBV infection, we finally review potential immune-therapeutic strategies that might achieve long-term viral control in the very many people with chronic HBV infection.

## BIOLOGY OF HBV

HBV is member of the Hepadnaviridae family of viruses (Fig. 1). Viruses closely related to HBV have been found in woodchucks (2) and ground squirrels (3). These viruses have about 70% homology with HBV but do not infect humans or other primates. More distantly related viruses with a similar genetic organization are found in ducks and geese. Owing to the limited host range of HBV (which infects only humans and great apes) and the lack of a *in vitro* system to infect normal human hepatocytes, these related viruses are currently used as a model system to characterize how hepadnaviruses replicate and as a disease model.

Hepatocytes are the only confirmed site of replication for HBV. Bile ductule, epithelial cells, or subsets of cells in the pancreas or kidney, or lymphoid cells may also be a target of infection, but the evidence of replication in these extrahepatic site is controversial, and at the moment these sites are not considered in the discussion of viral replication and pathogenesis (4). The replication of hepadnaviruses is characterized by the synthesis of an approx 3-kb partially double-stranded, relaxed circular DNA (rcDNA) genome by reverse transcription of an RNA intermediate, the pregenome (5). At initiation of infection, the viral rcDNA genome is converted into closed circle (cc)DNA. The ccDNA serves as the template for the

transcription of viral mRNAs. One of these mRNAs, called pregenome, is used to synthesize the core protein (nucleocapsid subunit) and the viral reverse transcriptase. The reverse transcriptase binds to its own mRNA templates and is packaged into the nucleocapsid, where viral DNA synthesis occurs.

Mature nucleocapsid containing the rcDNA is then enveloped in the endoplasmic reticulum and exported from the cell via the Golgi apparatus (Fig. 2) (reviewed in ref. 4).

The HBV genome contains four open reading frames (ORFs) that encode the viral nucleocapsid, polymerase, envelope, and X proteins (Table 1). Core and polymerase genes are essential for viral DNA replication, and the envelope protein, which consists of three polypeptides (S, M, L), is essential for envelopment of nucleocapsid. The function of hepatitis X protein is unknown; the protein is required for the establishment of infection *in vivo* (6) but is dispensable for viral replication in transfected cells (7).

The nucleocapsid ORF contains two start codons that define two overlapping proteins. The shorter of these proteins (hepatitis B core antigen [HBcAg]) is the viral capsid protein that assembles in the cytoplasm of the hepatocytes to form the icosahedral subviral particles that package the viral reverse polymerase and the pregenome (8). The longer protein (precore) is translocated into the endoplasmic reticulum, where it undergoes truncation of its carboxy-1 and amino-terminal residues and is secreted in the blood as hepatitis B early antigen (HBeAg) (9,10).

The presence of HBeAg in patient serum is a good marker of viral replication, but since HBeAg is not required for *in vivo* infection (11), its function is unknown. Experiments in mice suggest that HBeAg can cause depletion of Th1 helper cells (12), thereby suppressing antiviral immune responses. However, this possibility has not been tested in a natural host of hepadnavirus.

All the hepadnaviruses express three envelope components called S, M, and L. All three contain the smallest (226 amino acids long) S domain, called hepatitis B surface antigens (HBsAg). The two larger proteins contain S plus an amino acid extension containing the pre-S2 antigen (M protein, 226 + 51 amino acids long) or pre-S2 plus pre-S1 antigens (L protein, 226 + 51 + 163 amino acids long) (13,14) (Fig. 1). All three envelope proteins are found as components of the 42-nm-diameter infectious viral particles (Dane particle) (15). L and M constitute roughly 30% of the envelope protein content of the virus particle (16). S by itself and together with the larger envelope proteins also forms filamentous and spherical "surface antigen" particles that are secreted from infected cells in at least 100-fold excess over complete virions. Lacking viral nucleic acid, these particles are not infectious. Nevertheless, these spheres and filamentous particles can reach concentrations of several micrograms per milliliter of blood of HBV-infected patients (17). The reason for maintaining such a synthetic effort is still uncertain, but it could be connected to tolerization of immune responses and to the adsorption of neutralizing antibodies during the progression of infection. Binding of these particles with their cognate antibodies is probably responsible

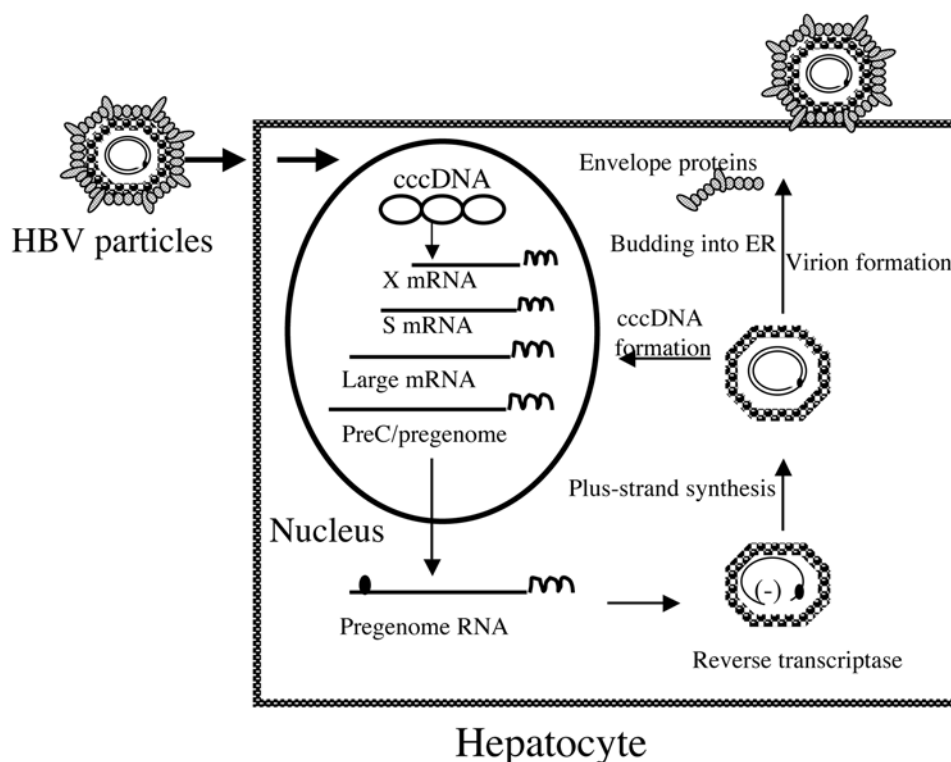


Fig. 2. Schematic representation of HBV life cycle.

for the immune complex syndromes that sometimes occur during HBV infection.

As already mentioned, the function of X protein is not completely understood, but it is essential for virus replication *in vivo* (6). Antibodies against X have been found in the sera of HBV-infected individuals (18,19), demonstrating its expression during natural infection. One other important point related to the replication of HBV is that the use of reverse transcriptase (5) results in a high rate of DNA mutations, owing to a lack of proofreading function by this enzyme.

### HOST-VIRUS RELATIONSHIP: PARAMETERS INFLUENCING DIFFERENT OUTCOMES

The variability in outcome of virus infections may depend on the balance between viral parameters that determine the ability of the virus to spread and persist and variables within the immune system that determine its efficiency in controlling virus replication (Table 2).

Viral parameters include the initial infectious dose, the kinetics of viral replication, and the ability to spread. These viral features are balanced by the variables of the immune system: kinetics, specificity, and duration of the humoral and cellular systems mediate immune response and other non-antigen-specific effector mechanisms such as activation of innate immune response and cytokine production.

The effect of these variables is usually difficult to study in humans but has been addressed in more detail in animal models of hepadnavirus infection.

Epidemiological data showed that the age of infection is a parameter that influences the outcome of HBV, with infection of neonates usually leading to persistent infection (20). Experimental work performed in ducks and woodchucks has confirmed the epidemiological data. Persistent infection with woodchuck hepatitis virus (WHV) followed the transmission of virus to neonatal animals, whereas infection of older animals is usually transient (21).

The effect of the virus dose on the outcome of infection is also supported by experiments in ducks and woodchucks. Higher doses of virus generally induced high rates of chronicity (21,22). However, the data here are less consistent. Infection with a single viral particle is sufficient to initiate persistent infection in ducks (23), and low doses of WHV have been shown to induce persistent infection in woodchucks (21,24). It is possible that the kinetics of viral replication, not tested in these studies, can influence the outcome of infection. Mathematical models of the relationship between the kinetics of virus replication and cytotoxic T-lymphocyte (CTL) expansion has indeed suggested that more slowly replicating viruses could induce a weaker CTL response (25). It is possible therefore that the replication speed of HBV, in addition to age at infection and quantity of initial inoculum, can influence the pathogenesis of HBV infection. In support of this hypothesis, HBV strains with enhanced viral replication have been demonstrated to be responsible for an epidemic of fulminant hepatitis (26,27). A further important point of the work performed in animals infected with hepadnaviruses is the fact that persistent



**Table 1**  
**Hepatitis B Virus Proteins**

<i>HBV proteins</i>	<i>Description</i>
Envelope or surface antigen (HBsAg)	Forms envelope of virions and noninfectious viral particles
Nucleocapsid antigen (HBcAg)	Assembles to form nucleocapsid
Antigen e (HBeAg)	Secreted protein that shares antigenic determinants with core antigen
X antigen	Essential for viral replication in vivo but dispensable for replication in vitro
Polymerase	RNA- and DNA-dependent DNA polymerase

**Table 2**  
**Parameters Influencing Outcomes of Infection**

<i>Viral parameter</i>	<i>Host parameter influencing immune response</i>
Dose of virus	Age of infection
Kinetics of viral replication	Genetic factors
Ability to spread	

infection does not evolve from a classical acute hepatitis. Chronicity in woodchucks appears in animals that, after infection, develop a diminished immune response with low production of cytokines and a low severity of acute hepatitis (28). This finding suggests that the initial strength of the immune response is a key factor that defines the outcome of infection.

## EARLY IMMUNE AND VIROLOGICAL EVENTS AFTER INFECTION

Innate immunity generally plays a role immediately after infection in limiting spread of the pathogen and initiating efficient development of an adaptive immune response. Innate host responses during the early phases of viral infections are mainly characterized by the production of type 1 interferon (IFN)- $\alpha/\beta$  cytokines and the activation of natural killer (NK) cells. Production of type 1 IFNs can be triggered directly by viral replication through cellular mechanisms that detect the presence of double-stranded RNA, whereas NK cells are activated by the recognition of stress-induced molecules and/or modulation of the quantity of MHC class I molecules on the surface of infected cells.

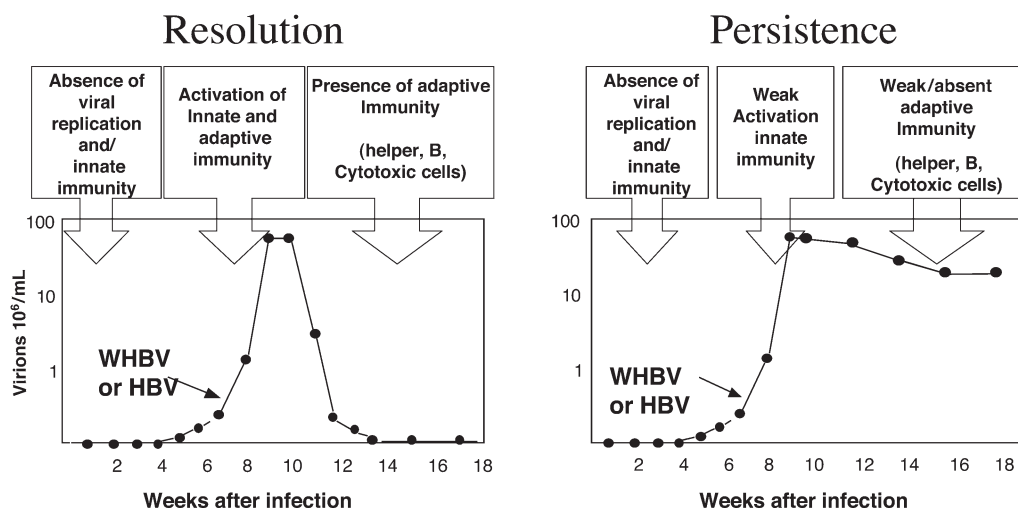
The general pattern of fast viral spread and subsequent rapid activation of innate immunity has been deduced primarily from mouse models of different viral infections (lymphocytic choriomeningitis virus [LCMV] and murine cytomegalo virus [MCMV]) (29) and holds true for many human viruses like HIV, cytomegalo virus (CMV), and Epstein-Barr virus (EBV). However, the simple observation of clinical, virological, and immunological phenomena that follow HBV infection depicts a completely different and unconventional pattern (Fig. 3).

Experimental data collected mainly in animal models, but also in humans (30), show that after inoculation, HBV does not immediately start to replicate efficiently. HBV DNA and HBV antigens are not detectable in serum or the liver until 4 to 7 wk post-infection (30–33). Following this period, HBV begins a logarithmic expansion phase that can be detected in the liver and serum, reaches levels of  $10^9$  to  $10^{10}$  copies/mL, and infects most hepatocytes (32–35).

The peculiarity of the kinetics of HBV replication has been largely ignored, and only recently has the comparison with HCV viral kinetics drawn attention to the unusual pattern of HBV replication (36,37). Rigorous experiments in chimpanzees showed that whereas HCV replication in the liver starts immediately after infection (38), larger doses of HBV inocula do not enter a logarithmic phase of replication until 4 to 5 wk after infection (33). The initial lag phase of HBV replication does not appear to be a consequence of HBV inhibition by elements of innate and adaptive immunity. HBV replication can be efficiently limited by IFN- $\alpha$  and - $\beta$  (39), but data on acutely infected chimpanzees suggest that such antiviral cytokines are not triggered by HBV replication (40). The activation of IFN- $\gamma$ , interleukin-2 (IL-2), and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and intrahepatic recruitment of inflammatory cells are delayed until the logarithmic expansion of HBV in experimentally infected woodchucks (28,41,42) and chimpanzees (32). Moreover, a recent elegant paper by Wieland et al. longitudinally analyzed the activation of cellular genes in three experimentally infected chimpanzees. In all three animals, no cellular genes were activated within the liver during the lag phase of infection, confirming that intrahepatic activation of innate immunity did not affect initial HBV spread (40).

It is possible to speculate that immediately after infection, HBV does not reach the liver but remains in other organs. Interestingly, longitudinal virological analysis of woodchuck hepatitis B virus (WHBV) infection showed that the initial site of WHBV infection was not the liver but the bone marrow (24). However, the lymphotropism of WHBV seems more pronounced and diffuse and to have greater pathological importance than that of HBV (24,43), and thus this possibility is attractive but still speculative in HBV infection. Alternatively, it is possible that HBV does target the liver but initially infects very few hepatocytes and, owing to a relatively slow doubling time, results in a lag phase between time of infection and detectable HBV DNA or proteins. However, at the moment, we cannot correctly delineate the fate of HBV in the first 4 wk after infection, and thus we have ignored the possibility that this apparent initial vanishing has an impact on the natural history of disease.

A further characteristic of HBV in relation to early host defence mechanisms resides in the lack of IFN- $\alpha$  and - $\beta$  production. Data on acutely infected chimpanzees suggest that such antiviral cytokines are not triggered by HBV replication (40). HBV might have evolved strategies to escape the initial antiviral defence mechanisms activated by the Toll-like receptor system. It has been proposed that because HBV replicates within nucleocapsid particles, the double-stranded RNA



**Fig. 3.** Coordinate activation of innate and adaptive response is necessary for HBV control. Data from: refs. 32, 33, 28, 51, and 21.

replicative intermediate, generally a strong activator of type I IFN genes, is protected from cellular recognition (40).

A note of caution should follow the analysis of these data. Hepatitis, after HBV infection, is generally mild in chimpanzees compared with humans, and it is possible that the inability to detect activation of genes related to innate immunity is a reflection of the mild profile of disease. Still, the striking difference between the early detection of type I IFN activation during early phases of HCV infection in chimpanzees (44,45) and its absence in HBV-infected animals is a further indication of the ability of HBV to sneak through the front-line host defence mechanisms.

### TRIGGERING HBV IMMUNITY

Immediately after the logarithmic phase of HBV expansion, chimpanzees able to control the virus show a typical acute phase of disease with robust activation of IFN- $\gamma$ , TNF- $\alpha$  (32) and many cellular genes linked to a Th1-type of cellular response (IFN- $\gamma$ , IFN- $\gamma$ -inducible protein-10 [IP-10], regulated on activation, T-cell expressed and secreted [RANTES]) (40). It is possible that this initial host response to HBV is primarily sustained by NK and NKT cells. Although we lack direct evidence of the role of NK and NKT cells during natural HBV infection, the experimental data are consistent with the possibility that the initial burst of IFN- $\gamma$  and the subsequent rapid inhibition of HBV could be mediated by these components of innate immunity. Activation of NKT cells in the transgenic mouse model of HBV infection can inhibit virus replication through the production of IFN- $\gamma$  (46,47). Here, NKT cell activation was a consequence of  $\alpha$ -galactoceramide stimulation rather than a response to the natural infection. However, recent results indicate that a population of nonclassical NKT cells can be directly activated when injected into mice expressing HBV antigens in the liver (48). Thus, NK and NKT cells could potentially be triggered during natural HBV infection, by the expression of stress signals either on infected hepatocytes

or liver dendritic cells (49) or possibly by direct recognition of viral components (48).

Work on acutely infected chimpanzees is again providing the strongest evidence that NK and NKT cells could be responsible for the initial control of HBV replication. In chimpanzees able to resolve the infection ultimately, a rapid drop in viral replication occurs in the presence of intrahepatic IFN- $\gamma$  production, before the massive recruitment of T cells (32). A sequence of events consistent with the contribution of NK cells in the initial inhibition of HBV replication was observed in patients studied during the incubation phase of acute hepatitis B. Increased numbers of circulating NK cells were concomitant with the peak of HBV replication, whereas, 2 to 4 wk later, HBV-specific CD8 T cells appeared when viral replication had already dropped (50).

A different pattern is observed when patients or animal models infected with *hepadnavirus* (WHBV) develop chronicity. Although virtually all patients who experience acute hepatitis B resolve the infection, development of chronicity is often associated with absent or mild symptoms of acute hepatitis. In line with these clinical observations, neonatally infected woodchucks that develop chronicity lack the large IFN- $\gamma$  and TNF- $\alpha$  production observed in resolved animals (28,41,42,51) and fail to develop an efficient antiviral specific immune response (Fig. 3).

Thus, activation of elements of innate immunity able to produce large quantities of IFN- $\gamma$  seems to be a factor that determines the subsequent efficient induction of adaptive immunity and ultimately the outcome of HBV infection. What is at the present unknown is what triggers this activation. Simple HBV quantity does not seem to be a separating criterion, since chronic patients ultimately reach HBV levels higher than are resolved. What seems to be well established is that the differences in the adaptive immune response to HBV that characterize chronic and resolved patients are heavily influenced by the immunological events occurring during the initial phase of HBV replication.

## PATTERNS OF ADAPTIVE IMMUNE RESPONSE IN RESOLVED VERSUS CHRONIC PATIENTS

The adaptive immune response is comprised of a complex web of effector cell types, all of which play key roles in development of immunity to HBV. CD4 T cells, classically referred to as helper T cells, are robust producers of cytokines and are required for the efficient development of effector cytotoxic CD8 T cells and B-cell antibody production. CD8 T cells go on to clear HBV-infected hepatocytes through cytolytic and noncytolytic mechanisms (52), reducing the levels of circulating virus, whereas B-cell antibody production neutralizes free viral particles and can prevent (re)infection (53).

There are clear differences in the adaptive immunity of patients with established chronic or resolved HBV infection. HBV-specific CD4 and CD8 T-cell responses with a Th type 1 profile of cytokine production are detectable in the blood of subjects with a favorable outcome. These helper and cytotoxic responses are quantitatively stronger than those found in patients with chronic infections, who are instead characterized by weaker or undetectable virus-specific T-cell responses (54–63). Whether the association between different outcomes of HBV infection and the vigor and breadth of the HBV-specific T-cell response has a causative effect has been difficult to demonstrate.

CD8 T-cell deletion experiments performed in HBV-infected chimpanzees have provided strong support for the concept that CD8 T cells are the main cellular subset responsible for viral clearance (33). Additional experiments in HBV patients or woodchucks demonstrate the importance of a coordinated helper and cytotoxic T-cell response in controlling hepadnavirus infection. In woodchucks, a reduced early expansion of virus-specific T cells was associated with virus persistence (51) whereas in patients studied during the incubation phase of acute HBV infections, expansion of virus-specific IFN- $\gamma$ <sup>+</sup> CD8 and CD4 T cells preceded complete virus clearance and was present only in subjects who controlled the infection (50). The importance of coordinated activation of CD4 and CD8 T cells has been further demonstrated by the recent analysis of one HBV-HCV acutely coinfecting patient who developed a chronic HBV infection. Longitudinal analysis of HBV-specific T-cell responses, from the time of infection to chronicity, showed the presence of a multispecific CD8 T-cell response in the absence of a CD4 T-cell response (64). It is likely that the absence of CD4 helped to prevent the maturation of a functionally efficient CD8 T-cell response. Another possibility is that cytotoxic T cells were directed toward HBV regions without protective values or prone to viral mutations that can escape CTL recognition. Additional indirect evidence that CD4 and CD8 T-cell responses are accountable for the immunological control of HBV is represented by the association of particular HLA class I and class II genetic profiles with resolution (65).

Even though the cellular immune response is a major contributor to HBV clearance, humoral responses also play a role in controlling HBV. HBV clearance is associated with the production of anti-envelope antibodies (66), and sera with high levels of antiviral antibodies (specific for the viral

envelope) can control HBV infection (53). Therefore, it is likely that the integrated activation of both the cellular and humoral arms of the adaptive immune response ultimately allows the host to control infection the different components being so interconnected that the failure of one of them clearly affects the expansion and protective efficacy of the others. A lack of CD4 T-cell help can impair CD8 T-cell activity and antibody production, whereas the inability to mount a virus-specific CD8 T-cell response results in a level of circulating virus that cannot be cleared by antibodies alone (67).

## IMMUNOLOGICAL HIERARCHY OF HBV-SPECIFIC CD4 AND CD8 T-CELL RESPONSES

### HELPER T-CELL RESPONSE

HBV-specific, HLA class II-restricted CD4 T-cell responses have been characterized mainly in patients with self-limited acute hepatitis (54,55,68). Multiple epitopes within the nucleocapsid protein are targeted by helper T cells of patients with self-limited hepatitis, and immunodominant core epitopes have been identified within a sequence covering region 50 to 69, which can stimulate helper T cells in 90% of patients tested, irrespective of HLA class II profile (69). The demonstration that increased core-specific CD4 responses are detectable during exacerbations of chronic hepatitis B, preceding HBeAg seroconversion (indicative of a reduced level of viral replication) (70), might represent an indication of the importance of the nucleocapsid-specific CD4 response in controlling HBV.

A different scenario is instead present for the envelope-specific CD4 T-cell response. In contrast to the immunogenicity of core antigen, the HBV envelope protein does not seem to expand an equally strong helper T-cell response during HBV infection (54,71). The limited expansion of envelope-specific CD4 cells does not imply that envelope is a generally weak immunogen. On the contrary, the HBV envelope protein elicits strong helper T-cell responses in subjects vaccinated with a plasma-derived or recombinant form of this antigen (71,72). The differential immunogenicity of envelope antigens in vaccine recipients and in patients with natural infection suggests that differences in antigen presentation and/or the presence of “natural” or synthetic adjuvant influences the immunogenicity of the responses in these two groups.

Even though most of the data have identified nucleocapsid-specific CD4 T cells as the dominant helper response correlating with HBV recovery, other aspects need to be considered. In particular, the helper T-cell response specific for the polymerase and X antigens have not been sufficiently investigated, and only recently have polymerase epitopes able to elicit CD4 T-cell responses been identified (73). These polymerase epitopes were conserved among the different HBV genomes, bound to the most common HLA-DR and induced, in resolved acute hepatitis B patients, a helper T-cell response comparable to that detected against core peptides.

### CYTOTOXIC T-CELL RESPONSE

Analysis of the HLA class I-restricted CD8 T-cell response to HBV has been severely hampered by the inability of HBV to be propagated in cell culture (74). The first definitive

characterization of CD8 T cells specific for HBV derived from the understanding that the sequence of the processed viral antigens presented by HLA class I molecules could be mimicked by synthetic peptides (58,75). Thus, CTLs specific for several viral epitopes within core (58,75,76), envelope (77), polymerase (57), and X (78) proteins of HBV were achieved using synthetic peptides, and not naturally processed epitopes, to expand memory CTLs *in vitro*. These initial studies demonstrated that the magnitude of the HBV-specific CD8 response is stronger in self-limited than in chronic infection (58,75), that the CTL response persists decades after clinical recovery from acute infection (79), and that it can also be observed after resolution of chronicity (80). These studies have been carried out using peptides able to bind specifically to HLA-A2 molecules, with the result that a disproportionate number of known HBV epitopes are HLA-A2 restricted. However, HBV-specific cytotoxic epitopes restricted by different HLA class I molecules (76,81–83) have also been identified.

The development of methods such as MHC/peptide tetramer staining, intracellular cytokine staining, and Elispot, which are able to quantify virus-specific CD8 cells directly *ex vivo*, has permitted a more accurate analysis of HBV-specific CD8 T cells during the different phases of HBV infection. These data confirmed the quantitative differences between self-limited and chronic infection (59,60) and demonstrated that the quantity of HBV-specific CD8 T cells correlated with HBV control and not with liver damage (84). This work also revealed that an epitope hierarchy exists within the HBV-specific CD8 T-cell responses that can be altered by viral persistence. Core 18 to 27 specific CD8 cells often represent the dominant response among the different A2-restricted epitopes tested in patients with acute hepatitis, but this is not absolute. In some patients, Pol 455 to 63, Env 183 to 91, or Env 335 to 43 specific CD8 T cells were found to dominate the CD8 T cell response quantitatively (50,62).

The cause of immunodominance of these sequences is likely linked to their good binding affinity to the HLA-A2 molecule. A further possible explanation of the dominance of these HLA-A2-restricted CD8 responses is the finding that some HLA class I epitopes are nested within helper T-cell epitopes. CD4 helper T cells are necessary for the maintenance of functional CD8 T cells, and the covalent linkage between helper and cytotoxic epitopes has been shown to be important for the induction of CTL responses (85). The well-characterized, often immunodominant, HBe18-27 epitope overlaps with an HLA class II-restricted epitope (86), and similar features have been described for new polymerase CD8 T-cell epitopes (73). It must, however, be stressed that the overall hierarchy of CTL responses is still incomplete, and there is no information available about competition among epitopes restricted by different HLA class I alleles.

Despite these limitations, the detailed analysis of HBV-specific CD8 responses has led to important information regarding the potential impact of different CTL specificities on HBV immunopathogenesis. Amino acid mutations within the core 18 to 27 region able to inhibit activation of the core 18 to 27 specific CD8 cells have been shown to occur in

patients with chronic hepatitis B (87). In contrast, mutations within polymerase and envelope epitopes are rare (88) and cannot be identified even in chronic patients who demonstrate the presence of envelope and polymerase-specific CD8 cells (62), suggesting that the antiviral pressure of the core 18 to 27 specific CD8 response is greater than the response against polymerase and envelope epitopes.

Longitudinal analyses of HLA-A2-restricted HBV-specific CD8 T cells in resolved and chronic hepatitis B patients have also revealed that the functional fate of epitope specificities differs markedly in chronic infection. A combined direct *ex vivo/in vitro* analysis of HBV-specific CD8 cells in chronic patients with different disease profiles demonstrated that core 18 to 27 specific CD8 T cells (often immunodominant in self-limited hepatitis) cannot be detected in the circulation and liver (either directly *ex vivo* or after *in vitro* expansion) when HBV-DNA levels are greater than  $10^7$  copies/mL (62).

Envelope and polymerase-specific CD8 T cells are the only specificities that can be demonstrated in chronic hepatitis B patients with concentrations of HBV DNA greater than  $10^7$  copies/mL (62,89). Their ability to persist in the face of high levels of HBV replication is associated with an apparent inability to display an antiviral function. Envelope-specific CD8 cells are characterized by an altered phenotype (tetramer/neg) (89), and their indifference to the dynamic fluctuations of HBV DNA levels is suggestive of a tolerant state. The persistence of polymerase-specific CD8 T cells could be the result of the low quantity of polymerase epitopes expressed *in vivo* by infected hepatocytes, as suggested by results obtained in the transgenic mouse model of HBV infection (90).

## THE COLLAPSE OF HBV-SPECIFIC T-CELL RESPONSE IN CHRONIC HBV PATIENTS

We have seen how the inability to control HBV infection and the establishment of chronicity lead to a state of relative collapse of virus-specific adaptive immunity. This state of HBV-specific T-cell tolerance is not absolute but appears to be mainly regulated by the quantity of HBV replication present in chronic hepatitis B patients. The impact of viral load on anti-viral T-cell responses has been precisely characterized in animal models of viral infections (like LCMV), all of which show that the sustained presence of viral antigens leads to a progressive functional decline of virus-specific CD8 responses (Fig. 4) and ultimately virus-specific T-cell deletion (91). Similarly, in HBV-infected patients, the frequency and function of circulating and intrahepatic HBV-specific CD8 T cells is inversely proportional to the level of HBV-DNA (61,62).

The factors that might contribute to the state of virus-specific T-cell collapse present in chronic hepatitis B patients are summarized in Fig. 5.

### HBeAg AND HBsAg

HBeAg, a secretory form of the nucleocapsid antigen, is produced in large excess during HBV replication (4). The tolerizing effect of HBeAg has been well characterized in mice (92) and likely contributes to the low level of core-

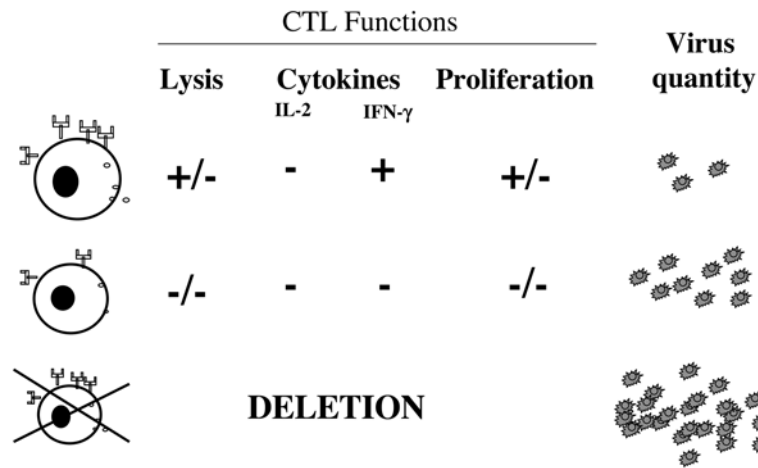


Fig. 4. Correlation of T cell with viral replication levels. Animal model. From ref. 191.

specific T-cell responses present in HBeAg<sup>+</sup> chronic patients. Clinical evidence supports the tolerogenic effect of HBeAg. Exacerbations of chronic hepatitis B are often associated with selection of HBV unable to produce HBeAg (93). In addition, HBV replication is linked to the production of excessive amounts of the soluble form of HBsAg. Particles composed of only surface antigen are present in 10<sup>3</sup> to 10<sup>6</sup> fold excess over whole virions (4). These particles are not infectious, but the evolution of such impressive levels of synthetic effort by HBV may deliberately cause a state of low T-cell response and T-cell deletion.

#### REGULATORY T CELLS (CD4<sup>+</sup> AND CD25<sup>+</sup>)

Studies in numerous experimental models have provided evidence that a population of specialized T cells is able to regulate the immune response. These cells reside mainly within a minor population of CD4 cells that express the phenotypic marker CD25. They have been shown to suppress immunological responses against self and foreign antigens through suppressive cytokines or direct cell-cell contact; however, the regulatory effects of CD4<sup>+</sup>/CD25<sup>+</sup> cells have not been fully elucidated (94). It is possible that CD4<sup>+</sup>/CD25<sup>+</sup> T cells are responsible for the weak HBV-specific T-cell response in chronic hepatitis B patients and may inhibit the expansion and function of HBV-specific CD8 T cells, precluding HBV clearance but also limiting immune-mediated liver damage.

The impact of circulating CD4<sup>+</sup>/CD25<sup>+</sup> T cells on HBV pathogenesis has recently been analyzed. Increased frequencies of circulating regulatory cells in patients with chronic hepatitis B have been reported in some (95) but not in other studies (96). Depletion of CD4<sup>+</sup>/CD25<sup>+</sup> cells increased the function of HBV-specific T cells (95,96), but such modulation was not HBV specific and could be observed in patients with resolved HBV infection (96). This casts doubts on the possible role of CD4<sup>+</sup>/CD25<sup>+</sup> regulatory cells in the pathogenesis of chronic HBV infection. However, these studies were limited to analysis of the CD4<sup>+</sup>/CD25<sup>+</sup> cells present in the blood, and a detailed analysis of the intrahepatic frequency and function of these

cells is likely necessary to reveal their role. Furthermore, it is possible that a population of HBV-specific regulatory cells, different from the CD4<sup>+</sup>/CD25<sup>+</sup> T-cell subset, analogous to the presence of IL-10-producing HCV-specific T cells (97), might be induced in chronic HBV infection (98).

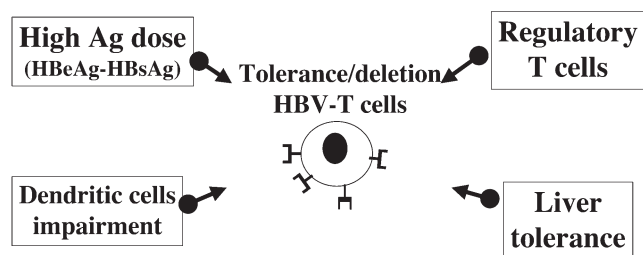
#### DENDRITIC CELLS

Dendritic cells represent a specialized antigen-presenting cell population necessary for the induction of an adaptive immune response (99). In relation to their crucial role in T-cell priming, functional alterations in dendritic cell populations could explain the state of T- and B-cell hyporesponsiveness present in chronic hepatitis B patients. However, even though dendritic cells are likely to be infected in animal models of *hepadnavirus* infection (43) productive HBV replication has recently been excluded in chronic hepatitis B patients (100), and the stimulatory defects seem minimal (101–104). Thus, the role of dendritic cell functional impairment in maintaining a state of HBV-specific T-cell tolerance is, at the moment, controversial.

#### LIVER ENVIRONMENT

The immunological features of the liver might contribute to the maintenance of immunological tolerance present in chronic HBV infection. Data produced mainly in animal models have shown that CD8 T-cell induction, expansion, survival, and antiviral function are altered following activation by antigens presented on hepatocytes. In mice, hepatocyte priming of CD8 T-cells preferentially induces tolerance and results in reduced CD8 T-cell clonal expansion (105–107). It has also been demonstrated that apoptosis of activated CD8 T cells preferentially occurs in the liver (108), although recent work in mice has shown that rapid activation of naive or effector CD8 T cells within the liver was followed by efficient expansion (109).

Hepatocytes express low levels of MHC class I and require nearly 100-fold higher peptide concentrations compared with other antigen-presenting cells to stimulate equivalent numbers of virus-specific CD8 T cells (Gehring et al., in preparation).



**Fig.5.** Possible causes of HBV-specific T cell tolerance during HBV persistence.

This would suggest that any pathogen infecting hepatocytes is less likely to be recognized by CD8 T cells and might allow HBV to avoid recognition when viral replication is reduced.

### PATHOGENESIS OF LIVER DAMAGE DURING HBV INFECTION

Since HBV is a noncytopathic virus, it has been assumed that the extent of liver damage is proportional to the recognition of infected hepatocytes by CTLs. However, the demonstration that large quantities of HBV can be cleared by noncytopathic mechanisms has challenged this model (110). Several studies have shown that when viruses infect hepatocytes, they are more likely to be controlled by intracellular inactivation mediated by cytokines than by direct killing. This noncytopathic mechanism of viral clearance is not peculiar to HBV but can also be seen in the liver clearance of MCMV (111) and *Listeria monocytogenes* (112) infections.

However, just because hepatocytes are capable of activating intracellular events leading to viral control through cytokine stimulation, this does not imply that they are completely resistant to direct CTL-mediated lysis. Hepatocytes are resistant to perforin/granzyme-mediated killing (113), but lysis of hepatocytes by Fas or perforin-mediated mechanisms has been clearly demonstrated (114), and an increase in transaminase level is always present during HBV clearance in patients with acute hepatitis (50). Furthermore, a degree of apoptosis and regeneration of hepatocytes occurs in acute and chronic liver damage in woodchuck hepatitis virus infection (115), showing that hepatocyte lysis also occurs during virus control.

The ability to identify antigen-specific T cells directly *ex vivo* using HLA class I tetramers has provided the opportunity to investigate the relationship between HBV-specific T cells and liver damage in humans (84). Chronic HBV-infected patients lacking evidence of liver damage but controlling HBV replication possess functionally active HBV-specific CD8 cells both in the circulation and in the liver. By contrast, patients with a high level of HBV replication and evidence of liver inflammation show a different pattern of virus-specific CD8 cells. The frequency of intrahepatic CD8 cells specific for core 18 to 27 was much lower in these patients owing to their dilution in a large infiltrate of apparently antigen-nonspecific T cells. However, the actual number of intrahepatic HBV-specific CD8 cells was similar to that seen in patients without liver disease,

taking into account the difference in the size of the total CD8 infiltrate. These results in chronic HBV infection show that comparable numbers of intrahepatic virus-specific CD8 cells could be associated with either protection or pathology (84).

Thus, the quantity of virus-specific cells does not appear to be the variable directly determining the extent of virus-induced liver pathology. Hepatic pathology could be the consequence of the large infiltrate of antigen-nonspecific mononuclear cells, since this is the one variable that correlates with the extent of liver inflammation. The importance of non-antigen-specific T-cell recruitment in the pathogenesis of liver damage has been shown in a transgenic mouse model of fulminant hepatitis (116) and in the concanavalin A-induced model of hepatitis (117,118). The recruitment of non-antigen-specific CD8 cells seems to be mediated by IFN- $\gamma$  (119). This cytokine should therefore be seen not only as an antiviral cytokine able to clear infection without causing liver damage, but also as a typical inflammatory cytokine, causing activation of macrophages and increased susceptibility to TNF-mediated hepatic damage (120) and initiating the recruitment of T cells, NK, or NKT cells (119).

A schematic model of HBV-mediated liver pathogenesis could be drawn from these data and is represented in Fig. 6.

### CONCLUDING REMARKS AND OPEN QUESTIONS

Increased knowledge of the virological and immunological events secondary to HBV infection allows us to define the mechanisms involved in viral clearance and persistence and disease severity. Analysis of early events following HBV infection has revealed that HBV fails to activate early immunological responses, which are delayed until the logarithmic phase of replication (40,50). Interestingly, the delayed kinetics of viral replication can explain why HBV vaccines are able to prevent infection, even if they are administered after exposure (121). Even though virus-specific CD8 T cells play a major role in HBV clearance (33), coordinated activation of the different branches of adaptive immunity seems necessary to achieve viral control. When chronicity develops, diffuse defects of helper and cytotoxic T-cell responses are apparent and are likely to be maintained by the concerted action of high levels of viral antigens, by the peculiar immunological features of the liver, and perhaps by the contribution of regulatory cells or dendritic cell defects. The immunological defects are proportional to the level of HBV replication, and inhibition of viral replication through antiviral treatment results in partial restoration of HBV-specific T-cell immunity (122,123), which, however, is inadequate to achieve viral clearance.

Vaccine treatments based on the concept of restoration of HBV-specific T-cell response in patients with chronic hepatitis B to the levels found in subjects controlling the infection have been actively tested in recent years in animal models (124) and in humans, with often limited therapeutic success (125–128).

It is likely that viral chronicity alters the repertoire of HBV-specific immunity to a level that makes its functional

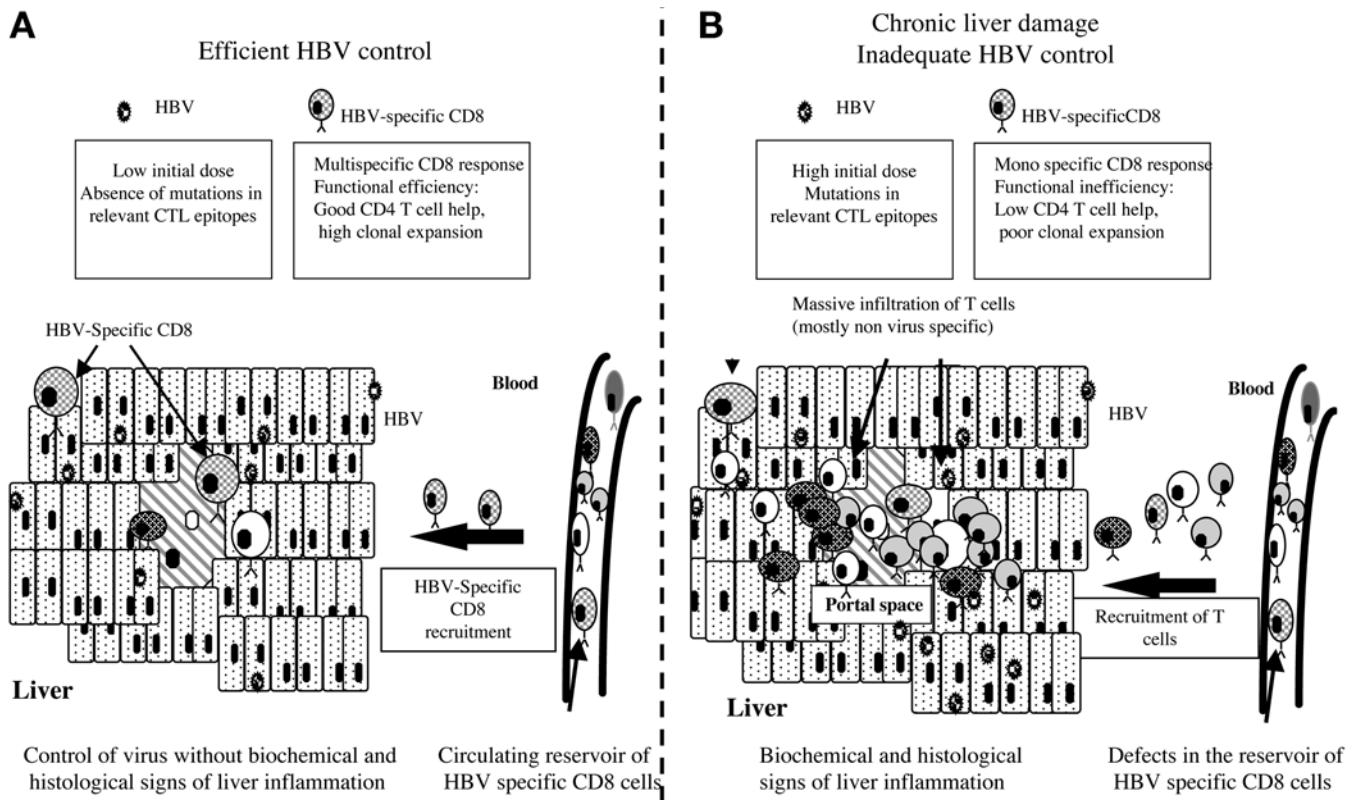


Fig. 6. Role of CD8 T cells in liver damage during chronic hepatitis B.

restoration very complex. Therapeutic vaccination combined with new potent antiviral drugs (129), which efficiently inhibit HBV replication, might achieve a better recovery of HBV-specific T-cell response and constitute a safer approach, owing to the problem of balancing the stimulation of specific immune response with the quantity of infectious virus.

Use of dendritic cells or production of potent cytotoxic and helper T cells through T-cell receptor transfer are strategies under investigation to improve the therapeutic chances of controlling this infection. It is hoped that our increasing understanding of the immunology of hepatitis B will lead to the development of immune-based therapies that, in conjunction with presently available therapies, may improve the prospects of long-term viral control for the very many people with chronic HBV infection.

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# 15 Immune Responses in Acute and Chronic Hepatitis C

## *Implications for Prognosis and Therapy*

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### KEY POINTS

- The hepatitis C virus (HCV) is a noncytopathic single-stranded RNA virus belonging to the Flaviviridae family. Worldwide, an estimated 130,000 million people are chronically infected with HCV.
- HCV RNA can be detected within 1 wk after infection. Spontaneous clearance of HCV RNA occurs in 10 to 50% of cases, depending on the severity of symptoms.
- Chronic hepatitis C is a major cause of end-stage liver disease and hepatocellular carcinoma. The risk of developing liver cirrhosis is highly dependent on coexisting host and environmental risk factors such as alcohol consumption, overweight, diabetes, and coinfections with other viruses and may range from 1 to 40% after 20 to 30 yr.
- Innate immune responses contribute to early control of viral replication. However, HCV may be partially resistant to type I interferons *in vivo* and may also directly inhibit NK cell function.
- A humoral immunity against HCV becomes detectable after 4 to 24 wk of infection in most but not all HCV-infected patients. Although antibodies with potential neutralizing capacity can develop, the clinical significance of these findings remains controversial. There is no long-term protective humoral immunity, and anti-HCV antibodies decline after recovery from acute infection, in some cases to undetectable levels 10 to 20 yr after recovery.
- A strong and broad HCV-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cell response is associated with spontaneous clearance of acute HCV infection. Functionally impaired HCV-specific CD8<sup>+</sup> T cells are detectable in patients with chronic hepatitis C. Possible explanations have been suboptimal IL-2 production, weaker stimulatory capacities of dendritic cells, HCV core-induced downregulation of C1q-mediated IL-12 production of macrophages, and host genetic factors.
- T cells with regulatory functions can be found at higher frequencies in the blood of chronic hepatitis C patients and also in HCV-infected livers, possibly contributing to weaker T-cell responses.
- Viral escape can occur and may affect antibody recognition, T-cell-receptor stimulation, MHC binding, and epitope processing.
- HCV-specific T cells have been induced by peptide and protein vaccination in chimpanzees and humans. Whether these T-cell responses confer protective immunity in humans remains to be shown. The first trials of therapeutic vaccination have been completed, showing an induction of humoral and cellular immune responses in chronically infected patients. However, significant changes in viral load have not been observed, so far.
- Heterologous immunity may significantly contribute to the outcome of acute and chronic HCV infection. CD8<sup>+</sup> crossreactivity between HCV and the influenza A virus has been shown and linked to cases with subfulminant acute hepatitis C.
- Early treatment of acute hepatitis C infection with (pegylated) interferon- $\alpha$  can prevent development of a chronic course in 80 to 98% of patients. T-cell responses decline during therapy of acute hepatitis C and are not correlated with the treatment outcome.
- Therapy of chronic hepatitis C with pegylated interferons and ribavirin leads to sustained virological responses in about 50% of patients infected with HCV genotype 1 and 80 to 90% of patients infected with HCV genotypes 2 and 3.
- Novel therapies including HCV-enzyme inhibitors and Toll-like receptor stimulators are currently in phase I to II trials.

### INTRODUCTION

The hepatitis C story involves molecular biologists, virologists, immunologists, mathematicians, epidemiologists, and clinicians. It is one of the most impressive examples in modern medicine in which a combination of expertise from very different fields helped to identify the cause of a major disease and led to the development of effective therapies.

In the seventies there was already much evidence that a non-A/non-B hepatitis virus must exist, and many research groups around the world were searching for the needle in the haystack. In 1989 the identification of the hepatitis C virus (HCV) by M. Houghton and co-workers (1) subsequently gave an enormous boost to research in the field. Now, 18 years after the discovery of HCV, we have detailed information on the prevalence, transmission, replication, natural history, and pathogenesis of the virus. Most importantly, the majority of infected patients can be treated successfully. For patients infected with HCV genotypes 2 or 3, HCV infection is a curable disease. The latest advances, include the development of novel antiviral and immunostimulatory treatment approaches; eradication of HCV seems to be an achievable goal for the future.

Since HCV is a noncytopathic virus in most circumstances, the immune response almost certainly plays a central role not only in control of replication but also in the pathogenesis of liver disease. Therefore, the main focus of this chapter is our current knowledge of adaptive and innate immune responses against HCV. In the second part, the latest developments in the treatment of acute and chronic hepatitis C are summarized.

## EPIDEMIOLOGY

An estimated 130 million people worldwide are infected with the hepatitis C virus (2). The prevalence of anti-HCV-positive individuals ranges from less than 0.2% in northern Europe to more than 15% in some African countries. Until 1990, HCV was frequently transmitted by blood transfusion. After the introduction of blood screening for anti-HCV antibodies, the epidemiology of HCV infection changed dramatically. The risk of acquiring HCV by blood products has been reduced to less than 1:500,000. Meanwhile, all blood products are screened for HCV RNA by polymerase chain reaction (PCR) and thus, the risk of becoming infected by transfusion is close to zero in developed countries (3). Nowadays, most patients with acute HCV infection are intravenous (IV) drug users. The prevalence of HCV in IV drug users has been reported to be up to 90% (4). Sexual transmission might occur; however, this route of infection is relatively inefficient, as sexual partners of HCV-infected persons in monogamous relationships become anti-HCV positive in only 1 to 3% of cases (5). On the other hand, recent studies of acute HCV infection demonstrated that sexual contact with an HCV-positive partner was the only risk factor to be identified in 20 to 30% of patients (6). The risk of vertical transmission of HCV is 1 to 5% (5). Other potential exposures (occupational, hemodialysis, household) account for about 10% of new infections.

## NATURAL HISTORY OF ACUTE AND CHRONIC HCV INFECTION

HCV RNA can be detected in the serum as early as 3 to 7 d after infection, which is in sharp contrast to HBV infection (see Chapter 14) (7). Symptoms such as abdominal pain and jaundice develop in 20 to 50% of infected patients after 4 to 8 wk; however, since acute hepatitis C is asymptomatic in many cases, the infection is often unrecognized. Thus, many studies are hampered by the fact that the true rate of infection after

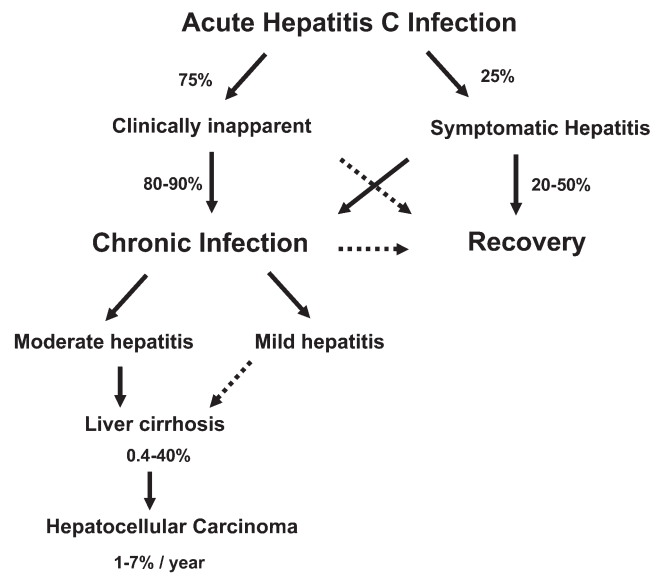


Fig. 1. Course of hepatitis C infection.

exposure is not known. Moreover, since anti-HCV antibodies do not develop in all patients, the number of patients with transient HCV infection may be underestimated. We recently performed systematic screening for HCV markers of more than 1100 inmates in the largest German young offender institution and identified 6 HCV RNA-positive/anti-HCV-negative patients (8). Four of those had cleared HCV spontaneously at further follow-up and remained anti-HCV negative. Thus, these individuals would never have been recognized as being viremic for HCV outside this systematic screening approach. These data are in line with the fact that the prevalence of HCV in health care workers is low in Western countries and was not higher in medical health professionals compared with nonmedical professionals in several studies (9–11). The risk of developing acute hepatitis C virus infection after exposure to HCV by an HCV-contaminated needle has been reported to be between 0 and 5% (5,12), with most studies reporting seroconversion rates below 1%. Acute HCV infection has a chronic course in 50 to 90% of cases (Fig. 1); the more symptomatic a patient is, the higher his or her chance of clearing the virus (13).

Although it is widely accepted that HCV infection is a major cause of end-stage liver disease and hepatocellular carcinoma, the natural history and hence the prognosis of chronic infection are still controversial (Table 1). An accurate determination of the natural history of hepatitis C is hampered by the facts that the initial onset of infection is usually devoid of signs and symptoms, that the disease course during the chronic phase is usually unaccompanied by symptoms, and that the duration to development of end-stage liver disease may exceed 30 to 40 yr. Chronic carriers usually have either only minimal or moderate hepatitis. The risk of developing liver cirrhosis may range from 0.4 to 40%. Transfusion-associated hepatitis C seems to be more aggressive, leading to cirrhosis in as many as 35% of the cases after 25 yr of infection if liver enzymes are persistently elevated

**Table 1**  
**Studies Investigating the Natural History of Hepatitis C Infection**

Author	Cohort	Number of patients (n)	Follow-up (yrs)	Cirrhosis
Vogt et al., NEJM 1999	Children after heart surgery	458	17	0.3%
Wiese et al. Hepatology 2000 J Hepatol 2005	Young women, Contaminated anti-D immune globulin, Germany	1980	20 25	0.4% 1.3%
Kenny-Walsh et al. NEJM 1999	Young women, Contaminated anti-D immune globulin, Ireland	710	17	2.0%
Seef et al., Hepatology 2001	Post-transfusion hepatitis	222	25	35% (if ALT is elevated)
Seef et al. Ann Int Med 2000	US military recruits	17	45–50	5.9%
Poynard et al. Lancet 1997	HCV patients undergoing liver biopsy	2235	Not prospective	33% after 20 yrs
Niederrau et al. Hepatology 1998	Large prospective cohort study	838	9–22	16%

(14). By contrast, the risk of progressive liver disease was much less in retrospectively identified cohorts of children or young adults infected with HCV, with rates of cirrhosis ranging from 0.4 to 5% after 17 to 45 yr (15–19). Paired biopsy studies have shown a progression rate of 0.1 to 0.2 fibrosis units per year, with more rapid progression in elderly patients (20). Once liver cirrhosis is present, hepatocellular carcinoma can develop in up to 7% per year (21).

The natural history of HCV is heavily influenced by co-existing factors such as alcohol consumption, HBV coinfection, HIV coinfection, genetics, and other liver diseases like hemochromatosis (Table 2). Coinfection with *Schistosoma mansoni* is a major problem in Egypt, with a prevalence of 18 to 43%, leading to significantly accelerated progression of liver disease associated with changes in the Th1/Th2 pattern of HCV-specific immunity (22). HIV-infected patients with chronic hepatitis C show more severe liver disease. After the introduction of highly active antiretroviral therapy for HIV infection, HCV-related liver mortality has become the leading causes of death in HIV/HCV-coinfected individuals (23). Similarly, liver disease is more severe in HBV/HCV coinfection, with higher fibrosis scores and more frequent cases of hepatocellular carcinoma compared with HBV mono-infected patients (24).

## THE HEPATITIS C VIRUS

The hepatitis C virus is a member of the Flaviviridae family of viruses, which consists of pestiviruses, flaviviruses, and hepaciviruses (Fig. 2). Viruses belonging to this family all have positive-sense single-stranded RNA genomes with a similar organization. The recently identified GB viruses A, B, and C have also been classified as members of the Flaviviridae and are most closely related to HCV.

The HCV genome consists of approx 9600 nucleotides (25). The open reading frame produces a polyprotein of about 3000 amino acids that is cleaved into at least ten structural and

**Table 2**  
**Determinants of Progression of Liver Disease in Chronic HCV Infection**

- Viral factors
  - genotypes (Genotype 2: Hepatitis flares; Genotype 3: steatosis?)
  - quasispecies (?)
  - mutations in T cell epitopes (?)
- Host factors
  - Age at infection (+)
  - Duration of infection (++)
  - Gender (+)
  - Other liver diseases (e.g., hemochromatosis) (+)
  - Diabetes (+)
  - Body mass (++)
  - Other genetic factors (KIRs, HLA-type, cytokine promotor polymorphisms, etc.)
- External factors
  - Alcohol (+++)
  - Diet (?)
  - Smoking (+)
  - Coinfections with HIV or HBV, Schistosomiasis, etc. (++)

nonstructural proteins by a combination of host and viral proteases (Table 3). A distinct characteristic of HCV is its genetic heterogeneity. Six major genotypes and more than 100 subtypes have been described. The impact of HCV genotypes on the long-term outcome of HCV infection is still controversial. Although many studies could not find a clear correlation between progression to cirrhosis and HCV genotype, there is increasing evidence that HCV genotype 3 infection causes liver steatosis (26) and that HCV genotype 2 can be linked to more frequent hepatitis flares (27). Moreover, it is well established that the response to interferon (IFN) therapy is very different between HCV genotypes (28).

HCV is an enveloped virus. The viral RNA is associated with the capsid protein, which is surrounded by a lipid-containing

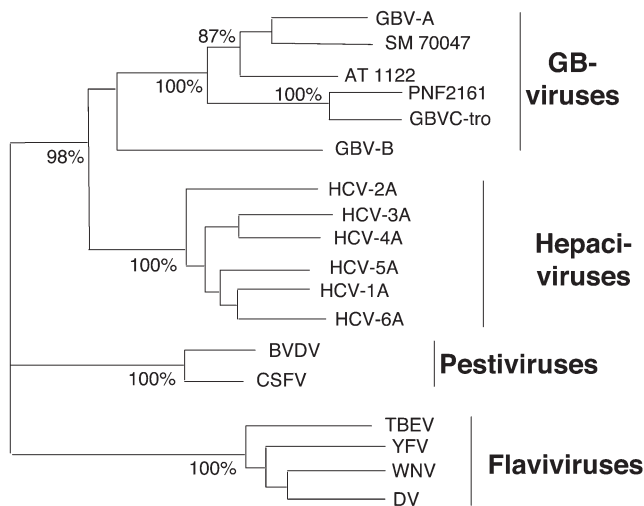


Fig. 2. The Flaviviridae family.

Table 3  
HCV Structural and Non-Structural Proteins

Protein	Molecular mass (kD)	Function
Core	21	Capsid protein
E1	31–35	Envelope protein
E2	68–72	Envelope protein, interaction with PKR + CD81
P7	7	Unknown, ion channel ?
NS2	23	Protease: NS2/3 cleavage cellular cofactor required
NS3	70	Serine protease: NS3/4A; 4A/4B; 4B/5A; 5A/5B cleavage
NS4A	8	NTPase, RNA-helicase
NS4B	27	Forms complex with NS3, cofactor for NS3 protease
NS5A	58	unknown
NS5B	68	Unknown; contains ISDR; interaction with PKR
		RNA-dependent RNA polymerase

Abbreviations: ISDR, interferon-sensitive determining region; PKR, interferon-induced cellular protein kinase.

envelope formed by the two viral glycoproteins E1 and E2. HCV is about 30 to 60 nm in diameter. The mechanism by which HCV enters cells to initiate infection is not known. Binding of the HCV E2 protein to the second extracellular loop of CD81 has been demonstrated (29). This loop is heterogeneous in sequence among different animal species but conserved between humans and chimpanzees, which could explain why the virus is infectious only in these species. However, CD81 is expressed on almost all nucleated cells, which does not explain the hepatotropism of HCV. Even if binding of E2 to CD81 does not lead to HCV entry into hepatocytes, E2-CD81 interaction seems to be important for the regulation of innate immune responses via the inhibition of natural killer (NK) cell activity (30,31). Another mechanism for HCV cell entry proposes that HCV forms complexes with very low-density lipoproteins or low density lipoproteins

(LDLs), thus suggesting endocytosis of HCV via the LDL receptor (32). Other HCV receptor candidates are scavenger receptor class B type I, the mannose-binding lectins DC-SIGN and L-SIGN, LDL receptor, heparan sulfate proteoglycans, and the asialoglycoprotein receptor (33). An infectious cell culture system was recently developed, allowing the study of HCV entry and early steps in HCV infection (34,35).

The function of the p7 protein is largely unknown. Interestingly, HCV virus-like particles (HCV-VLP) lacking p7 generated a higher cellular immune response with a more Th1-like profile than particles without p7 in BALB/c mice immunized with HCV-VLP (36), indicating a potential role for this short protein in the regulation of HCV-specific cellular immune responses. Moreover, HCV-p7 has an ion channel-like structure and may also be a target for novel antiviral approaches. It has been suggested that amantadine exerts some antiviral effects by blocking p7 (37).

Although the hydrophobic NS2 protein together with the N-terminal part of NS3 forms a protease mediating the cleavage of the NS2/NS3 junction, the NS3 protein has multiple functions. The carboxy terminus of NS3 contains an NTPase providing energy and an RNA-helicase unwinding duplex RNA during genomic replication. A serine protease cleaving several downstream junctions is formed by a stable complex of NS3 and NS4A. The HCV serine protease NS3-NS4A blocks the production of type I interferons in vitro via inhibition of interferon regulatory factor-3 (IRF-3) and thus may be of great importance for regulating innate immune responses (38). The NS5A protein has been implicated in the modulation of the host's IFN-mediated antiviral response. Mutations in a region called the IFN-sensitive determining region (ISDR) correlate with response to IFN therapy (39). The interaction of NS5A with an IFN-induced cellular protein kinase (PKR) could represent a mechanism to explain this observation. PKR may also interact with specific sequences within the HCV E2 protein (40). Proposed functions of structural and nonstructural proteins are summarized in Table 3.

The crystal structures of NS3, the NS3 protease-NS4A complex, and NS5B have been determined, allowing the generation of inhibitors of HCV replication. Phase I/II trials investigating HCV protease and polymerase inhibitors are ongoing. Some of the novel compounds showed a remarkable 2 to 4 log<sup>-10</sup> suppression of HCV replication after only a few days of treatment (41–43). Future trials should investigate the resistance profile of enzyme inhibitors and explore combination therapies with IFN and immunomodulatory approaches.

## IMMUNOPATHOGENESIS OF HEPATITIS C

Since HCV is a noncytopathic virus in most circumstances, the immune response almost certainly plays a central role not only in control of infection but also in the pathogenesis of liver disease. On the one hand, symptomatic patients with acute HCV infection are more likely to recover than asymptomatic patients (13). Symptoms are caused by the host immune system, suggesting that stronger cellular immune responses are associated with viral clearance. On the other hand, patients with more severe hepatitis have a greater chance of developing liver cirrhosis

and hepatocellular carcinoma (Fig. 1). The histological activity of the disease is determined by qualitative and quantitative assessment of the cellular infiltrate in the liver. This infiltrate consists mainly of T cells and NK/NKT cells; thus an immune response to the virus is of disadvantage for the host.

The immune response against HCV is complex and is generated by various cell types and tissues. Understanding in more detail how these immune responses are regulated during the different stages of the infection may lead to alternative treatment options and to the development of an anti-HCV vaccine that is still not available.

### INNATE IMMUNE RESPONSE

Innate immune responses are first-line defense mechanisms involving a complex network of natural antibodies, granulocytes, monocytes, NK cells, NKT cells,  $\gamma\delta$  T cells, and dendritic cells. Tissue-specific macrophages can be found in various organs, in the liver they are known as Kupffer cells. Cells of the innate immune response, in particular NK cells and NKT cells, can be found at higher frequencies in the liver than in the peripheral blood and are therefore of special interest in viral hepatitis. For detailed reviews of the lymphocyte repertoire of the liver and intrahepatic T cells in viral infections, see Chapters by 1, 3, and 6.

Once a pathogen has infected a cell, an immediate response is initiated. In viral infections, the expression of type I IFNs represents one of the earliest characteristics of an innate immune response. IFN- $\alpha$  and - $\beta$  cause a wide range of effects on the infected cell and immune cells: on the one hand protein synthesis of the infected cell may be inhibited via induction of the cellular protein kinase PKR (which also interacts with the HCV E2 and NS5A proteins); on the other hand, MHC expression of antigen-presenting cells and on target cells is enhanced. Viral replication is inhibited by activation of Mx protein or 2'5' oligoadenylate synthetase-induced RNase, and the functions of NK cells, CD8<sup>+</sup> T cells, and dendritic cells are stimulated. IFNs also induce cell death by upregulating the expression of a variety of apoptosis-inducing molecules (e.g., FasL, TRAIL). It has long been evident that type I IFNs must have an effect on hepatitis C replication since recombinant IFN- $\alpha$  has been used for the treatment of HCV infection for almost 15 yr and a decline in HCV RNA in serum can be observed as early as a few hours after injection of IFN- $\alpha$  (44). The inhibitory effect of IFN- $\alpha$  on HCV replication has also been shown in vitro using the HCV replicon system (45).

The analysis of early innate immune responses in HCV infection is hampered by the fact that patients can usually be studied only when disease is already present; thus the infection has already been ongoing for several weeks or even months. The chimpanzee is the only animal that is both susceptible to HCV infection and available for study. Using this model, microarray analyses of serial liver biopsies demonstrated early changes in the expression of a wide variety of genes including type I IFN (46,47). Importantly, the early induction of IFN-response genes preceded the expression of T-lymphocyte markers by several weeks and was associated with a decline in HCV RNA but not with a resolution of infection. Mechanisms associated with the relative in vivo resistance of HCV to type I

IFNs include the interaction of the NS3-NS4A protein with IRF-3 (38), the interaction of HCV-E2 and NS5A sequences with PKR (40), and loss of function of NK cells.

NK cell activity was shown by 1997 to be impaired in chronic HCV infection (48). In 2002, it was demonstrated by two groups that binding of the HCV-E2 protein to CD81 inhibited NK cell activation, cytokine production, cytotoxicity, and proliferation but had no effect on T-cell function (30,31). Interestingly, this effect was mediated by distinct negative signaling pathways associated with NK cell-inhibitory receptors for MHC class I. Moreover, it was shown that HCV core enhances MHC class I expression and thereby inhibits NK cell activity (49). Finally, the presence of a specific NK cell receptor has been associated with recovery from HCV infection, suggesting that different activation thresholds of NK cells contribute to the course of HCV infection (50).

In contrast, another cell type of the innate immune system, the  $\gamma\delta$  T-cell, has been shown to be activated by E2-CD81 crosslinking. Livers of patients with viral hepatitis contain elevated numbers of T cells expressing  $\gamma\delta$  from the T-cell receptor. These cells are cytotoxic against hepatocytes and produce tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interleukin-8 (IL-8), but do not display specific immune responses against HCV proteins or target cells infected with recombinant HCV-expressing vaccinia viruses. However, activated by HCV-E2/CD81 crosslinking, these cells secreted significant amounts of IFN- $\gamma$  and TNF- $\alpha$  and thus may contribute to HCV-related liver disease (51).

In summary, innate immune responses play an important role in early control of HCV replication and also in IFN-induced clearance during antiviral therapy. Different mechanisms including CD81/HCV-E2 crosslinking may interact with the functions of different immune cells, suggesting interesting targets for novel immunotherapies (Table 4).

### HUMORAL IMMUNE RESPONSE

Anti-HCV antibodies usually develop between mo 2 and 8 of acute HCV infection, which is quite late compared with other viral infections. In sharp contrast to hepatitis B, the humoral immune response against HCV does not allow us to discriminate between different stages of the infection (like anti-HBe<sup>+</sup> vs anti-HBe<sup>-</sup>). Antibodies against epitopes from all HCV proteins are detectable in acute infection, in chronic infection, and after recovery from HCV. No specific antibody pattern is associated with recovery or a specific level of replication.

Antibodies against epitopes within the hypervariable region of the E2 protein (HVR-1) with potentially neutralizing capacity have been detected by several groups; however, their contribution to viral clearance or evolution of disease is still a matter of debate. An early antibody response against the NH2 terminus of the HVR-1 has been associated with a self-limiting course of infection (52). Since there is a high variability of the virus in this region, it seems possible that escape from efficient humoral immunity might occur the longer viremia lasts. Subsequently, a more heterogeneous humoral immunity against HVR-1 is associated with chronicity (53). It has also been demonstrated that HCV may be cleared even in the absence of any humoral immunity against envelope proteins (8,54,55). Very, recently,

**Table 4**  
**Function and Role of Different Cell Types in HCV Infection**

<i>Cell Type</i>	<i>Function</i>	<i>Findings in HCV infection</i>
CD4+ T cells	<ul style="list-style-type: none"> <li>• Provide "help" to B cells and CD8+ T cells</li> <li>• Th1 cells produce: IFN-<math>\gamma</math></li> <li>• Th2 cells produce: IL-4, IL-5, IL-10</li> </ul>	Multispecific Th1 responses targeted against various HCV proteins are associated with viral clearance during acute hepatitis C.
CD8+ T cells	<ul style="list-style-type: none"> <li>• Cytotoxic effector functions</li> <li>• Production of cytokines (IFN-<math>\gamma</math>, TNF-<math>\alpha</math>, among others)</li> </ul>	Strong and multispecific responses during acute infection are associated with sustained control of HCV. Contribution to chronic liver disease?
NK cells	<ul style="list-style-type: none"> <li>• Cells of the innate immune system</li> <li>• Unspecific first-line defense</li> <li>• Cytotoxicity; cytokine release</li> </ul>	Increased frequency in chronic hepatitis. Impaired function in chronic hepatitis C. Expression of KIR2DL3 is associated with recovery from HCV infection
NK-T cells	<ul style="list-style-type: none"> <li>• Cells expressing NK- as well as T-cell markers</li> <li>• High numbers within the liver</li> <li>• Cytotoxicity; cytokine release</li> </ul>	Increased frequency in chronic hepatitis. Impaired in chronic HCV?
$\gamma\delta$ T cells	<ul style="list-style-type: none"> <li>• Cells expressing the <math>\gamma\delta</math> form of the T cell receptor</li> </ul>	Higher intrahepatic frequency in chronic HCV. No specific cytotoxicity against HCV proteins. Cross-linking with CD81 induced significant IFN- $\gamma$ production
Dendritic cells	<ul style="list-style-type: none"> <li>• "Professional" antigen presenting cells</li> <li>• Key function in inducing cellular immune responses</li> </ul>	Impaired function of myeloid DCs in chronic hepatitis C (?) Lower frequency and reduced IFN- $\alpha$ production of plasmacytoid DCs in acute hepatitis C

rapid induction of neutralizing antibodies in the early phase of infection has been suggested to contribute to control of HCV infection (55a).

There seems to be no long-lasting protective humoral immunity against HCV. In contrast, anti-HCV antibodies decline after recovery from acute HCV infection to undetectable levels even after two decades (56). Moreover, HCV antibody titers decline during and after antiviral therapy for acute hepatitis C (57) (Fig. 3).

#### ADAPTIVE CELLULAR IMMUNE RESPONSES

**Dendritic Cells and Hepatitis C Infection** Cellular immune responses are induced by dendritic cells (DCs), which present antigens to CD4<sup>+</sup> and CD8<sup>+</sup> T cells (58). DCs are widely distributed in both lymphoid and nonlymphoid tissues. They capture antigen at the site of infection, undergo maturation, and migrate to the draining lymph node, where priming of the cellular immune response takes place. Lymphocytes subsequently enter the bloodstream and home back to the site of infection, in this case the liver. The strength of the immune response is largely dependent on the stimulatory function of DCs, which is determined by antigen processing, MHC expression, and costimulation. In vitro derived DCs loaded with specific antigens are already used for the therapy of cancers and their role in the treatment of viral infection is currently being explored.

There is evidence that HCV may replicate in DCs (59,60), and thus cellular proteins might interfere with DC function. Interestingly, distinct "DC-tropic" HCV quasispecies seem to be present in this extrahepatic site of replication (61). Conflicting data on the function of DCs in chronic HCV infection have been presented. Earlier studies showed that the allostimulatory

capacity and IL-12 production of monocyte-derived DCs seem to be impaired in patients with chronic hepatitis C infection, although the cells displayed normal morphology, phenotype, and capacity to capture antigen (61,62). However, these findings were not confirmed by others (63), who demonstrated normal DC function. The latter finding was consistent with clinical and immunologic data showing that the deficit in the patient's immune repertoire is HCV specific. Reasons for these difference are not clear. Our own findings also suggest a weaker allostimulatory capacity of DCs from chronic HCV patients. Nevertheless, future studies with in vivo models are required to investigate this important question further.

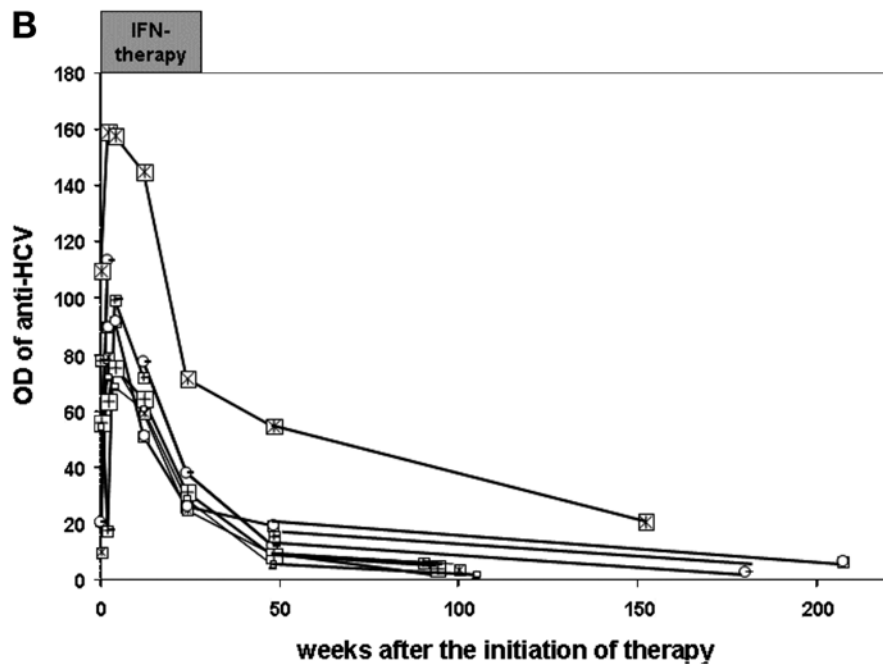
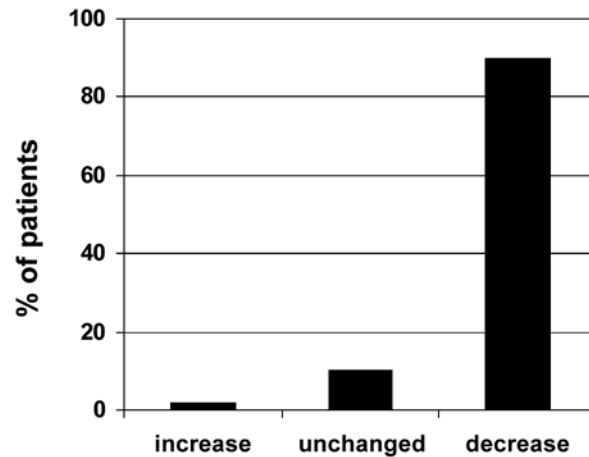
Besides antigen presentation, other DC subtypes may display additional functions including the production of IFN- $\alpha$  by plasmacytoid DCs. The frequency and IFN- $\alpha$ -producing capacity of peripheral blood plasmacytoid DCs is reduced in acute hepatitis C, whereas in chronic hepatitis C an incomplete recovery of plasmacytoid DC function was found (64). Other effector functions of DCs including cytokine production and cytotoxicity are subjects of current investigations.

In summary, knowledge of the role of DCs in viral hepatitis is increasing but is still limited. Evaluation of this cell type in more detail will be an essential step in understanding the immunopathogenesis of HCV infection and will perhaps lead to the generation of cell-based immunotherapies.

**HCV-Specific CD4<sup>+</sup> and CD8<sup>+</sup> T-Cell Responses in Acute and Chronic HCV Infection** Several groups have consistently found an association between a multispecific, strong, and maintained HCV-specific CD4<sup>+</sup> and CD8<sup>+</sup> T-cell response and viral clearance during acute HCV infection (Fig. 4)



### A Change of antibody titers between 10 and 18 years after recovery



**Fig. 3.** Humoral immune response to hepatitis C virus (HCV). (A) Change in HCV antibody titers between 10 and 18 yr after recovery, according to Takaki et al. (56). (B) Change in anti-HCV titers in selected patients during and after therapy for acute hepatitis C. IFN, interferon. (Data from ref. 57.)

(65). The CD4<sup>+</sup> response is maintained for several years after recovery. The CD8<sup>+</sup> response remains also detectable, but there are conflicting data as to what extent the CD8<sup>+</sup> response decreases over time after recovery (56,66).

Noncytolytic inhibition of viral replication by antiviral cytokines is a major mechanism for the control of HBV infection. Similarly, HCV replication seems to be inhibited by IFN- $\gamma$ . Thimme and colleagues investigated virological and immunological features of patients exposed to HCV after needlestick injury (7). First, viremia was detectable in all subjects as early as 1 to 2 wk after injury, which is quite early compared with

#### Intensity and diversity of T-cell responses

Acute infection – recovery	++++
Acute infection – chronic course	++/-
Long-term recovered patients	++/+
Chronically infected patients	+/-

**Fig. 4.** Cellular immune response to HCV.

**Table 5**  
**A. Immune Responses During Acute HCV Infection: Recovery**

	<i>Incubation phase</i>	<i>Acute disease</i>	<i>Viral clearance</i>	<i>Recovered</i>
Time (Week)	0–4	4–12	10–14	>14
HCV-RNA	Positive by week 1	++	+/-	-
ALT	Normal	↑↑	↑	Normal
Intrahepatic cellular infiltration	-	++	+	-
HCV-specific CD4 T cells	-	+	++	++
HCV CD8+ T cells cytotoxic	-	+++	++	-/+
HCV CD8+ T cells IFN- $\gamma$ producing	-	-	+++	++
Innate Immune response (type I interferons)	++	++	+	-

**B. Chronic course**

	<i>Incubation phase</i>	<i>Acute disease</i>	<i>Chronification</i>	<i>Chronic infection</i>
Time (Week)	0–4	4–12	10–14	>14
HCV-RNA	Positive by week 1	++	+	+
ALT	Normal	↑	↑	↑
Intrahepatic cellular infiltration	-	+	+	+
HCV-specific CD4 T cells	-	+	+	+/-
HCV CD8+ T cells cytotoxic	-	+	+	-/+
HCV CD8+ T cells IFN- $\gamma$ producing	-	-	+/-	Fluctuating Tc1/Tc2 cells
Innate Immune response (type I interferons)	+ (?)	+ (?)	+	+

**C. HCV-Specific T Cell Responses During Therapy of HCV Infection**

	<i>Sustained responder</i>	<i>Relapser</i>	<i>Nonresponder</i>
Prior to therapy	++	+	-
Week 1–4	+/-	+/-	-
Month 2–12	++	+/-	+/-
Follow up	+	+/-	-

HBV. Second, liver disease as measured by an increase in ALT was accompanied by the appearance of activated cytotoxic CD8<sup>+</sup> T cells that did not secrete IFN- $\gamma$  after 4 to 6 wk. HCV viral load did not decrease significantly at this time, indicating that destruction of infected hepatocytes is not sufficient alone to control HCV replication. Later on, a functional switch of HCV-specific CD8<sup>+</sup> T cells was observed: the cells lost the activation marker CD38 and started to secrete IFN- $\gamma$ . At the same time, HCV-RNA in serum rapidly declined, and a strong HCV-specific CD4<sup>+</sup> response was detectable, suggesting that HCV-specific CD4<sup>+</sup> T cells may have contributed to CD8<sup>+</sup> maturation. In contrast, in patients in whom chronic infection evolved, no IFN- $\gamma$  secreting CD8<sup>+</sup> T cells were observed, and the CD4<sup>+</sup> response was much weaker (Table 5). These results suggest that noncytolytic mechanisms are important to control HCV replication and that distinct effector T-cell populations contribute to different aspects of HCV pathogenesis. A direct role of IFN- $\gamma$  in suppressing viral replication is supported by *in vitro* data derived in the replicon system. The findings by Thimme et al. are in line with other reports demonstrating

“stunned” HCV-specific CD8<sup>+</sup> T cells in the acute phase of HCV infection followed by a functional switch associated with viral clearance (67,68).

Once chronic HCV infection has been established, the persisting HCV may maintain an inefficient Tc1 (IFN- $\gamma$ -producing CD8<sup>+</sup> T-cell) response. Activated CD8<sup>+</sup>-positive T cells can be found at 30 times greater frequencies in the liver than in the peripheral blood of chronically infected patients (69,70) and potentially contribute to liver disease. Downregulation of cytotoxic T-lymphocyte (CTL) effector function may be important for host in this stage of the infection. Several possible mechanisms have been proposed for downregulation of T-cell function in acute and chronic hepatitis C (Table 6). HCV core protein causes a reduced IL-2 production, inhibiting CTL differentiation (71). Weaker stimulatory capacities of DCs have already been discussed. HCV core-induced downregulation of C1q-mediated IL-12 production of macrophages could be another mechanism (72). Programmed death-1 (PD-1) has recently been shown to be expressed on exhausted CD8 T cells in mouse lymphocytic choriomeningitis virus

**Table 6**  
**Potential Mechanisms Leading to Down-Regulation**  
**of HCV-Specific CD8<sup>+</sup> T Cell Responses**

- Insufficient IL-2 production caused by HCV core
- Impaired stimulatory capacities of dendritic cells (?)
- HCV core-induced downregulation of C1q
- PD-1 expression on exhausted CD8 T cells
- Host genetic factors such as cytokine polymorphisms, HLA types (e.g., HLA-B27), chemokine receptor genes
- Regulatory T cells (CD4<sup>+</sup>CD25<sup>+</sup>)
- HCV-specific CD8<sup>+</sup> T cells with suppressor function
- Escape mutations affecting epitope processing, MHC binding and T-cell-receptor stimulation

Abbreviations: PD-1, programmed death 1.

(LCMV) infection (73) and was also shown to be present on HCV-specific CD8<sup>+</sup> T cells in chronic hepatitis C (74). In addition, several host genetic factors such cytokine polymorphisms (75), HLA types (e.g., HLA-B27 [76]), and chemokine receptor genes (77) may influence T-cell function and thereby contribute to the outcome of infection. Finally, higher frequencies T cells with a regulatory function (CD4<sup>+</sup>/CD25<sup>+</sup>) have been described in chronic hepatitis C infection in both chimpanzees (78) and humans (79,80), and HCV-specific CD8<sup>+</sup> T cells with suppressor function were detected in the livers of patients with chronic hepatitis C (81).

HCV has a high replication rate, and the lack of proofreading may lead to escape mutations. This process may be facilitated by the delayed appearance of adaptive immune responses. Viral variants may be associated with reduced T-cell receptor (TCR) stimulation (82–84), impaired MHC binding (85), and altered epitope processing (86), but they also effect viral fitness (87).

The importance of intrahepatic HCV-specific CD8<sup>+</sup> T cells was exemplified by studies demonstrating that sustained responses to IFN- $\alpha$  treatment were associated with detectable HCV-specific cytotoxic activity of liver-derived lymphocytes (88) and that multispecific and vigorous CTL responses were detected in the livers of chimpanzees who cleared acute HCV infection (89,90). Whether clonal expansion of HCV-specific T cells occurs in the liver is still a matter of debate (91). Nevertheless, the specificity of HCV-specific T-cell responses seems not to differ between the peripheral blood and the liver (69,70).

**HCV-Specific CD4<sup>+</sup> and CD8<sup>+</sup> T-Cell Responses and Interferon Therapy in HCV Infection** The decline of HCV RNA during therapy with IFN occurs in two phases. The first phase (24–48 h) is believed to be owing to direct inhibition of HCV replication, and the slower second phase is thought to be mediated by cellular immune responses (44). Finally, maintenance of response after the end of therapy should be mediated by memory effector T cells.

Several investigators studied CD4<sup>+</sup> T-cell responses prior to and during therapy for hepatitis C. Overall, responses before therapy were stronger in patients who showed a complete sustained response than in nonresponder or relapsed patients (92,93). A combination of IFN- $\alpha$  with ribavirin has been shown

to enhance Th1-like cellular immune responses. During the early phase of therapy, HCV-specific T cells disappear from the peripheral blood (Fig. 5), supporting the hypothesis that HCV-specific cells become activated and home to the site of infection, the liver (6). After 1 to 2 mo, HCV-specific cells reappear in the blood, and thereafter, HCV-specific responses are stronger in patients who maintain the response than in nonresponder patients (Table 5). These findings support the concept that therapy might be improved by a combination of inhibition of viral replication with boosting of cellular immune responses. However, in treatment of acute hepatitis C, there is no clear correlation between the kinetics of T-cell responses and treatment outcome (94,95).

#### **Induction of HCV-Specific T-Cell Responses by Vaccination**

There is evidence that subinfectious doses of HCV can induce HCV-specific T cells in seronegative individuals with potential exposure (55,96–98). Moreover, drug users who have cleared HCV previously and who are reexposed to HCV are more likely to clear the infection than drug users who are exposed to HCV for the first time (99). Thus, attempts have been made to induce HCV-specific T-cell responses by vaccination in chimpanzees and humans. Data from chimpanzees show that vaccination with structural proteins does not confer sterilizing immunity but leads to a reduction in chronic courses of HCV infection (100). In humans, phase I studies using recombinant E1 protein (101) and HCV peptides have been completed (102). Both studies demonstrated the induction of T-cell responses in healthy volunteers, and both vaccines have also been explored in chronic hepatitis patients. Whereas the E1 vaccine was suggested to cause a halt in fibrosis progression (103), which, however, was not yet confirmed in the first placebo-controlled study (104), the peptide vaccine IC41 enhanced CD4<sup>+</sup> and CD8<sup>+</sup> T-cell responses in chronic HCV infection, which, however, led only to transient changes in HCV RNA in single cases (105). Further trials are ongoing. These first trials have shown that timing of vaccination, dose, and route of administration as well as selection of antigens/peptides still need to be optimized.

#### **HETEROLOGOUS IMMUNITY AND HCV INFECTION**

Memory immune CD8 T cells are part of a continuously evolving intricate immune network. Every new infection will alter the frequencies, distributions, and activities of these memory cells. This concept is known as heterologous immunity (106). CD8 T-cell crossreactivity, whereby a CD8 TCR can degenerate and recognize multiple antigens, is one example. Crossreactive expansion of CD8 T cells can alter hierarchies of T-cell responses and even influence protective immunity or immunopathology (107). A heterologous antigen may only activate a small part of an existing memory T-cell repertoire, resulting in great variability in immune hierarchies and T-cell repertoires among different individuals depending on the private specificity of the preexisting memory immune response (108) (Fig. 6A–C). This may reflect the high inconsistency of CD8 T-cell immune hierarchies among patients infected with HCV (109). Considering the importance of the CD8 T-cell

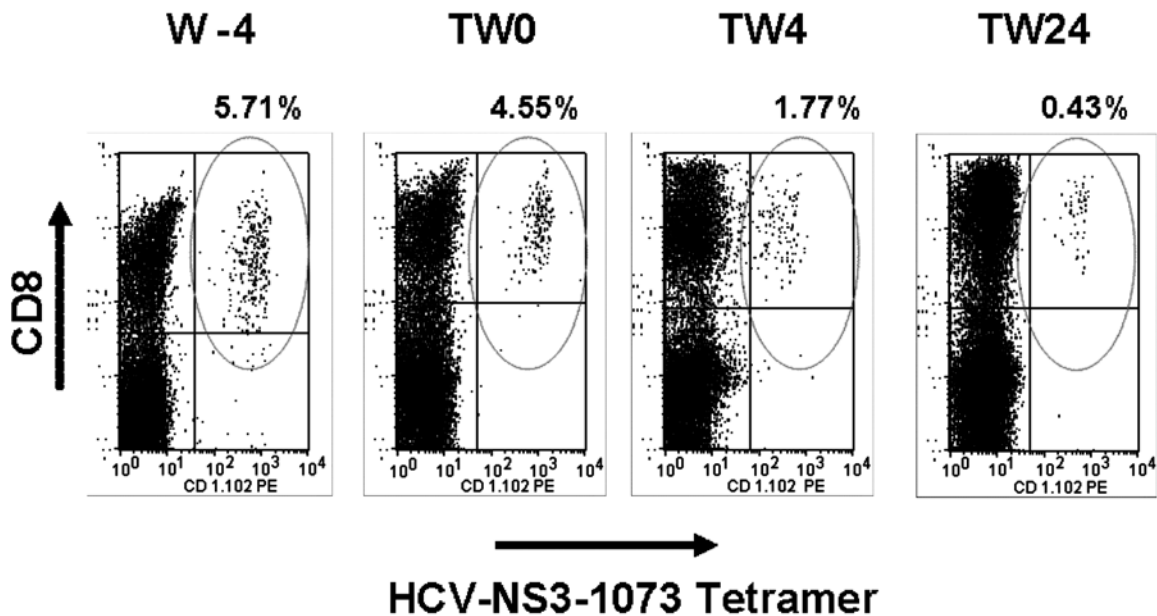


Fig. 5. Decline of HCV-specific CD8<sup>+</sup> T-cell response during interferon- $\alpha$  therapy in a patient with acute hepatitis C.

response during acute HCV infection, we suggest that reactivation of crossreactive memory T cells participating in the immune response during acute HCV infection is a common event that, depending on the private T-cell repertoire, may influence not only immune hierarchies but also disease outcome. This may contribute to the differences in the natural course of HCV infection between different individuals (Figs. 1 and 6). Indeed, crossreactive HCV-specific CD8 T-cell responses have already been documented (110). Wedemeyer et al. described CD8 T-cell responses to HCV NS31073 in healthy blood donors that were shown to be mediated by memory CD8 T cells specific to influenza A (IV) NA231 (110). In a more recent study, Kennedy et al. noted crossreactive CD8 T-cell responses between HCV NS5B2816 and HHV1 UL5529 (111).

When a memory CD8 T-cell pool encounters a crossreactive antigen, e.g., HCV, the high frequency of memory cells and the activation state give them an advantage over naïve cells that may lead to a preferential proliferation of the crossreactive cells. This may be helpful in boosting a memory response. However, it may also select for a lower affinity response or result in an oligoclonal response, which may allow for immune evasion (112). Heterologous immunity and CD8 T-cell cross-reactivity can be beneficial (protective immunity) or detrimental (immune pathology) for the host during a viral infection. Reactivation of crossreactive memory CD8 T cells either from a previous infection with an unrelated virus such as influenza A or generated after prior exposure to heterologous HCV strain may participate in the immune response during acute HCV infection. The consequences of this proliferation may depend on the private T-cell repertoire and the quality of the crossreactive CD8 T-cell response. For example, Urbani et al. reported on two patients with severe subfulminant hepatitis C, which is usually extremely rare. Both patients had a strong HCV-specific

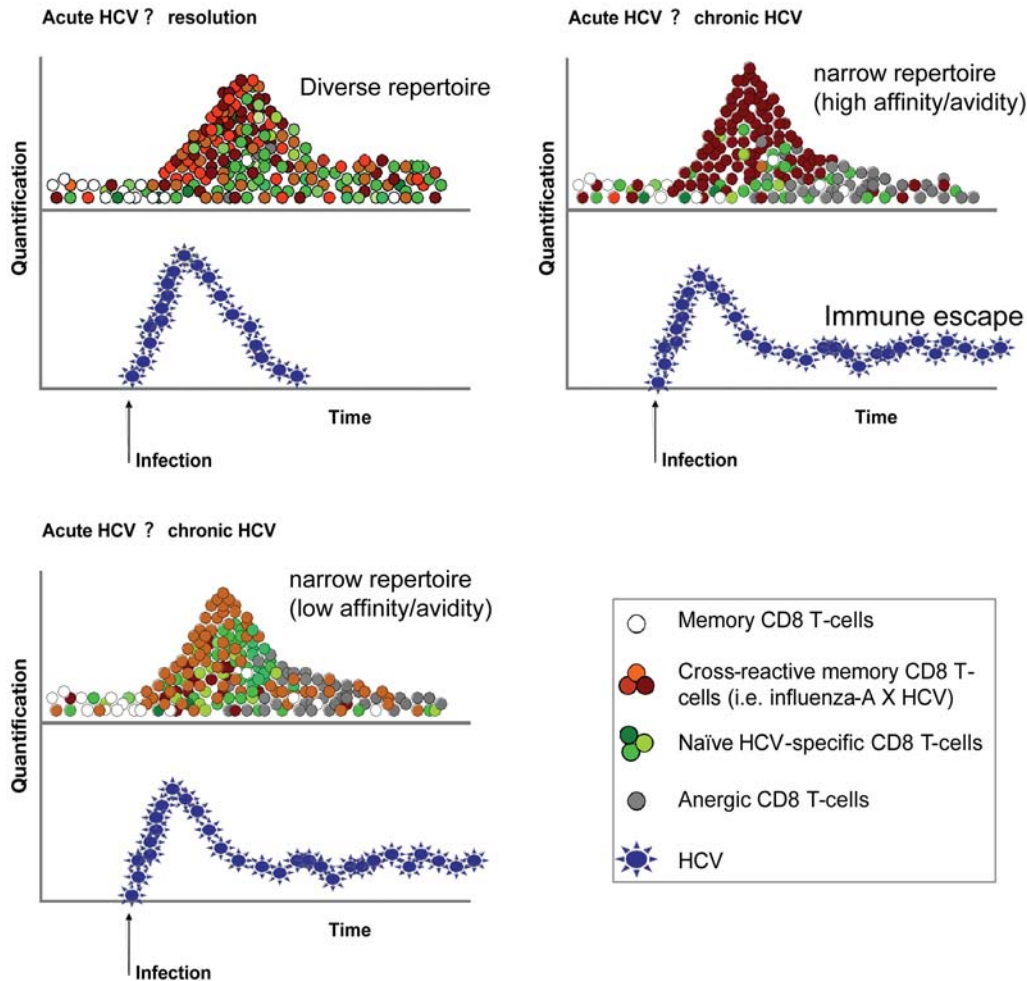
CD8 T-cell response focused on a single determinant, the HCV NS31073 epitope. Both patients with subfulminant hepatitis C but not two controls with milder acute HCV infection also had responses to IV NA231. Crossreactive CD8 T-cell responses could be confirmed by showing that a part of the HCV NS31073 tetramer-positive CD8 T cells produced IFN- $\gamma$  upon IV NA231 in vitro stimulation (113). The exclusive IV NA231 responses in both patients with subfulminant hepatitis suggests that the private T-cell repertoire of the patients determined the proliferation of crossreactive CD8 T cells and immunodominance of the HCV NS31073 response, which may have led to the severe immunopathology. Interestingly, both patients with severe symptoms developed a persistent infection despite a strong HCV-specific CD8 T-cell response (as shown in Fig. 6B and C). We would suggest that not only the quantity of the CD8 T-cell response but also the quality of this response determined disease outcome.

Future studies need to investigate the role of heterologous immunity for acute and chronic HCV infection in more detail. It is strongly recommended to take heterologous immune responses into account in the development of HCV vaccines to prevent unexpected adverse events.

## THERAPY FOR ACUTE AND CHRONIC HEPATITIS C INFECTION

### ACUTE INFECTION

Several studies have evaluated the efficacy of IFN- $\alpha$  therapy for acute HCV infection, and nearly all reported a beneficial effect of treatment (114). The German Hep-Net Acute HCV-I trial investigated the efficacy of recombinant IFN- $\alpha$ 2b in 44 patients with acute hepatitis C. The average time from infection to the first signs or symptoms of hepatitis was 54 d, and the average time from infection until the start of therapy was 89 d.



**Fig. 6.** Heterologous immunity. Naïve and crossreactive memory CD8 T cells participate in the HCV-specific immune response during acute HCV infection. Proliferation of crossreactive CD8 T cells may occur owing to their memory phenotype. Depending on the private specificity of the immune T-cell repertoire, this may lead to different CD8 T-cell response patterns. (A) Diverse T-cell repertoire including low, medium, and high affinity/avidity crossreactive as well as naïve CD8 T cells, which may result in HCV clearance. (B) Narrow T-cell repertoire dominated by a high affinity/avidity crossreactive CD8 T-cell response, which may result in immune evasion. (C) Narrow T-cell repertoire dominated by a low affinity/avidity crossreactive CD8 t-cell response, which may result in immunopathology but also may lead to chronicity. The concept of heterologous immunity should be taken into consideration for the design of therapeutic vaccines.

At the end of both therapy and follow-up, 43 patients (98%) had undetectable levels of HCV RNA in serum and normal serum ALT levels (Fig. 7) (115). In a larger second trial, 6-mo treatment with pegylated IFN- $\alpha$ 2b produced a slightly lower response rate. Moreover, adherence to therapy was a significant problem, leading to a much lower response rate in the intent-to-treat analysis (Fig. 7B) (116). Thus, these studies demonstrated that chronic HCV can be prevented by early treatment of acute HCV infection with IFN- $\alpha$  monotherapy for just 6 mo. Importantly, no combination with ribavirin was necessary. However, we have to take into account that about 20 to 40% of patients would have had self-limited disease (13). Therefore, there has been some debate on optimal timing of therapy. We are currently performing a nationwide randomized trial comparing early and delayed therapy for acute hepatitis C in the German Hep-Net. Until these data are available, we would suggest timing treatment according

to HCV genotype, severity of symptoms, and HCV kinetics (Fig. 8) (114).

### CHRONIC INFECTION

Treatment of chronic hepatitis is based on a combination of IFN- $\alpha$  and ribavirin (117). The combination of pegylated IFN- $\alpha$  plus ribavirin led to sustained virological response rates (negative HCV RNA in serum 6 mo after the end of therapy) in about 50% of patients infected with HCV genotype 1 treated for 1yr and in 80 to 90% of patients infected with genotype 2/3 (Fig. 9) treated for 16 to 24 wk (28). HCV genotype 4 should also be treated for 48 wk (118). The mode of action of ribavirin is still not completely understood. Alterations in immune responses (119) and mutagenic effects (120) have been discussed. Future concepts may use a combination of IFN- $\alpha$  with novel antivirals (121), therapeutic vaccination (103,122), or

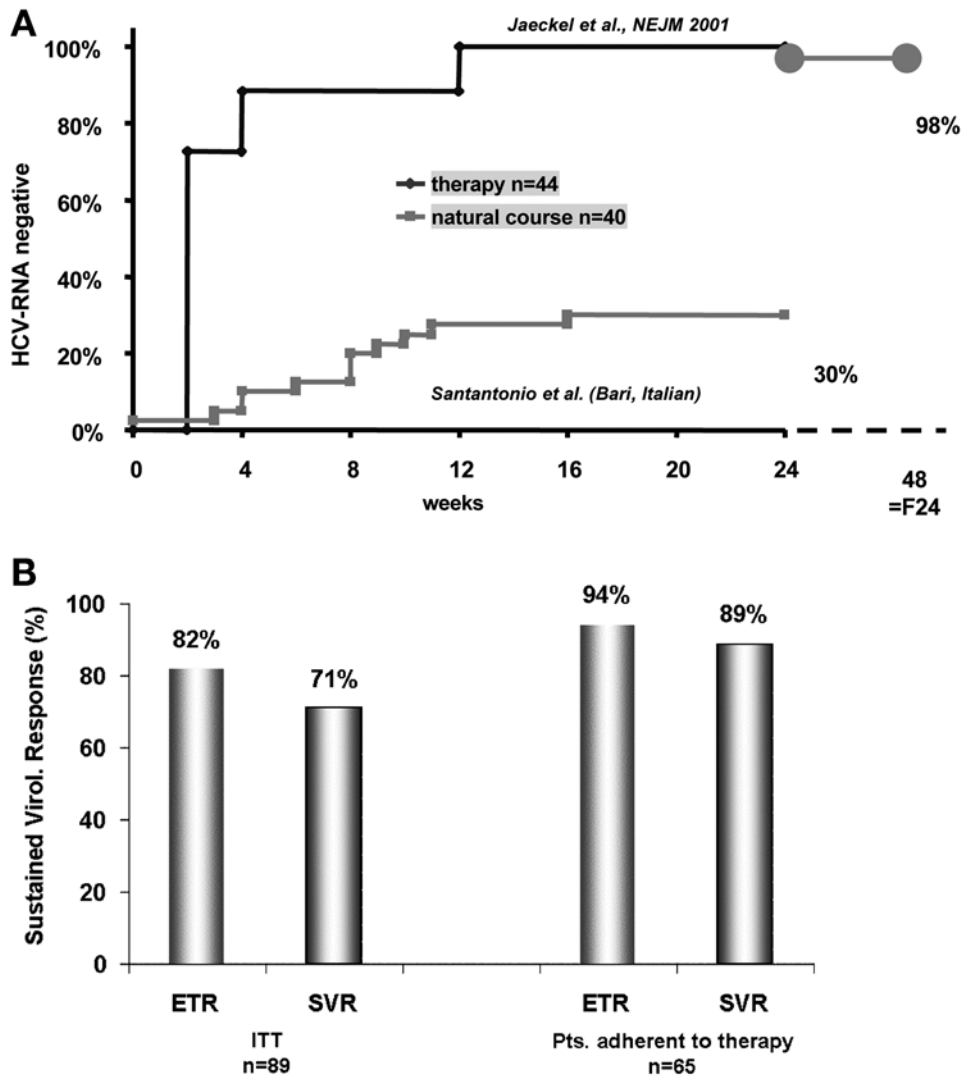


Fig. 7. Early treatment of acute hepatitis c virus (HCV) infection. (A) The Hep-Net Acute HCV-I Study (Jaeckel et al. [115]). (B) Therapy with pegylated interferon (Wiegand et al. [116]). ETR, end of treatment response; SVR, sustained virological response.

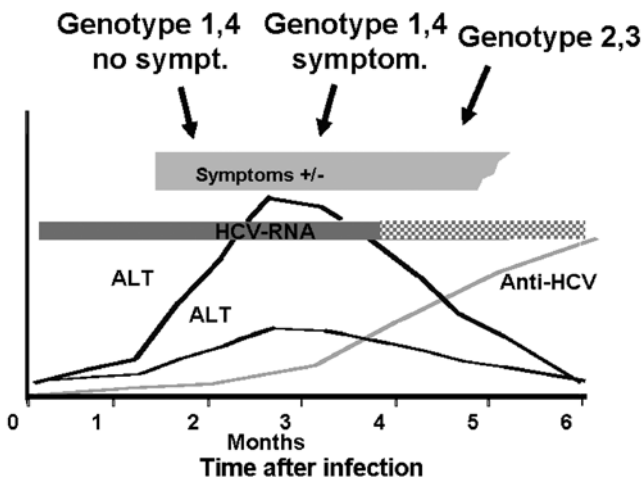


Fig. 8. Timing of treatment in acute hepatitis C (HCV).

other immunomodulatory approaches such as T-cell-like receptor stimulation (123,124).

Tolerance induction rather than enhancement of immune responses to downregulate inflammatory activity might be another reasonable way to treat some patients with chronic hepatitis if no virus eradication can be achieved. A pilot trial with IL-10 was conducted in IFN- $\alpha$ -nonresponding chronic hepatitis C patients (125). Interestingly, not only hepatic inflammation but also liver fibrosis decreased in 14 of the 22 patients. However, no further trials using IL-10 for chronic hepatitis C have been reported.

**CONCLUDING REMARKS**

The outcome of HCV infection is determined by innate and adaptive immune responses contributing not only to protection and spontaneous clearance but also to inflammatory activity in chronic infection and progression to liver cirrhosis and

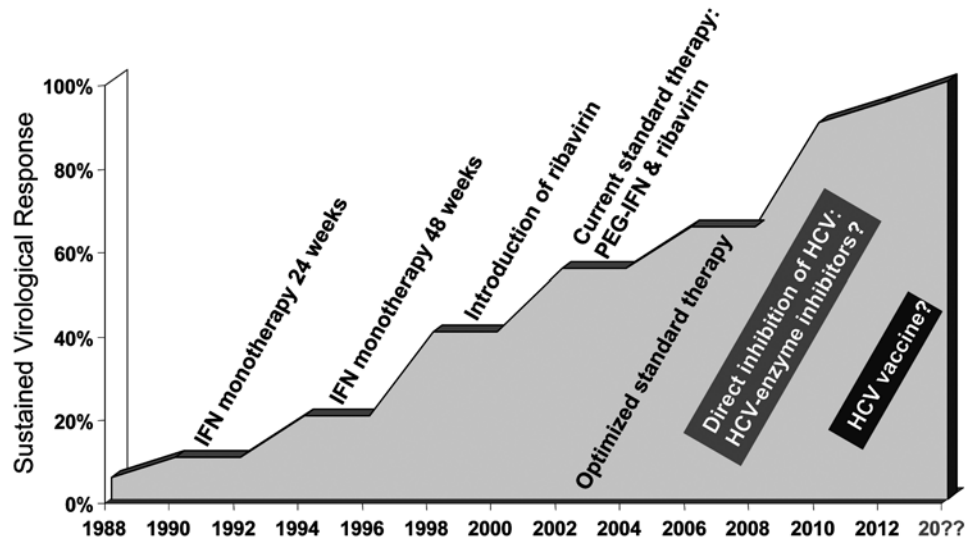


Fig. 9. Present and future therapy of chronic hepatitis C (HCV). IFN, interferon; PEG, pegylated.

hepatocellular carcinoma. HCV has developed different strategies to circumvent immune responses, which have significant implications for the development of future immunotherapies. However, the ultimate goal must be the prevention of new infections by developing a vaccine for hepatitis C. The first vaccine trials have been completed — the challenge will be to prove in humans that these vaccines are also effective in terms of protection or at least for preventing progression to chronic infection. Heterologous immune responses have to be considered in the pathogenesis of HCV infection and also in the development of prophylactic and therapeutic vaccines.

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# 16 Immunopathogenesis of Extrahepatic Manifestations in HAV, HBV, and HCV Infections

## *Importance of Recognition and Therapy*

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### KEY POINTS

- Extrahepatic symptoms associated with viral hepatitis can affect different organs during acute or chronic hepatitis.
- Extrahepatic manifestations are often but not always associated with immune complexes or autoantibodies.
- Different immunopathogenetic mechanisms have been considered to cause extrahepatic symptoms. In addition, direct viral effects on nonliver cells have been described.
- In hepatitis A, extrahepatic manifestations are infrequent.
- In hepatitis B, polyarteritis nodosa and glomerulonephritis are the most important extrahepatic manifestations.
- In hepatitis C, a wide variety of symptoms can occur that are frequently caused by cryoglobulinemia.
- Autoantibodies targeting different organs are more prevalent in hepatitis C than in HCV-negative controls.
- A specific treatment is not necessary in hepatitis A since HAV infection is self-limited, and associated extrahepatic manifestations are usually not sustained.
- Treatment of extrahepatic manifestations in hepatitis B requires an individual strategy for each patient including immunosuppression, interferon- $\alpha$ , and antiviral treatment with nucleoside or nucleotide analogs.
- Treatment of extrahepatic manifestations in hepatitis C is based on both immunosuppression and antiviral therapy with (pegylated) interferon and ribavirin.
- Treatment should only be given by experienced physicians since extrahepatic symptoms may worsen during interferon therapy and may require immediate intervention.

### INTRODUCTION

Hepatitis A, B, and C virus (HAV, HBV, and HCV) infections can be associated with various extrahepatic manifestations, which can be seen in both acute and chronic infections.

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Extrahepatic manifestations in acute hepatitis are often seen in hepatitis B, less often in hepatitis C, and only occasionally in hepatitis A. Clinically important extrahepatic manifestations are most often seen in chronic hepatitis C infection. Therefore some of the causative pathomechanisms are explained in more detail for HCV, e.g., the autoimmune syndromes and cryoglobulinemia.

### HEPATITIS A

Extrahepatic manifestations of hepatitis A are uncommon and are only rarely seen in the clinical routine. If they occur, they are acute and recede upon resolution of acute hepatitis A.

#### ASSOCIATED DISEASES

Symptoms involving extrahepatic organ systems in acute HAV infection are arthralgia, diarrhea, renal failure, red cell aplasia, generalized lymphadenopathy, and pancreatitis (Table 1). There are only a few fatal cases, owing to extrahepatic manifestations like pericarditis and renal failure (1). An association of hepatitis A infection with cryoglobulinemia has been reported but is also rarely seen (2). Arthralgia is most often seen and has an incidence of 11% in hepatitis A patients. Cutaneous vasculitis can occur on the lower extremities. In some of these cases, skin biopsies revealed IgM antibodies against HAV and complement in the vessel walls. Unlike hepatitis B and C, renal involvement is extremely rare, and there are only few case reports showing acute renal failure associated with HAV (3–6).

### HEPATITIS B

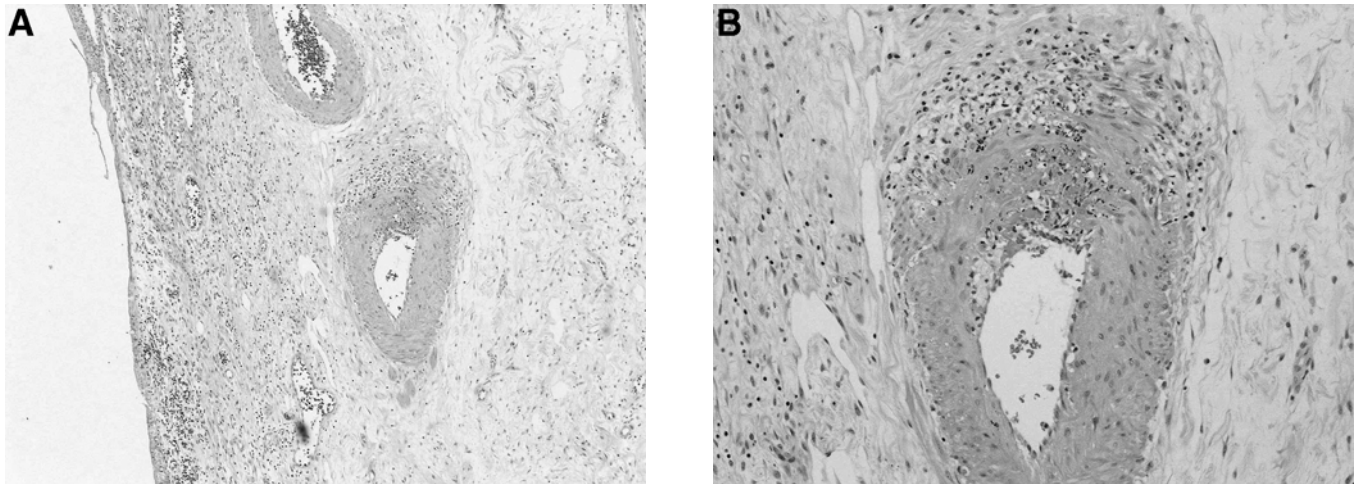
Extrahepatic manifestations have been described in acute and chronic hepatitis B in up to 10 to 20% of cases (Table 2). No correlations between HBV genotypes and the presence of extrahepatic manifestations have been found in adult patients (7). Some studies, however have shown an association between the subtype ayw and the Gianotti-Crosti syndrome, a special skin manifestation of hepatitis B in children. Most of the extra-

**Table 1**  
**Hepatitis A-Associated Diseases**

Arthralgia  
Diarrhea  
Renal failure  
Glomerulonephritis  
Red cell aplasia  
Lymphadenopathy  
Pancreatitis  
Cutaneous vasculitis  
Cryoglobulinemia

**Table 2**  
**Hepatitis B-Associated Diseases**

Splenomegaly  
Lymph node enlargement  
Serum sickness  
Polyarteritis nodosa  
Glomerulonephritis  
Mixed cryoglobulinemia  
Gianotti-Crosti syndrome  
Rare manifestations (aplastic anemia, pancreatitis, pericarditis, Raynaud-syndrome, peripheral neuropathy, Guillain-Barre syndrome)



**Fig. 1.** (A) and (B) Necrosis of polyarteritis nodosa in a duodenal arter. (Kindly provided by Dr. med F. Laenger, Institute for Pathology, MHH, Hannover Germany.)

hepatic manifestations seem to be immune mediated and can be linked to circulating autoantibodies or circulating immunocomplexes of viruses and immunoglobulins. Some studies suggested that HBV replication in extrahepatic tissues might lead to organ manifestation. In most cases, however, extrahepatic replication occurs without any visible cytopathic or immune-related tissue damage. Suppression of viral replication with antiviral therapy or spontaneous viral clearance positively correlates with resolution of extrahepatic symptoms. Immunosuppressive therapies for most of the severe manifestations were therefore replaced by specific antiviral therapies.

#### ASSOCIATED DISEASES

**Splenomegaly and Lymph Node Enlargement** Splenomegaly is found in 5 to 10% of patients with acute hepatitis B. Mild lymph node enlargement can be seen in acute hepatitis.

**Serum Sickness** One systemic syndrome in the acute phase of hepatitis B infection is serum sickness. In most cases, symptoms precede the onset of jaundice by a few days to up to 4 wk. Typical symptoms like polyarthralgia, arthritis, joint edema, fever, and skin rash usually develop suddenly at the beginning of an acute infection and resolve during a short period within 20 d (8,9). Joint involvement is usually symmetrical and is more commonly observed in

females. In some patients, hepatitis B surface antigen (HBsAg) can be detected in synovial membranes and sometimes in synovial fluid. The synovial fluid is often non-inflammatory and contains reduced levels of complement (10,11).

**Polyarteritis Nodosa** Polyarteritis nodosa (PAN) is a severe vasculitis associated with HBV (Fig. 1). Ten to 50% of polyarteritis patients are HBsAg-positive, but only 1 to 5% of patients with chronic hepatitis B will develop PAN (12,13). The association between chronic HBV infection and PAN is relatively strong in North America and Europe, where HBV is typically acquired later in life, whereas the association is weaker in Asia, where HBV is more often acquired perinatally (9,14).

This vasculitis affects the small- and medium-sized vessels in many organs and can cause various symptoms like arthritis, pericarditis, hypertension, cardiac failure, mononeuritis and involvement of the central nervous system, skin rashes, hematuria, proteinuria, fever, anemia, and especially gastrointestinal problems like abdominal pain. In most patients, early symptoms include abdominal pain, hypertension, eosinophilia, weight loss, and polyarthritis. The arthralgia usually affects the small joints of the hands, with morning stiffness, and does usually not lead to joint deformities. Gastrointestinal complications of perforation or bleeding are seen in 46%, malignant hypertension

in 30%, and renal infarction and orchiepididymitis in 26% of cases (9,15).

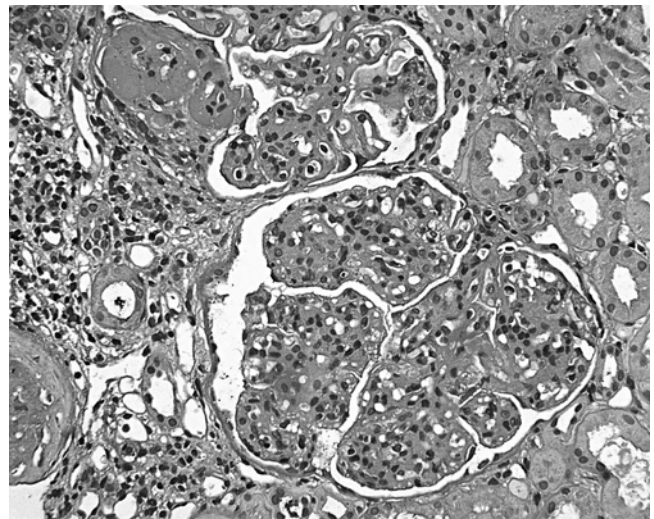
The arterial lesions of PAN are characteristically segmental and include the bifurcations and branchings of the vessels. The diagnosis relies on angiographic findings and characteristic arterial lesions on tissue biopsy. Histologically, the vasculitis shows Fibrinoid necrosis and perivascular inflammation of the vessels; in the acute phase, polymorphonuclear leukocytes infiltrate the vessel wall, and in chronic cases mononuclear cells infiltrate the walls with possible severe complications, like occlusion, thrombosis, ischemia, and finally necrosis (Fig. 1A, B). A typical laboratory finding is a low level of serum complement. In PAN without HBV infection, serum antineutrophil cytoplasmic antibodies can often be found, which are usually not seen in PAN caused by HBV infection (16).

The disease can evolve gradually into a chronic debilitating disorder. Untreated, the mortality rate reaches 40% within 3 yr. HBV-associated circulating immune complexes have been suggested to cause PAN but the exact mechanisms are still controversially discussed (17–19). There is a strong correlation between the levels of circulating immune complexes and disease activity of HBV-associated PAN (17).

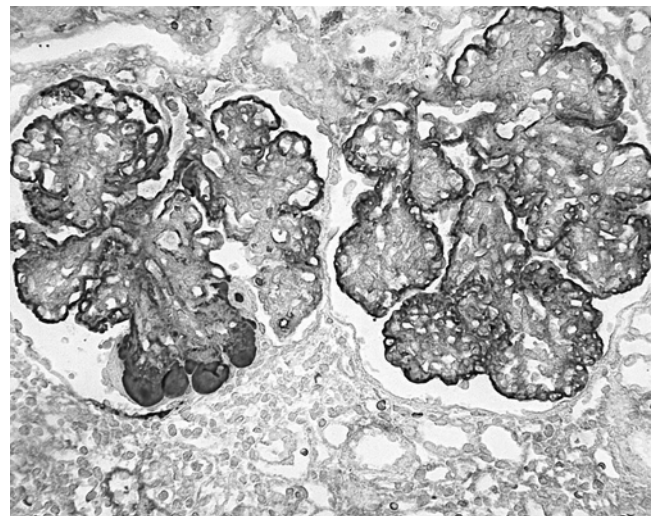
**Glomerulonephritis** Glomerulonephritis is the most common renal complication seen during chronic HBV-infection, but membranoproliferative, mesangial proliferative, and membranous glomerulonephritis can also be found, typically presenting as nephritic syndrome (Figs. 2 and 3). The development of HBV-associated glomerulonephritis seems to be dependent on environmental and genetic factors (20). Membranous glomerulonephritis is often seen in infected children and resolves spontaneously in almost 50% of cases. Membranoproliferative glomerulonephritis occurs more often in adult patients. In general, the prognosis is more severe, and 10% of patients will develop renal insufficiency requiring hemodialysis.

The mechanisms are not completely defined, but the following possibilities are discussed: a direct cytopathic effect by infection with HBV, tissue deposition of immune complexes, damage to the kidney by virus-stimulated T lymphocytes or antibodies, and an indirect effect on renal tissue by cytokines or proinflammatory mediators (20). In membranoproliferative glomerulonephritis, mesangial and capillary wall deposits containing HBsAg can be seen. In membranous glomerulonephritis, capillary wall deposits of HBeAg have been found. Hepatitis B early and core antigens (HBeAg and HBcAg) can be detected in glomeruli, but in most studies immune complexes containing HBsAg, anti-HBs-antibodies, and complement components have been found in glomerular basement membrane and are believed to be the main causative factor for the development of HBV-associated glomerulonephritis. The differences in the origins of HBV-associated membranous glomerulonephritis and membranoproliferative glomerulonephritis are not yet clear.

**Mixed Cryoglobulinemia** Cryoglobulins are circulating immunoglobulins that reversibly precipitate at temperatures



**Fig. 2.** Membranoproliferative glomerulonephritis. (Kindly provided by PD Dr. med. M. Mengel, Institute for Pathology, MHH, Hannover, Germany).



**Fig. 3.** Immunofluorescence for membranoproliferative glomerulonephritis (IgM) (kindly provided by PD Dr. med. M. Mengel, Institute for Pathology, MHH, Hannover, Germany).

below 37°C. They can be occasionally found in patients with hepatitis B and much more often in patients with hepatitis C (21). In some studies HBV DNA was detected in these cryoprecipitates. Mixed cryoglobulins, which precipitate in small blood vessels (venules, capillaries, arterioles), can cause a vasculitis that clinically manifests as a systemic disease with glomerulonephritis, arthritis, and purpura, (Fig. 4). There are cases reported in which cryoglobulinemia is associated with Raynaud's phenomenon.

**Gianotti-Crosti Syndrome** Gianotti-Crosti syndrome is a papular acrodermatitis seen in children infected with HBV. It mainly involves the face, buttocks, and limbs and is characterized by erythematous, maculopapular eruptions without



**Fig. 4.** Necrotic skin lesion caused by cryoglobulinemia. (Kindly provided by Dr. med. S. Schnarr, Department of Rheumatology, MHH, Hannover, Germany).

pruritus. Sometimes these signs are associated with enlarged lymph nodes. An association between this skin manifestation and the HBV- subtype ayw has been reported.

**Aplastic Anemia, Pancreatitis, Neurological Manifestations, and Other Rarely Seen Problems** Isolated cases of pancreatitis (22) and aplastic anemia have been reported during acute and chronic HBV infection. Sometimes in the early phase of acute hepatitis B, a severe aplastic anemia can be seen (23).

An association between HBV infection and peripheral neuropathy, Raynaud's syndrome, Guillain-Barre Syndrome, or some kind of pericarditis has been reported in some studies and case reports. Sometimes these phenomena are linked to cryoglobulinemia; sometimes such symptoms can be found without any detectable serological abnormalities.

## HEPATITIS C

HCV can affect many other organ systems besides the liver, and approx 39% of patients with chronic hepatitis C infection have one or more extrahepatic manifestations (24), for which long-term infection is usually necessary. Some of these are of autoimmune origin. Compared with other hepatotropic viruses, HCV has been implicated in many different extrahepatic and autoimmune manifestations (Table 3). The best well documented associations are mixed cryoglobulinemia and associated autoimmunopathies caused by the presence of autoantibodies and serological markers like rheumatoid factor. Lymphoma, renal disease, neuropathy, and Sjögren's syndrome manifest incomplete overlaps with the cryoglobulinemic syndrome. In recent years a link between hepatitis C infection and some associated syndromes like membranoproliferative glomerulonephritis, mixed cryoglobulinemia, or porphyria cutanea tarda has been confirmed in several studies. The relationship between HCV and non-Hodgkin's lymphoma, Sjögren's syndrome, lichen planus, idiopathic pulmonary fibrosis, or

**Table 3**  
**Hepatitis C-Associated Diseases**

Glomerulonephritis
Autoimmunity
Cryoglobulinemia
Lymphatic system-associated diseases
Skin lesions
Pulmonary manifestations
Cardiovascular manifestations
Diabetes

thyroiditis remains controversial. One important pathomechanism of HCV in this context is the ability to replicate outside the liver in lymphocytes and macrophages.

### ASSOCIATED DISEASES

**Membranoproliferative Glomerulonephritis** Renal involvement is common and is usually caused by membranoproliferative glomerulonephritis. Membranous nephropathy (8.3%) and IgA nephropathy (1.7%) are only rarely associated with HCV. The prevalence of membranoproliferative glomerulonephritis is more common in Japan than in France or the United States; 15% of U.S. case and 60% of Japanese cases might be related to HCV infection. Liver disease in patients with mixed cryoglobulinemia-related membranoproliferative glomerulonephritis may be occult (25), and 40% have other systemic manifestations of mixed cryoglobulinemia. Therefore the diagnosis of membranoproliferative glomerulonephritis should always be followed by investigation of markers of HCV infection.

Clinically, patients with membranoproliferative glomerulonephritis suffer from edema, systemic arterial hypertension, and weakness. Sometimes cryoglobulins and rheumatoid factor are present. Patients usually present with proteinuria of more than 3.5 g/d. Often the serum albumin is less than 3 g/dl, with mild renal insufficiency. A few patients progress to dialysis. Sometimes decreased complement levels can be seen. Renal biopsy usually shows distinct morphological features consistent with immune complex disease.

The pathogenesis of membranoproliferative glomerulonephritis and HCV infection is not completely understood. A deposition of immunocomplexes might be causative for the renal dysfunction. The fact that precipitates of IgG, IgM, and C3 can be found in glomeruli of HCV-infected patients with glomerulonephritis supports this hypothesis (26). On the other hand, a direct effect of HCV has been suggested. It has been shown that the HCV c22 antigen is located at the region of glomerular damage, but HCV RNA or antibodies are not often found at areas of glomerular damage.

**Autoimmunologically Triggered Syndromes** Acute and chronic hepatitis C infections have been implicated in the pathogenesis of numerous autoimmune diseases (Table 4). Autoimmune diseases are defined by loss of tolerance of the adaptive immune response to self-antigens. A large variety of extrahepatic manifestations of this disease are seen, most of which are caused by lymphoproliferation or show characteristics of an autoimmune nature.

**Table 4**  
**Autoimmunity Associated With HCV Infection**

<b>Antigen-specific</b>
Thyroiditis
<b>Autoantibodies</b>
Type 1 diabetes
Antiphospholipid syndrome
Vitiligo
Autoimmune thrombocytopenic purpura
<b>Antigen-nonspecific/B-cell stimulation</b>
Mixed cryoglobulinemia
Membranoproliferative glomerulonephritis
Leukocytoclastic vasculitis
Sialoadenitis (Sjögren's-like)
Arthralgia, neuropathy, and pulmonary vasculitis
B-cell lymphoma/MALT lymphoma
<b>Antigen-nonspecific/unknown mechanism</b>
Lichen planus
Polyarteritis nodosa
Sicca syndrome
Mooren's corneal ulcer
<b>Others</b>
Porphyria cutanea tarda

**Table 5**  
**Prevalence of Autoantibodies in Chronic HCV**

<i>Autoantibody</i>	<i>Prevalence (%)</i>
<b>Strong association</b>	
Antinuclear (ANA)	9–38
Smooth muscle actine (SMA)	5–91
Liver-kidney microsome1 (LKM-1)	0–10
Liver cytosol type 1 (LC-1)	0–?
Rheumatoid factor	8–76
Antithyroid	9–20
<b>Weak or no association</b>	
IgG and IgM anticardiolipin (ACA)	
Antineutrophil cytoplasmic (ANCA)	
Antigastric parietal cells (GPC)	

The occurrence of autoantibodies is relatively common in patients with chronic hepatitis C infection (27). Although initial studies might contain some sampling bias and may not represent the autoantibody prevalence in the general population of HCV-infected patients, the prevalence of patients with autoantibodies is higher in chronic hepatitis C infection than it is in chronic hepatitis B infection. However, there is a significant variation in the prevalence of patients with positive autoantibodies, which might represent ethnic or geographic differences. In addition, the determination of autoantibodies is not standardized between laboratories. Most autoantibody titers in chronic HCV infection are lower than those reported in organ-specific autoimmune disease, and their relevance for the course of HCV infection, the response to antiviral therapy, and the development of organ-specific autoimmunity is generally low. There is, however, the chance that patients with the propensity to develop an autoimmune disease and patients with undiagnosed autoimmune diseases become

infected with HCV. In these cases therapy with interferon- $\alpha$  might worsen the underlying autoimmune disorder. This is especially important if the autoantibodies are indicative of autoimmune liver disease, as deterioration of liver inflammation may occur despite reduction in viral load.

Various autoantibodies are found in autoimmune liver disease associated with hepatitis C (Table 5). Antinuclear and anti-smooth muscle actin autoantibodies have the highest prevalence in chronic HCV infection. Although these autoantibodies are the hallmark of autoimmune hepatitis (AIH) type 1, they are frequently found in other autoimmune diseases and in chronic inflammation. Their titer in chronic HCV is usually lower than in AIH. The ANAs usually show a nonhomogenous immunofluorescence staining pattern, and most-SMAs are not reactive with actin-containing microfilaments, both diagnostic features of AIH. The exact antigen-specificity of ANAs in chronic HCV remains unknown. It has been suggested that autoantibody-positive patients have a more severe course of infection. However, these findings have not been recapitulated by others.

Autoantibodies to liver and kidney microsomes (LKMs) reactive with cytochrome P450 IID6 are one major diagnostic determinant in patients with AIH type II, an autoimmune disease preferentially affecting children. However, it has become clear that substantial proportions of anti-LKM-1 positive patients are infected with HCV and do not suffer from AIH type 2. The overall prevalence of anti-LKM-1 in chronic HCV is low in adult patients (0–6%) and tends to be higher in children (8–11%). Anti-LKM-1 autoantibodies are less often seen in American and Japanese populations with HCV. The prevalence data of autoantibodies might be skewed by a selection bias and varying expertise in autoantibody testing, as the highest prevalence has been reported from centers involved in studies of autoimmune liver diseases. Prevalence in unselected populations may be around 1%. Development of anti-LKM is not linked to viral genotypes. Anti-LKM-1 autoantibodies in chronic HCV usually have titers similar to those seen in AIH type 2. However, they less frequently recognize the linear epitope of amino acid 257 to 265 of CYP450 2D6, which is recognized by over 60% of HCV-negative AIH type 2 patients. It thus seems that AIH type 2 and anti-LKM-1-positive HCV infection present diverse disease entities calling for different therapeutic regimens. However, there might be an overlap between both entities in rare patients, who probably have an undiagnosed autoimmune liver disease in addition to HCV infection. In addition, anti-liver cytosol antibodies (LC-1) and anti-LKM-3 autoantibodies directed against UGT-1.1 have been reported in rare cases of patients with HCV infection.

**Cryoglobulinemia** The clinical signs of purpura, arthralgia, and weakness were originally described as Meltzer's triad. However, the disease manifestations caused by this systemic vasculitis are more diverse. Lymphoma, renal disease, neuropathy, and Sjögren's syndrome have an incomplete overlap with the cryoglobulinemic syndrome. However, many HCV patients with mixed cryoglobulinemia are asymptomatic, others are severe by affected. The prevalence of cryoglobulinemia

**Table 6**  
**Prevalence of Cryoglobulinemia in HCV-Infected Individuals**

Country	HCV-positive (no.)	Cryoglobulinemia-positive (%)	RF-positive (%)
Sweden	21	0	ND
Israel	90	11	44
Germany	132	28	42
France	58	36	70
France	321	56	38
Korea	49	59	14



**Fig. 5.** Severe cryoglobulinemia with critical skin necrosis. This patient had nearly complete recovery after corticosteroid therapy; a plasmapheresis was not necessary (Kindly provided by Dr. med. S. Schnarr, Department of Rheumatology, MHH, Hannover, Germany).

shows regional differences (Table 6). The classical sign of leukocytoclastic vasculitis is palpable purpura of the lower extremities, although other locations, like the hands, might be involved as well (Fig. 5). A severe systemic vasculitis such as that seen in polyarteritis nodosa, often associated with HBV infection, is rarely seen.

Mixed cryoglobulinemia-related arthritis is usually an intermittent, non-destructive mono- or oligoarthritis affecting the interphalangeal, and metacarpophalangeal joints and the knees. Occasionally joint pain might be precipitated by exposure to cold. It is important to note that arthralgias are common in HCV, whereas mixed cryoglobulinemia-related arthritis is not. Likewise, weakness might be caused by HCV infection rather than mixed cryoglobulinemia-related symptoms. Peripheral neuropathies caused by mixed cryoglobulinemia have frequently been under-recognized. They usually present as a peripheral moderate axonal sensory polyneuropathy involving bilateral nerves symmetrically or multiple isolated nerves. They are often painful long before motor deficits develop. Compared with polyarteritis nodosa, motoneuropathies are less common, lesions are distally by symmetrical without necrotizing vasculitis. As involvement is discontinuous, lesions may be missed by biopsy.

The pathology of mixed cryoglobulinemia is caused by vascular deposits of cryoprecipitate containing HCV- RNA, low-density lipoprotein, IgG, and a highly restricted IgM with rheumatoid factor (RF) activity. Virus and anti-HCV concentrations are 10- and 100-fold higher in cryoprecipitates than in serum, respectively. Of the monoclonal IgMs found in HCV patients, 80% share a major complementarity region named WA (initials of the patient in whom these were first described). This WA cross-idiotype is associated with a high degree of rheumatoid activity (formation of immune complexes by avid binding to IgG). These antibodies often express a VK light chain derived from a single germinal gene (K325 VL gene). Thus the repertoire is highly limited, with the same cross-reactive idiotype, and is encoded by few genes, probably owing close antigenic stimulation. Most of the IgM RFs are generated in the liver and bone marrow. It is of interest that formation of intrahepatic lymphoid follicles is a characteristic feature of chronic HCV infection and that most intrahepatic mononuclear cells in chronic HCV are B-cell-expressing IgM. In addition to a controversial lymphotropism of HCV the interaction of CD81 with E1/E2- proteins may contribute to the generation of B-cell activation and production of IgM by lowering the activation threshold of B cells. In this regard, it is interesting that the CD81 expression of B cells is increased in chronic HCV and is highest in patients with mixed cryoglobulinemia also, this certain amino acid sequences within the E2 region possessing a high binding affinity to CD81 in vitro and are associated with the development of mixed cryoglobulinemia. By itself, this process could lead to a type III (polyclonal) mixed cryoglobulinemia. Emergence of a dominant clone would subsequently result in a type II (monoclonal) mixed cryoglobulinemia. Therefore type III mixed cryoglobulinemia might be the precursor of mixed cryoglobulinemia II in some patients.

The emergence of a dominant B-cell clone might be owing to alterations enhancing B-cell survival. Translocation of the *bcl-2* gene from chromosome 18 to 14 results in overexpression of the anti-apoptotic *bcl-2*. This translocation has been found in 88% of patients with HCV-related mixed cryoglobulinemia compared with 8% in HCV-positive patients without mixed cryoglobulinemia and 2 to 3% in control populations of chronic liver or autoimmune disease. A further genetic alteration by a stochastic hit like a *c-myc* mutation might then be sufficient to transform the B-cell into a malignant lymphoma blast.



**Lymphoproliferative Diseases** Viral etiologies of different lymphomas have been described. Epstein-Barr virus and human T-cell leukemia viruses I and II have been associated with lymphoproliferative diseases. The number of hepatitis C-patients with fever and lymphadenopathy is small. These patients might present progression from the mixed cryoglobulinemia-syndrome to non-Hodgkin lymphoma.

Although the etiological role of HCV in the development of B-cell non-Hodgkin's lymphoma (B-NHL) is controversial, it was shown that up to 56% of chronic HCV patients with mixed cryoglobulinemia present with abnormal bone marrow morphology (28). A recent meta-analysis estimated the HCV prevalence in patients with B-NHL to be approximately 15%, higher than that reported not only in the general population (1.5%) but also in patients with other hematological malignancies (2.9%), suggesting a role of HCV in the etiology of B-NHL. The striking geographic variation in this association suggests that genetic and/or environmental factors are also involved in the pathogenesis of this disorder (29). Extranodal involvement is common, with significant overrepresentation of the liver and salivary glands. Another extranodal site is the stomach, and HCV has been suggested to be a possible cause of mucosa-associated lymphoid tissue lymphoma (MALT). The development of anemia or lymphadenopathy in chronic hepatitis C patients with mixed cryoglobulinemia may demonstrate an underlying lymphoproliferative disorder and should be monitored.

**Skin Manifestations** Skin manifestations in HCV include pruritus, lichen planus, urticaria, erythema nodosum, erythema multiforme, and especially porphyria cutanea tarda, which is the most common form of porphyria. The reported prevalence of HCV in patients with porphyria cutanea tarda varies considerably but averages around 45%.

The pathogenesis of the disease is not autoimmune. HCV may be the trigger for clinical expression but is by itself insufficient to cause metabolic porphyrin derangements. Alcohol consumption is an important cofactor with HCV for the development of porphyria. Also, hepatic iron and fat accumulation as well as increased oxidative stress during chronic HCV infections might be involved in the pathogenesis.

**Pulmonary and Cardiovascular Manifestations** Pulmonary manifestations have been reported in patients with HCV. These include direct effects of HCV on the lung as well as secondary effects in the settings of progressive liver disease and treatment for HCV (30). Direct effects might lead to a worsening of lung function, especially in patients with pre-existing lung diseases such as chronic obstructive pulmonary disease or asthma. The exact pathomechanisms leading to declining pulmonary function in HCV are not well understood. Several mechanisms have been proposed, and the chronic immune activation and inflammation induced by HCV infection might play the most important role.

**Diabetes Mellitus** It is now clear that HCV conveys a risk of developing diabetes mellitus, type 2 in particular (31). Hepatic steatosis insulin resistance, and oxidative stress caused by HCV might be involved in the pathogenesis, which is not

autoimmune. In terms of type 1 diabetes, antibodies against  $\beta$ -cells (anti-GAD65, and anti IA-2) and against adrenals (anti-21OH) have been found in patients with HCV infection, and their titers increased during therapy. However, none of these patients developed clinical disease. Although there are single case reports of type 1 diabetes under interferon (IFN) therapy, these cases seem to be rare (32) and rather represent HCV infection in patients with underlying  $\beta$ -cell autoimmunity.

## THERAPY

### EXTRAHEPATIC MANIFESTATIONS OF HEPATITIS A

Patients who have hepatitis A with extrahepatic manifestations almost always have complete recovery, so treatment is usually not necessary. Only severe complications should be treated symptomatically.

### EXTRAHEPATIC MANIFESTATIONS OF HEPATITIS B

The optimal treatment of HBV-associated polyarteritis nodosa is a combination of antiviral and immunosuppressive therapies. In the past, HBV-associated polyarteritis nodosa was treated like non-virus-related polyarteritis nodosa, with corticosteroids, cyclophosphamide, and/or plasma exchange. However immunosuppression without antiviral therapy has often increased HBV replication. Recent studies show a positive effect for the treatment of HBV-associated polyarteritis nodosa if a combination of antiviral and immunosuppressive therapies is used. IFN can be used alone or combined with nucleoside analogs or combined with plasma exchange or immunosuppressive therapy (33–35).

Glomerulonephritis is usually self-limited in children and does not progress to renal failure, in contrast to adult patients, in whom glomerulonephritis can be more aggressive. Immunosuppressive therapy is not recommended in HBV-related glomerulonephritis, but antiviral therapy with INF- $\alpha$  has shown promising results and should be given to avoid renal failure. Several studies have proved the effectiveness of nucleoside analogs for HBV infection therapy; however, there are only case reports showing an effect on HBV-associated renal manifestations. In one study, the authors reported that two cases of HBV-related nephrotic syndrome were successfully treated with lamivudine (36).

**Extrahepatic Manifestations of Hepatitis C** Before chronic HCV was discovered as the major cause of mixed cryoglobulinemia, symptomatic disease was treated with plasmapheresis and/or steroids cyclophosphamide to decrease production of cryoglobulins and inhibit vascular inflammation. More than 50% of patients with HCV-related mixed cryoglobulinemia will respond to antiviral therapy (IFN- $\alpha$ ) with decreased cryocrit and RF levels and improvement in symptoms. However, in almost all patients not achieving a sustained response (lasting HCV clearance), mixed cryoglobulinemia symptoms will recur after therapy is stopped.

The treatment response to IFN in terms of viral clearance is independent of the presence of mixed cryoglobulinemia. Most studies were performed with IFN monotherapy using 3 million units. Large studies with pegylated interferons and

ribavirin do not exist so far. However, in most small trials improvement of symptoms was usually linked to suppression of viral replication. It can therefore be assumed that response rates to modern therapeutic regimes might be substantially better than those seen in IFN monotherapy. Although a small trial reported an effect of ribavirin monotherapy on symptoms of mixed cryoglobulinemia (37), these results have never been confirmed by others. Long-term IFN therapy is effective in controlling symptoms of mixed cryoglobulinemia in partial virological responders, particularly for symptoms of cutaneous vasculitis. In the latter patients, a combination of IFN with steroids does not improve results compared with IFN monotherapy. However, combined antiviral and immunosuppressive therapy may be indicated in patients with severe renal disease.

A suggested therapy scheme based on different studies has been developed (38). Initially IFN with or without ribavirin should be used. For nonresponders and patients, a combination of the first-line-medications with corticosteroids, cyclophosphamide, or plasmapheresis could be helpful.

For the lymphoproliferative diseases associated with hepatitis C, there are only few studies. One study demonstrated a complete response of a lymphoma after treatment with IFN with or without ribavirin (39). All patients in this study whose lymphomas regressed lost detectable HCV RNA.

The influence of HCV treatment on the course of HCV cryoglobulinemic membranoproliferative glomerulonephritis is controversial. A recently published study demonstrated that after first-line treatment with prednisone, furosemide, or plasmapheresis, antiviral therapy with standard or pegylated IFN- $\alpha$  and ribavirin improved proteinuria and stabilized creatinine clearance in sustained virological responders (40). However, in the presence of acute cryoglobulinemic glomerulonephritis, IFN does not prevent progression of renal damage. Instead, combination therapy with cytotoxic and antiinflammatory drugs, and sometimes plasma exchange, is recommended. Combined antiviral and immunosuppressive therapy may be indicated in patients without sustained virological response. IFN monotherapy is promising for the therapy of HCV-associated cryoglobulinemia (38). IFN monotherapy has caused significant improvements in insulin sensitivity in HCV-patients with diabetes mellitus (38). Thus interferon can be used safely in diabetics, but the results of these studies are still discussed controversially.

Various other extrahepatic manifestations respond differently to antiviral therapy. Cutaneous vasculitis usually responds well to antiviral therapy. Skin lesions disappear in sustained viral responders and improve significantly in the rest under long-term therapy. For hepatitis C-associated lichen planus, Mooren's corneal ulcer, or porphyria cutanea tarda, there are only very small studies or case reports showing an effect of antiviral therapy.

## CONCLUDING REMARKS AND OPEN QUESTIONS

Acute and chronic viral hepatitis are associated with and may trigger or exacerbate a wide range of extrahepatic manifestations.

Thus it is important that all HCV and HBV carriers be investigated for extrahepatic manifestations. On the other hand, patients with typical symptoms of extrahepatic manifestations should be tested for viral hepatitis, even if liver function tests are normal.

Several possible mechanisms causing extrahepatic manifestations in viral hepatitis have been described; nevertheless, many questions about the immunopathogenesis of extrahepatic symptoms remain to be answered. Future studies need to investigate to what extent HBV and HCV can directly damage extrahepatic tissues and which symptoms are only caused by immune responses. Furthermore, more studies on the role of T-lymphocyte activation, apoptosis, and cytokine/chemokine responses are necessary to unravel the pathways of damage, with the aim of developing novel treatment strategies.

Since current treatments use either immunosuppression or IFN- $\alpha$ , which may also activate immune responses, it will be extremely important to identify patients at risk for worsening of symptoms during interferon therapy. The current standard combination therapy for hepatitis C (pegylated IFN plus ribavirin) frequently triggers autoimmune thyroiditis, skin rashes, or hemolytic anemia.

Direct antiviral therapies should be applied in particular when symptoms are caused by virus-induced damage of extrahepatic tissues or by immune complexes containing viral particles. In contrast, if a pathological immune response is believed to be the main cause of disease, immunosuppressive therapy is recommended. There are many situations in which both antiviral and immunosuppressive therapies will be needed in combination. An individualized strategy is usually required for the therapy of extrahepatic manifestations, balancing potential risks and benefits of treatment.

Long-term therapy with lamivudine and (pegylated) IFN plus ribavirin has been shown to suppress viral replication in patients with chronic hepatitis B and C and subsequently to reduce the incidence of adverse clinical outcomes of liver disease. However, the treatment of extrahepatic manifestation requires further investigations since almost no large trials including sufficient numbers of patients and comparing different treatment strategies have been performed.

Finally, the value of new antiviral drugs currently being explored for hepatitis B and C will have to be studied in terms of treatment of extrahepatic manifestations. The portfolio of antiviral drugs against hepatitis B has significantly improved in recent years, and more drugs are close to being licensed; thus the problem of drug resistance should be minimized in hepatitis B by combination therapies in the near future. In hepatitis C, several new direct antivirals targeting HCV enzymes such as the HCV protease and polymerase are currently in phase II/III trials, opening completely new treatment options in HCV-associated extrahepatic diseases. Since IFN- $\alpha$  may be avoided. However, resistance to HCV enzyme inhibitors will evolve much more rapidly than in hepatitis B, and thus primary combination therapies will be required; it will still take several years until the new substances will be available for use without IFN.

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# **AUTOIMMUNE LIVER DISEASES**

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**III**

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# 17 Immunogenetics of Autoimmune Liver Disease

## *Risk Factors for Susceptibility and Progression*

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PETER TICKELL DONALDSON

### KEY POINTS

- The common autoimmune liver diseases (type 1 autoimmune hepatitis, primary biliary cirrhosis, and primary sclerosing cholangitis) do not exhibit simple Mendelian inheritance attributable to a single gene locus.
- These autoimmune diseases are “genetically complex”, arising from the interaction of both environmental factors and one or more host genes.
- The alleles that are permissive for autoimmunity are common in the “healthy” population and by themselves are neither necessary nor sufficient for disease to occur.
- Most of the evidence for a genetic component in the pathogenesis of autoimmune liver disease is based on case-control (association) studies. Informative (multiplex) families are rare, and conventional linkage data are not available.
- The most consistent data we have suggest strong links with the major histocompatibility complex (MHC) on chromosome 6p21.3. Possible links with other susceptibility alleles on a number of chromosomes are more speculative.
- Genetic effects on both disease susceptibility/resistance (i.e., disease risk) and disease progression (i.e., phenotype) have been documented. Identification of the former provides the necessary background for a better understanding of the disease pathogenesis. Identification of the latter alleles may be more immediately useful in developing predictive indices for disease prognosis.
- Overlap syndromes and comparison with other autoimmune diseases indicate that there may be shared (common) disease susceptibility alleles acting as non-(disease)-specific promoters of autoimmunity. These findings indicate the activation of common pathways in the pathogenesis of autoimmunity and the processes underlying tolerance breakdown.
- Current knowledge of the genetics of autoimmune liver disease is incomplete, but the Human Genome Project has

identified an astounding degree of polymorphism in our genes; 5 yr on, we still face a major challenge in integrating the “new genetics” into medical practice.

- The key issues for future investigators will be: defining the genetic mechanisms whereby self-tolerance is broken; defining the genetic mechanisms that determine the rate of disease progression; and identifying genetic markers to predict both progression and malignancy.
- The same HLA genes and haplotypes that are important in autoimmune liver diseases are also implicated in susceptibility and resistance to infectious liver disease, opening a new avenue for future investigations.

### INTRODUCTION

Autoimmune liver diseases are not classical Mendelian autosomal or sex-linked genetic traits. However, there is considerable evidence that our genes play a significant role in determining individual susceptibility to (and progression of) these diseases. In the absence of a “simple” pattern of inheritance, attributable to a single gene locus, autoimmune liver diseases are classified as “genetically complex.” Variation at a gene locus gives rise to a number of alleles. When alleles are rare within a population (less than 1%), they are referred to as mutations. When alleles are common, they are referred to as polymorphisms. To the geneticist, “complex traits” are those in which one or more genes (alleles) acting alone or in concert increase or reduce the risk of a disease or syndrome (1). In Mendelian diseases, the permissive alleles are rare in the normal population (i.e., mutations), whereas in complex diseases, the permissive alleles are common (i.e., polymorphisms). Furthermore, it appears that alleles that are permissive for autoimmunity are not themselves abnormal and may be present in a large proportion of the “healthy” population. This finding suggests that inheritance of a specific allele or group of alleles is neither necessary nor sufficient for disease genesis but will simply increase (or reduce) the likelihood (risk) of disease.

Investigations of the genetic basis of complex disease hold three promises: (1) they will aid disease diagnosis, especially for near-Mendelian diseases; (2) they will identify alleles that

may inform disease management and therapy (in this respect pharmacogenetics is particularly important); and (3) they will identify alleles that inform the debate on disease pathogenesis. In the context of autoimmune liver diseases, the third promise is one that is most likely to bear fruit and here immunogenetics is the key.

Immunogenetics is concerned with the genes that regulate the immune response. Investigators in the mid-20th century, driven by clinical need, discovered complex systems in both mice and humans that govern the outcome of transplanted tissues: the major histocompatibility complexes (MHCs). For a considerable time it was thought that MHC genes were the only immune response (IR) genes. However, following completion of the human genome mapping project, we now know that nearly all human genes are polymorphic, and therefore any gene expressed in lymphoid tissue has the capacity to influence the immune response and thus (in the context of this chapter) disease risk. In the post-genome mapping era, understanding the role of host genes in autoimmune disease presents a major challenge. Approximately 11,000 of the 33,000 human genes may be expressed in lymphoid tissues, and there are more than 10.4 million variations in the genome (2). The most widespread of these are single-nucleotide polymorphisms (SNPs), which account for 90% of the human genetic polymorphisms. A comprehensive database of human genomic variations, dbSNPs, can be found at: <http://www.ncbi.nlm.nih.gov/SNP/> (2).

## INVESTIGATING COMPLEX DISEASE GENETICS

As the terminology implies, identifying disease-promoting mutations and polymorphisms (DPMs and DPPs) in “complex” diseases is not as simple as it may be for most Mendelian diseases. Classical approaches such as linkage analysis require multiplex families or sibling pairs and are most effective in near-Mendelian complex traits, in which there are few susceptibility loci and high levels of penetrance (1). Association analysis is the method of choice for diseases in which penetrance is low, onset is late, and/or families are rare (1,3–5). Almost all the work in autoimmune liver disease has been through association studies, a choice dictated by the relative paucity of multiplex families for study (5). Consequently, there are no linkage data from either genome scanning or large-scale family studies for any of the three diseases discussed here.

In association studies, two different genetic effects can be identified. Possession of an allele may increase or reduce the risk of disease (i.e., render an individual susceptible to or confer protection from the disease), or possession of an allele may determine the clinical phenotype, for example, disease severity or progression. These two effects are not exclusive, and one susceptibility allele may modify the effect of another.

The key to success with association analysis is adequate numbers (6). Thus the statistical power of any study to identify DPMs and DPPs is directly proportional to the number of patients and controls studied and the number of different

candidate alleles assessed. Other common errors in association studies include use of poorly matched controls, analysis of multiple subgroups, and over-interpretation of the data (5–8). Association studies must be designed to include large, well-established patient series and appropriate controls. Calculations of statistical power should be performed prior to any study to determine the necessary sample size, and ideally all findings should be replicated in a second series prior to publication (5–8).

Two other factors that have a strong influence on our understanding of the genetics of complex diseases in general are strong publication bias in favor of significant probability values (making non-significant data difficult, or even impossible, to publish) and “case ascertainment bias,” which can arise when association studies are performed (as they most frequently are) at national and regional referral centers (5,6). Referral centers often see a higher proportion of “unusual” cases (most often a higher proportion of severe cases), and the case load at such centers rarely reflects the total disease population. Consequently, alleles may be falsely identified as “susceptibility alleles,” when their true role is in determining the disease phenotype. A recent example of this phenomenon was the identification of *DRB1\*0801* as a determinant of disease progression (severity) in primary biliary cirrhosis (9), which is discussed below.

## WHERE TO LOOK: SELECTION OF CANDIDATE GENES

Gene loci for investigation are usually selected on the basis of either a known or potential functional role in disease pathogenesis or prior knowledge of linkage to or associations with other (similar) autoimmune diseases. As stated above, there are no linkage data for the three diseases discussed here, and knowledge of disease pathogenesis is patchy. Therefore, the latter criterion is the most frequently applied in the selection of candidate genes. The justification for this approach is based on the understanding that autoimmune diseases share common immune response pathways and often have similar genetic associations (10). It is reasonable to assume that only a proportion of the DPPs identified in any disease will be disease specific and that the remainder (even the majority), although no less important, may be nonspecific promoters of autoimmunity (10).

In autoimmunity, most studies have concentrated on polymorphism in the genes that control the adaptive immune response, centered on the role of T and B cells and the maintenance of immune homeostasis (tolerance to self). However, there is a growing interest in role of genes involved in innate immunity and the interaction with bacterial pathogens. This new interest in innate immunity has been fuelled by a number of factors. **First**, with the completion of the Human Genome Project, we have a much better knowledge about non-MHC IR genes in general. **Second**, recent studies in inflammatory bowel disease have been very successful in identifying major susceptibility loci outside the MHC (11). All of these genes (*CARD15*, *CARD4*, and *CARD8*) are important determinants

of the innate immune response to bacterial antigens. **Third**, there has been a revolution in our understanding of liver immunology indicating that the liver is home to a large population of nonconventional immune cells regulated by non-HLA receptor-ligand interactions.

Even so, when it comes to candidate selection in autoimmune liver disease, the most frequently examined genes have been those involved in antigen presentation, especially the MHC-encoded human leukocyte antigens (HLAs) on chromosome 6p21.3. Although historically it has always been convenient to study HLA and rather difficult to study other IR genes, it is also important to remember that HLA molecules have critical roles in both innate and adaptive immunity, and this makes them “prime candidates” in autoimmune disease.

More recently, studies of autoimmune diseases have concentrated on non-MHC immunoregulatory genes including genes encoding accessory molecules, which provide second signals in antigen presentation; genes encoding the cytokines and chemokines that regulate the inflammatory milieu; genes encoding proteins involved in wound healing and repair; and genes whose expressed products are important in redressing the immunological balance and restoring immune homeostasis.

## AN INTRODUCTION TO THE MAJOR HISTOCOMPATIBILITY COMPLEX

The full gene map for the human MHC was published in 2004 and is more complex than previously envisaged (12). Accurate and up-to-date gene maps for the MHC and other genes referred to herein can be found on [www.ensembl.org/](http://www.ensembl.org/). The extended-MHC (xMHC) maps to 7.6 Mb of chromosome 6p21.3 and encodes 421 gene loci, of which 252 are expressed genes, 30 are classified as transcripts, and 139 are pseudogenes. The xMHC is characterized by extreme linkage disequilibrium and a very high degree of polymorphism (56 of the 252 expressed xMHC genes are known to be polymorphic). This polymorphism includes single nucleotide polymorphisms (SNPs), deletion/insertion polymorphisms (DIPs), and two large regions of duplication. The high level of polymorphism is best exemplified by human leukocyte antigens (HLAs), for which there are now more than 1000 registered alleles (updates on HLA nomenclature can be found on [www.anthonynolan.com/](http://www.anthonynolan.com/) HIIH/nomenclature) (13).

Within the xMHC, it is possible to recognize clusters and super-clusters, which appear to have arisen from both small- and large-scale segmental duplication. Currently six clusters and six superclusters are recognized. Among these are three that are of immediate interest in the context of MHC genes in autoimmune liver disease. These are the HLA class I supercluster, the tumor necrosis factor (TNF) cluster, and the HLA class II cluster. This new terminology pays homage to (but replaces) the historic and practical division of the MHC into three subregions, class I, class II, and class III. This early terminology which was based on both position and relative function, is no longer applicable to most genes encoded within

these regions. According to the “old order,” the MHC class I and class II regions encode the classical transplantation antigens (HLA A, B, Cw and DR, DQ, DP, respectively), and the class III (for brief time referred to as class III and class IV) region encodes an assortment of immune response genes including TNF; complement proteins C2, C4A, C4B, Bf; several members of the HSP-70 family of heat shock proteins; and the genes encoding the MHC class I chain-related proteins MIC $\alpha$  and  $\beta$  (*MICA* and *MICB*).

According to the “new order,” the HLA class I supercluster comprises the classical HLA A, B, and Cw gene loci; the non-classical HLA E, F, and G genes; the class I-like genes *MICA* and *MICB*, as well as the more distant *HFE* locus and 12 pseudogenes. The products of the classical HLA class I (A, B, and Cw) gene loci form heterodimers together with  $\beta$ -2-microglobulin that present short antigenic peptides (eight to nine amino acids) to CD8<sup>+</sup> T cells. In addition, both classical and nonclassical HLA class I gene products are involved in the natural killer (NK) cell-mediated immune responses, through both CD8<sup>+</sup> T-cell activation and recognition of the leukocyte receptor or NK complexes including the killer immunoglobulin-like receptors (KIRs) on NK cells. Among the other HLA class I supercluster genes, the expression profile of the class-I-like *MICA* and *MICB* genes indicates a possible role in mucosal immunity. The products of the *MICA* gene, MIC $\alpha$  molecules, are important in regulation of  $\gamma\delta$  T cells, CD56<sup>+</sup> (NK) cells and T cells expressing the NKG2D-DAP10 activatory receptor (14). All these cell types are found in large numbers in the “normal” liver (15).

The TNF gene cluster comprises the genes encoding the cytokines TNF- $\alpha$ , lymphotoxin- $\alpha$ , and lymphotoxin- $\beta$ , (*see* Cytokine Gene Polymorphisms below).

The HLA class II cluster comprises the classical HLA DP, DQ, and DR genes and the nonclassical HLA DM and DO genes. The products of the DP, DQ, and DR genes form heterodimers that present short antigenic peptides (13–23 amino acids) to CD4<sup>+</sup> T cells. The nonclassical class II genes DM and DO are involved in peptide exchange and loading into class II molecules. Interestingly although there are many class I-like genes in the genome, there are (as yet) no class II-like genes outside the xMHC (12).

Although we now know there are many more potential IR genes in the MHC, few of them have been investigated in autoimmunity. Our understanding of the role of the genes in this region in autoimmune disease is currently very simple, and yet when we consider the role of HLA molecules in the immune response and the functional relevance of inherited variations in HLA gene sequences, this simplification has turned out to be very appropriate. In most cases the disease risk associated with various HLA alleles has been shown to be an order of magnitude greater than that associated with any non-MHC susceptibility genes so far identified. There are two reasons for this: first, the functional relevance of HLA polymorphism; and second, the strong linkage disequilibrium across this region. HLA class I and class II antigens are critical for T, B, and also NK cell immunity. MHC-peptide interaction constitutes

**Table 1**  
**Six HLA Haplotypes Associated With Primary Sclerosing Cholangitis<sup>a</sup>**

Haplotype	No.
<i>B8-MICA*008-MICB*24-TNFA*2-DRB3*0101-DRB1*0301-DQA1*0501-DQB1*0201</i>	1
<i>DRB3*0101-DRB1*1301-DQA1*0103-DQB1*0603</i>	2
<i>MICA*008-DRB5*0101-DRB1*1501-DQA1*0102-DQB1*0602</i>	3
<b><i>DRB4*0103-DRB1*0401-DQA1*03-DQB1*0302</i></b>	4
<b><i>DRB4*0103-DRB1*0701-DQA1*0201-DQB1*0303</i></b>	5
<b><i>MICA*002</i></b>	6

<sup>a</sup>Haplotypes 1, 2, and 3 are associated with increased risk (susceptibility).

Haplotypes 4, 5, and 6 (in bold) are associated with reduced risk (resistance).

one-half of the immune synapse and is an essential element in adaptive immunity, whereas NK-KIR interaction may be an important mechanism in immune surveillance for tumor cells and viral infections.

The peptide that is bound and presented by an HLA molecule is determined by the structure of the MHC binding site. The expressed molecule comprises a series of  $\beta$ -pleated sheets that support two opposing  $\alpha$ -helices, forming a cleft or groove in which antigenic peptides are bound. The cleft has nine pockets that accommodate the side chains on the antigen peptide. Up to 90% of inherited variations in the HLA genes (HLA alleles) results in amino acid variation in and around the peptide binding cleft. The HLA alleles an individual inherits determine the menu of peptides that will preferentially bind and be presented to the T-cell receptor (TCR; the other half of the immune synapse). In addition, HLA Cw, B, and some HLA A molecules have motifs that are recognized by KIR on NK cells, and appropriate KIR-MHC interaction downregulates NK cell activity. These two essential processes (MHC-peptide and MHC-KIR interaction) illustrate the functional basis by which allelic variation in the HLA system may determine individual susceptibility and resistance to autoimmune liver disease.

## MHC GENE POLYMORPHISM IN AUTOIMMUNE LIVER DISEASE

### TYPE 1 AUTOIMMUNE HEPATITIS

Early studies in autoimmune hepatitis identified significant associations with both HLA A1 and B8 (16) and the mixed lymphocyte culture-determined antigen Dw3 (17) (which is essentially identical to DR3). Later works confirmed that these three alleles are inherited as a single unit or haplotype (referred to as A1-B8-DR3 or the ancestral 8.1 haplotype) and identified DR4 (later *DRB1\*0401*) as a second susceptibility allele in DR3-negative (older) patients and *DRB1\*1501-DQB1\*0602* as a protective haplotype (18–20). A series of studies from 1994 onward then mapped susceptibility/resistance to the *DRB1* locus and excluded HLA Cw (21) *TNFA* (22,23), and (HLA *DQA1 DQB1* and *DPB1* (19,20,24) as the primary susceptibility loci. Meanwhile, investigations outside of Europe and North America identified different susceptibility alleles at *DRB1* including *DRB1\*0405* in Japan (25,26), *DRB1\*0404* in Mexico (27), *DRB1\*0405* in adult patients from Argentina (28),

and *DRB1\*1301* in South American children (29). More recently, transmission disequilibrium-based analysis of a mixed group of French and French-Canadian pediatric patients, comprising 35 with type 1 autoimmune hepatitis (AIH) and 15 with type 2 AIH, identified highly significant levels of transmission disequilibrium for both *DRB1\*0301* and *DRB1\*1301* (30). Exactly what these different genetic associations may tell us about the pathogenesis of type 1 AIH is discussed below.

### THE RELATIONSHIP WITH DISEASE PHENOTYPE AND DISEASE PROGRESSION

Analysis suggests that HLA alleles may play a significant role in disease severity in type 1 AIH. Patients with B8 and *DRB1\*0301* are significantly younger than those with *DRB1\*0401* (18–20); have more severe disease with higher serum aspartate aminotransferase and bilirubin levels and a greater degree of liver necrosis and cirrhosis (31); are less likely to enter remission on corticosteroid therapy; are more prone to relapse after therapy; and are more likely to require transplantation for end-stage disease than those without these markers (18,19,31). Interestingly, the *DRB1\*0301* genotype is also associated with the presence of antibodies to soluble liver antigen/liver-pancreas (anti-SLA/LP) (32). Whereas patients with *DRB1\*0401* are more likely to have smooth muscle antibodies (SMAs) and higher titers of antinuclear antibodies (ANAs) and are also more likely to have overlapping immune diseases (33). This latter observation may reflect a higher degree of epitope crossreactivity associated with the DR4 molecule and may also be an indication of the activation of common pathways in the generation of autoimmune diseases.

### PRIMARY SCLEROSING CHOLANGITIS

The history of HLA associations in primary sclerosing cholangitis (PSC) has been reviewed (34). Briefly, studies in the early 1980s described increased frequencies of HLA B8 and DR3 and a lower frequency of B44 in PSC patients compared with healthy controls (34). All these findings were later confirmed (except the protective effect of B44) and extended to include a secondary association with DR2 in DR3-negative patients (34,35). Subsequent investigations using various molecular genotyping techniques have identified six different HLA haplotypes associated with PSC (Table 1) (36–46). Five of these six haplotypes have been now been confirmed.



**Table 2**  
**Key HLA Susceptibility Haplotypes in Primary Biliary Cirrhosis<sup>a</sup>**

Population	Haplotype	Ref.
Japan	<i>DRB1*0803-DQ3-DPBI*0501</i>	56–59
Europe NEC	<i>DRB1*0801-DQA1*0401-DQB1*0402</i>	9,51–53,61, 62
USA NEC	<i>DRB1*0801-DQA1*0401-DQB1*0402</i>	54,55
USA NEC	<b><i>DRB5*0101-DRB1*1501-DQA1*0102-DQB1*0602</i></b>	54
Europe NEC	<b><i>DRB3*-DRB1*11-DAQ1*0501-DQB1*0301</i></b>	60, 61
Europe NEC	<b><i>DRB3-DRB1*13-DQA1*0102-DQB1*0603</i></b>	60–62

Abbreviation: NEC northern European Caucasoid.

<sup>a</sup>Protective haplotypes are in **in bold font**. The association with ***DRB5\*0101-DRB1\*1501-DQA1\*0102-DQB1\*0602*** has not been confirmed in other series; the association with ***DRB3\*-DRB1\*11-DAQ1\*0501-DQB1\*0301*** has only been found in Italian PBC patients so far; the association with ***DRB3-DRB1\*13-DQA1\*0102-DQB1\*0603*** is a weak protective association that is stronger in southern compared with northern Europe.

Haplotypes 1 and 2 are strongly associated with disease susceptibility (36,39,40). Haplotype 3 has a weak positive association (35–37,40). Haplotypes 4, 5, and 6 all have strong negative (protective) associations (35,36–44).

Almost all the published data on HLA and PSC is from a single racial group (European Caucasoid), but even so there is a marked variation in the strength of the reported associations. Consequently, the associations have been interpreted differently by different groups (35–46). Overall, there are three key questions in this debate:

1. Which is the primary susceptibility locus on these haplotypes?
2. Do haplotypes 1, 2, and 3 (i.e., the susceptibility haplotypes) encode a common (shared) allele or amino acid motif not found on haplotypes 4, 5, and 6 (i.e., the protective haplotypes)?
3. Can we construct (based on the answers to 1 and 2 above) a unified hypothesis to explain HLA-encoded susceptibility to PSC?

This debate is well rehearsed (34,42–44) and has been going on for some time without resolution. In summary, the critical issues to be resolved are: whether MHC-encoded susceptibility to PSC maps to the (old order) class II region and if so to which locus—*DRB1*, *DRB3*, or *DQB* (or a combination); whether MHC-encoded susceptibility maps elsewhere within the MHC—*TNFA* (46) and *MICA* (42) have both been proposed as alternative candidates to *DR/DQ*; and whether there is more than one susceptibility allele on each haplotype, of which some may be common (shared) and others may haplotype specific. Whatever the answer, resolving this debate is of major importance in terms of the impact of these genetic studies on our understanding of disease pathology in PSC (see below).

**The Relationship Between Disease Phenotype and Disease Progression** In all studies to date, the relationship between MHC genes and disease progression/severity, the presence of ulcerative colitis or malignancy (cholangiocarcinoma) has been a minor consideration only, and claims that DR3 (35) *DRB3\*0101* (36) and *DRB1\*04* (37,47) influence disease progression remain controversial (34,39,40). More

recently, heterozygous status for the *DRB1\*0301* haplotype has been associated with accelerated disease progression (48). This observation partially confirms earlier reports that patients with DR3 have more severe disease (35).

The relationship with inflammatory bowel disease (IBD) in PSC, mostly ulcerative colitis, which occurs in 80 to 100% of patients and is usually mild, has been mostly overlooked. Overall, PSC occurs in less than 4% of IBD patients, and although there are weak HLA associations with IBD (*DRB1\*0103* and *DRB1\*1502*), no strong HLA associations have been described. Even so, a recent report suggested that some of the HLA associations in PSC may be restricted to the subgroup of patients with IBD (44). Although this latter hypothesis remains to be confirmed, clinical-genetic heterogeneity may explain some of the difficulties in mapping HLA-encoded genetic susceptibility to a specific MHC locus in PSC and variations in the strengths of reported associations.

#### PRIMARY BILIARY CIRRHOSIS

In contrast to both type 1 AIH and PSC, the HLA associations in primary biliary cirrhosis (PBC) are relatively weak (Table 2). Although early studies (reviewed in ref.49) identified genetic associations with a variety of different serologically defined HLA DR specificities including DR2, DR3, DR4 and later with DR8 (49), the only consistently reported association was with DR8 (49,50). This association accounts for 11 to 36% of patients in northern Europe and North America (NEC) and 36 to 79% of patients in Japan. More recent molecular genotyping studies have suggested that *DRB1\*0801* and *DRB1\*0803* are the primary susceptibility alleles in NEC patients (9,51,55) and Japanese patients respectively (56–59) and that associations with specific *DQA1*, *DQB1*, and *DPBI* alleles (Table 2) are due to linkage disequilibrium with *DRB1\*08*, (9,38,51). These studies have identified a number of novel protective associations including associations with members of the *DRB1\*11* and *DRB1\*13* families of alleles (60–62).

Based on the available data, it is tempting to consider the matter of MHC association in PBC closed, with *DRB1* as the primary susceptibility locus on all haplotypes. However, even

**Table 3**  
**Molecular Models of MHC-Encoded Disease Susceptibility in Type 1 Autoimmune Hepatitis**

Original population	Model: Motif or amino acid	Risk	Other populations	Ref.
Japanese NEC	Histidine-13		None	25,26
UK	LLEQKR-lysine-71		—	19
	ILEQAR-alanine-71		—	19
USA	LLEQKR-lysine71		—	20
	ILEQAR-alanine71		—	20
	LLEQRR-arginine 71		Japan	25,26
	LLEQRR-arginine 71		Mexico	27
	LLEQRR-arginine 71		Argentina (adults)	28
South America (children only)	Valine/glycine-86			29
	Valine/glycine-86		NEC-UK-PSC (adults only)	— <sup>a</sup>

Abbreviation: NEC, northern european caucasoid.

<sup>a</sup>See Table 1.

in comparison with type 1 AIH and PSC, our knowledge of MHC gene polymorphism in PBC is relatively poor. Early studies of PBC investigated HLA A and B serotypes and found no significant associations. Consequently, the focus of interest moved to the (old order) class II region. However, the possibility of stronger associations within the HLA class I genes, in particular the HLA Cw locus, should not be excluded until there have been comprehensive investigations using molecular genotyping techniques. Furthermore, these investigations should also be extended to include the (old order) MHC class III region, in which investigators in the 1980s identified strong associations with the complement C4 alleles *C4B\*2* (63) and *C4A\*Q0* (64) and where later studies of the *TNFA* promoter A/G SNPs at positions -238 and -308 produced conflicting data that remain controversial to this day (49,65).

### HOW DO THESE STUDIES OF MHC ASSOCIATIONS INFORM THE DEBATE ON DISEASE PATHOGENESIS?

One of the major long-term promises of the human genome mapping project was that once the map of the genome was complete, studies identifying DPMs and DPPs would aid in the understanding of disease pathogenesis. Here I will use examples from each of the three diseases discussed above to illustrate how this promise may be fulfilled.

#### TYPE 1 AUTOIMMUNE HEPATITIS

By translating the basic data from studies of HLA alleles into amino sequences and comparing the distribution of these in patients and controls, we can develop molecular models of disease susceptibility/resistance. These models are based on shared sequences or epitopes in and around the peptide binding cleft of the expressed HLA molecule, and this allows us to formulate hypotheses about the nature of antigenic peptides involved in disease pathogenesis. This has been applied with some success in type 1 AIH and to a lesser extent in PSC.

In type 1 AIH, three different models have been developed (Table 3). Comparing and contrasting these models offers

two different interpretations. In the discussion below, the standard interpretation is illustrated by the first two models and a contrast with the third model offers an alternative possibility.

The first model is based on histidine or other basic amino acid residues at position 13 of the DR $\beta$ -polypeptide and may account for up to 100% of Japanese patients (25,26), but it does not account for disease susceptibility in any other population. The second model is based on possession of the six-amino acid epitope LLEQKR at positions 67 to 72 of the DR $\beta$ -polypeptide, a sequence shared by the two major susceptibility alleles in Europeans and their North American cousins (19,20). Further analysis comparing the amino acids encoded by the *DRB1* alleles associated with an increased risk versus those with a reduced risk, in these and other populations, indicates that the key amino acids in this sequence are either lysine (K) or arginine (R) at position 71. Both lysine and arginine are basic, highly charged amino acids. *DRB1* alleles that confer a reduced risk of disease (e.g., *DRB1\*1501*) encode neutral non-polar amino acids such as alanine at position 71. Exchange of alanine for either lysine or arginine at position 71 would have a major effect on the antigen-binding characteristics of the expressed HLA DR molecule. This type of model has been used to explain MHC-encoded disease susceptibility for rheumatoid arthritis (66). In all cases these models imply a strong and direct involvement of the susceptibility alleles in disease genesis, either through a relative failure in immune tolerance (at the level of thymic selection or in the periphery) or by directly promoting presentation of self-antigen.

The third model, based on valine/glycine dimorphism at position 86 of the DR $\beta$ -polypeptide, was developed from studies of children in Argentina (29). In this series, the primary associations were with *DRB1\*0301* and *DRB1\*1301*. The proposed model does not fit any of the other published series and initially met with some concern from those in the field. The major difference between studies of patients in northern Europe and North America was the strong association with *DRB1\*1301* in South American children, which was not seen elsewhere. However, a possible explanation for this

“out of phase” association came from a second set of observations by same group. *DRB1\*1301* was identified as a major determinant of susceptibility to chronic infection with hepatitis A virus (HAV) (67), a virus that is endemic among the studied populations (68).

Taken together, the two sets of observations from South America offered an alternative explanation for HLA associations that would embrace reports of different genetic associations in different populations. According to this possibility, susceptibility alleles may be key elements in the interaction with environmental triggers of autoimmunity. These triggers may vary between populations depending on local conditions. This may lead us to the hypothesis that the HLA associations are themselves markers or “molecular footprints” of prevailing infections, the HLA susceptibility allele(s) identified in each population being selected by the molecular characteristics of the disease-causing environmental factor(s), for example, HAV. According to this hypothesis, different triggers (viral or otherwise) may be responsible for disease initiation in different populations, giving rise to different genetic associations. In this context, but outside the scope of this chapter, it is particularly important to consider the relationship between IR genes and infectious, particularly viral, liver diseases.

### PRIMARY SCLEROSING CHOLANGITIS

Not all MHC-encoded disease susceptibility/resistance maps clearly to the (old order) class II region. PSC provides a good illustration of how important it may be to map these genetic associations precisely. In PSC, the essential question is whether disease risk is determined by HLA *DR/DQ* or by *MICA* polymorphism. MHC class II associations mostly indicate inadequate regulation of the adaptive immune response either through immune tolerance or response to infection (*see above*), whereas an association with *MICA* may indicate a failure of innate immunity. Several molecular models based on shared amino acids have been proposed for the MHC class II associations in PSC, including DR $\beta$ -leucine 38 and valine 86, DQ $\beta$ -arginine 55, and phenylalanine-87 (44). However, AIH provides a better illustration of how disease risk associated with MHC class II can influence our understanding of disease pathogenesis, and therefore I will concentrate on *MICA* only in PSC.

MIC $\alpha$  molecules appear to be exclusively expressed on gastrointestinal (including the biliary epithelium) and thymic epithelia and may be induced by stress and heat shock (14,69). The MIC $\alpha$  molecule has been identified as a ligand for  $\gamma\delta$  T cells and NK (CD56<sup>+</sup>) cells, and both “normal” (15) and PSC livers (70) have a large resident population of these cells. There are two independent associations with *MICA* in PSC: an increased frequency of *MICA\*008* homozygotes and a very significantly reduced frequency of *MICA\*002* (haplotype 6, Table 1) (42,43). If *MICA* is the primary MHC susceptibility locus in PSC, then it may have profound implications for our understanding of the pathogenesis of this idiopathic disease. The *MICA\*008* allele encodes a MIC $\alpha$  molecule that has a short cytoplasmic tail, and this is thought to result in a less stable molecule than that encoded by other *MICA* alleles (71).

If PSC were to occur as a result of infection, this may provide the catalyst for heat shock induction of MIC $\alpha$  molecules on biliary epithelium, leading to the activation of intrahepatic  $\gamma\delta$  T cell and NK cells with subsequent cytokine secretion and cytolytic effector functions. In individuals homozygous for *MICA\*008*, the unstable MIC $\alpha$  molecule may permit persistent immune activation, leading to autoimmunity or failed immune activation with the consequence of persistent infection and an increased risk of autoimmunity. If *MICA\*008* is associated with a loss of function, then this may explain why only those with two copies of this allele are at an increased risk of PSC, whereas a single copy of *MICA\*002* protects from the disease (42).

### PRIMARY BILIARY CIRRHOSIS

The current data in PBC illustrate a different concept. One of the most difficult observations to explain, with respect to the *DRB1\*0801* association in PBC, has been the wide variation in the reported strength of this association within the same racial group (NEC) (9,51–55,60–62). One explanation that has been offered for this phenomenon is “case ascertainment bias.” There are two probable examples of this in the PBC-HLA literature. In one report patients were divided into those with either early- or late-stage disease based on histology. The *DRB1\*0801* association was confined to those with late-stage disease (27%), and there was no significant risk associated with *DRB1\*0801* in those with early-stage disease (5% compared with 3% of “healthy” controls) (9). More recently, a second study in Italy failed to find any association with the *DRB1\*0801* allele in 158 PBC patients (60). However, follow-up studies in Italy indicate that this “peculiar” association may be due to case ascertainment bias (61). Perhaps these latter cases were less severe. The hypothesis that *DRB1\*0801* is a marker of disease progression in PBC has not been universally accepted and remains controversial (49,55), but this observation, which is not without precedent, could have profound implications for the conduct of disease association studies and may also have practical value. If we can identify patients with a “severe clinical phenotype,” we may be able to manage or monitor their disease more closely, and this may lead to more economic use of (increasingly scarce) health care resources.

Before we leave the MHC, a final word of caution about molecular models: in analyzing and reanalyzing the above associations and in revising these models, we must not forget that the expressed MHC molecule is made up of several hundred amino acids and substitutions along the length of the molecule can influence both protein folding and also the characteristics of the peptide binding groove. Concentration on a single epitope or amino acid is therefore naive. It is likely that disease susceptibility is affected by more than one of the amino acid variations peculiar to each susceptibility allele. For example, further analysis of the models referred to above for type 1 AIH indicates that positions including tyrosine DR $\beta$ 26 and arginine DR $\beta$ 74 are also significantly associated with disease susceptibility in European and North Americans (19,20,33,72); valine at DR $\beta$ 11 is associated with

susceptibility in Japan (25,26); other residues (including glutamic acid at DR $\beta$ 9, tyrosine at DR $\beta$ 10, serine at DR $\beta$ 11, serine at DR $\beta$ 13, aspartic acid at DR $\beta$ 28, phenylalanine at DR $\beta$ 47, and aspartic acid at DR $\beta$ 57, in various combinations with valine at DR $\beta$ 86) are associated with susceptibility in South American children (29); and asparagine at DR $\beta$ 37 has been proposed as a possibility to explain susceptibility in French and French Canadian children (30).

## NON-MHC IMMUNOREGULATORY GENES IN AUTOIMMUNE LIVER DISEASE

The number of immunoregulatory genes outside the MHC is very large. In this section of the chapter, I will consider only a selection of the more commonly investigated genes according to the subdivisions identified in Where to Look: Selection of Candidate Genes above.

### CYTOTOXIC T-LYMPHOCYTE-ASSOCIATED ANTIGEN-4

Over the past 5 yr there has been considerable interest in the role played by non-MHC immunoregulatory genes in autoimmune disease, especially the T-cell regulatory gene cytotoxic T-lymphocyte-associated antigen-4 (*CTLA4*) on chromosome 2q33. In particular, *CTLA4* polymorphisms have been proposed as “nonspecific determinants of disease risk” in a variety of autoimmune diseases including type 1 diabetes, Graves’ disease, and many others (73).

CTLA-4 (CD152) is expressed exclusively on CD25<sup>+</sup> T cells and binds to the same ligands (B7.1 or CD80 and B7.2 or CD86) as CD28. The CD28-B7 interaction is one of the critical costimulatory events required for initiation and progression of the T-cell immune response. CTLA-4, expressed on CD25<sup>+</sup> T cells, has a 20 to 50-fold higher affinity for B7 and appears to downregulate immune activation by competing with CD28 (73–75).

Extensive investigations have revealed more than 108 SNPs in and around the *CTLA4* gene on chromosome 2q33 (76). Early studies in type 1 diabetes and autoimmune thyroid disease concentrated on the *CTLA4* A+49G SNP, which encodes a threonine-to-alanine substitution at position 17 in the first exon of the CTLA-4 protein (73). This SNP, which is the only polymorphism leading to an amino acid substitution within the gene, became the focal point for studies of *CTLA4* in many different autoimmune diseases (73,76). However, more recent studies have revealed that not only is this SNP unlikely to be a DPP in type 1 diabetes and Graves’ disease, but the risk associated with the *CTLA4*-encoded DPP is much smaller than originally suggested (76). The current focus for investigators is an A/G SNP in the 6.1-kb region 3’ of *CTLA4* (76), referred to as CT60. Inheritance of this SNP is thought to be associated with variation in the efficiency of the splicing and production of soluble (s) compared with full-length (fl) isoforms of CTLA-4 mRNA. Furthermore, the disease susceptibility allele *CT60*\*G is associated with lower levels of sCTLA-4 mRNA production. It has been proposed that lower levels of sCTLA-4 in serum could lead to reduced efficiency

of blocking of CD80/CD86, permitting increased or prolonged activation of CD28 T cells (76).

The first studies of the *CTLA4* gene in autoimmune liver disease suggested that the *CTLA4* +49\*G allele is a significant risk factor for both type 1 AIH (77) and PBC (78,79). However, more recent extensive studies, based on larger numbers of patients and controls both in PSC (80) and PBC (Donaldson, May 2006, unpublished observations), categorically show that there are no major associations with this gene in the northern European patients. This major revision of the claims for *CTLA4* in PBC follows some controversy about the role of specific *CTLA4* SNPs in autoimmune disease (73,76).

Currently the position on type 1 AIH remains unchanged, but also unchallenged. The identification of *CTLA4* as the “second” susceptibility allele in type 1 AIH (77) has yet to be confirmed, and, bearing in mind the current position on this gene in PBC and PSC, confirmation is a matter of some urgency. In keeping with other autoimmune diseases (73), the association reported in type 1 AIH is relatively weak (maximum odds ratios 2.12 and 2.45), and it may be due to linkage disequilibrium with other *CTLA4* SNPs (for example CT60) and/or with other immunoregulatory genes in and around 2q33. (Candidates include CD28.) It is also possible that *CD28* and *CTLA4* polymorphisms may act in synergy and that the current data for all three of these diseases represent only half the picture. In the future, the emphasis should be on analysis of polymorphisms across complete biological systems, not on single isolated candidates, as here and also below.

### CYTOKINE GENE POLYMORPHISMS IN AUTOIMMUNE LIVER DISEASE

The various components of the cytokine network are obvious candidates for investigation in autoimmune liver disease. However, not all of the current published studies are of good quality, and, as with *CTLA4* (discussed in the previous section), many of the findings of these studies are controversial. In this section I will discuss the three most frequently considered cytokine genes: *TNFA*, *IL1*, and *IL10*. Currently identified genetic polymorphisms in the cytokine genes are summarized on the worldwide web at: <http://www.nanea.dk/cytokinesnps/>

#### TUMOR NECROSIS FACTOR

Initial interest in cytokine genes in autoimmune liver disease was prompted by a need to map MHC-encoded susceptibility to a specific gene locus. Thus, it was not long before investigators turned their attention to the *TNF* gene cluster, which maps close to *HLA B* telomeric of the *DRB1* locus (12). The *TNF* gene cluster exhibits extensive polymorphism and has been widely studied in autoimmune and infectious diseases with mixed results. TNF production is one of the earliest events in response to liver injury, and it triggers a cascade of inflammation, cell death, and fibrosis (81). Because of these actions, TNF is an excellent positional and functional candidate in liver disease. Of the many SNPs in the TNF cluster, only two, at positions –238 and –308 in the *TNFA* gene, have been investigated. As expected, there was a strong link between *TNFA*\*2

(the *TNFA-308 A* allele) and disease susceptibility in both type 1 AIH and PSC (22,46). Current opinion suggests that this association is due to linkage disequilibrium with the HLA 8.1 haplotype rather than the direct influence of *TNFA\*2* on disease pathogenesis (22,23,46,82,83). Current data on the role of these two polymorphisms in PBC are controversial (49,65).

### INTERLEUKIN-1

Interleukin-1 (IL-1) is a proinflammatory cytokine and is fundamental in health and disease, with wide-ranging roles in both innate and adaptive immunity and in the generation of inflammatory responses by a variety of different target cells. IL-1 also has an important role in collagen synthesis by stellate cells (81). The regulation of IL-1 cytokines is complex but serves as a useful paradigm. The three original members of the IL-1 family are IL-1 $\alpha$ , IL-1 $\beta$ , which have agonist activity, and the IL-1 receptor antagonist (IL-1Ra). More recently, at least six novel proteins have been added to the family. These six new IL-1 proteins have agonist and antagonist activities, and the restricted expression of some of them may suggest specialized functions in particular tissues.

The major part of the *IL1* gene family (including all of the above) maps to a 350- to -450-kb segment on chromosome 2q12-22. The genes for two functional IL-1 receptors (*IL1R1* and *IL1R2*), are encoded 10 Mb centromeric of *IL1B*, the gene encoding IL-1 $\beta$ . A recent study identified 95 polymorphisms within a 350-kb segment focused on *IL1RN* (84). The biology and the genetics of IL-1 regulation are highly complex, and studies of *IL1* genes have simply not taken this complexity into account. However, just as with the MHC, in which studies began before our knowledge of the system was complete, there are some very interesting findings from these limited studies.

Of all the possible *IL1* gene family polymorphisms, only two have been investigated in autoimmune liver disease: these are the *IL1B* SNP at position +3953 and the 86-bp VNTR (variable number tandem repeat/micro- or minisatellite) in the *IL1RN* gene. So far, studies of type 1 AIH and PSC have proved entirely negative (22,85). In contrast, there appears to be a strong association between *IL1RN* and *IL1B* and both disease susceptibility and progression in patients with PBC (9,86). Whether this association is due to these SNPs themselves or to the others in the region remains to be determined. However, members of the *IL1* family represent good functional candidates in PBC; interestingly, recent microarray analysis of PBC livers reported a fourfold increase in *IL1A* mRNA transcripts (87).

The *IL1* allele associations (just described), together with preliminary evidence for an association with the gene-encoding divalent cation transporter (NRAMP1; correct gene name *SLC11A1*) (88), which is also encoded on chromosome 2q, may suggest that 2q is a hot spot for PBC susceptibility. Although the study of NRAMP microsatellites in PBC generated an increased risk of 4.4, it was based on only 53 patients and remains to be confirmed. Once again, the candidate is a strong functional candidate, playing a central role in activation

of the MHC, TNF, interferon- $\gamma$  (IFN- $\gamma$ ), IL-1 $\beta$ , and being linked with susceptibility to *Mycobacterium* infection (89), a bacterium that has been suggested by some to be a potential environmental trigger for PBC (90).

### INTERLEUKIN-10

IL-10 is an antiinflammatory cytokine that controls the balance between Th1 and Th2 immune responses. Furthermore, IL-10 may have antifibrotic properties and therefore is a good candidate in autoimmune liver disease. The IL-10 gene maps to chromosome 1q31-q32, but thus far only three SNPs (C-592A, C-819T, and G-1082A) have been investigated in autoimmune liver disease, and all these investigations have failed to find any association with susceptibility, treatment failure, or disease phenotype in type 1 AIH, PSC, or PBC (22,82,85,86,91).

### GENETIC POLYMORPHISM AND FIBROSIS IN AUTOIMMUNE LIVER DISEASE

Progression in PBC and PSC in particular is related to fibrosis, which is a complex process resulting from the excess production of extracellular matrix (approximately 5 – 10-fold increase) and reduced matrix degradation. These processes are regulated by the metalloproteinases (MMPs); the naturally occurring tissue inhibitors of metalloproteinases, and several cytokines. The cytokines most commonly associated with matrix metabolism are transforming growth factor- $\beta$ , (TGF- $\beta$ ) and TNF- $\alpha$ , which have profibrotic activity, and IL-10 and IFN- $\gamma$ , which have antifibrotic activity (92).

Currently there is evidence that polymorphism of at least two genes involved in regulation of collagen synthesis and fibrosis (93,94) may be important in PSC. Both genes are located on chromosome 11q. The first polymorphism is a commonly occurring 5A or 6A repeat sequence at position -1171 in the gene encoding stromelysin (*MMP3*). The *MMP3\*5A* allele has been associated with an increased risk of portal hypertension (93) and ulcerative colitis in PSC (94). The second polymorphism is a dimorphism (or G insertion) at position -1607 in the promoter region of the MMP-1 gene, which has been associated with a high risk of cholangiocarcinoma in PSC (94). Although there is clearly more work to be done on these two genes, these data are promising. Both genes are reasonable candidates for PSC: MMP-3 degrades type II, IV, and IX collagens, laminins, fibronectin, gelatins, and elastin and may activate other metalloproteinases; MMP-1 degrades fibrillar collagen types I and II, which are abundant in the gut. The 5A variant of *MMP3* has been linked with lower levels of gene transcription, and the 2G *MMP1* genotype has been associated with higher levels of *MMP1* expression (93,94). The findings with respect to *MMP1* and *MMP3* in PSC illustrate how important studying genes involved in fibrosis may be in liver disease. Therefore, it is disappointing to note that of all the other potential candidate genes only one, *TGFBI*, has been investigated. Two different *TGFBI* SNPs (G+74C, which results in an arginine-for-proline substitution in codon 25) and a C/T SNP at position -509 in the promoter region of the gene have been investigated, but

**Table 4**  
**Summary of Immune Response Gene Associations in Autoimmune Liver Disease**

<i>Population</i>	<i>Gene locus</i>	<i>Allele/motif or amino acid</i>	<i>Chromosome</i>	<i>Ref.</i>
<b>Type 1 AIH</b>				
All	Female sex	Unknown	X	—
NEC	<i>HLA DRB1</i>	Lysine-71	6p21.3	19,20
		Alanine-71		
Japan	<i>HLA DRB1</i>	Histidine-13	6p21.3	25,26
South America (children)	<i>HLA DRB1</i>	Valine-86	6p21.3	29
NEC	<i>CTLA4</i>	AG/GG	2q33	77
NEC	<i>TNFRSF6</i> (Fas)	AG/GG	10q24	98
<b>PSC</b>				
All	Male sex	Unknown	Y	—
NEC	<i>HLA DRB1</i>	0301,1301, 1501	6p21.3	35–41, 44
NEC	<i>HLA DRB1</i>	0401	6p21.3	35–41, 44
NEC	<i>HLA DRB1</i>	0701	6p21.3	44
NEC	<i>HLA DRB1</i>	Leucine-38	6p21.3	36,44
NEC	<i>HLA DRB1</i>	Valine-86	6p21.3	44
NEC	<i>TNFA</i>	<i>TNFA</i> *2	6p21.3	46,82
NEC	<i>MICA</i>	<i>MICA</i> *008 (5.1)	6p21.3	42,43
NEC	<i>MICB</i>	<i>MICB</i> *24	6p21.3	43
NEC	<i>MMP1</i>	GG	11q	94
NEC	<i>MMP3</i>	5A,5A	11q23	93,94
<b>PBC</b>				
All	Female sex	Unknown	X	—
NEC	<i>HLA DRB1</i>	0801	6p21.3	9,51–55
Japan	<i>HLA DRB1</i>	0803	6p21.3	56–59
NEC	<i>CTLA4</i> (A49G)	A,G/G,G	2q33	78
Japan	<i>CTLA4</i> (A49G)	G,G	2q33	79
NEC	<i>IL1B</i> (+3953)	1,1	2q12-22	9
NEC	<i>IL1RN</i>	Allele 2	2q12-22	9,86
NEC	<i>SLC11A1</i> (nramp)	Allele 5	2q35	88
NEC	<i>APOE</i>	Allele 4	19q13.2	103

Abbreviations: AIH, autoimmune hepatitis; NEC, Northern European caucasoid; PBC, primary biliary cirrhosis; PSC, primary sclerosing cholangitis.

neither polymorphism was associated with PBC or PSC (Donaldson, 2000, unpublished observations).

### GENETIC POLYMORPHISM AND APOPTOSIS IN AUTOIMMUNE LIVER DISEASE

Recent identification of several members of the *CARD* gene family as major determinants of susceptibility to Crohn's disease (18–21) has brought apoptosis (programmed cell death) as a mechanism of immune regulation under the genetics spotlight. Although there is no suggestion of any association between *CARD15* (frequently referred to as NOD2) and autoimmune liver disease, other apoptosis regulators have been investigated including *CASP8* in PBC and Fas (gene name *TNFRSF6*) in all three diseases. Fas is constitutively expressed on hepatocytes, leading to the suggestion that the liver may play an important role in immune regulation through induction of apoptosis in resident and surveying immunocytes (95,96). *CASP8* encodes a key member of the cysteine protease family of enzymes caspase-8. The caspase-8 gene is located in close proximity to the *CTLA4* gene on chromosome 2q33, and the enzyme recognition sequence includes the motif LETD, which

is remarkably similar to the sequence in the PDC-E2 inner lipoyl domain, which is thought to be a critical autoantigenic epitope in PBC (97).

So far only one of several Fas gene polymorphisms (an A/G SNP at position –670 in the promoter) has been investigated, and there are no significant associations with disease susceptibility in any of the three autoimmune liver diseases (Donaldson, May 2006, unpublished observations). However, in type 1 AIH, preliminary data did suggest that there may be a relationship between this SNP in the *TNFRSF6* gene and severity of liver inflammation (98).

The *CASP8* study in PBC was based on 351 PBC patients and 390 controls and used the haplotype tagging (ht) approach based on analysis of four key SNPs (including a T/C SNP in intron 2, a G/C SNP in exon 9, a G/T SNP in intron 9, and a G/C SNP in exon 10), to identify all the common *CASP8* haplotypes (99). This “ht” method represents an efficient and innovative approach to candidate gene analysis in complex diseases, which reduces genotyping load without compromising the analysis (99). The study, which is currently unpublished work, found that there were categorically no associations

with *CASP8* alleles, genotypes, and haplotypes in PBC or in any clinical subgroups of PBC patients (100).

## CONCLUDING REMARKS AND OPEN QUESTIONS

This chapter poses more questions than it answers. The strong genetic associations so far described in autoimmune liver disease are those with sex and the MHC. Other associations with *CTLA4* and Fas in type 1 AIH, *IL1* and *SLC11A1* in PBC, and with *MMP3* and *MMP1* in PSC, should all be considered as interesting but preliminary until widely confirmed. This is also true for some of the other genes, which I have not mentioned. Among these there are claims of genetic associations with the genes for ICAM-1 in PSC (101) and vitamin D receptor (102), APO-E (103), mannose binding lectin, CD40 ligand, and CD14 in PBC (49). Many of the latter have an immunoregulatory function or potential, but few of these associations have been widely confirmed, and in many cases there are unpublished negative data that refute the original findings. Overall, our current knowledge of the genetic basis of autoimmune liver disease remains incomplete (Table 4). The story so far illustrates many of the problems with candidate gene association studies. In each case (including the MHC), a complex biological system has been reduced, thus overlooking both the complexity and redundancy within the system. Nearly all the studies cited here illustrate the need for better study design, large collections, and comprehensive analysis of candidate genes. Haplotypes should be assessed, as opposed to single SNPs, and systems rather than isolated receptors or ligands. The completion of a high-quality comprehensive sequence of the human genome in 2003 heralded a new era of genomics, systems biology, and bioinformatics and a revolution in technologies for genetic research (104) that should remedy this situation. Overall, the information gathered to date may be best used as a guide for future investigators, indicating where to look and which genes or systems are most likely to yield informative results.

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# 18 Primary Biliary Cirrhosis and Autoimmune Cholangitis

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CARLO SELMI, ANA LLEO, PIETRO INVERNIZZI, AND M. ERIC GERSHWIN

## KEY POINTS

- Primary biliary cirrhosis is (PBC) an enigmatic liver disease characterized by the destruction of small intrahepatic bile ducts with portal inflammation.
- PBC features include a striking female predominance and high-titer serum autoantibodies to mitochondrial antigens (AMAs).
- The presence of serum AMAs and autoreactive T and B cells, in conjunction with the co-occurrence of other autoimmune diseases, implies an autoimmune pathogenesis for PBC.
- PBC is to be considered as a model autoimmune condition.
- The etiology of PBC remains enigmatic even though several theories have been proposed that include a complex genetic background and one or more environmental triggers as common traits.
- The diagnosis of PBC is based on three criteria: detectable serum AMA (titer more than 1:40), increased plasma cholestasis enzymes (alkaline phosphatase) for longer than 6 mo, and a compatible or diagnostic liver histology.
- The most common symptoms accompanying PBC at diagnosis in precirrhotic stages are classically defined as fatigue and pruritus, although we are witnessing a dramatic change in patient presentation patterns.
- In most cases, PBC slowly progresses over years.
- Several medical treatments have been investigated in patients with PBC. Among these, ursodeoxycholic acid (UDCA) appears to reduce disease progression rate, whereas liver transplantation is the only definitive treatment although recurrences are common.
- The use of immunosuppressants is not encouraged in PBC.

## INTRODUCTION

Primary biliary cirrhosis (PBC) is a chronic cholestatic liver disease of unknown etiology characterized by high-titer serum antimitochondrial autoantibodies (AMAs) and an

autoimmune-mediated destruction of the small and medium-sized intrahepatic bile ducts. From a clinical standpoint, PBC is a peculiar, yet representative, autoimmune disease (Table 1). It affects women more frequently than men, with a female-to-male ratio of 9 to 1, and the average age at diagnosis is within the fifth and sixth decades of life, with exceptional cases described in pediatric ages. Epidemiological data indicate a geographical pattern of PBC prevalence and incidence rates, which are higher in northern countries (England, Scandinavia, northern United States). The diagnosis of PBC is made when two of three criteria are fulfilled, i.e., presence of serum AMAs, increased enzymes indicating cholestasis (i.e., alkaline phosphatase) for longer than 6 mo, and a compatible or diagnostic liver histology. Clinical symptoms include fatigue, pruritus, and jaundice. The progression of PBC varies widely for unknown reasons, as represented by certain patients remaining asymptomatic and others reaching liver failure at young ages. Several clinical and experimental findings strongly imply an autoimmune pathogenesis for PBC, whereas the disease onset recognizes two necessary components in a permissive genetic background and an environmental trigger.

The first description of biliary cirrhosis, albeit possibly secondary, can be traced back to the work of the Italian pathologist Giovanni Battista Morgagni from Padua in 1761; the first report of nonobstructive biliary cirrhosis was by Addison and Gull in 1851. Subsequently, the term PBC was accepted in the medical literature (1), and in 1959 Dame Sheila Sherlock described the first series of patients affected by PBC who had been followed over the previous decade and noted that patients presented with pruritus as well as the signs and symptoms of end-stage liver disease including jaundice (2). The association between serum AMAs and PBC was first recognized as specific in 1965 by Walker and colleagues (3); in 1987, the AMA antigens were cloned and identified by the senior author of this chapter as subunits of the pyruvate dehydrogenase complex (PDC) located on the inner mitochondrial membrane (4). This discovery led to the development of more sensitive assays for the determination of AMAs, although indirect immunofluorescence remains the method of routine testing in most clinical centers.

**Table 1**  
**Clinical and Pathological Profiles of Primary Biliary Cirrhosis**

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Predominantly middle-aged women (M/F ratio 9:1)
Recurrent pruritus, fatigue, and progressive jaundice
Elevation of serum alkaline phosphatase, $\gamma$ -glutamyltranspeptidase, and IgM
Antimitochondrial antibodies (AMA) titer > 1:40
Associated with other autoimmune diseases: Sjögren's syndrome, scleroderma, autoimmune thyroid disease, and others
Classified histologically into four stages:
1. Inflammatory destruction of intrahepatic small bile ducts
2. Proliferation of bile ductules and/or piecemeal necrosis
3. Fibrosis and/or bridging necrosis
4. Cirrhosis

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## CLINICAL AND PATHOLOGICAL FEATURES

### DIAGNOSIS

As mentioned in the previous section, the diagnosis of PBC is based on three objective criteria. A classification proposed by a British group (5) suggests a "probable" diagnosis when two of the three criteria (most often AMA positivity and compatible liver histology but normal liver enzymes) are present. Accordingly, a "definite" diagnosis can be made in the presence of all three states. This classification may be seen as strict since it can be assumed that the vast majority of asymptomatic AMA-positive individuals (particularly when serum reactivities are found using sensitive and specific methods) will eventually develop a classical picture of PBC during follow-up. Moreover, patients lacking detectable AMAs, (especially when indirect immunofluorescence is used) but otherwise presenting signs of PBC should be regarded as affected by "AMA-negative PBC" (or *autoimmune cholangitis*), as they appear to follow a similar natural history compared with their AMA-positive counterparts (6).

The use of liver histological assessment remains a hot topic of discussion in PBC. We believe that a liver biopsy specimen provides an important tool to determine the stage of the disease, both at presentation and during follow-up (7). It should be pursued in those in whom the diagnosis is suspected but serum AMAs are undetected or alkaline phosphatase levels are within the limits. Conversely, performing a liver biopsy is not recommended when the other two diagnostic criteria are met. The differential diagnosis of PBC includes other colestatic diseases. First, primary sclerosing cholangitis (PSC) may be considered, particularly in patients with inflammatory bowel disease; however, colitis rarely occurs in PBC, and AMAs are seldom detectable in PSC. Sarcoidosis is also associated with cholestasis and granulomatous involvement of the liver. Several drugs have been reported to induce cholestasis, cholangitis, and ductopenia (8).

### ASYMPTOMATIC/SYMPTOMATIC PBC

The number of asymptomatic patients at the time of diagnosis has been steadily increasing since the earlier series descriptions when most patients were diagnosed when jaundice was already present (1). At present, the diagnosis of PBC is established in

the absence of symptoms indicating a liver condition or cholestasis in the vast majority of cases (9). The increasing number of symptomless patients most likely also represents the growing awareness of the syndrome as well as, perhaps more importantly, the availability of more sensitive noninvasive tests. In a similar fashion, we cannot rule out at present that higher prevalence rates are in fact secondary to prolonged survival of affected individuals.

We note, however, that during extended clinical follow-up, most AMA-positive patients will eventually develop PBC-associated symptoms (5). The most common symptoms accompanying PBC are fatigue and pruritus; classically described physical findings may include skin hyperpigmentation, hepatosplenomegaly, and (rarely) xanthelasmas (caused by deposition of cholesterol). End-stage symptoms are those common to all liver etiologies of cirrhosis and include jaundice, ascites, encephalopathy, and upper digestive bleeding. Importantly, endoscopic signs of portal hypertension, such as esophageal varices or portal hypertensive gastropathy, can be encountered at histologically, proven early-stage PBC, i.e., without evidence of liver cirrhosis, and are thought to be secondary to presinusoidal fibrosis and inflammation induced by granulomas (10).

### CLINICAL FEATURES

**Fatigue** Fatigue is an incompletely defined, nonspecific symptom that is believed to affect up to 70% of patients with PBC while often being overlooked by patients and physicians. Importantly, the severity of fatigue is independent of the stage of PBC or its other features (pruritus or severe cholestasis), nor does it depend on psychiatric factors. More importantly, the specificity of the symptom is still debated, as well-controlled studies are lacking to define the importance of chronic liver disease *per se*. Morphological abnormalities of the central nervous system owing to accumulation of manganese have been postulated as putative causes of fatigue in PBC (11). No medical treatment has been shown to be effective in alleviating this symptom, although fatigue has never been included as an end point in any of the large controlled clinical trials.

Similar prevalence rates can be observed in other autoimmune conditions including systemic lupus erythematosus, in which, however, fatigue often correlates with depression rather than with immunological markers or inflammation.

**Pruritus** Pruritus is considered the second most common presenting symptom of PBC. Longitudinal data show that the vast majority of patients will experience this symptom during progression of the disease, and its appearance most commonly precedes jaundice by months or years. Pruritus can be localized or diffuse, but at the time of onset it more frequently worsens at night, following contact with certain fabrics (wool) or in warm climates. The bases of PBC-associated pruritus are not clear, and two hypotheses have been proposed, i.e., serum bile-acid retention secondary to chronic cholestasis or, alternatively but not exclusively, an amplified release of endogenous opioids (12).

Finding an effective medical treatment for pruritus in PBC is often challenging. Trials of antihistamines or phenobarbital

for the treatment of the symptom have proved these medications to be ineffective, whereas the use of cholestyramine (4 g before and after the first meal) ameliorates pruritus. In selected cases poorly responsive to resins, rifampicin has been used to achieve rapid symptom relief; its prolonged use, however, is not recommended. Experimental evidence indicates that the opioid neurotransmitter system, rather than bile acid retention alone, might mediate pruritus in chronic cholestasis; a central mechanism has been proposed. This hypothesis is supported by experimental data demonstrating that opioid receptor ligands with agonist properties mediate pruritus and that endogenous opioid-mediated neuromodulation in the central nervous system is increased in chronic cholestasis. Based on this theory, treatment with an opiate antagonist such as naltrexone (50 mg/d) is currently used, with limited adverse effects; its efficacy has been assessed in one controlled clinical study that has also indicated that side effects were temporary and usually did not require specific treatment (13). The recently proposed use of sertraline is encouraged by promising preliminary data but warrants further evaluation. In patients with intractable pruritus, liver transplantation is the ultimate therapeutic option.

**Portal Hypertension** As mentioned above, portal hypertension is a common finding in patients with PBC, but significantly fewer patients now present with acute digestive bleeding or other signs of portal hypertension, compared with the first reported series of affected individuals. Interestingly, portal hypertension in PBC does not imply the presence of liver cirrhosis. Longitudinal studies indicate that about 58% of untreated patients will eventually develop endoscopic signs of portal hypertension over a 4-yr follow-up (14). The prevention and treatment of PBC-associated portal hypertension is not different from other chronic liver diseases and is based mostly on the use of  $\beta$ -blockers.

**Reduction in Bone Density** A metabolic bone disease is found in PBC, with accelerated bone loss owing to reduced bone deposition being noted in patients compared with sex- and age-matched healthy individuals. These findings are still somewhat contentious, and conflicting data have been reported. A mild reduction in bone density (osteopenia) is present in about 30% of patients, and frank osteoporosis is diagnosed in 10% of patients. The bone loss can, moreover, worsen after liver transplantation, possibly owing to the administration of specific immunosuppressive drugs and steroids. The mechanisms leading to metabolic bone alterations are not completely understood, as no significant changes in the metabolism of calcium and vitamin D can be found in patients with PBC. The current treatment of bone loss in PBC, similar to non-PBC cases, includes oral calcium supplementation, weight-bearing activity, and oral vitamin D replacement (if a deficiency is present). Postmenopausal hormone replacement therapy should be considered as effective and as prone to cause long-term side effects in women with PBC as in the general population. However, as estrogens have been associated with worsening of the cholestatic pattern, jaundice and signs of liver failure should be monitored closely, particularly during the first months of treatment.

Most recently a large improvement in the femoral bone mineral density (BMD) of patients treated with alendronate has been observed. BMD changes were independent of concomitant estrogen therapy, and oral alendronate appeared to be well tolerated (15). Larger studies are needed to evaluate formally the safety and efficacy of other proposed treatments.

**Hyperlipidemia** Alteration in the blood lipid profile is a common finding in PBC (up to 85% of patients present with hyperlipidemia) and often precedes the diagnosis. Both serum cholesterol and serum triglyceride levels can be raised as the result of chronic cholestasis, but it seems that these patients are not exposed to greater cardiovascular risk; in fact, these alterations do not correlate with increased incidence of cardiovascular events or early atherosclerotic lesions (16). Treatment with ursodeoxycholic acid may reduce blood lipid levels via unknown mechanisms, and the use of statins is still debated.

**Steatorrhea and Malabsorption** Long-standing cholestasis leads to steatorrhea by inducing bacterial overgrowth syndrome in the gut. The mechanism is mediated by the impaired flow of bile acids to the small intestine and is commonly found in advanced stages of PBC (17). Oral replacement of medium-chain triglycerides for long-chain compounds, along with an overall reduction of fat in the diet can be offered as the treatment for symptoms. Pancreatic enzyme replacement medications can also improve the symptoms when pancreatic insufficiency is suspected. Empirical antibiotic regimens can treat the bacterial overgrowth, but their use, particularly when prolonged, should be carefully evaluated.

Malabsorption of fat-soluble vitamins is commonly found in advanced stages of PBC (18). The most common deficiency, involving vitamin A, although almost always symptomless, is present in 20% of cases. Oral replacement therapy can overcome impaired absorption, and monitoring of serum concentrations is recommended after 6 to 12 mo to avoid potential hepatotoxicity or overcorrection. In less common deficiencies such as vitamin E (potentially leading to ataxia), vitamin K (influencing coagulation), and vitamin D (*see* Reduction in Bone Density), oral or parenteral supplementations are safe and effective.

**Associated Conditions** Various disorders, particularly other autoimmune syndromes, have been reported to be associated with PBC. According to our most recent data, as many as 33% of patients with PBC will present with another autoimmune disease (19). Table 2 illustrates the most commonly associated diseases and conditions and their prevalence in PBC. Among the autoimmune conditions found in PBC, Raynaud's (12%) and Sjögren's syndrome (10%) are most frequently observed, but also scleroderma comorbidity is not uncommon.

**Malignancies** Like other chronic liver conditions that lead to cirrhosis, PBC at the stage of cirrhosis can be complicated by the occurrence of hepatocellular carcinoma (HCC), and patients should be periodically monitored (20). From a clinical perspective, this implies that in PBC patients with cirrhosis, screening for HCC should be performed using ultrasonography (and computed tomography in selected cases)

**Table 2**  
**Prevalence of Disorders Associated With Primary Biliary Cirrhosis**

	<i>No. of cases (%) (n = 1032)</i>	<i>No. of controls (%) (n = 1041)</i>	<i>Unadjusted p value</i>
Rheumatoid arthritis	103 (10)	83 (8)	0.1292
Systemic lupus erythematosus	27 (3)	5 (0.5)	<0.0001
Autoimmune thyroid disease	93 (9)	11 (1)	<0.0001
Raynaud's syndrome	118 (12)	23 (2)	<0.0001
Sjögren's syndrome	102 (10)	5 (0.5)	<0.0001
Scleroderma	24 (2)	0	<0.0001
Polymyositis	6 (0.6)	1 (0.1)	0.0684
Any of the above	323 (32)	131 (13)	<0.0001
Diabetes mellitus	99 (10)	119 (11)	0.1744
Hypercholesterolemia	582 (58)	445 (46)	<0.0001
History of urinary tract infections	612 (59)	536 (52)	0.0003
History of breast cancer	31 (3)	45 (5)	0.1277
Asthma	124 (12)	141 (14)	NS
Hay fever	141 (14)	186 (18)	0.0113

Data derived from the authors' most recent study (19).

twice a year to estimate the prognosis and to choose among therapeutic alternatives, particularly when orthotopic liver transplantation (OLT) is being evaluated. Apart from liver cirrhosis, there do not seem to be any PBC-specific risk factors for the development of HCC. The treatment of HCC in PBC should follow the same guidelines as in other chronic liver diseases. No association between PBC and cholangiocellular carcinoma or breast cancer is found.

### NATURAL HISTORY

The progression of PBC varies widely, as represented by patients remaining asymptomatic for decades and others reaching liver failure at young ages. The factors influencing the severity and progression of the disease remain unknown, although data seem to indicate that genetic factors other than those inducing the disease ("second hit") might play a role. In general terms, the natural history of the disease can be divided into three time periods preceding liver failure, i.e., asymptomatic, symptomatic, and pre-liver failure. The duration of these periods can vary significantly, but we note that the first stage might last for decades and the third is usually very rapid. The diagnosis of PBC is currently most commonly made within the first stage; patients presenting with symptoms or advanced disease are significantly less frequent compared with older reports.

Interestingly, however, symptomless patients are commonly older than symptomatic ones, which possibly implies differences in the progression of PBC in these two groups (21).

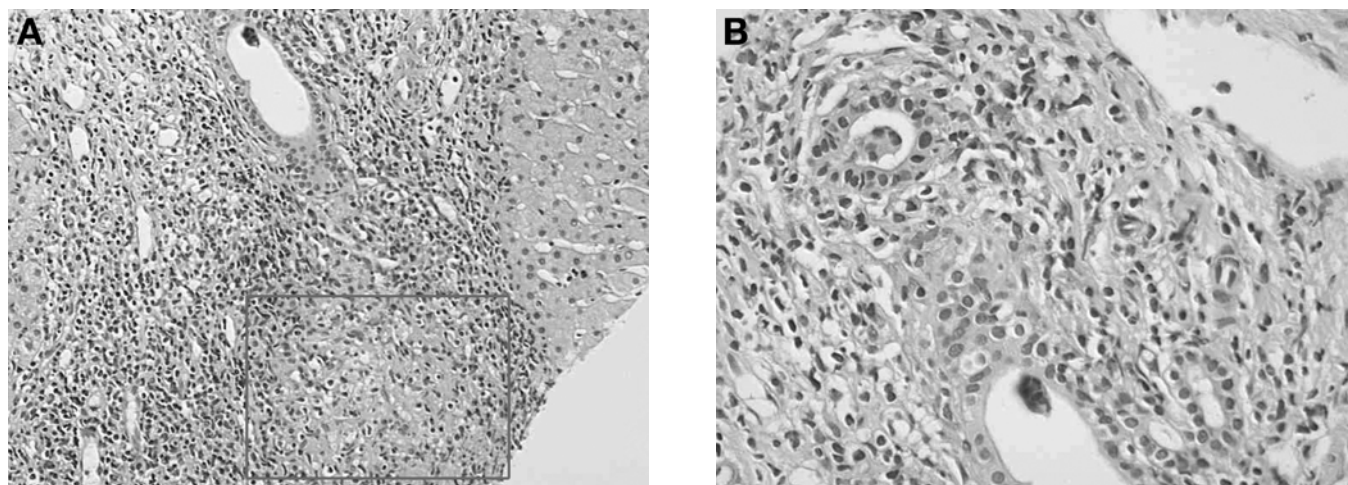
Having symptoms at presentation is considered the major factor determining survival rates of patients with PBC. In fact, symptomless PBC is accompanied by 10-yr survival rates similar to those of the general population. On the other hand, 67% of precirrhotic patients will develop liver cirrhosis over a 7-yr observation period, whereas 70% of asymptomatic patients will develop symptoms. Accordingly, more recent regression models indicate that asymptomatic patients with PBC

have significantly lower survival than the general population. Based on the somewhat conflicting data, it has been hypothesized that survival rates of asymptomatic patients with PBC are shorter than those of the general population if symptoms develop during follow-up (22). An additional confounding factor is provided by the rate of non-liver-related deaths that appears to cause the reduced survival of asymptomatic patients (23). Further studies on large populations and longer follow-up periods are warranted.

Patients with symptomatic PBC show a more rapid progression to late-stage disease and a worse prognosis than their asymptomatic counterparts; survival time among symptomatic subjects is 6 to 10 yr. Older age at diagnosis and signs of advanced disease (clinical, histological, or biochemical) are also associated with a worse prognosis. The establishment of accurate prognostic models to predict survival in patients with PBC is of obvious importance in clinical practice. The model based on the Mayo score is the only validated one and also the most widely utilized (24); it is based on clinical (age, presence of ascites) and biochemical variables, as represented by cholestasis (bilirubin levels) and liver function (prothrombin time, albumin). We submit that this model is a static representation of a dynamic entity and has a lower accuracy for patients with noncirrhotic disease. Recently, it has been reported that PBC-specific serum ANAs, albeit found in a minority of patients, can predict a more aggressive disease, as indicated by longitudinal data on long follow-up periods (25).

### LIVER HISTOLOGY

According to Ludwig's classification (26), histology identifies four PBC stages. Stage I is characterized by portal tract inflammation with predominantly lymphoplasmacytic infiltrates, resulting in vanishing septal and interlobular bile ducts (diameter less than 100  $\mu$ m). At this stage, bile duct obliteration and granulomas (possibly found at all stages) are strongly suggestive of PBC. In stage II a periportal inflammatory



**Fig. 1.** Histological findings in early stages of primary biliary cirrhosis following hematoxylin & eosin staining. (A) Mixed lymphocytic and plasma cell periductular inflammation with bile duct infiltration and granulomatous reaction (square). (Original magnification  $\times 200$ .) (B) Detail of bile duct disruption with lymphocytic and plasmacellular periductular and intraepithelial infiltration. (Original magnification  $\times 400$ .) (Courtesy of Dr. Marco Maggioni, Human Pathology Service, San Paolo Hospital, Milan, Italy.)

infiltrate is observed, and signs of cholangitis, granulomas, and florid proliferation of ductules are typical. Stage III is characterized by septal or bridging fibrosis, with ductopenia (over half of the visible interlobular bile ducts having vanished), and copper deposition in periportal and paraseptal hepatocytes can be seen. Stage IV corresponds to frank cirrhosis. Peculiar characteristics of PBC that can be found at any histological stage include epithelioid granulomas with no signs of caseous necrosis such as in tuberculosis. A large retrospective study has demonstrated that 23.8% cases of granulomas encountered in unselected liver biopsies could be attributed to PBC. The mechanisms leading to granuloma formation are still largely unknown, although experimental findings suggest that Gram-positive bacteria through lipoteichoic acid might initiate the process (27), and osteopontin might also mediate the recruitment of mononuclear cells (28). The observation of eosinophils in the portal tract is a specific finding in PBC histology (29), although its significance, along with a peripheral hypereosinophilia, is currently poorly understood (30).

Finally, the possibility of a sampling error should be considered when one is evaluating histology in PBC; in the case of variable staging within one biopsy, the highest stage should be accepted. Figure 1 illustrates the histological findings in a representative case of early PBC.

## EPIDEMIOLOGY

### EPIDEMIOLOGY AND GEOEPIDEMIOLOGY OF PBC

Most of the epidemiologic data used to determine the incidence and prevalence rates of PBC are descriptive (31). Some studies have methodological flaws, including ambiguous precise or nonuniform case definition. PBC is considered to be most prevalent in England, Scandinavia, and specific areas of the United States, although a factitious prevalence owing to

more exhaustive epidemiological studies from these countries compared with other areas cannot be excluded. Table 3 gives a synopsis of the epidemiological data available.

Prevalence rates for PBC vary widely in different geographical areas and have been reported to be as high as 402/million. PBC should be considered a rare disease, based on its prevalence in the United States of 4/10,000 (32). Accordingly, less than 200,000 affected individuals should be expected in the general U.S. population, thus fulfilling the criteria of the 2002 Rare Disease Act. As very few studies of PBC have been conducted in non-European countries, the incidence and prevalence rates of the disease in many parts of the world such as Asia and Africa are unknown.

### SEX DIFFERENCES AND GRAVIDITY

As observed for most autoimmune diseases, women, primarily those of middle age, are found to outnumber men by as much as 22:1 among those afflicted with PBC. Although gender ratios are variable in different epidemiological studies, the average can be estimated to be 9:1 (Table 3). Some controversial earlier evidence would suggest that the natural history of PBC differs in males and females, with early-onset asymptomatic PBC apparently being more common among men, accompanied by symptoms often not as severe as those seen in women. In an attempt to explain the female preponderance, the prevailing view is that this gender difference may involve the effects of sex hormones on the immune system. Sex hormones are believed to influence the onset and severity of autoimmune disease by modulating lymphocytes at various stages in life. Although specific studies are lacking on the influence that sex hormones have on the occurrence of PBC in either sex, such studies have been conducted for other autoimmune conditions, mostly in animal models. In humans, several case reports have shown an exacerbation of systemic lupus erythematosus and rheumatic diseases with administration of oral contraceptives.

**Table 3**  
**Synopsis of Population-Based Epidemiological Studies of Primary Biliary Cirrhosis**

<i>Year</i>	<i>Location</i>	<i>No. of cases</i>	<i>Annual incidence (per million)</i>	<i>Prevalence (per million)</i>	<i>Gender ratio (M/F)</i>
1980	Sheffield, UK	34	5.8	54	1:16
1980	Dundee, UK	21	10.6	40.2	1:9.5
1983	Newcastle, UK	117	10	37–144	1:14
1984	Malmoe, Sweden	33	4–24	28–92	1:3
1984	Western Europe	569	4	23 (5–75)	1:10
1985	Orebro, Sweden	18	14	128	1:3.5
1987	Glasgow, UK	373	11–15	70–93	—
1990	Umea, Sweden	111	13.3	151	1:6
1990	Ontario, Canada	225	3.26	22.4	1:13
1990	Northern England	347	19	129–154	1:9
1995	Victoria, Australia	84	—	19.1	1:11
1995	Estonia	69	2.27	26.9	1:22
1997	Newcastle, UK	160	14–32	240	1:10
2000	Olmsted County, MN (USA)	46	27	402	1:8

Data from ref. 31.

Sex differences in PBC have recently been addressed by genetic studies on the X chromosome, and results are promising (*see Sex Chromosomes*).

### AUTOIMMUNE FEATURES

Several clinical and experimental findings strongly imply an autoimmune pathogenesis for PBC, being both a model and a paradox for autoimmune conditions. The former is indicated by the characteristics of PBC that are common to other conditions, such as the female predominance, the genetic predisposition, or the presence of specific autoantibodies in the vast majority of cases. Such autoantibodies, however, in the case of PBC also constitute the basis for the disease being a paradox, as their direct pathogenetic role is still poorly defined (33). PBC is characterized by the presence of detectable AMAs in approx 90% of affected individuals, although we note that patients lacking AMAs can present with a similar disease picture and progression as found in AMA-positive subjects, seemingly arguing against a pathogenic role for these autoantibodies. Autoreactive T cells, both CD4 and CD8, have been identified in AMA-negative PBC, and such lymphocytes and AMAs recognize overlapping epitopes within the mitochondrial antigens (34).

Second, autoantibodies should interact with the target antigen, the passive transfer of autoantibodies should reproduce the clinical features, and experimental immunization with the antigen should produce a model disease. An intriguing feature of PBC, and of certain other autoimmune diseases, is that the immunologic offense is organ specific but the autoantigen is not tissue specific. As just mentioned, no direct proof has yet been provided for a direct pathogenic role of AMAs in the bile duct injury observed in PBC. Similarly, no convincing animal model has been described, although AMAs can be generated in experimental animals following immunization.

Third, in autoimmune diseases the reduction in autoantibody levels should ameliorate the disease; this criterion is poorly fulfilled in PBC, in which there is no correlation

between the pattern or titer of AMAs and progression or severity of disease (35). Finally, it is well established that most autoimmune diseases are responsive to immunosuppressive therapy. In PBC, all immunosuppressive agents have proved relatively ineffective.

### AMAs

AMAs are highly specific for PBC and can be detected in nearly 100% of patients. When AMAs are tested with more recently developed techniques, based on the use of recombinant mitochondrial antigens (with immunoblotting), the sensitivity and specificity of the test are significantly higher (36). In most clinical settings, however, indirect immunofluorescence techniques are used for initial screening of cases and might provide false-positive or -negative results.

AMAs are directed against components of the 2-oxoacid dehydrogenase complex (2-OADC) family of enzymes within the mitochondrial respiratory chain, most frequently the E2 and E3 binding protein (E3BP) components of the PDC and the E2 components of the 2-oxo glutarate dehydrogenase and branched-chain 2-OADCs. In all three antigens, epitopes contain the motif DKA, with lipoic acid covalently bound to the lysine (K) residue. The role of lipoic acid in epitope recognition by AMAs is unclear. The pathogenic role of AMAs remains debated, since no clinical correlation can be found and animal models developing serum AMAs do not manifest PBC-like liver lesions.

### ANAs

As many as 50% of patients with PBC have detectable serum antinuclear antibodies (ANAs), most commonly producing “nuclear rim” or “multiple nuclear dots” patterns, based on recognition by the autoantibodies of gp210 and nucleoporin 62 (within the nuclear pore complex) as well as Sp100 and polymorphonuclear leukocytes (PMLs) (possibly also crossreacting with small ubiquitin-like modifiers, [SUMOs]), respectively (37). Rim-like ANAs, on the other hand,

react against proteins of the nuclear pore complexes (NPCs), supramolecular structures that include gp210 (a 210-kDa transmembrane glycoprotein involved in the attachment of NPC constituents within the nuclear membrane), p62 (a nuclear pore glycoprotein), and the inner nuclear membrane protein lamin B receptor (LBR). Serum anti-gp210 ANAs are detected in about 25% (10–40%) of AMA-positive and up to 50% of AMA-negative patients (in both cases with high specificity). Autoantibodies reacting with p62 or LBR are found in about 13 and 1% of patients with PBC, respectively. Interestingly, the presence of anti-gp210 and anti-p62 ANAs in the same serum is rare.

ANA-positive patients are more frequently AMA negative, possibly because of the lack of a masking effect of these latter antibodies in such sera. The pathogenic role of ANA in PBC remains enigmatic, although cross-sectional and longitudinal data demonstrate an association between ANA positivity and a worse prognosis. Finally, we note that patients with PBC and limited systemic sclerosis have detectable serum anticentromere antibodies in 10 to 15% of cases.

### AUTOACTIVE T CELLS

A number of mononuclear cells can be found in the area surrounding damaged bile ducts in PBC. T-helper (CD4<sup>+</sup>) T-cell receptor (TCR)  $\alpha\beta^+$  and CD8<sup>+</sup> T cells are most commonly seen among such cells, perhaps secondary to high levels of interferon- $\gamma$  (IFN- $\gamma$ ) acting as a chemotactic stimulus. Autoreactive T cells have been well characterized in PBC from both the liver and peripheral blood of affected patients. PDC-E2-specific autoreactive CD4 T-cell (T-helper) clones were isolated by *in vitro* stimulation of intrahepatic or peripheral lymphocytes to PDC-E2 (38). The autoepitope for T cells overlaps with the B-cell (AMA) epitope and includes the lipoyl-lysine residue located at amino acid residue 174 of the inner lipoylated domain of the protein. Interestingly, a specific 100- to 150-fold increase in the frequency of PDC-E2163-176-specific CD4 T cells in the hilar lymph nodes and liver (compared with that in the periphery) is observed in PBC. Autoreactive cytotoxic T lymphocytes (CTLs) are also well characterized in PBC and are currently considered major effectors in the tissue injury encountered in PBC. The MHC class I restricted epitope for CTLs, namely, amino acids 159 to 167, also maps in close vicinity to the epitopes recognized by CD4<sup>+</sup> cells and by AMAs. Similarly to CD4<sup>+</sup> cells, moreover, the recent use of tetramer technology has shown a 10-fold higher prevalence of PDC-E2159–167-specific CTLs in the liver compared with peripheral blood of patients with PBC.

### GENETIC FEATURES

#### FAMILIAL PBC AND GENETIC FACTORS

PBC is more frequent in relatives of affected individuals, and the term *familial PBC* has been coined to indicate families that have more than one case. Variable rates of familial PBC are seen in different geographical areas, possibly owing once again to different methods of case definition. In general, data indicate that 1 to 6% of PBC cases have at least one family member presenting with the disease, and our most recent data

indicate that 6% of cases have a first-degree affected relative (19). Such familial prevalence rates are significantly higher than general population prevalence estimates, thus indicating a genetic predisposition to the disease. However, the difficulty in evaluating these data is that prevalence rates in the general population are still uncertain, and control groups are not always included in the family studies.

#### TWIN STUDIES

The pairwise concordance rate observed among monozygotic twins for PBC is 63%, among the highest reported in autoimmunity, reinforcing the idea of an important role of genetics in disease susceptibility (39).

#### GENETIC ASSOCIATION STUDIES

Several studies have attempted to identify genes associated with PBC. No family study of genetic linkage has been performed, possibly because PBC is a relatively rare disease, and it is therefore difficult to obtain DNA samples from a large number of representative families. All available studies were designed in a controlled, cross-sectional fashion but were prone to multiple sampling errors and biases of incorrect estimations. A multihit genetic model seems to apply to PBC, with different genetic variants conferring susceptibility (first hit) and others influencing disease progression (second hit). For this reason, most authors investigating genetic factors in PBC have studied the role of such factors in susceptibility to the disease (comparing allele and genotype frequencies in patients and controls), as well as in its severity (through the analysis of clinical characteristics of patients carrying different genotypes or alleles). No definitive association of PBC susceptibility or progression could be identified in these studies (40). When an association has been found, in fact, it has proved to be weak or limited to specific geographical areas. We note, moreover, that this also applies to study of MHC variants (including type I, II, and III loci), in which, different from most autoimmune diseases, reported associations were often weak or limited to specific geographical areas (41).

Similar findings were also reported from the study of the genetic variants of immunomodulatory molecules (such as chemokines and receptors), enzymes producing vasoactive compounds, and bile-acid transporters. In the future, definitive indications for the genetics of PBC may be obtained using methods based on inheritance by descent techniques on large series of affected and nonaffected family members and should be encouraged. Such an approach will in fact lead to more reliable findings compared with the use of cross-sectional association studies based on the comparison of allelic frequencies in cases and controls.

#### SEX CHROMOSOMES

Similar to other autoimmune diseases more commonly diagnosed in women following the reproductive years, fetal microchimerism has been suggested to play a role in PBC, with the hypothesis of a higher prevalence of small amounts of detectable fetal (i.e., paternal) DNA found in mothers with PBC. The evidence is conflicting, and this hypothesis has not been cumulatively confirmed (42).



**Table 4**  
**Risk Factors for Primary Biliary Cirrhosis (PBC)<sup>a</sup>**

	<i>Risk</i>	<i>OR</i>	<i>95% CI</i>	<i>p</i>
Family history of PBC	1.1868	10.736	4.227–27.268	<0.0001
Family history of systemic lupus erythematosus	0.4019	2.234	1.261–3.957	0.0059
History of urinary tract infections	0.2065	1.511	1.192–1.915	0.0006
Ever smoked > 100 cigarettes	0.2252	1.569	1.292–1.905	<0.0001
Ever used hormonal replacement	0.2185	1.548	1.273–1.882	<0.0001
Age of first pregnancy	-0.0470	0.9541	0.9331–0.9755	<0.0001

<sup>a</sup>In all the models used, household income was significantly correlated with PBC ( $p < 0.0001$ ). Data from ref. 19.

Genes mapping on the X chromosome are critical to the maintenance of physiological sex hormone levels and, more importantly, of immune responsiveness. Invernizzi and colleagues reported an age-dependent enhanced monosomy X in the peripheral white blood cells of women with PBC (43). This observation seems to indicate a polygenic model for PBC, with an X-linked major locus of susceptibility in which genes escaping inactivation are the major candidates. On the other hand, it can also be hypothesized that susceptibility to PBC is the result of a multigenic complex inheritance model where Y-linked genes might exert a protective role.

## ENVIRONMENTAL INFLUENCES

### RISK FACTORS

Although genetics should be regarded as the major determinant in susceptibility to PBC, several other factors have been proposed. Our epidemiological study has demonstrated that a high risk of developing PBC is associated with a positive family history for PBC, a history of urinary or vaginal infections, comorbidity with other autoimmune diseases, lifestyle factors such as smoking, and previous pregnancies (Table 4). Furthermore, we observed that the frequent use of nail polish also slightly increased the risk of having PBC (19).

### PROPOSED ENVIRONMENTAL FACTORS

The lack of strong genetic associations for PBC has meant that environmental factors have received attention as possible triggers of autoimmunity in PBC. Attention has focused on two main agents, infectious (bacteria and viruses) and chemical (xenobiotics). The ability of infectious agents, particularly bacteria, to induce autoimmune responses in experimental settings has been documented, and molecular mimicry is the most widely studied mechanism explaining these observations (44).

Briefly, this paradigm suggests that microbes present peptides sharing different degrees of homology with self-proteins, thus leading to a promiscuous antibody and cell-mediated immune response capable of reacting with both microbial and self-epitopes. T-cell activation produces crossreacting T cells, leading to self-tissue destruction and thus perpetuating the autoimmune response, possibly through degeneracy of the TCR and cross-priming. Of the bacterial strains suggested to lead to PBC through molecular mimicry (45), the greatest amount of evidence has been reported for *Escherichia coli*, mostly based on the reports

of an increased prevalence of urinary tract infections in patients with PBC (19).

We also note that, based on serum crossreactivity, several infectious agents have been proposed for the initiation of PBC, including *Klebsiella pneumoniae*, *Proteus mirabilis*, *Staphylococcus aureus*, *Salmonella minnesota*, *Mycobacterium gordonae*, *Neisseria meningitidis*, and *Trypanosoma brucei*. More recently, the common commensal yeast *Saccharomyces cerevisiae* has also been investigated in PBC, based on the expression of AMA antigens in extramitochondrial sites but, serological studies have indicated that the reactivity against the yeast (presence of anti-*S.cerevisiae* antibodies [ASCAs]) was not specific for the disease (46). Interestingly, contrasting evidence has been collected on the role of *Chlamydia pneumoniae* in the pathogenesis of PBC. Abdulkarim and colleagues detected the bacterial antigen and RNA in 100% of PBC explanted liver sections compared with 8.5% of controls (47); a different immunological and molecular approach could not confirm this hypothesis (48). Finally, our group has recently provided serological data suggesting that a ubiquitous xenobiotic-metabolizing the Gram-negative bacterium *Novosphingobium aromaticivorans* is the best candidate yet for the induction of PBC, as it elicits a specific antibody reaction (estimated to be 100- to 1000-fold higher than that against *E. coli*) and its 16S rRNA-specific sequences were detected in human fecal samples (49).

For completeness, we also note that a novel human  $\beta$ -retrovirus has been identified in lymph nodes and other samples from patients with PBC, thus suggesting that this mouse mammary tumor virus (MMTV)-like virus might play a role in the pathogenesis of PBC. However, our laboratory failed to confirm such a hypothesis using a different molecular and immunological approach in a large series of patients and controls (50), therefore discouraging the idea of the usefulness of any antiretroviral therapy in PBC.

Xenobiotics are foreign compounds that may either alter or complex to defined self- or non-self-proteins, inducing a change in the molecular structure of the native protein sufficient to induce an immune response. Such immune responses may then result in cross-recognition of the self form, which could in turn perpetuate the immune response, thus leading to chronic autoimmunity. A role for specific compounds has been proposed in a number of autoimmune conditions. Interestingly,

most xenobiotics are metabolized in the liver, thereby increasing the potential for liver-specific alteration of proteins. Experiments showed that specific organic structures attached to the mitochondrial antigens were recognized by sera from PBC patients with a higher affinity than native forms of such antigens (51). Such findings indicated for the first time that an organic compound may serve as a mimotope for an autoantigen, thus further providing evidence for a potential mechanism by which environmental organic compounds may cause PBC. One halogenated compound was able to induce AMA production in animal models (52). This model did not lead to production of liver lesions, however. It appeared to be a model of the stage of the disease in humans in which AMA is present prior to the appearance of liver damage. The AMA positivity, moreover, was reversible after the immunization ceased.

The vast majority of data on molecular mimicry in PBC have been obtained from the study of humoral immunity (i.e., AMAs), either in patient sera or in animal models, whereas the study of cellular autoimmunity is limited. An extensive study of autoreactive CD4<sup>+</sup> T-cell clones by Shimoda and colleagues (53) has demonstrated that molecular mimicry takes place between T-cell epitopes of PDC-E2 and gp210 (an ANA antigen in PBC), thus suggesting that immunospreading may occur from mitochondrial proteins to nuclear proteins, similar to what is hypothesized for bacterial antigens.

### ANIMAL MODELS

The development of an animal model would be extremely helpful in elucidating the undoubtedly multifactorial causation and progression of PBC. Several models, mostly murine, have been proposed. A complete discussion of these models is beyond the aims of this chapter. In 2000, a British group reported that immunization of SJL/J mice with PDC-E2 led to autoimmune cholangitis associated with T cells that displayed a mixed cytokine profile similar to what is observed in PBC. Such findings were later proved to be nonspecific, as bile duct inflammation was also found after immunization with control peptides under the same conditions. Furthermore, our group has reported that immunization of rabbits with a specific halogenated compound coupled with albumin could elicit AMA development (52) but not liver lesions. Recently, a variant of the NOD mouse model has been described as presenting autoimmune cholestasis and PBC-specific serology (54).

### PATHOGENIC MECHANISMS

Several theories have been proposed for the pathogenesis of the immune-mediated tissue injury observed in PBC. In all cases, such theories should not be regarded as independent from other etiological factors leading to PBC susceptibility (i.e., genetic background and environmental triggers) but rather as effector mechanisms leading to the clinical manifestations.

#### ABERRANT EXPRESSION OF PDC-LIKE ANTIGENS ON CHOLANGIOCYTES

The hypothesis for the selective destruction of biliary epithelial cells (BECs) states that the immunodominant AMA autoantigen, the E2 subunit of pyruvate dehydrogenase

(PDC-E2), which is normally located in the mitochondrial inner membrane, could be aberrantly expressed on the cell surface and thus be recognized by specific antibodies. Several experimental results seem to support this possibility. First, although *in situ* hybridization studies of PDC-E2 mRNA showed no significant difference in the amount of PDC-E2 transcript present in PBC liver compared with other liver diseases, PDC-E2 may be selectively overexpressed in small bile duct BECs. Second, variants of PDC-E2 may cause an abnormal turnover of the molecule, leading to the accumulation of PDC-E2 in these subpopulations of cells. It is possible that toxic substances disposed of by the liver may accumulate in the biliary epithelium and potentially modify the PDC-E2 molecule locally, leading to the production of such variants.

Third, altered PDC-E2 mRNA could be produced by the abnormal transcription of PDC-E2. For example, it is possible that abnormal splicing during synthesis of PDC-E2 mRNA would substitute an endoplasmic reticulum-targeting signal instead of a mitochondria-targeting signal. Thus, PDC-E2 may potentially be delivered into the endoplasmic reticulum and Golgi apparatus via a secretory route, to be expressed on the cell surface of biliary ducts, instead of mitochondria. Although direct evidence supporting these mechanisms is currently lacking, it remains possible that the molecules expressed and identified on the ductular surface of BECs and recognized by anti-PDC-E2 antibodies may not be PDC-E2 itself but rather PDC-E2 mimics that crossreact with human PDC-E2. Some experimental data seem to support this hypothesis.

### IMMUNOGLOBULINS

Another hypothesis that might explain the selective targeting of bile ducts in PBC is that the autoantigen-specific immunoglobulin A (IgA) antibody plays a role. IgA is the principal isotype of immunoglobulin in epithelial surfaces, including biliary epithelium. If AMA-IgA autoantibodies are responsible for the specific destruction of BECs in PBC, it is possible that this occurs by disruption of cell metabolism whereby the AMA-IgA bound to the mitochondrial antigen induces cellular dysfunction and hence tissue specificity. Interestingly, in our experiments, IgA from PBC patients colocalized with PDC-E2 inside the cells and on the apical membrane of BECs (49), as demonstrated by dual staining with antihuman IgA and anti-PDC-E2 mouse monoclonal antibody, whereas no colocalization was found when IgA from healthy controls was used. These data support the idea that both the aberrant polar expression of PDC-E2 and the trafficking of IgA in BECs are possible mechanisms for selective damage of BECs. However, the apical staining of anti-PDC-E2 monoclonal antibodies could also be accounted for by the presence of an immune complex formed by secreted IgA and mitochondrial enzymes.

### MOLECULAR MIMICRY

Crossreactivity of AMAs with prokaryotic antigens (particularly with the microbial respiratory chain enzymes) has been reported for a number of microbes, including *E. coli*. This crossreactivity is not particularly surprising given the conserved sequence of PDC-E2 across all species, from eubacteria to

mammals. Indeed, it is proposed that mitochondria originated following uptake of bacteria into the precursors of eukaryotic cells and maintenance as intracellular symbionts. Thus it becomes difficult to tease out a causal role for microbial proteins in pathogenesis, given their phylogenetic relationship to the human autoantigen.

One line of argument we have taken is that the breaking of tolerance and induction of autoimmunity would be more likely to occur when the microbial protein is extremely similar in sequence. We have recently suggested that a Gram-negative ubiquitous bacterium, *Novosphingobium aromaticivorans*, sharing the highest homology with human PDC-E2 yet described and capable of metabolizing xenobiotics, is the best candidate so far identified for the induction of PBC via molecular mimicry (55). A necessary requirement for such a scenario would be the exposure of the patient to the candidate bacteria, either accompanied by overt signs of infection or not. A number of studies have searched for bacterial species within the liver and biliary tract of patients with PBC, but data have so far failed to define bacteria specific only to PBC liver. *A priori*, it is not clear that the bacteria would necessarily need to be present in these tissues, as infection and tolerance breakdown may occur anywhere, including in the urinary tract. Furthermore, the bacteria may well have disappeared by the time the patient presents with PBC, complicating the search even more with a "hit and run" model. Recently, researchers from one group have reported the presence of a  $\beta$ -retrovirus in the liver and lymph nodes of some patients with PBC and also that the culture of normal BECs in the presence of homogenate of such PBC lymph nodes induced the expression of a PDC-E2-like antigen on the cell membrane. Although intriguing, this latter observation has not been confirmed.

Briefly, we can summarize our theory on molecular mimicry in PBC as follows. The microorganism (possibly the ubiquitous *N. aromaticivorans*) enters the human system through the digestive mucosa. Bacterial mimics containing lipoic acid residues at this point might be modified by xenobiotics to form immunoreactive adducts. This modification would then be sufficient to trigger the innate immune system to initiate a cascade of local inflammatory events, via Toll-like receptors, for example, thus resulting in local dendritic cell activation and antigen processing. Mucosal antigen-presenting cells (APCs) in turn activate autoreactive T and B cells that are directed to the liver through the portal system. T cells participate directly in the autoimmune injury and/or further recruit autoreactive lymphocytes. B cells, on the other hand, secrete AMAs, particularly of the IgA type. AMA IgA is then transported to the vascular side of the bile duct cell, where they react with the PDC-E2-like molecules located on the luminal surface cell membrane. This binding then initiates the apoptotic signaling cascade. Ultimately, the immune complexes of postapoptotic PDC-E2 and IgG-AMA and the direct cytopathic effects of autoreactive T cells (and possibly AMAs) contribute to the tissue injury observed in PBC.

## REGULATORY T CELLS

Recent studies have pointed out the critical role of CD4<sup>+</sup>/CD25<sup>high</sup> regulatory T cells (T-regs) in the prevention of autoimmune disease in murine models. An important role for CD4<sup>+</sup>/CD25<sup>high</sup> T-regs in the prevention of autoimmunity and maintenance of self-tolerance has also been hypothesized. Some studies have demonstrated that the transfer of T cells lacking the CD4<sup>+</sup>/CD25<sup>high</sup> T-reg subset into athymic nude mice results in the development of various T-cell-mediated autoimmune diseases. Experimental data demonstrate that PBC patients display significantly lower frequencies of CD4<sup>+</sup>/CD25<sup>high</sup> T-regs as percentages of total TCR- $\alpha$ <sup>+</sup>/CD4<sup>+</sup> T cells, which may contribute to the breakdown of tolerance in PBC (56).

## TREATMENT AND OUTCOME

Several medical treatments have been investigated in patients with PBC. Currently, ursodeoxycholic acid (UDCA) is the only accepted therapy and has received US Food and Drug Administration approval.

### UDCA

UDCA accounts for 4% of the bile acid pool in human bile. Compared with other bile acids, such as chenodeoxycholic and deoxycholic acids, UDCA is more hydrophilic. Its absorption (30–60% following an oral dose) occurs mainly in the small intestine, and its presence decreases cholesterol secretion into bile, possibly lowering its conversion to bile acids. The mechanism of action of UDCA in PBC is incompletely understood, but it has been hypothesized that it is based on different factors, including modification of the bile acid pool, reduction in proinflammatory cytokines, effects on apoptosis and on vasoactive mediators, and modification of the bile acid pool. However, since UDCA's antiinflammatory effects are found only in bile ducts, it has been assumed that its effect is mediated by modification of the bile acid pool.

Doses ranging from 13 to 15 mg/kg of UDCA are currently used and lead to optimum bile enrichment. Accordingly, a metaanalysis demonstrated that increased survival is obtained only when a dose greater than 13 mg/kg is prescribed (57), even though a complete biochemical response to UDCA (normalization of serum liver tests in the absence of cirrhosis) is achieved in approx 40% of treated patients (58). Pares et al. have recently demonstrated that biochemical response to UDCA after 1 yr is associated with a survival similar to that of the matched control population, clearly supporting the favorable effects of this treatment in PBC (59).

### OTHER MEDICAL TREATMENTS

Based on success rates observed in other autoimmune diseases, the use of immunosuppressive drugs was attempted in PBC, but efficacy was poor. Immunosuppressive drugs used in PBC have included corticosteroids, azathioprine, cyclosporin, methotrexate, penicillamine, and colchicine. Their use is currently encouraged only in combination with UDCA in selected cases. In the event of an unsatisfactory response to UDCA alone, these drugs are still considered, but the lack of efficacy and the risk of serious side effects make their use highly debat-

able. Definitive data are still awaited on the efficacy of UDCA plus bezafibrate (60), mycophenolate mofetil (61), methotrexate (62), budesonide (63), and tamoxifen (64,65).

### LIVER TRANSPLANTATION

Liver transplantation is the ultimate treatment for end-stage PBC, with survival rates of 92 and 85% at 1 and 5 yr after transplant, respectively. Recurrence is common, and rates seem to be influenced by certain immunosuppressive regimens; the use of UDCA for recurrence is safe and recommended. Interestingly, the frequency of OLT for PBC in a large series from the United Kingdom was reported to have decreased over the past decades, along with increased age at the time of transplantation. Cumulatively, such data could once again indicate that the natural history of PBC might be influenced by earlier diagnosis or medical treatment. The use of UDCA in transplanted patients is currently considered safe, and no contraindications have been identified so far.

### OVERLAP SYNDROMES

Autoimmune hepatitis (AIH)-PBC overlap syndrome is found in 10% of adults with AIH or PBC. Besides overlaps, transitions are also possible in rare cases from PBC to AIH or AIH to PBC. Thus, the clinical management of overlap syndromes is based on single diseases, whereas medical treatment is empiric. Therefore, UDCA is used for chronic cholestasis, immunosuppressants (steroids and azathioprine) are used for AIH, and liver transplantation is indicated for end-stage disease.

### AUTOIMMUNE CHOLANGITIS

The term *autoimmune cholangitis* was first introduced to indicate AMA-negative PBC, possibly with serum ANAs (66). However, a broader significance has been suggested more recently, to include: (1) serum ANA and/or smooth muscle actin (SMA) antibody positivity and/or hypergammaglobulinemia, (2) serum AMA negativity by immunofluorescence, (3) biochemical and/or histological features of cholestatic and hepatocellular injury, and (4) exclusion of chronic viral, metabolic, or toxic liver disease (67). This definition possibly includes PBC with nontypical presentation, small duct PSC, idiopathic adulthood ductopenia, and transitional stages of the classic diseases. Consensus is still awaited on this issue. In summary, autoimmune cholangitis is now considered a disease of unknown cause that typically displays serum ANAs with or without SMA antibodies in serum and cholestatic clinical, laboratory, and/or histological changes in the absence of AMAs.

Currently, this entity has no established niche in the spectrum, it is a disease that lacks uniform diagnostic criteria, and its characterization is still evolving.

It is probable that autoimmune cholangitis is a cholestatic liver disease with a natural history similar to that of AMA-positive PBC despite some differences in serology and that it should be treated similarly, that is, with UDCA. Perhaps after all it would be less confusing if autoimmune cholangitis was referred to as "AMA-negative PBC."

### CONCLUDING REMARKS AND OPEN QUESTIONS

PBC should be considered a unique disease within the range of autoimmunity. Future efforts should be dedicated to overcoming some of the conceptual and logistic difficulties. First, only study of a very large number of representative families will unravel the genetic basis of PBC. Given the relatively rare prevalence of the disease, only a worldwide effort will allow the collection of a population of families large enough (and with two or three generations available) to guarantee enough statistical power for a linkage analysis. Second, the role of xenobiotics and infectious agents in the onset of PBC should be further probed, particularly with respect to the development of an animal model and the use of detailed epidemiological studies to ascertain the exposure to specific environmental factors. Third, it is crucial to determine the pathogenic role of AMAs in the bile duct damage of PBC. Once again, the development of an animal model appears to be the only way to provide a clear demonstration of such a pathogenic mechanism. Finally, from a clinical standpoint, new clinical trials are needed to identify novel therapies in the long-term treatment of PBC. Together with the already present trend toward an earlier diagnosis of the disease, more effective medical treatment, possibly using specific monoclonal antibodies or hematopoietic stem cell transplant, will be the cornerstone in reducing the need for OLT in patients affected by PBC.

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# 19 Sclerosing Cholangitis

## *Primary and Secondary*

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SUE CULLEN AND ROGER CHAPMAN

### KEY POINTS

- Primary sclerosing cholangitis (PSC) is not a classical autoimmune disease, but as immune mechanisms play an important role in the pathogenesis of the disease, it could be described as an immune-mediated inflammatory disease (IMID).
- The term “secondary sclerosing cholangitis” refers to a disease that is histologically similar to PSC but the causative agent is known. Little information regarding the immunology of secondary sclerosing cholangitis is available.
- There is epidemiological evidence of a genetic component in the pathogenesis of PSC, and a number of haplotypes have now been defined that confer susceptibility or resistance to the development of PSC.
- The response of the innate immune system to bacterial antigen is likely to be an initiating step in the pathogenesis of the disease.
- PSC is associated with changes in peripheral lymphocyte subsets and a T-lymphocyte portal tract infiltrate. These lymphocytes have been shown to be functional.
- The role of Th1 and Th2 cytokines in the pathogenesis of primary sclerosing cholangitis is not yet clearly defined, but there is evidence of their involvement in many aspects of the progression of the disease.
- A range of autoantibodies can be detected in the sera of PSC patients. The most specific is p-ANNA (antineutrophil nuclear antibody), but this antibody does not appear to have a role in the pathogenesis of PSC.
- Despite the close association of PSC with inflammatory bowel disease (IBD), the clinical course of PSC is independent of the activity of IBD and, indeed, can present for the first time after a colectomy for ulcerative colitis.
- Abnormal homing of gut-activated memory T lymphocytes to the liver, as a result of the aberrant expression of adhesion molecules and chemokines by cholangiocytes, may explain the lack of relationship between the course of inflammatory bowel disease and PSC.

- Differentiation of PSC from autoimmune pancreatitis is important, as the latter responds well to steroid therapy.

### INTRODUCTION

Sclerosing cholangitis comprises a spectrum of chronic cholestatic disease of the hepatobiliary system characterized by hepatic inflammation, biliary strictures, and fibrosis. The best studied form is primary sclerosing cholangitis (PSC), which is a slowly progressive disorder eventually resulting in concentric obliterative fibrosis of the bile ducts, biliary cirrhosis, and, in approximately 30% of patients, cholangiocarcinoma. There is a strong association between PSC and inflammatory bowel disease (IBD), with between 75 and 80% of PSC patients of northern European origin having underlying IBD (1,2). Ulcerative colitis is the most common form of IBD associated with PSC, and, interestingly, when considering the pathogenesis of the disease, those PSC patients who have Crohn’s disease almost invariably have disease predominantly affecting the colon.

The term *secondary sclerosing cholangitis* (SSC) is used for a disease with similar clinical features to PSC but for which the causative agent for the pathological process is known. These agents includes choledocholithiasis with intraductal stones, surgical damage to bile ducts, ischemia from hepatic artery occlusion, infections, and chemical agents such as drugs. Table 1 gives a list of possible causes of SSC as well as the conditions that can mimic sclerosing cholangitis on cholangiography. There are few good data on the natural history of SSC, which has tended to enter the literature as case reports. A comparative study of SSC and PSC comprising two groups of 31 patients with each disease found similar survival or requirement for orthoptic liver transplantation. Nine of the SSC patients and seven of the PSC required liver transplantation, and four SSC patients died compared with seven PSC patients (3). SSC is usually managed with multiple endoscopic retrograde cholangiopancreatography (ERCP) and balloon dilatation, stenting, and sphincterotomies for biliary strictures. Some patients proceed to surgery, which normally involves a hepaticojejunostomy with roux en Y or choledochoduodenostomy. Little information regarding the immunological processes occurring during the progression of SSC is known, although liver biopsies often show similar changes to those of PSC, with

Table 1

Causes and Mimics of Secondary Sclerosing Cholangitis (SSC)	
Cause or mimic	Ref.
<b>Causes of secondary sclerosing cholangitis</b>	
Surgical trauma to bile ducts	116
Ischemic injury, e.g., after transplantation	117
Hepatic arterial chemotherapy, e.g., floxuridine	118–121
Intraductal gallstones	3
Viral or bacterial infection, e.g., cytomegalovirus or cryptosporidiosis	122–124
Caustic injury, e.g., formalin treatment of hydatid disease	125,126
Congenital abnormalities, e.g., cystic fibrosis	127
<b>Conditions mimicking sclerosing cholangitis on imaging</b>	
Malignancy, e.g., metastatic carcinoma	128,129
Hypereosinophilic syndrome	130
Choledochal cyst	131

ductopenia and patchy inflammation. The rest of this chapter therefore concentrates on the liver immunology of PSC.

The etiology and pathogenesis of PSC is not yet well understood, although clues continue to accumulate. The insidious onset of the disease makes identification of an etiological factor particularly difficult. This chapter discusses the evidence that immunopathogenic mechanisms are involved with the development of the clinical syndrome of PSC and considers the relative contributions of humoral and cellular immunity and the role of the biliary epithelial cells.

### EVIDENCE OF A GENETIC COMPONENT IN THE PATHOGENESIS OF PSC

Few papers have reported incidents of familial cases of PSC in the English literature (2,4–7). Until Bergquist et al.'s paper in 2005 (8), a total of only seven affected families had been reported, and in all reports the affected family members were siblings. The most striking of these reports is that of Jorge et al. (1987), which describes an Argentinian family with 15 siblings, 4 of whom had well-documented PSC on cholangiography and liver biopsy, with a further brother suffering from chronic cholestasis that might have been caused by undiagnosed PSC (5). Bergquist et al. recently published the first large study of the familial occurrence of PSC, using a group of 145 PSC patients (8). A PSC prevalence of 0.7% in first-degree relatives of PSC patients was demonstrated, which represents an impressive 100-fold increased risk for these relatives compared with the general population in Norway. Even this figure is probably an underestimation, as the average age at diagnosis in this population is 32 to 42 yr, so few of the patients had children old enough to have developed the disease.

The importance of genetic predisposition in the pathogenesis of PSC seems well established despite the lack of genetically informative families. This is as a result of the work performed over the last 25 yr on the genes of the major histocompatibility complex (MHC). This area is the most obvious candidate for investigation of autoimmune disease, as it encodes the HLA

molecules, which are highly polymorphic cell-surface heterodimeric glycoproteins that are essential for cell-cell recognition. Investigation into HLA in the context of PSC has resulted in the development of a number of extended HLA haplotypes associated with susceptibility and resistance to the disease. The genetics of autoimmune liver diseases and PSC are discussed in more detail in Chapter 17.

### IS PRIMARY SCLEROSING CHOLANGITIS AN AUTOIMMUNE DISEASE?

PSC has been described as an “atypical autoimmune disease” owing to the presence of autoantibodies, an association with “autoimmune” HLA haplotypes, and a close association with IBD. However, PSC lacks a specific autoantigen, affects predominantly men rather than women, and does not appear to respond well to immunosuppressive medication. Although PSC cannot be regarded as a classical autoimmune disease, there is a substantial body of evidence that immune mechanisms play an important role in the pathogenesis of the disease. The most striking difference is in the gender of patients, with a male-to-female ratio in PSC of 2:1 compared with the female predominance usually found in autoimmune disease.

PSC is known to be associated with other autoimmune diseases. This phenomenon has been studied in 119 patients by Saarinen et al., who compared PSC patients with patients who had IBD alone to determine whether the increased frequency of autoimmune disease noted in PSC patients could be ascribed to the close association of PSC to IBD (9). This comprehensive study demonstrated that patients with PSC and IBD were more likely to have other autoimmune liver diseases outside the liver and colon than patients with IBD alone. Twenty-five percent of the PSC patients had one or more other autoimmune diseases, compared with only 9% of the IBD patients. The most common autoimmune diseases in the PSC group were diabetes mellitus and Grave's disease. Interestingly, this paper found no difference in class II typing between PSC patients with or without other autoimmune diseases outside the liver and colon. This observation suggests that the association of PSC to autoimmune diseases is not secondary to the HLA autoimmune haplotype but is a primary phenomenon in PSC. Rheumatoid arthritis has also been described in association with PSC. In three of the four cases reported, the liver disease was rapidly progressive and this condition may be a marker for patients at high risk for the development of cirrhosis (10).

The *immune-mediated inflammatory disease* (IMID) model appears to describe the clinical features of the disease better. This group of diseases, which are now thought to include inflammatory bowel disease IBD, rheumatoid arthritis, and psoriasis, appears to be mediated by T cells and macrophages (11). A comparison of PSC with classical autoimmune diseases and IMID is shown in Table 2. The trigger is more likely to be an environmental antigen than a self-antigen, and this trigger produces an inappropriately aggressive immune response, resulting in inflammation and tissue damage. The response to the trigger is genetically determined with multiple genes controlling the extent, site, and nature of the immune reaction; this produces a



**Table 2**  
**Features of Primary Sclerosing Cholangitis Compared With Classical Autoimmune Disease**

<i>Characteristic</i>	<i>Classical autoimmune disease</i>	<i>Immune-mediated inflammatory disease</i>	<i>Primary sclerosing cholangitis</i>
Age	Children and adults	Children and adults	Children and adults
Sex	Female predominance	No gender predilection	Male predominance
Autoantigens	Yes	No	No
Autoantibodies	Yes (pathogenic)	Yes (markers)	Yes (probably markers)
Associated autoimmune disease	Yes	Yes	Yes (particularly strong association with IBD)
HLA associations (class I and II)	Yes	Yes	Yes
Response to immunosuppression	Usually good	Often good	Good in children Poor in adults

Abbreviation: IBD, inflammatory bowel disease.

genetically “complex” disease with multiple genes and mutations appearing to influence the final phenotype.

### ROLE OF BACTERIA AND INNATE IMMUNITY

The association of PSC and IBD led at an early stage to the hypothesis that the hepatobiliary lesion was secondary to the bowel disease, caused by intestinal or toxic substances absorbed through a leaky colonic mucosa (12). Portal bacteremia has been described in 24 of 90 patients with ulcerative colitis who underwent colectomy (13). The innate immune system, mediated by macrophages (including Kupffer cells), dendritic cells, natural killer (NK) cells, and natural killer T (NKT) cells, provides the first line of defence against microbial pathogens. Macrophages and dendritic cells recognize invariant microbial molecules known as pathogen-associated molecular patterns (PAMPs) and activated complement molecules on opsonized pathogens. This recognition occurs through a variety of pattern recognition receptors (PRRs) expressed by macrophages and dendritic cells and leads to activation of the cells with consequent phagocytosis and the production of cytokines and chemokines. The PRRs include the family of Toll-like receptors, CD14, and complement receptors. As PAMPs are only molecular components of bacteria, they can activate the innate immune response in the absence of viable organisms. PAMPs of relevance in the pathogenesis of PSC probably include lipopolysaccharide (LPS) and lipoteichoic acid from the cell walls of Gram-negative and Gram-positive bacteria, respectively, peptidoglycans, and unmethylated bacterial dinucleotide motifs.

Interest in the importance of the innate immune system increased after identification of the nucleotide-binding oligomerization domain 2 (NOD2/CARD15) gene as a susceptibility gene for Crohn’s disease, together with the significant role of defensins in the pathogenesis of both ulcerative colitis and Crohn’s disease (14–16). There appears to be no association between NOD2 polymorphisms and susceptibility to PSC (17). The focus on the innate immune system produced by these fascinating discoveries may well lead to further insights into the pathogenesis of IBD-associated diseases such as PSC.

A number of animal models using T-cell receptor and interleukin-10 (IL-10) knockout mice have indicated that

immune responses to bacterial antigens are involved in the generation of colitis and perinuclear antineutrophil nuclear antibody (p-ANNA), the antibody most closely associated with PSC (18–20). The hypothesis that bacterial antigens may produce deleterious immune responses in immunogenetically susceptible individuals has been investigated using a rat model of small bowel bacterial overgrowth, (21–23). Small bowel bacterial overgrowth was induced by creating jejunal self-filling blind loops in five strains of rat. Female Lewis rats and both male and female Wistar and Sprague-Dawley rats developed hepatic injury 4 to 16 wk after surgery. In contrast, neither Fischer nor Buffalo rats developed any injury after the same procedure, indicating a genetic difference in susceptibility. Histopathology demonstrated inflammatory lesions in portal tracts with bile duct proliferation and fibrosis. Cholangiograms demonstrated abnormal thickened, tortuous, and irregular bile ducts reminiscent of human PSC (22). The pathogenetic mechanism underlying this model is not completely understood. Bacterial cell wall components (principally endogenous peptidoglycan-polysaccharide [PG-PS]) appear to induce hepatic macrophage (Kupffer cell) cytokine secretion (24). Circulating levels of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) was most significantly correlated with hepatobiliary injury, (25). It should be borne in mind when interpreting this model that adult humans with anaerobic small bowel bacterial overgrowth do not develop hepatic abnormalities. A small study has recently been conducted on the role of small bowel bacterial overgrowth and abnormal intestinal permeability in PSC patients. Only 1 of 22 PSC patients had evidence of small bowel bacterial overgrowth (compared with none of 18 control patients), and there was no significant difference in intestinal permeability between PSC patients and controls (26).

Another rat model has been used for investigating the role of *N*-formyl/L-methionine/L-leucine/L-tyrosine (fMLT), a proinflammatory peptide secreted by *Escherichia coli*. fMLT is known to undergo enterohepatic circulation and is a strong chemoattractant for macrophages and neutrophils. A dilute fMLT solution was instilled intrarectally in mice with acetic acid-induced chemical colitis. fMLT appeared in the bile within 3 h. Serial histopathology revealed macrophage and neutrophil infiltration into the small bile ducts, followed by the appearance of CD4 and CD8 T cells in the peribiliary infiltrate. The model

demonstrated that bacterial chemotactic peptides can cause small duct cholangitis if they gain access to the portal circulation via an inflamed colonic mucosa. Pretreatment with carageenan to reduce the number of macrophages led to reduced cholangitic lesions, indicating that activated macrophages are responsible for the T-cell attraction, activation, and function (27–29).

Another model of interest is that of Kuroe et al., who described granulomatous enterocolitis in rabbits induced by administering muramyl dipeptides emulsified with Freund's incomplete adjuvant submucosally (30). Interestingly, this also induced inflammation of the bile ducts, with periductal fibrosis. The authors suggested that continuous stimulation with bacterial cell wall fragments may be involved in chronic intestinal inflammation and extraintestinal disease such as PSC.

A study of explanted livers revealed positive bacterial cultures from 21 of 36 PSC patients compared with none of 14 PBC patients (31). The cause of this bacterial load was thought to be possible contamination from cannulation of the bile duct from ERCP. Interestingly, however,  $\beta$ -hemolytic streptococci accounted for 46% of the bacterial strains identified in PSC patients. This is at odds with other published data, which have identified *E. coli*, Enterobacteriaceae organisms, and enterococci as being the bacteria most often found in patients with biliary tract disease, suggesting that  $\beta$ -hemolytic streptococci may have an etiopathogenetic role in the disease. Bjornsson et al. cultured bile from PSC patients who were ERCP naïve and compared them with PSC patients who had had prior ERCP, as well as with patients with choledocholithiasis and with biliary obstruction (32). Positive cultures were demonstrated in 3 of 10 PSC patients (25%) who were ERCP naïve, compared with 6 of 10 PSC patients (60%) who had had prior ERCP. Sixty-four percent of patients with choledocholithiasis and 56% with biliary obstruction also had positive cultures. These data do not suggest a causative role for bacterial infection of bile in the etiopathogenesis of PSC and does not exclude the possibility that episodes of bacterial infection may alter the progression of the disease.

Recent molecular studies have shown an increased prevalence of *Helicobacter pylori* and other nongastric *Helicobacter* species in cholestatic liver diseases compared with healthy controls and noncholestatic liver disease. In PSC, the presence of *H. pylori* was associated with higher levels of serum alkaline phosphatase, prothrombin complex factors, and concurrent ulcerative colitis. The lack of disease specificity, however, makes it unlikely that this organism plays a role in the pathogenesis of PSC (33).

Ponsioen et al. have suggested an association between PSC and previous *Chlamydia* infection after an increase in seroprevalence of *Chlamydia* anti-LPS antibodies was found in PSC patients. No viable *Chlamydia* organisms were found in liver tissue, however, and the significance of this finding remains unclear (34).

Vierling has proposed a hypothesis for the pathogenesis of PSC in which the initial event is the reaction of an immunogenetically susceptible host to bacterial cell wall products (35). This reaction results in the production of hepatic macrophages

by TNF- $\alpha$  and endotoxin. The exposure to bacterial components and increased gut permeability would be increased by the presence of IBD but could also, in theory, occur during episodes of gut infection. The resulting increase in peribiliary cytokine and chemokine secretion would attract activated neutrophils, monocyte/macrophages, T cells, and fibroblasts. He further postulated that the deposition of concentric fibrosis could result in atrophy of biliary epithelial cells (BECs) secondary to ischemia. The resulting bile duct loss would lead to progressive cholestasis, fibrosis, and secondary biliary cirrhosis. This hypothesis does not explain the relative scarcity of patients with Crohn's colitis and the association of PSC with pancreatic duct abnormalities. It also does not take into account the strong circumstantial evidence of immune mediation and autoimmunity discussed above.

## HUMORAL IMMUNITY IN PSC

Although a range of humoral immune abnormalities have been described in PSC, many are associated primarily with cholestasis rather than PSC, specifically. For example, high levels of circulating immune complexes have been demonstrated in PSC, but this phenomenon has also been found in other liver diseases (36). Complement activation associated with circulating immune complexes was reported by Senaldi et al., who found that both C3d and C4d were elevated in patients with PSC compared with patients with extrahepatic obstructive cholestasis and normal controls (37). Currently there is no evidence of complement activation in the liver (38).

## AUTOANTIBODIES IN PSC

Various autoantibodies may be detected in the sera of patients with PSC (Table 3) (39). These antibodies are unlikely to be implicated in disease pathogenesis but may indicate an altered state of immune responsiveness or immune regulation.

Antineutrophil-specific antibodies are detected in up to 88% of patients with PSC. The labeling pattern of neutrophils produced by these antibodies is different from that produced by antineutrophil cytoplasmic antibodies (ANCA) in vasculitic disease. Work by Terjung et al. demonstrated that the target antigen in PSC is localized to the periphery of the nucleus and suggested that the antineutrophil antibody in PSC therefore be renamed p-ANNA (antineutrophil nuclear antibody) (40). The antigen was identified by the same group in 2005 as myeloid-specific tubulin- $\beta$  isotype 5 (41). The role of p-ANNA in the immunopathogenesis of PSC remains unclear, particularly as the myeloid-specific tubulin autoantigen is recognized by autoantibodies from patients with both PSC and autoimmune hepatitis (AIH). However, the titers of p-ANNA do not change after liver transplantation, which suggests that they are not merely an epiphenomenon.

Animal studies have suggested that pANCA might be induced by immune responses to bacterial PAMPs or antigens crossreactive with enteric antigens. Most patients with PSC have antibodies against enterobacterial proteins, and 36 to 46% of PSC patients have antineutrophil cytoplasmic antibodies directed against the bactericidal/permeability increasing protein (BPI) (42). This protein is found mainly in the granules

**Table 3**  
**Antibodies Associated With Primary Sclerosing Cholangitis**

<i>Antibody</i>	<i>Target</i>
ANA	
Anticardiolipin	IgA/IgG and/or IgM
	$\mu_2$ -GPI IgG
ANCA	h-Lamp-2 IgG
	Proteinase 3 IgG
	Bactericidal/permeability-increasing protein IgG5
Thyroperoxidase	
Rheumatoid factor	

Abbreviations: ANA, antinuclear antibody; ANCA, antineutrophil cytoplasmic antibody.

Adapted from ref. 39.

of neutrophils and, to a lesser extent, eosinophils and has potent antimicrobial properties with special effectiveness against Gram-negative bacteria. The presence of BPI-ANCA has been associated with inflammation and tissue damage, and it has been suggested that BPI-ANCA might promote innate immune reactions by preventing clearance of lipopolysaccharide (43).

Only a few studies have reported a correlation between the presence of antineutrophil antibodies and clinical parameters in PSC. Pokorny et al. (44) found that biliary tract complications were more common in patients with PSC who had anti-neutrophil antibodies. Bansi et al. (45) demonstrated a correlation between the presence or absence of antineutrophil antibodies and the involvement of intrahepatic or extrahepatic bile ducts, respectively. Mulder et al. (46) investigated the development of cirrhosis in PSC and found that this was associated with high-titer antineutrophil antibodies. Titers of antineutrophil antibodies remain unchanged after liver transplantation (47). Currently there is not enough evidence to make the presence or absence of antineutrophil antibodies a useful prognostic indicator in clinical practice or to conclude that this antibody plays any role in the pathogenesis of the disease.

#### ANTICOLON AND OTHER AUTOANTIBODIES

It has been proposed that autoantibodies reacting with colonic antigens might be implicated in the pathogenesis of PSC. Cangemi et al. found anticolon antibodies in 62% of patients with ulcerative colitis and PSC compared with only 17% of patients with ulcerative colitis alone (48). These anticolon antibodies did not react with hepatobiliary tissue. Subsequent studies, however, have demonstrated shared epitopes on colonic and biliary epithelial cells that might act as a target for the immune response (49,50). Patients with PSC also exhibit an increased frequency of other autoantibodies that are unlikely to be related to the pathogenesis of PSC but might reflect a state of immune hyperreactivity or compromised immunoregulation (Table 3) (39).

Autoantibodies to BECs have also been detected in PSC patients. Autoantibodies reacting with antigens on healthy BECs were detected in 63% of PSC patients, compared with 37% of patients with primary biliary cirrhosis (PBC), 16% of

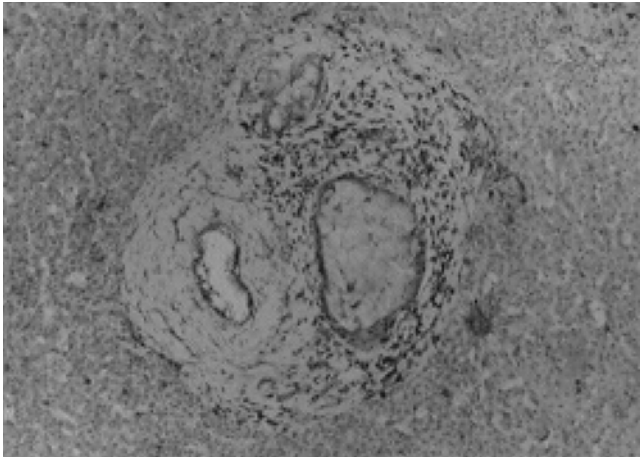
AIH patients, and 8% of healthy controls (51). This study went on to show that only the anti-BEC antibodies from the PSC and PBC patients had the capacity to induce cultured BECs to secrete IL-6, a proinflammatory cytokine that can stimulate cholangiocyte proliferation and inhibit apoptosis. Furthermore, the IgG and IgM autoantibodies from PSC patients alone could induce expression of CD44 cell adhesion molecules on BECs. CD44 has a role in the recruitment of lymphocytes to sites of inflammation in AIDS and IMiDs

#### CELLULAR IMMUNE ABNORMALITIES IN PSC

Initiation and maintenance of the immune cascade is determined not only by MHC recognition but also by the presence of accessory cells and molecules that provide costimulatory signals and the production of cytokines that amplify or modify the immune response.

Because of the central role of lymphocytes in the immune response, a number of studies have investigated changes in circulating lymphocyte subsets in PSC patients. Although initial studies were suggestive of an increase in the ratio of CD4<sup>+</sup> to CD8<sup>+</sup> (helper/suppressor) T cells and an overall reduction in the number of circulating T lymphocytes, further studies have not confirmed these findings (52–56). Methodological differences in quantifying lymphocytes (flow cytometry, rosetting, blood smears, immunofluorescence) probably explain some of the discrepancies between the studies. Although early-stage disease does not appear to be associated with abnormal T- and B-cell populations, there does appear to be evidence that CD8<sup>+</sup> levels fall as the disease progresses. Lindor et al. noted that PSC patients with cirrhosis had significantly higher CD4<sup>+</sup>/CD8<sup>+</sup> ratios than noncirrhotics and that the fall in CD8<sup>+</sup> T cells with progressive disease was accompanied by an increased number of circulating B lymphocytes (57). As these changes in peripheral lymphocyte subsets are only seen in advanced disease, they are unlikely to be involved in the pathogenesis of the disease. More recently, Panasiuk et al. demonstrated a significant increase in both activated lymphocytes and NK cells in the peripheral blood of PSC patients compared with controls (58). In a mouse model of dextran sulfate sodium-induced colitis associated with PSC-like hepatobiliary changes, stimulation of NKT cells was found to modify the Th1/Th2 balance (59). A single stimulation of NKT cells produced a Th1-dominant immune response associated with increased inflammation around bile ducts, whereas repeated NKT cell stimulation produced a Th2-dominant response that tended to lead to an improvement in hepatic inflammation.

Studying the circulating lymphocyte populations may not be as relevant as looking at the cellular infiltrate at the site of tissue injury. Infiltration with T lymphocytes is a characteristic finding in several organ-specific autoimmune diseases, and PBC and AIH are both associated with a T-cell infiltrate (60,61). A number of immunohistochemical studies have attempted to identify the nature of the cellular infiltrate in PSC (52,53,61). All these studies agree that the portal tract mononuclear cell infiltrate in patients with PSC is predominantly composed of T lymphocytes (Fig. 1) There is, however, no consensus about



**Fig. 1.** Monoclonal antibody stain for CD3 from portal tract of patient with primary sclerosing cholangitis showing marked infiltration with T lymphocytes.

the relative importance of CD4<sup>+</sup> and CD8<sup>+</sup> cells in the portal infiltrate. The study of Hashimoto et al., probably the most comprehensive published to date, found that CD4<sup>+</sup> cells were more common in the portal tracts, with CD8<sup>+</sup> cells predominating in areas of interface hepatitis (61). NK cells were reported in this study to constitute around 10% of the portal infiltrate. The variation in the findings of studies in this area probably reflect the focal nature of the disease, with small biopsies being of limited value in predicting the immunohistopathological changes in the whole organ. The stage of the disease is clearly also important, as the cellular infiltrate may change as the disease progresses. Mast cells have also been demonstrated in relatively high numbers in the portal tracts of PSC patients and may play a role in fibrogenesis (62).

Although the studies just discussed have clearly described the presence of T lymphocytes in the portal infiltrate, it is also necessary to determine whether they are of functional significance in the pathogenesis and progression of PSC or are merely acting as a marker for the presence of the disease. T-cell antigen recognition is immediately followed by clonal expansion of antigen-specific T-cells and the differentiation of antigen-specific memory T-cells. This mechanism allows an enhanced immune response after subsequent exposure to the antigen. Activated and memory T cells express a range of surface markers that aid in their identification.

The expression of T-cell activation markers has been studied by Martins et al. using dual-color flow cytometry (63). Elevated levels of the markers HLA-DR, CD25(IL25), and CD71 and the memory marker CD45RO were found in the peripheral blood of PSC patients compared with controls. There was no correlation between the levels of activated or memory cells in these patients and disease stage, biochemical profile, or HLA status. Similar results were found in patients with PBC and AIH. A monoclonal antibody technique for immunohistochemical analysis of the portal mononuclear infiltrate in frozen liver sections reported similar results (64). A preponderance of HLA-DR<sup>+</sup> (activated) and CD45<sup>+</sup>

(memory) cells was found in the portal infiltrate. These two studies demonstrate the presence of functional T lymphocytes in the liver and in the peripheral blood of patients with PSC. These cells are present at all disease stages and are therefore more likely to be a cause rather than an effect of disease progression.

#### T-CELL RECEPTOR

The TCR is, necessarily, a highly diverse structure, to enable it to recognize the wide variety of antigens it encounters. The TCR usually consists of two disulfide-linked polypeptides, termed  $\alpha$  and  $\beta$ . In the past 13 yr, a group of T cells has been identified that carries an alternative receptor termed  $\gamma\delta$ . The role of these  $\gamma\delta$  cells in the normal immune response is unclear, but they appear to be strong candidates for involvement in the phenomenon of autoimmunity (65). An increased number of  $\gamma\delta$  T cells has been found in the peripheral blood and portal infiltrates of patients with PSC and AIH compared with controls. It was noted, however, that the predominant cell type within the liver was still  $\alpha$  cells. There was no specific concentration of the  $\gamma\delta$  cells in the bile ducts or in areas of interface hepatitis. This makes it less likely that  $\gamma\delta$  T cells have a primary role in the pathogenesis of PSC, but it is still possible that they might function by modulating  $\alpha\beta$  T-cell activation or by regulating antibody or autoantibody production from B cells (66).

Although TCR gene rearrangements serve to generate genetic diversity, a particular V $\alpha\beta$  gene segment can play a dominant role in the recognition of certain peptide-MHC complexes. Expanded T-cell populations using restricted sets of TCR V gene segments have been identified in areas of inflammation in diseases such as rheumatoid arthritis and Sjögren's disease. This suggests the presence of a specific antigen with the ability to drive the production of T cells with this restricted V $\beta$  segment product (67,68). Studies of Broome et al. indicated that the hepatic, but not peripheral, T cells in PSC preferentially have V $\beta$  3 T-cell repertoires (69). An oligoclonal expansion was not demonstrated in this study, but oligoclonal TCRs that proliferate in culture with enterocytes and are cytotoxic to enterocyte cell lines have also been reported in PSC (70).

#### CYTOKINES

Cytokines are protein hormones that mediate the effector phase of both humoral and cell-mediated immunity via the activation, proliferation, and differentiation of lymphocytes. Cytokines are secreted from CD4 Th cells. Th1 cells secrete IL-2, interferon- $\gamma$  (IFN- $\gamma$ ) and tumor necrosis factor- $\alpha/\beta$  (TNF- $\alpha/\beta$ ) which promote immunopathology through delayed-type hypersensitivity, cytotoxic T cells, and activated macrophages. CD4 Th2 cells are induced by IL-4 to secrete IL-4, IL-5, IL-6, IL-10, and IL-13 (71). These cytokines stimulate B-cell secretion of IgG1 and IgE antibodies and activate eosinophils and mast cells. Th1 (via IFN- $\gamma$ ) and Th2 (via IL-10) cross-regulate each other's proliferation and function, and their dynamic equilibrium is an essential element of the development of immunopathology.

Most studies of cytokines in the context of PSC have been based on measurement of cytokine production from peripheral

lymphocytes. There has been a suggestion in some abstracts that, compared with healthy controls, the cytokine profile is shifted toward the production of Th1-derived cytokines in PSC (72). This has also been demonstrated in a rat model of the disease (73).

A study by Bansal et al. demonstrated elevated levels of IL-10 and IL-8 but not IFN- $\gamma$  in patients with PSC compared with controls (74). Broome et al., however, found no differences in the spontaneous production of cytokines from colonic lymphocytes between patients with PSC and ulcerative colitis, ulcerative colitis alone, or controls, although some differences were elicited after stimulation of the cells with purified protein derivative (75). Overall, little work has been published in this area, and the picture is not completely clear. It is probably more physiologically relevant to study the presence and activity of liver-derived lymphocytes anyway. Studies performed by Mitchell et al. comparing the peripheral and intrahepatic expression of Th1 and Th2 cytokines at the mRNA level have shown an increased expression of both Th1 and Th2-type cytokines within the liver of PSC patients compared with patients who had disease (alcoholic liver disease and large duct obstruction) and healthy controls. Downregulation of IL-10 mRNA expression in PSC and PBC was also demonstrated. These changes were reversed after treatment with ursodeoxycholic acid. Liver-derived T cells from PSC patients have been shown to have greater intracytoplasmic TNF- $\alpha$  levels compared with those from patients with PBC or autoimmune hepatitis, and anti-TNF- $\alpha$  antibody enhances the usually blunted proliferative response of these cells (76).

An abnormal cytokine repertoire and the high expression of cytokine mRNA in the early stages of PSC suggest that Th1 and Th2 cytokines may play a pathogenic role. Cytokines could have an influence on many aspects of the progression of PSC, including cytotoxic T-cell development, aberrant expression of class II MHC molecules on BECs, and matrix and metalloproteinase gene expression in fibroblasts (77). There is some evidence to suggest that biliary epithelial cells are induced to produce nitric oxide (NO) as a result of the synergistic action of TNF- $\alpha$  with IFN- $\gamma$ . The NO produced inhibits cAMP-dependent HCO<sub>3</sub><sup>-</sup> secretion and thus contributes to ductal cholestasis (78). The true role of cytokines in the development and progression of PSC has yet to be clearly defined.

## LYMPHOCYTE HOMING

Both IBD and PSC are characterized by an influx of destructive inflammatory cells into the tissue. Most extraintestinal manifestations of IBD occur at the same time as a flare in the bowel disease. PSC, in contrast, appears to run a course entirely independent of the associated bowel disease and can even present for the first time after a colectomy. In a series of elegant studies, Grant et al. have developed an immunological hypothesis to explain this clinical phenomenon (79–82).

Antigen entering via the gut is processed by the mucosal immune system, generating active and memory T lymphocytes. The memory lymphocytes circulate continuously between blood and tissue and provide immune surveillance. Each memory lymphocyte expresses specific receptors for

endothelial adhesion molecules. The expression of these molecules and of associated chemokines varies according to tissue type, with some adhesion molecules, e.g., intracellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule (VCAM-1) being involved in lymphocyte recruitment to many tissues. Other molecules have a more restricted expression and these are known as *addressins*, as they provide a specific site of action for the lymphocytes. The interaction between the adhesion molecule on the endothelium and the receptor on the lymphocyte allows the lymphocyte to be exposed to chemokines (produced as a response to tissue injury or stress) and integrins, producing firm adhesion of the lymphocyte to the endothelium. The lymphocyte can now migrate across the endothelial wall and into the target tissue. This system of transendothelial migration ensures that memory T lymphocytes can perform surveillance of the tissues where they originally encountered their cognate antigen.

During episodes of gut inflammation (for example, relapses of IBD), memory T cells are produced. Grant et al. demonstrated that there is aberrant expression of an adhesion molecule, mucosal addressin cell adhesion molecule (MAdCAM-1), on the endothelial cells of the portal vein and sinusoids. This adhesion molecule is usually restricted to the gut, and this finding therefore suggested the existence of an enterohepatic recirculation of memory T cells between the gut, and liver. This might have developed to enable the immune system to respond to gut antigens entering via the portal circulation.

MAdCAM-1 allows adhesion of T lymphocytes expressing an  $\alpha_4\beta_7$  integrin. MAdCAM-1 was previously thought to be exclusive to the gut but has been shown to be expressed on hepatic endothelium in the context of chronic inflammation. These T cells also carry the chemokine receptor CCR9, which binds to the chemokine ligand CCL25, which is also aberrantly expressed on hepatic endothelium. The source of CCL25 might be activated BECs. Additionally, vascular adhesion protein-1 (VAP-1), which is constitutively expressed on both vascular and sinusoidal endothelium in the liver, has also been found to occur on the vascular endothelium in IBD. The lymphocyte receptor for VAP-1 is not known. These studies have gone on to show that MAdCAM-1 is functionally important by demonstrating the presence of  $\alpha_4\beta_7^+$ , CCR9<sup>+</sup> lymphocytes in liver tissue and clarifying that the imprinting of memory T lymphocytes with these molecules occurs in mesenteric lymph nodes rather than locally in the liver (80,83).

The hypothesis proposed therefore, is that T cells activated in the gut during attacks of IBD will differentiate into effector cells with the ability to bind to both gut and hepatic endothelium. Some will also persist as memory T cells. Any condition causing hepatic inflammation would then allow recruitment of these T cells to the liver owing to the expression of VAP-1, MAdCAM-1, and CCL25 on the hepatic endothelium. Subsequent clonal expansion of memory T cells could lead to the development of inflammatory liver disease such as PSC. The pivotal role of memory T cells could explain why IBD and PSC do not necessarily occur together and indeed why PSC can occur many years after colectomy for ulcerative colitis (82).

Currently it is still unclear whether the expression of these adhesion molecules on hepatic endothelium occurs prior to the onset of PSC, as, for example, a manifestation of a genetic predisposition to the development of PSC, or whether expression occurs as a consequence of hepatic inflammation. The recurrence of PSC in transplanted livers suggests that expression is inducible. It has been noted that patients who undergo colectomy prior to liver transplantation are less likely to develop recurrent PSC, and it would be interesting to know whether these adhesion molecules can be identified in the transplanted livers of such patients (84).

Some unanswered questions remain around this hypothesis. It has been shown, for example, that more than 90% of lymphocytes in the small bowel express  $\alpha_4\beta_7$  integrin and CCR9 and that mice deficient in either  $\alpha_4\beta_7$  integrin or CCR9 have disrupted mucosal lymphocyte compartments (85–87). The role of  $\alpha_4\beta_7$  integrin and CCR9 in the homing of lymphocytes to the colon, however, is less well documented, and although CCR9 expression has been shown on up to 25% of T cells isolated from the human colon during episodes of gut inflammation, CCL25 expression has not been demonstrated in colonic tissue, and animal models have not shown entry of CCR9<sup>+</sup> lymphocytes into colonic tissue (88–90). This suggests that PSC should be more clearly associated with small bowel rather than colonic inflammation. Interestingly, a phenotype of IBD associated with PSC has been recently described, and the term “PSC-IBD” has been adopted by some authors (91). PSC-IBD appears to be characterized by a pancolitis with a particularly high prevalence of involvement of the distal small bowel, known as “backwash ileitis.”

## CONTRIBUTION OF BILIARY EPITHELIAL CELLS

The biliary epithelium appears to be both a target for immune-mediated injury and an active participant in the immune response. The mechanism of bile duct targeting is currently obscure, as the BECs demonstrate only modest expression of CD58 (lymphocyte function-associated antigens), CD80 (B7BB1), and CD95 (Fas), which are the usual essential epitopes that constitute the targets of cell mediated immunity (92).

### IMMUNOMODULATORY ROLE

BECs have been shown to respond to PAMP ligands, via Toll-like receptors, and to proinflammatory cytokines e.g., TNF- $\alpha$ , IL-1 $\beta$ , IL-6, and IFN- $\gamma$ . This interaction leads the BECs to secrete chemokines and cytokines along with matrix metalloproteinases and growth factors (93,94). The secretion of these proinflammatory and fibrogenic factors by the BECs determines the composition of peribiliary inflammation, including the recruitment of gut-primed lymphocytes.

### EXPRESSION OF HLA CLASS II MOLECULES ON BILE DUCT EPITHELIUM

Aberrant expression of HLA molecules on target cells is important in the pathogenesis of autoimmune diseases (60,95). Normal bile duct epithelial cells express HLA class I antigens but not class II antigens (96). Class II molecules expressed on the bile duct epithelium have the potential to

initiate an immune reaction by binding autoantigens or exogenous antigens and presenting the peptides to class II-restricted T lymphocytes (60). The HLA class II antigens HLA-DR, -DQ, and -DP have all been found to be expressed by BECs from patients with PSC (97,98). This phenomenon does not appear to be disease specific, however, as BEC HLA-DR expression has also been found in patients with extrahepatic biliary obstruction and various inflammatory disorders (96,99). The increased HLA expression may be induced by a secondary response to inflammation, possibly through the action of IL-2 (100).

The hypothesis that HLA class II molecules participate in a first step in the induction of PSC requires the demonstration of activated class II-restricted T cells in the portal tracts of PSC patients. This has not so far been achieved, and doubt has been cast on the hypothesis that BECs function as antigen-presenting cells by the finding that BECs lack the costimulatory molecules necessary to activate T cells (101,102). A small Japanese study has, however, identified the presence of costimulatory factor B-27 on some BECs in PSC patients (103). It seems likely that BECs are less active antigen-presenting cells than dendritic cells or macrophages.

## HEPATOBIILIARY TRANSPORTERS IN PSC

Defects in the hepatobiliary transport system have been shown to be the cause of a number of hereditary cholestatic disorders, e.g., progressive familial intrahepatic cholestasis and BSEP (bile salt export pump) (104). This system is responsible for the hepatocellular uptake and excretion of bile salts into bile canaliculi. Defects in the transport system can result in bile duct injury.

Knockout mice for the *Mdr2* (*Abcb4*) gene, which corresponds to human MDR3/ABCB4, spontaneously develop sclerosing cholangitis with features similar to those of human PSC (105). A nonfunctional MDR3 (multidrug resistance 3) protein leads to the formation of a “toxic” bile with increased concentration of free, nonmicellar bile acids that cause BEC injury, pericholangitis, periductal fibrosis, and, eventually, sclerosing cholangitis. Studies in PSC patients, however, did not find MDR3 variations (106). Similarly, the role of the cystic fibrosis transmembrane conductance regulator (CFTR) remains controversial (107–109). The potential role of other hepatobiliary transporters, e.g., BSEP, or AE2, in the pathogenesis of PSC remains to be explored. As defects in these systems are known to cause bile duct injury and cholangitis, they are excellent candidates for further investigation.

## AUTOIMMUNE PANCREATITIS (LYMPHOPLASMACYTIC SCLEROSING PANCREATITIS)

Autoimmune pancreatitis (AIP) was first described by Sarles in 1961 and is an increasingly recognized benign condition of the pancreas (110). Abnormalities and sclerosing changes in the bile ducts are well recognized in AIP and can cause diagnostic confusion with PSC. Correct diagnosis is important, as AIP responds well to corticosteroid therapy and tends to have a

**Table 4**  
**Comparison of Primary Sclerosing Cholangitis (PSC) and Autoimmune Pancreatitis-Sclerosing Changes (AIP-SC)**

<i>Parameter</i>	<i>PSC</i>	<i>AIP-SC</i>
Gender	M:F = 2:1	Probably some male predominance (132,133)
Clinical presentation	Usually insidious; sometimes with obstructive jaundice secondary to cholangiocarcinoma	Mild abdo/back pain Sometimes with short history of obstructive jaundice owing to common bile duct stricture
Associated inflammatory bowel disease	Yes	No
Cholangiographic findings	Diffuse changes throughout intra- and extrahepatic bile ducts	Pancreatic duct strictures or narrowing; often stricture of distal 1/3 of common bile duct
Blood chemistry data	Abnormalities in pancreatic duct common Often cholestatic but bilirubin usually near normal	Intrahepatic duct changes less common May be cholestatic; bilirubin often high
Autoantibodies	Atypical pANCA plus range of others	Antibodies to carbonic anhydrase II plus range of others (132,134,135)
Immunoglobulins	IgG4 levels normal	IgG4 levels usually elevated (136)
Histology	Absence of plasma cells positive for IgG4 on immunostaining	gG4-positive plasma cells present in bile ducts and portal tracts (114)
Liver biopsy staging	Range of Ludwig staging including higher stages e.g., III or IV	Ludwig staging usually only I or II (137)
Treatment	Ursodeoxycholic acid ± biliary drainage for dominant strictures	Systemic steroid therapy usually leads to complete resolution of symptoms and signs of disease. Occasionally patients relapse and require longer courses of steroids

**Table 5**  
**Evidence for the Influence of Immune Mechanisms on the Etiology of Primary Sclerosing Cholangitis**

<i>Immune mechanisms</i>	<i>Effect</i>
Humoral immunity	Increased circulating immune complexes Elevated immunoglobulin levels (IgG and IgM) Low titers of non-organ-specific autoantibody (ANA and SMA) High titers of antineutrophil nuclear antibody (ANNA)
Cell-mediated immunity	Decreased levels of circulating peripheral CD8 <sup>+</sup> T cells Portal T-cell and NK-cell infiltrate Increased activated and memory T cells Restricted T-cell receptor repertoire (Vβ3) Aberrant expression of HLA-DR on BECs Coexpression of costimulatory molecules and HLA-DR on BECs Abnormal expression of adhesion molecules on biliary epithelial cells Abnormal expression of chemokine ligands on biliary epithelial cells
Immune effector mechanisms	Enhanced cytokine expression in the liver
Immunogenetic mechanisms	HLA associations

Abbreviations: ANA, antinuclear antibody; BEC, biliary epithelial cells; SMA, smooth muscle antibody.

significantly better prognosis than PSC (111–113). The association of AIP and sclerosing changes in the bile ducts has been termed AIP-SC (114). Diagnostic criteria for AIP have been proposed and developed by the Japan Pancreas Society (115). These criteria consist of the finding of a diffuse narrowing of the pancreatic duct on imaging studies and either a laboratory finding of an abnormally elevated serum  $\gamma$ -globulin, IgG, or IgG4 or the presence of autoantibodies or classical histopathological features of the disease, i.e., fibrotic changes with lymphocyte or plasma cell infiltration. The differences between the two conditions are summarized in Table 4.

## CONCLUDING REMARKS AND OPEN QUESTIONS

The evidence presented in this chapter suggests that PSC is immune mediated but is not a classical autoimmune disease (Table 5). A wide range of abnormalities in the usual immune response has been demonstrated in patients with PSC, however, it is still difficult to be sure about the sequence of events and to establish whether the immune reactions seen in PSC are the cause or consequence of the tissue injury. The triggering factors in an immunogenetically susceptible host are still uncertain, although the suggestion that bacterial products gain access to the portal circulation via a diseased and leaky bowel is an attractive hypothesis. Important work on mucosal adhesion molecules links IBD and PSC through the mechanism of long-lived memory T cells homing aberrantly to the liver rather than the gut and setting up inflammatory change. This also produces the first explanation for the observation that the clinical courses of IBD and PSC run independently of each other. Many questions remain unanswered, however, including the triggering factors for the development of PSC, the immunomodulatory influence

of the innate immune system, the role of autoantibodies and cytokines in the course of the disease, and predisposing factors for the development of cholangiocarcinoma. It may be that in attempting to answer these questions it will be possible to define new therapeutic modalities for this progressive, and ultimately terminal disease.

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# 20 Autoimmune Hepatitis

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DIEGO VERGANI AND GIORGINA MELI-VERGANI

## KEY POINTS

- Autoimmune hepatitis (AIH) is characterized by a histological lesion called interface hepatitis in which mononuclear cells infiltrate the portal tracts and invade the parenchyma, disrupting the limiting plate.
- A set of inclusion and exclusion criteria for the diagnosis of AIH has been established by the International Autoimmune Hepatitis Group.
- There are two main types of AIH: type 1, positive for antinuclear (ANA) and/or antismooth muscle (SMA) antibodies, and type 2, positive for anti-liver-kidney-microsomal antibody type 1 (LKM-1).
- Autoantibodies should be tested by indirect immunofluorescence (IIF) at an initial dilution of 1:40 on a freshly prepared rodent substrate that includes kidney, liver, and stomach to allow simultaneous detection of all reactivities relevant to AIH.
- Anti-LKM-1 antibody is often confused with anti-mitochondrial antibody (AMA) if only rodent kidney is used as substrate in IIF.
- The identification of the molecular targets of anti-LKM-1 and AMA has led to the establishment of immunoassays based on the use of the recombinant or purified antigens.
- Perinuclear antinuclear neutrophil antibodies (p-ANNA) are an additional marker of AIH-1; anti-liver cytosol type 1 (LC-1) antibody is an additional marker of AIH-2; and anti-soluble liver antigen (SLA) antibodies can be present in AIH-1 and AIH-2 and are associated with a more severe clinical course.
- Predisposition to AIH-1 is conferred by the possession of HLA-DR3 in young patients and HLA-DR4 in older patients, whereas susceptibility to AIH-2 is conferred by possession of HLA-DR7.
- Patients with AIH respond well to immunosuppressive treatment, even in the presence of cirrhosis, and have an excellent long-term prognosis.
- In AIH-2, the key autoantigen has been identified as cytochrome P4502D6 (CYP2D6).

- All arms of the immune system, including CD4, CD8, and B lymphocytes are involved in the pathogenesis of AIH-2.

## INTRODUCTION

Autoimmune hepatitis (AIH) is an inflammatory liver disease with a strong female preponderance, characterized by elevated levels of transaminases and immunoglobulin G (IgG), seropositivity for organ and non-organ-specific autoantibodies, and a histological picture of interface hepatitis. The major pathogenic mechanism is believed to be immune reaction against host liver antigens. AIH responds well to immunosuppressive treatment. The diagnosis should be made as soon as possible because symptomatic AIH, if left untreated, progresses to liver failure requiring transplantation. The development of a panel of marker autoantibodies has allowed the subdivision of AIH in distinct types, type 1 (AIH-1) being positive for antinuclear (ANA) and/or anti-smooth muscle antibodies (SMA) and type 2 (AIH-2) being positive for anti-liver-kidney microsomal antibody type 1 (anti-LKM-1).

## HISTORY AND EPIDEMIOLOGY

AIH is a recently recognized disease, having been first described by Waldenström in 1950 (1). Seropositivity for ANA, the hallmark of systemic lupus erythematosus, led Mackay to call it “lupoid hepatitis” (2), a term no longer used. Since the disease frequently presents acutely, the term “chronic active hepatitis,” is similarly obsolete this term implying that the disease should be chronic, i.e., of at least 6-mo duration, before institution of treatment. Before the efficacy of immunosuppression was established, untreated severe AIH had a mortality of 50% at 5 yr and 90% at 10 yr (3,4).

The prevalence of AIH is unknown. Most of the information available was collected before the introduction of the International Autoimmune Hepatitis Group (IAIHG) Scoring System (5,6), and therefore no standardized way of evaluating patients was used. Moreover, early studies did not exclude hepatitis C. A study in a Norwegian population reports a mean annual incidence of 1.9 cases of AIH per 100,000 and a point prevalence of 16.9 cases per 100,000 population (7). The rates of AIH found in this study are about twice those found in studies of idiopathic chronic active hepatitis in Iceland and of AIH within patients with chronic active hepatitis in Sweden and

England (8). Other reported prevalences range from 1 per 200,000 in the US general population (9) to 20 per 100,000 in females over 14 yr of age in Spain (10), although probably both figures are underestimates.

The prevalence of AIH-2, which affects mainly children and young adults, is unknown, also because the diagnosis is often overlooked. At the King's College Hospital tertiary pediatric hepatology referral center, there has been a seven fold increase in the incidence of AIH over the last decade (unpublished data).

## AUTOANTIBODIES

A key component of the diagnostic criteria developed by the IAIHG (5,6,11) is detection by indirect immunofluorescence of autoantibodies to constituents of the nuclei (ANA), smooth muscle (SMA), and liver-kidney microsome type 1 (anti-LKM-1) (Table 1 and Fig. 1). Autoantibody detection not only assists in the diagnosis but also allows differentiation of AIH in to type 1 and type 2. ANA and SMA which characterize AIH-1, and anti-LKM-1, which defines AIH-2, are practically mutually exclusive; in those rare instances in which they are present simultaneously, the clinical course is similar to that of AIH-2. Recognition and interpretation of the immunofluorescence patterns is not always straightforward. The operator dependency of the technique and the relative rarity of AIH explain the not infrequent occurrence of errors in reporting, particularly of less frequent specificities such as anti-LKM-1. Problems exist between laboratory reporting and clinical interpretation of the results that are partly dependent on insufficient standardization of the tests but also partly dependent on a degree of unfamiliarity of some clinicians with the disease spectrum of AIH. In regard to standardization, a lead has been taken by the IAIHG, which has established an international representative committee to define guidelines and develop procedures and reference standards for more reliable testing (11).

The basic technique for the routine testing of autoantibodies relevant to AIH is indirect immunofluorescence on a freshly prepared rodent substrate that should include kidney, liver, and stomach to allow the detection of ANA, SMA, and anti-LKM-1, as well as anti-liver cytosol type 1 (anti-LC-1), but also of antimitochondrial antibody (AMA), the serological hallmark of primary biliary cirrhosis. Positive sera should be titrated to extinction; the pattern of nuclear staining for ANA-positive patients may be further characterized by the use of HEp2 cells. Of particular importance is the plan of section and orientation of the kidney because both anti-LKM-1 and AMA stain renal tubules, but with different patterns distinguishable only in the presence of both proximal and distal tubules. Thus AMA stains preferentially the distal tubules, which are smaller in size, whereas anti-LKM-1 stains characteristically the third portion of the proximal tubules. The sections of liver, kidney, and stomach should be dried in air and used without further fixation. Commercially available sections are of variable quality because, to lengthen shelf life, they are treated with fixatives (acetone, ethanol, or methanol), which readily result in enhanced background staining that may hinder the recognition of diagnostic autoantibodies, especially when these are present at low titer.

Since healthy adults may show reactivity at the conventional starting serum dilution of 1:10, the arbitrary dilution of 1:40 has been considered clinically significant by the IAIHG in this age group.

## ANTINUCLEAR ANTIBODY

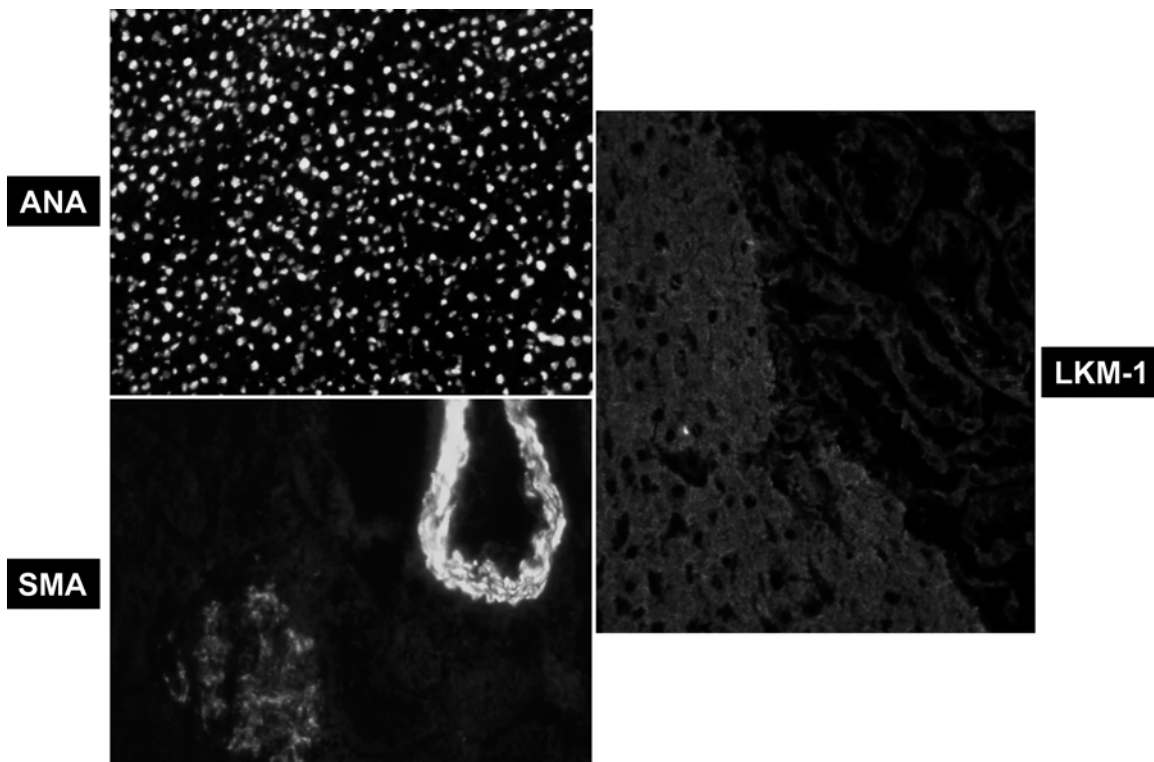
ANA is readily detectable as a nuclear staining in kidney, stomach, and liver. On the latter in particular, the ANA pattern may be detected as homogeneous, or coarsely or finely speckled. In most cases of AIH, but not in all, the pattern is homogeneous. To obtain a much clearer and easier definition of the nuclear pattern, HEp2 cells that have prominent nuclei should be used. HEp2 cells, however, should not be used for screening purposes, because nuclear reactivity to these cells is frequent at low serum dilution (1:40) in the normal adult population (12). For ANA, likely molecular targets include nuclear chromatin and histones, akin to lupus, but there are probably several others. The advent of new techniques using recombinant nuclear antigens and immunoassays will allow a better definition of ANA target antigens, an assessment of their specificity for diagnosis, and their possible role in the pathogenesis of AIH-1.

## SMOOTH MUSCLE ANTIBODY

SMA is detected on kidney, stomach, and liver, where it stains the walls of the arteries. In the stomach it also stains the muscularis mucosa and the lamina propria. On the renal substrate, it is possible to visualize the V, G, and T patterns; V refers to vessels, G to glomeruli, and T to tubules (11,13). The V pattern is also present in non-autoimmune inflammatory liver disease, in autoimmune diseases not affecting the liver, and in viral infections, but the VG and VGT patterns are more specific for AIH. The VGT pattern corresponds to the so-called F actin or microfilament (MF) pattern observed using cultured fibroblasts as substrate. Neither the VGT nor the anti-MF patterns are, however, entirely specific for the diagnosis of AIH-1. Although the VGT-MF pattern has been suggested to be owing to a specific antibody uniquely found in AIH-1, it may just reflect high-titer SMA. The molecular target of the microfilament reactivity that is observed in AIH-1 remains to be identified. Although "anti-actin" reactivity is strongly associated with AIH-1, some 20% of SMA-positive AIH-1 patients do not have the F-actin/VGT pattern (14). The absence, therefore, of anti-actin SMA does not exclude the diagnosis of AIH.

## ANTI-LIVER-KIDNEY MICROSOMAL ANTIBODY

Anti-LKM-1 stains brightly the liver cell cytoplasm and the P3 portion of the renal tubules but does not stain gastric parietal cells. Anti-LKM-1 is often confused with AMA, since both autoantibodies stain the liver and kidney. Compared with LKM-1, AMA stains the liver more faintly and the renal tubules more diffusely, with an accentuation of the small distal ones. In contrast to anti-LKM-1, AMA also stains the gastric parietal cells. In the context of AIH, there can be positivity for AMA in a small subset of patients (3–5%) in whom there are overlapping features with primary biliary cirrhosis (15). The identification of the molecular targets of anti-LKM-1, i.e., cytochrome P4502D6 (CYP2D6), and of AMA, i.e., enzymes of the 2-oxo-acid dehydrogenase complexes, has led to the



**Fig. 1.** Immunofluorescence pattern of antinuclear (ANA), smooth muscle (SMA), and anti-liver-kidney microsomal type 1 (LKM-1) autoantibodies on renal and liver rodent sections. SMA stains the small artery and the glomerulus in the renal section, ANA the nuclei in the liver section, and anti-LKM-1 the cytoplasm of hepatocytes and proximal renal tubules.

**Table 1**  
Methods, Associations, and Reactants for Autoantibodies in Liver Diseases

<i>Autoantibody</i>	<i>Conventional method of detection</i>	<i>Molecularly based assays</i>	<i>Disease association</i>	<i>Molecular target(s)</i>
ANA	IIF	N/A	AIH-1; overlap syndromes	Multiple targets, particularly chromatin
SMA	IIF	N/A	AIH-1; overlap syndromes	Microfilaments (actin?), intermediate filaments (vimentin and others)
Anti-LKM-1	IIF <sup>a</sup>	ELISA, IB, RIA	AIH-2; HCV infection (5%)	Cytochrome P450 2D6
Anti-LC-1	IIF, DID, CIE	ELISA, RIA	AIH-2	Formiminotransferase cyclodeaminase
SLA/LP	Inhibition ELISA	ELISA, IB, RIA	AIH-1; AIH-2 and AIH negative for other reactivities	tRNP(Ser)Sec ( <i>see text</i> )
Atypical p-ANCA (p-ANNA)	IIF	N/A	AIH-1; sclerosing cholangitis	Unidentified antigen(s) at nuclear periphery
AMA	IIF	ELISA, IB, RIA	Primary biliary cirrhosis	E2 subunits of 2-oxo-acid dehydrogenase complexes, particularly PDC-E2

Abbreviations: ANA, antinuclear antibody; SMA, anti-smooth muscle antibody; LKM-1, anti-liver-kidney microsomal antibody type 1; LC-1, anti-liver cytosol type 1 antibody; SLA/LP, anti-soluble liver antigen/liver-pancreas antibody; p-ANCA, perinuclear antineutrophil cytoplasmic antibody; p-ANNA, perinuclear antineutrophil antibody; AMA, anti-mitochondrial antibody. IIF, indirect immunofluorescence (recommended cutoff titer for positivity is 1:40 except in children—*see text*); DID, double dimension immunodiffusion; CIE, counter-immunoelectrophoresis; ELISA, enzyme-linked immunosorbent assay; IB, immunoblot; RIA, radio-immunoprecipitation assay; AIH, autoimmune hepatitis; HCV, hepatitis C liver.

<sup>a</sup>Anti-LKM-1 and AMA both stain renal tubules and are frequently confused (*see text*).

Modified from ref. 11.

establishment of immunoassays based on the use of the recombinant or purified antigens. Commercially available enzyme-linked immunosorbent assays (ELISAs) are accurate for detection of anti-LKM-1, at least in the context of AIH-2, and are reasonably accurate for the detection of AMA. Therefore, if a doubt remains after examination by immunofluorescence, this can be resolved by the use of molecularly based immunoassays.

#### **VARIANT LIVER MICROSOMAL ANTIBODIES: ANTI-LM AND ANTI-LKM-2 AND -3**

These antibodies are mostly directed against P450 cytochrome isoforms different from 2D6. Anti-LM antibodies stain only the liver cytoplasm, react with liver-specific cytochrome P4501A2, and occur in dihydralazine-induced hepatitis and in hepatitis associated with autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED), a monogenic disorder with a variable phenotype that includes about 20% of AIH cases (16). An anti-LKM-1-like pattern of immunofluorescence is given by autoantibodies to P4502A6 that occur in APECED and occasionally in hepatitis C. The term anti-LKM-2 was originally applied to LKM-1-like microsomal antibodies produced during hepatitis induced by the no longer marketed antihypertensive tienilic acid and are directed against cytochrome P4502C9 (17). Anti-LKM-3, which targets members of the 1 family of UDP-glucuronosyltransferases (UGT), also gives an immunofluorescent pattern similar to that of anti-LKM-1, but it occurs mainly in hepatitis D (delta) (18).

#### **ANTI-LIVER CYTOSOL TYPE 1**

This antibody was originally described either in association with anti-LKM-1, or in isolation, in both instances defining a clinical entity resembling AIH-2 (19). Later, anti-LC-1 was also found occasionally in association with the serological markers of AIH-1 and in patients with chronic hepatitis C virus (HCV) infection (20). Anti-LC-1 can be detected by indirect immunofluorescence using the standard tissue panel composed of rodent liver, kidney, and stomach. It stains the cytoplasm of liver cells, with relative sparing of the centrilobular area, but it is usually obscured by the concurrent presence of anti-LKM-1. In the presence of anti-LKM-1, anti-LC-1 can be detected by the use of liver cytosol in double-dimension immunodiffusion, or counterimmunoelectrophoresis, and a positive reference serum. In Western blot, anti-LC-1 reacts with a 58 to 60 kD protein when human liver cytosolic fraction is used as substrate. The molecular target has been identified as formimino-transferase cyclodeaminase (FTCD) (21). The clinical relevance of anti-LC-1 is currently being assessed by the use of molecularly based immunoassays. The presence of anti-LC-1 in isolation scores positively toward a diagnosis of AIH-2, allowing prompt initiation of treatment.

#### **ANTI-SOLUBLE LIVER ANTIGEN/LIVER-PANCREAS ANTIGEN**

Anti-SLA and anti-LP, earlier described separately in AIH, target the same antigen and are therefore the same autoantibody (22). Anti-SLA was thought to identify a third type of AIH in which tests for conventional autoantibodies were negative (23). However, early reports predated the publication

of the IAIHG recommendations and used a cutoff point for conventional autoantibody levels higher than that currently used for the diagnosis of AIH. Several patients considered to have AIH-3 were probably positive for conventional autoantibodies and therefore had type 1 or 2 AIH. Screening of cDNA expression libraries using high titer anti-SLA serum has allowed to identify the molecular target antigen as UGA tRNA suppressor-associated antigenic protein (tRNP[Ser]Sec) (22,24). Molecularly based diagnostic assays have become available, but their full evaluation is still under way. Although anti-SLA/LP is found occasionally in patients with AIH who are negative for ANA, SMA, and anti-LKM-1, it is also frequently present in typical cases of AIH-1 and AIH-2 and also in AIH/sclerosing cholangitis overlap syndrome (25) (*see* Chapter 21, Unique Aspects of Autoimmune Hepatitis in Children). Anti-SLA appears to be highly specific for the diagnosis of AIH. Its detection at the time of diagnosis identifies patients with more severe disease and worse outcome (25).

#### **ANTIBODIES TO LIVER-SPECIFIC LIPOPROTEIN COMPLEX AND ITS COMPONENTS**

In the mid-seventies it was reported that antibodies to the liver-specific lipoprotein (LSP)—a macromolecular complex present on the hepatocyte membrane—are present in AIH and also that their titer correlates with the biochemical and histological severity of the disease (for a review, *see* ref.26). A similar relationship to disease severity was later observed for the titers of an antibody to a well-characterized component of LSP, the asialoglycoprotein receptor (ASGPR). More recently antibodies to alcohol dehydrogenase (ADH), a second well-defined component of LSP, have been described in patients with AIH (27). The measurement of these autoantibodies, however, is confined to research laboratories, in view of the difficulties involved in setting up and standardizing their detection assays.

#### **ANTINEUTROPHIL CYTOPLASMIC ANTIBODY**

Antineutrophil cytoplasmic (ANCA) autoantibodies are directed at cytoplasmic components of neutrophils and give either a perinuclear (p-ANCA) or a cytoplasmic (c-ANCA) pattern. c-ANCA mainly reacts with proteinase 3 and is found in Wegener's granulomatosis; p-ANCA reacts with myeloperoxidase and is frequently detected in microscopic polyangiitis. In AIH-1, akin to primary sclerosing cholangitis and inflammatory bowel disease, p-ANCA are frequently detected, but they are atypical, since they react with peripheral nuclear membrane components (perinuclear antinuclear neutrophil antibodies, [p-ANNAs]) (11). In contrast to AIH-1, p-ANNAs are virtually absent in AIH-2. Detection of p-ANNA can act as an additional pointer toward the diagnosis of AIH, particularly in the absence of other autoantibodies (6).

#### **DIAGNOSIS AND CLINICAL FEATURES**

The diagnosis of AIH is based on the presence of autoantibodies, elevated transaminase and IgG levels, and interface hepatitis on liver biopsy. The latter is needed to confirm the diagnosis and to evaluate the severity of liver damage. The levels

of transaminases and IgG do not reflect the extent of the histological inflammatory activity, nor do they indicate the presence or absence of cirrhosis. Other hepatic disorders that may share some of the above features need to be considered in the differential diagnosis. These include viral hepatitis (in particular B and C), Wilson disease, and drug-induced liver disease (minocycline, nitrofurantoin, isoniazid, propylthiouracil, diclofenac, pemoline, atorvastatin, and  $\alpha$ -methyl dopa). Females are three times more likely to be affected than males. A family history of autoimmune diseases is present in some 40% of the patients.

Associated autoimmune disorders are present at diagnosis or develop during follow-up in at least one-fifth of the patients and include thyroiditis, ulcerative colitis, insulin-dependent diabetes, vitiligo, nephrotic syndrome, hypoparathyroidism, and Addison's disease, the latter two being observed in particular in young patients with AIH-2 or in children with APECED. Typically AIH responds to immunosuppressive treatment, which should be instituted as soon as diagnosis is made. The onset of AIH is often ill defined, and it frequently mimics acute hepatitis, particularly in young patients. The distinction in type 1 and type 2 AIH is particularly relevant in pediatrics (*see* Chapter 21), since anti-LKM-1-positive disease is quite rare, although not absent, in adults. Although most patients with AIH are symptomatic, some are asymptomatic and are diagnosed after incidental discovery of abnormal liver function tests.

The criteria for the diagnosis of AIH have been defined and revised by the IAIHG. This diagnostic system, which includes positive and negative scores, was devised mainly for comparative and research purposes (5,6) (Table 2), since in most instances clinical, laboratory, and histological features allow the diagnosis of AIH to be made without a need for the scoring system. In the IAIHG scoring system, differences between a definite and probable diagnosis of AIH relate mainly to the degree of serum  $\gamma$ -globulin or IgG elevation, levels of ANA, SMA, or anti-LKM-1, and exposures to alcohol, medications, or infections that can cause liver injury. Cholestatic laboratory and histological changes carry a negative score. In rare cases, the presence of nonstandard autoantibodies, such as anti-ASGPR, anti-LC-1, anti-SLA, and p-ANNA, supports a probable diagnosis in the absence of conventional autoantibodies. Response to steroids weighs strongly toward the diagnosis of AIH and has been incorporated into the scoring system, because this condition typically enters remission during corticosteroid therapy and frequently relapses after drug withdrawal. A definite diagnosis before steroid treatment requires a score greater than 15, whereas a definite diagnosis after steroid treatment requires a score greater than 17 (Table 2).

The diagnostic criteria for children are slightly different from those of adults. In view of the fact that autoantibodies are very rarely positive in healthy children, the presence of autoantibody titers as low as 1:20 for ANA and SMA and 1:10 for anti-LKM-1 is compatible with the diagnoses of type 1 and type 2 AIH, respectively (*see* Chapter 21). Also, in adults, autoantibodies are sometimes present at low titer or even absent, the titer rising or becoming detectable during follow-up. Seronegative individuals, therefore, classified at presentation

**Table 2**  
**IAIHG Scoring System for the Diagnosis of Autoimmune Hepatitis**

Parameter	Feature	Score
Principal parameters		
Sex	Female	+2
ALP-AST (or ALT) ratio	>3	-2
	1.5-3	0
	<1.5	+2
Serum globulins or IgG (times above normal)	>2.0	+3
	1.5-2.0	+2
	1.0-1.5	+1
ANA, SMA, or anti-LKM-1 titers	<1.0	0
	>1:80	+3
	1:80	+2
	1:40	+1
AMA	<1:40	0
	Positive	-4
Viral markers of active infection	Positive	-3
	Negative	+3
Hepatotoxic drug history	Yes	-4
	No	+1
Average alcohol	<25 g/d	+2
	>60 g/d	-2
Histological features	Interface hepatitis	+3
	Plasma cells	+1
	Rosettes	+1
	None of above	-5
	Biliary changes <sup>a</sup>	-3
	Atypical changes <sup>b</sup>	-3
Optional additional parameters		
Seropositivity for other defined autoantibodies	Anti-SLA/LP, actin, LC-1, ASGPR, p-ANCA	+2
	HLA	DR3 or DR4
Response to therapy	Remission	+2
	Relapse	+3
Interpretation of aggregate scores		
<i>Pretreatment</i>		
	Definite AIH	>15
	Probable AIH	10-15
<i>Posttreatment</i>		
	Definite AIH	>17
	Probable AIH	12-17

Abbreviations: IAIHG, International Autoimmune Hepatitis Group; ALP, alkaline phosphatase; AST, aspartate aminotransferase; ALT, alanine aminotransferase; IgG, immunoglobulin G; ANA, antinuclear antibody; SMA, smooth muscle antibody; LKM-1, liver-kidney microsomal antibody type 1; AMA, antimitochondrial antibody; SLA/LP, soluble liver antigen/liver-pancreas; LC-1, liver cytosol type 1; ASGPR, asialoglycoprotein receptor; p-ANCA, perinuclear antineutrophil cytoplasmic antibody; HLA, human leukocyte antigen; AIH, autoimmune hepatitis.

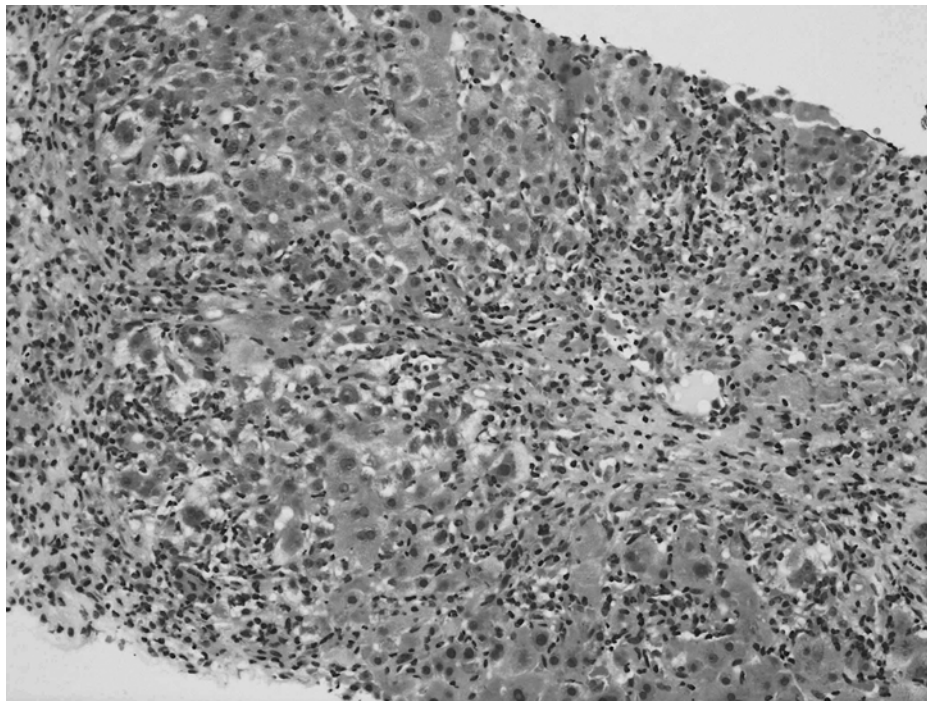
<sup>a</sup>Including granulomatous cholangitis, concentric periductal fibrosis, ductopenia, and marginal bile duct proliferation with cholangiolitis.

<sup>b</sup>Any other prominent feature suggesting a different etiology.

Modified from ref. 6.

as having cryptogenic chronic hepatitis, may later be firmly diagnosed when conventional markers appear or when autoantibodies that are not generally available are tested.





**Fig. 2.** Portal and periportal lymphocyte and plasma cell infiltrate, extending to and disrupting the parenchymal limiting plate (interface hepatitis). Swollen hepatocytes, pyknotic necroses, and acinar inflammation are present. Hematoxylin & eosin staining. (Picture kindly provided by Dr. Alberto Quaglia.)

## HISTOLOGY

Interface hepatitis (hepatitis at the portal-parenchymal interface) is characteristic, but not exclusive, to AIH (28). Other lesions typically present in AIH are periportal lymphocytic or lymphoplasmacytic infiltration, hepatocyte swelling, and/or pyknotic necrosis (Fig. 2A). Lymphocytes, plasma cells, and histiocytes surround individual dying hepatocytes at the portal/parenchymal interface and in the lobule. Although plasma cells are usually abundant at the interface and throughout the lobule, their presence in low number does not preclude the diagnosis. In AIH presenting acutely or relapsing, panlobular hepatitis is often present, associated with bridging necrosis and, in the case of a fulminant presentation, to massive necrosis (Fig. 2B). Although sampling variation may occur in needle biopsy specimens, especially in the presence of cirrhosis, the severity of the histological appearance is usually of prognostic value. However, even patients with cirrhosis at presentation respond well to immunosuppressive treatment. Inflammatory changes surrounding the bile ducts, which may be present in a small proportion of patients with AIH, suggest an overlap with sclerosing cholangitis, as reported more frequently in the pediatric setting (29) (*see* Chapter 21).

## ANIMAL MODELS

Research on the pathogenesis of AIH has been hampered by the lack of animal models reproducing the human condi-

tion faithfully (30,31). In early studies aimed at characterizing the nature of the liver antigens responsible for the formation of hepatic mononuclear cell infiltrates in experimental hepatitis, liver cell necrosis and periportal infiltration, reminiscent of the histological changes seen in human chronic hepatitis, were obtained by multiple immunization of rabbits over a period of several months with allogeneic liver extracts in complete Freund's adjuvant. Further studies identified two hepatocyte antigens, one located in the plasma membrane and the other in the cytosolic fraction, that are targets of autoantibody-containing sera from rabbits with experimental hepatitis, induced by repeated immunizations with human liver antigen over several months. The membrane-associated antigen, which was found to be a lipoprotein, was called LSP and later was also identified in human liver. However, the liver autoantibodies present in serum and on hepatocytes did not correlate with histological liver damage, which raised questions as to their pathogenic relevance and indirectly implied a role for cell-mediated immune damage. Subsequent *in vitro* studies in the rabbit model did find lymphocyte-proliferative responses against liver antigen.

*In vivo* evidence of cell-mediated liver damage was provided in a murine model whereby experimental hepatitis could be induced through immunization with syngeneic liver antigen and adoptively transferred to naïve mice with nylon wool adherent lymphocytes (mainly T cells) (32). Interestingly, this study showed that the susceptibility to liver damage was

strain dependent, implying a genetic influence. Splenocytes from the animals with experimental hepatitis were also able to suppress liver-specific and non liver-specific immune responses (33). A balance between effector and regulatory cells may explain the chronic relapsing course of AIH in humans. A widely studied model of experimental hepatitis is that induced by concanavalin A (34). Although this model does not reflect the pathological entity of AIH in humans accurately, it has provided evidence that liver damage mainly occurs within a T-helper 1 (Th1) scenario, with the involvement of activated CD4+ T cells and release of the proinflammatory cytokines interferon- $\gamma$  (IFN- $\gamma$ ) and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) against a specific genetic background.

All the models just described, although informative regarding single steps leading to liver inflammation and damage, do not mimic the chronic relapsing course of human AIH. In fact, they demonstrate the difficulty in breaking tolerance toward liver antigens and the involvement of regulatory mechanisms in maintaining it. More recently, researchers have focused on animal models of AIH type 2, since in this condition the autoantigens are well defined.

The model produced by Alvarez's group (35) is based on C57BL/6 female mice immunized every 2 wk for three times with a plasmid containing the antigenic region of human CYP2D6, the target of anti-LKM-1, and FTCD, the target of anti-LC-1, together with the murine end-terminal region of cytotoxic T-lymphocyte antigen 4 (CTLA-4). The latter was added to facilitate antigen uptake by antigen-presenting cells (APCs). In a parallel set of experiments, a plasmid containing the DNA encoding interleukin-12 (IL-12) a Th1-skewing proinflammatory cytokine, was also used. When autoantigens and IL-12 were used to break tolerance, antigen-specific autoantibodies were produced, a relatively modest elevation of transaminase levels at 4 and 7 mo was observed, and a portal and periportal inflammatory infiltrate composed of CD4 and CD8 T cells and, to a lesser extent, B cells was demonstrated 8 to 10 mo after the third immunization. When the same immunization protocol was used in different mouse strains, either a mild hepatitis or no inflammatory changes were observed, indicating the importance of a specific genetic background (36).

Another model of AIH type 2 uses CYP2D6 transgenic mice and aims at breaking tolerance with an adenovirus-CYP2D6 vector 51 (Mrs. Christen, personal communication). Although focal hepatocyte necrosis was seen in both mice treated with the adenovirus-CYP2D6 vector and control mice treated with adenovirus alone, only the former developed chronic histological changes, including fibrosis, reminiscent of AIH. The hepatic lesion was associated with a specific immune response to an immunodominant region of CYP2D6 and a cytotoxic T-cell response to adenovirus-CYP2D6 vector-infected target cells. Although these two experimental approaches provide useful information on the possible pathogenic mechanisms leading to AIH-2, a model closely mimicking AIH in humans is still missing.

## PATHOGENESIS

### GENETICS

AIH is a "complex trait" disease, i.e., a condition not inherited in a Mendelian autosomal dominant, autosomal recessive, or sex-linked fashion. The mode of inheritance of a complex trait disorder is unknown and involves one or more genes, operating alone or in concert, to increase or reduce the risk of the trait, and interacting with environmental factors.

Susceptibility to AIH is imparted by genes within the histocompatibility leukocyte antigen (HLA) region on the short arm of chromosome 6, especially those encoding DRB1 alleles. These class II major histocompatibility complex (MHC) molecules are involved in peptide antigen presentation to CD4 T cells, suggesting the involvement of MHC class II antigen presentation and T-cell activation in the pathogenesis of AIH.

In Europe and North America, susceptibility to AIH-1 is conferred by the possession of HLA DR3 (*DRB1\*0301*) and DR4 (*DRB1\*0401*), both heterodimers containing a lysine residue at position 71 of the DRB1 polypeptide and the hexameric amino acid sequence LLEQKR at positions 67 to 72 (37,38). In Japan, Argentina, and Mexico, susceptibility is linked to *DRB1\*0405* and *DRB1\*0404*, alleles encoding arginine rather than lysine at position 71, but sharing the motif LLEQ-R with *DRB1\*0401* and *DRB1\*0301* (39). Thus, K or R at position 71 in the context of LLEQ-R may be critical for susceptibility to AIH, favoring the binding of autoantigenic peptides, complementary to this hexameric sequence. However, an alternative model based on valine/glycine dimorphism at position 86 of the DR- $\beta$  polypeptide has been proposed, better representing the key HLA associations in patients from Argentina and Brazil (37,38). In a study from Japan, patients with AIH-1 were found to have DRB1 alleles that encode histidine at position 13 (37,38). There appears, therefore, to be at least three different models, suggesting that different genetic associations are present in different populations and that the peptides presented by HLA class II molecules to the T-cell receptors are different and may derive from different antigens. Thus, these HLA associations may be the molecular footprints of the prevailing environmental triggers that precipitate AIH-1 in different environments. In this context, it is of interest that in South America possession of the HLA *DRB1\*1301* allele, which predisposes to pediatric AIH-1 in that population, is also associated with persistent infection with the endemic hepatitis A virus (40).

The lysine-71 and other models for AIH-1 cannot explain the disease completely, since, for example, in European and North American patients the presence of lysine-71 is associated with a severe, mainly juvenile, disease in those *DRB1\*0301* positive, but with a mild, late-onset disease in those *DRB1\*0401* positive (37,38). Other genes within and/or without the MHC are, therefore, likely to be involved in determining the phenotype. Possible candidates are the MHC-encoded complement and TNF- $\alpha$  genes, mapping to the class III MCH region, and the MHC class I chain-related A and B genes.

Susceptibility to AIH-2 is conferred by the possession of HLA DR7 (*DRB1\*0307*) and DR3 (*DRB1\*0301*), patients

positive for *DRB1\*0307* having a more aggressive disease and worse prognosis (41).

A form of AIH resembling AIH-2 affects some 20% of patients with APECED, a condition also known as autoimmune polyendocrine syndrome 1. APECED is a monogenic autosomal recessive disorder caused by homozygous mutations in the *AIRE1* gene and characterized by a variety of organ-specific autoimmune diseases, the most common of which are hypoparathyroidism and primary adrenocortical failure, accompanied by chronic mucocutaneous candidiasis (42,43). The *AIRE1* gene sequence consists of 14 exons containing 45 different mutations, with a 13-bp deletion at nucleotide 964 in exon 8 accounting for more than 70% of APECED alleles in the United Kingdom (43). The protein predicted to be encoded by *AIRE1* is a transcription factor. *AIRE1* is highly expressed in medullary epithelial cells and other stromal cells in the thymus involved in clonal deletion of self-reactive T cells. Studies in a murine model indicate that the gene inhibits organ-specific autoimmunity by inducing thymic expression of peripheral antigens in the medulla, leading to central deletion of autoreactive T cells.

Interestingly, APECED has a high level of variability in symptoms, especially between populations. Since various gene mutations have the same effect on thymic transcription of ectopic genes in animal models, it is likely that the clinical variability across human populations relates to environmental or genetic modifiers. Of the various genetic modifiers, perhaps the most likely to synergize with *AIRE* mutations are polymorphisms in the HLA region. HLA molecules are not only highly variable and strongly associated with multiple autoimmune diseases but are also able to affect thymic repertoire selection of autoreactive T-cell clones. Carriers of a single *AIRE* mutation do not develop APECED. However, although the inheritance pattern of APECED indicates a strictly recessive disorder, there are anecdotal reports of mutations in a single copy of *AIRE* being associated with human autoimmunity of a less severe form than classically defined APECED (42,43). The role of *AIRE1* heterozygote state in the development of AIH remains to be established.

### PATHOGENETIC MECHANISMS

Various mechanisms have been proposed to account for the onset of an autoimmune liver response, with no single initiating event being able to explain all instances of autoimmunity. Two general conditions, however, should prevail: self-reactive B and T lymphocytes must exist in the immunological repertoire and autoantigens must be presented in conjunction with MHC class II molecules by APCs.

**Humoral Autoimmunity** Titers of antibodies to LSP, a macromolecular complex present on the hepatocyte membrane, and to its well-characterized components ASGPR and ADH, correlate with the biochemical and histological severity of AIH (44). Immunofluorescence studies on monodispersed suspensions of liver cells obtained from patients with AIH show that these cells are coated with antibodies *in vivo*. A pathogenic role for these autoantibodies has been indicated by cytotoxicity assays demonstrating that autoantibody-coated hepatocytes from patients with AIH are killed when they are incubated with

autologous or allogeneic lymphocytes. The effector cell was identified as an Fc receptor-positive mononuclear cell (44).

In AIH-2, the target of the disease-defining antibody, anti-LKM-1, is CYP2D6, a member of the hepatic P450 cytochrome family. Since CYP2D6 is expressed on the membrane of the hepatocytes and is readily accessible (45), anti-LKM-1 antibodies are likely to play a pathogenic role. In AIH-2, anti-LKM-1 antibodies recognize linear regions of CYP2D6 in a hierarchical manner. The principal linear B-cell epitope, CYP2D6<sub>193-212</sub> is recognized by 93% of patients, CYP2D6<sub>257-269</sub> by 85%, CYP2D6<sub>321-351</sub> by 53%, and two additional minor epitopes CYP2D6<sub>373-389</sub> and CYP2D6<sub>410-429</sub> are recognized by 7 and 13%, respectively (46). Intriguingly, anti-LKM-1 antibodies are also found in up to 10% of patients with HCV infection, in whom they appear to correlate with increased disease severity and adverse reactions to IFN- $\alpha$  treatment. The major CYP2D6 epitope recognized by patients with AIH-2, CYP2D6<sub>193-212</sub>, is also recognized by 50% of patients with anti-LKM-1-positive HCV infection. Interestingly, these patients have antibodies that crossreact with homologous regions of HCV (NS5B HCV<sub>2985-2990</sub>) and CYP2D6 (CYP2D6<sub>204-209</sub>), and also of cytomegalovirus (exon CMV<sub>130-135</sub>) (46).

Cross-reactive mechanisms to explain the emergence of CYP2D6-specific autoimmunity have also been suggested for other sequences of CYP2D6 that share homologies with HCV and herpes simplex virus (HSV) (47), such as the sequence spanning amino acids 310 to 324 of E1 HCV and amino acids 156 to 170 of IE175 HSV1, which share homology with the CYP2D6 region comprising amino acids 254 to 271. As anti-LKM-1 antibodies crossreact with homologous regions of CYP2D6, HCV, HSV, and CMV, a "multihit" mechanism for the generation of these antibodies and possibly of AIH-2 may be envisaged. In this model, multiple exposures to CMV or HSV, common viral pathogens, may establish permissive immunological conditions, by priming a crossreactive subset of T cells, in a genetically predisposed host. Depending on the degree of immunological priming, the degree of genetic susceptibility (particularly at the HLA locus and coding regions for "innate" components of immunity), and the antigenic dose of the infecting pathogens, a minority of individuals may progress to autoimmune disease. It is therefore conceivable that an as yet unknown virus infection may be at the origin of the autoimmune attack in AIH, in agreement with the concept expressed by Rolf Zinkernagel that an autoimmune disease is a viral disease in which the virus is unknown (48).

**Molecular Mimicry** The central function of the adaptive immune system is to generate T and B lymphocytes that can specifically recognize a potentially infinite number of non-self-antigens without any prior information as to their structure. This is achieved by randomly generating a large number of T- and B-cell specificities (via their respective antigen receptors, the T-cell receptor and the antibody) that are then able to expand clonally and recruit effector mechanisms on recognition of their specific antigen. It is, however, becoming clear that even this system cannot cope with the extent of non-self-antigenic diversity, and in the past decade convincing

evidence for cross reactivity as an inherent property of immune ontogeny has emerged (44). This has been studied primarily in the context of T lymphocytes, in which it is clear that altered peptide ligands (APLs)—peptides similar in structure to the peptide antigen initially encountered—are able to induce both stimulatory and inhibitory T-cell responses, and, indeed, endogenous APLs operate in selecting the T-cell repertoire in the thymus. This implies that a single T cell, rather than responding to a single antigen specificity, is able to respond crossreactively to a number of antigens, thus expanding the antigenic specificities of the immune system to a level that reflects the antigenic diversity of the external environment.

This inherent potential for crossreactivity, while allowing efficient responses to a vast array of pathogens, also provides the immune system with the potential to crossreact with self, leading to autoimmunity. This concept has been termed “molecular mimicry”: immune responses to external pathogens become directed toward structurally similar self-components. Molecular mimicry has been demonstrated to be a dominant mechanism in the pathogenesis of autoimmune disease, both in experimental models and in the human setting at the level of both T and B cells (44).

**Cellular Autoimmunity** The histological picture of interface hepatitis, with its striking infiltrate of lymphocytes, plasma cells, and macrophages, was the first to suggest an autoaggressive cellular immune attack in the pathogenesis of AIH. Whatever is the initial trigger, this massive recruitment of activated inflammatory cells is likely to cause damage. Immunohistochemical studies have identified a predominance of T lymphocytes mounting the  $\alpha/\beta$  T-cell receptor (49). Among the T cells, most are positive for the CD4 helper/inducer phenotype, and a sizeable minority for the CD8 cytotoxic phenotype. Lymphocytes of non-T-cell lineage are fewer and include (in decreasing order of frequency) natural killer (NK) cells (CD16/CD56 positive), macrophages, and B cells. The involvement of NK T cells is the focus of ongoing studies.

There are different possible pathways that an immune attack can follow to inflict damage on hepatocytes (Fig. 3). These are discussed below.

**Impairment of T-Regulatory Cells** An impairment of immunoregulatory mechanisms, which would enable the autoimmune response to develop, has been repeatedly reported. Thus, in early studies it was shown that patients with AIH have low levels of circulating T cells expressing the CD8 marker and impaired suppressor cell function, which segregates with the possession of the disease-predisposing HLA haplotype B8/DR3 and is correctable by therapeutic doses of corticosteroids (50,51). Furthermore, patients with AIH have been reported to have a defect in a subpopulation of T cells controlling the immune response to liver-specific membrane antigens (52). Recent experimental evidence confirms an impairment of the immunoregulatory function in AIH.

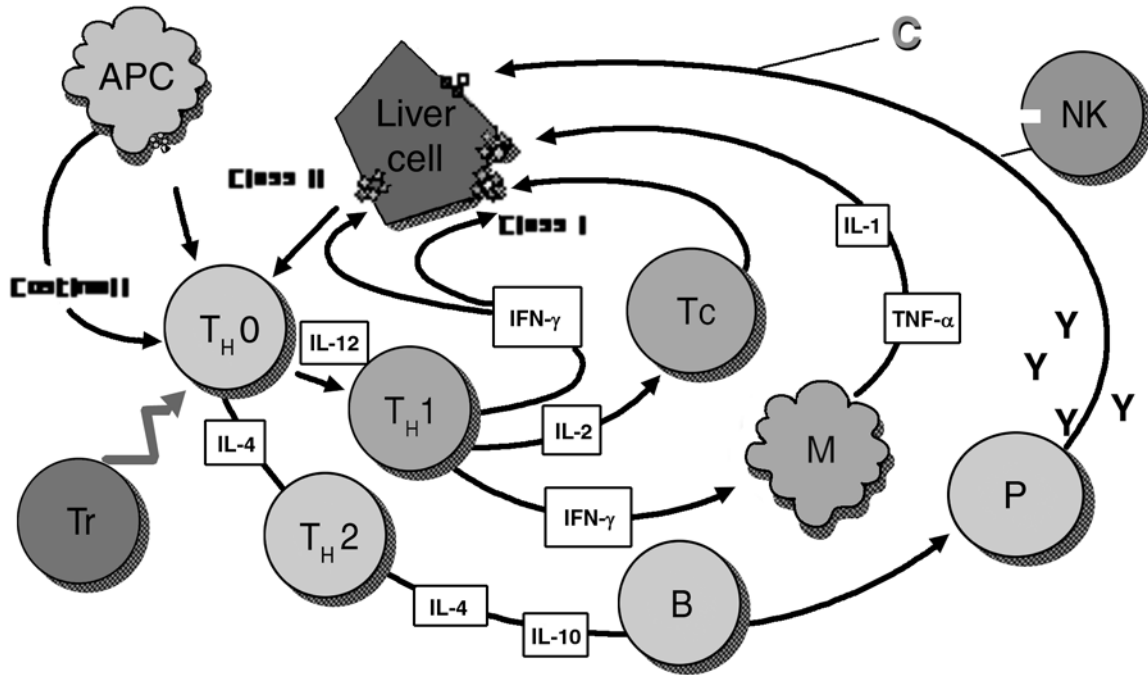
Among recently defined T-cell subsets with potential immunosuppressive function, CD4<sup>+</sup> T cells constitutively expressing the IL-2 receptor  $\alpha$ -chain (CD25 T-regulatory cells [T-regs]) have emerged as the dominant immunoregulatory lymphocytes.

These cells, which represent 5 to 10% of the total population of peripheral CD4<sup>+</sup> T cells in health, control the innate and the adaptive immune responses by preventing the proliferation and effector function of autoreactive T cells. Their mechanism of action mainly involves a direct contact with the target cells and to a lesser extent the release of immunoregulatory cytokines, such as IL-10 and transforming growth factor  $\beta$ 1. In addition to CD25, which is also present on T cells undergoing activation, T-regs express a number of other markers such as the glucocorticoid-induced TNF receptor, CD62L, CTLA-4, and the forkhead/winged helix transcription factor FOXP3, whose expression has been associated with the acquisition of regulatory properties. In patients with AIH, T-regs are defective in number and function compared with normal controls; and this impairment relates to the stage of disease, being more evident at diagnosis than during drug-induced remission (53–55).

The percentage of T-regs inversely correlates with markers of disease severity, such as anti-SLA and anti-LKM-1 autoantibody titers, suggesting that a reduction in T-regs favors the serological manifestations of autoimmune liver disease. If loss of immunoregulation is central to the pathogenesis of autoimmune liver disease, treatment should concentrate on restoring the ability of T-regs to expand, with consequent increase in their number and function. This is at least partially achieved by standard immunosuppression, since T-reg numbers increase during remission.

**CD4 Autoreactive T Cells** To trigger an autoimmune response, a peptide must be embraced by an HLA class II molecule and presented to uncommitted T-helper (Th0) cells by professional APCs, with the costimulation of ligand-ligand (CD28 on Th0, CD80 on APC) interaction between the cells (Fig. 3). Once the autoimmune response has been initiated and in the absence of effective immunosuppressive treatment, tissue damage ensues and persists. Hepatocytes from patients with AIH, in contrast to normal hepatocytes, express HLA class II molecules (26). Although lacking the antigen-processing machinery typical of APCs, these hepatocytes may present peptides through a bystander mechanism. In the presence of impaired immunoregulation and inappropriate expression of HLA class II antigens on the hepatocytes, an autoantigenic peptide could be presented to the helper/inducer cells leading to their activation. Although no direct evidence exists as yet that an autoantigenic peptide is presented by hepatocytes and recognized by CD4 T-helper cells, activation of these cells has been documented in AIH (26). Liver autoantigen-specific T-cell precursors are also found in normal subjects, but in AIH their frequency is at least 10-fold higher (49). This finding suggests that the pool of liver-autoreactive T cells undergoes a significant expansion in patients with AIH and may be involved in the initiation and perpetuation of the immune attack to the liver.

Given that T cells recognize antigens in a precise fashion, studies in the early 1990s were conducted at a single T-cell level in order to characterize antigen-specific T-cell recognition. T-cell clones generated from the peripheral blood were mainly CD4<sup>+</sup>  $\alpha/\beta$  T cells, whereas a large proportion of liver-derived clones were either CD4<sup>-</sup>/CD8<sup>-</sup>  $\gamma/\delta$  or CD8<sup>+</sup>  $\alpha/\beta$  T cells (49). Both  $\alpha/\beta$  and  $\gamma/\delta$  T-cell clones proliferated in the presence



**Fig. 3.** Autoimmune attack to the liver cell. A specific autoantigenic peptide is presented to an uncommitted T-helper ( $T_H0$ ) lymphocyte within the HLA class II molecule of an antigen-presenting cell (APC).  $T_H0$  cells become activated, and, according to the presence in the microenvironment of interleukin (IL)-12 or IL-4 and the nature of the antigen, differentiate into  $T_H1$  or  $T_H2$  and initiate a series of immune reactions determined by the cytokines they produce:  $T_H2$  secrete mainly IL-4 and IL-10 and direct autoantibody production by B lymphocytes;  $T_H1$  secrete IL-2 and interferon- $\gamma$  (IFN- $\gamma$ ), which stimulate T cytotoxic (Tc) lymphocytes, enhance expression of class I, and induce expression of class II HLA molecules on hepatocytes and activate macrophages; activated macrophages release IL-1 and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ). If regulatory T cells (Tr) do not oppose, a variety of effector mechanisms are triggered: liver cell destruction could derive from the action of Tc lymphocytes; cytokines are released by  $T_H1$  and recruited macrophages; complement activation occurs or engagement of Fc receptor-bearing cells such as natural killer (NK) lymphocytes by the autoantibody bound to the hepatocyte surface.

of liver membrane antigens,  $\alpha/\beta$  being more reactive than  $\gamma/\delta$  clones. Some of the liver membrane reactive clones also proliferated in the presence of LSP and/or ASGPR, responded in an HLA class II-restricted fashion and helped autologous B cells to produce immunoglobulins, in particularly autoantibodies to LSP and ASGPR (49).

T-cell ligands are best studied in AIH-2, since the target of anti-LKM-1 has been characterized as CYP2D6. CYP2D6<sub>262-285</sub> specific T-cell clones generated from liver tissue and peripheral blood express a Th1 CD4<sup>+</sup> phenotype (56,57). In contrast to the latter study, which focused on a short antigenic sequence of CYP2D6, a systematic approach based on the construction of overlapping peptides, covering the whole CYP2D6 molecule, was recently adopted to define the specificity of ex vivo CYP2D6-reactive T cells in patients with AIH-2 (41). This study has shown that T cells from patients positive for the predisposing HLA allele *DRB1\*0701* recognize in a proliferation assay seven regions of CYP2D6, four of which are also partially recognized by T cells of *DRB1\*0701* negative patients. Whereas distinct peptides induce production of IFN- $\gamma$ , IL-4, or IL-10, peptides inducing IFN- $\gamma$  and proliferation overlap. There is also an overlap between sequences inducing T- and B-cell responses. The number of epitopes rec-

ognized and the quantity of cytokine produced by T cells are directly correlated to biochemical and histological markers of disease activity. These results indicate that the T-cell response to CYP2D6 in AIH-2 is polyclonal, involves multiple effector types targeting different epitopes, and is associated with hepatocyte damage (41).

**CD8 Autoreactive T cells** In addition to the unfolding role of CYP2D6-specific CD4 T cells in AIH-2, there is growing evidence implicating an HLA class I-restricted CD8 response in the pathogenesis of autoimmune liver damage. In the early 1990s, CD8 T-cell clones specific for ASGPR were described in patients with AIH (49). Studies currently in progress have identified CYP2D6-specific CD8 T cells capable of secreting IFN- $\gamma$  and of exerting cytotoxicity after recognition of CYP2D6 epitopic sequences in an HLA class I-restricted fashion.

Taken together, the data just presented suggest that a failure of immune homeostatic processes, normally keeping the response against self-antigens under control, is involved in the pathogenesis of AIH. The prime mechanism for tolerance breakdown remains to be elucidated. There is some experimental evidence that molecular mimicry mechanisms between viral and self-mimicking sequences may be involved, and such mechanisms are the focus of ongoing studies.

## TREATMENT AND OUTCOME

Immunosuppressive treatment is beneficial in patients with severe symptomatic disease, and it should be started as soon as possible, without waiting for 6 mo as suggested in early studies. Most patients, including those with cirrhosis (58), will achieve remission on 30 mg prednisolone daily for 1 mo, after which azathioprine can be introduced at 1 mg/kg/d and the dose of prednisolone reduced to 5 to 15 mg/day to maintain the aminotransferase activity within the normal range. Although some authors define remission as transaminase levels up to twice the upper limit of normal, a better outcome has been reported when normal transaminase levels are attained and maintained (59). If the patient develops steroid side effects, the dose of azathioprine can be increased to 2 mg/kg/d and a complete withdrawal of steroids considered.

In the 1980s and 1990s, a number of studies addressed two important questions: whether immunosuppression could be safely withdrawn after obtaining remission and whether steroid-free maintenance could be achieved (58,60). It was shown that the great majority of cases relapse rapidly upon immunosuppression withdrawal, but that steroid-free maintenance could be achieved with azathioprine alone provided that its dose is increased to 2 mg/kg/d. The optimal duration of treatment is unknown. It is prudent not to attempt withdrawal of immunosuppression within 2 yr of diagnosis. During withdrawal attempts, it is essential to monitor liver function tests closely since relapse may be severe and even fatal. Patients who have successfully stopped immunosuppression should be followed up long term, since relapse may occur even 10 yr later.

A question frequently asked is whether treatment can be safely continued during pregnancy. Although experience is limited, there do not appear to be adverse events for mother and baby (61). In particular, no teratogenic effects have been described with azathioprine in humans, although for women concerned about its use, treatment with steroids alone can be considered.

It is now clear that there are patients with a milder form of the disease who may be asymptomatic or pauci-symptomatic and who are detected incidentally, during routine checkups. For these patients, the approach to treatment is less clear. The benefit of therapy is undefined, and it may be so low that the risk of corticosteroid side effects is unjustified. This is particularly relevant to postmenopausal women and elderly patients.

The most common side effect of steroid treatment is cushingoid changes, which affect most patients after prolonged treatment. Less common but severe side effects include osteoporosis, vertebral collapse, diabetes, cataract, hypertension, and psychosis. Only 13% of treated patients develop complications that necessitate dose reduction or premature drug withdrawal, the most common reasons for treatment withdrawal being cosmetic changes or obesity, osteopenia with vertebral collapse, and brittle diabetes (58).

Side effects of azathioprine are uncommon, affecting less than 10% of patients and include cholestatic hepatitis, veno-

occlusive disease, pancreatitis, nausea and vomiting, rash, and bone marrow suppression. Usually these complications subside upon drug withdrawal (58). A theoretical long-term complication of continuous immunosuppressive therapy is the development of malignancies. The risk of extrahepatic cancer in AIH has been reported to be 1.4-fold higher than that of an age-and-sex matched normal population (62). Akin to other chronic liver diseases, the risk of primary hepatocellular cancer is related mainly to the presence of cirrhosis and is generally reported to be uncommon.

Liver transplantation is the ultimate treatment for most patients who present with fulminant hepatic failure and those who reach end-stage chronic liver disease. Transplantation in AIH has an excellent prognosis, with a 5-yr patient and graft survival between 80 and 90%. Before transplantation is considered, however, it is important to remember that even patients presenting with decompensated cirrhosis can respond to immunosuppressive treatment and avoid surgery for a long time (58). AIH may recur after transplant.

### RECURRENCE OF AIH AFTER TRANSPLANT

Recurrence of AIH after liver transplant has been shown in several studies (63,64). The diagnosis is based on reappearance of clinical symptoms and signs, histological features of periportal hepatitis, elevation of transaminases and circulating autoantibodies, and elevated IgG, associated with response to steroids and azathioprine. Possession of the HLA DR3 allele appears to confer predisposition to disease recurrence, as it does to the original AIH, although this has not been universally confirmed. Recurrence has been noted in both adult and pediatric series, and although the rate of this complication increases with the posttransplant interval, it may appear as early as 1 mo post surgery. Most transplant recipients with recurrent AIH respond to an increase in the dose of corticosteroids and azathioprine, but, in a few, recurrence can lead to graft failure and to the need for retransplantation. Care should be taken in weaning immunosuppression in patients who undergo transplantation for AIH since discontinuation of corticosteroid therapy may increase the risk for recurrent disease.

### DE NOVO AIH AFTER TRANSPLANT

Tissue autoantibodies after liver transplantation, in particular ANA and SMA, are also common in patients transplanted for nonautoimmune liver disease (63). Anti-LKM-1 is the third most frequently reported antibody, but its fluorescence pattern is at times atypical, staining preferentially the renal tubules and sparing the liver. The described prevalence of post liver transplant autoantibodies is variable, probably reflecting different techniques used for their detection, the cutoff point above which the autoantibodies are considered positive, the time post transplant at which they are tested, the nature of the clinical condition leading to transplantation, and the presence or absence of posttransplant complications. In the late 1990s, it was observed that AIH can arise *de novo* after liver transplantation in patients who had not been transplanted

for autoimmune liver disease (65). After the original report in children, *de novo* AIH after liver transplant was confirmed by several studies in both adult and pediatric patients (64). Importantly, treatment with prednisolone and azathioprine, using the same schedule as for classical AIH, is also effective in *de novo* AIH, leading to excellent graft and patient survival. It is of interest that these patients do not respond satisfactorily to standard antirejection treatment, making it essential to reach an early diagnosis to avoid graft loss.

The recurrence of AIH post transplant can be readily explained. The recipient's immune system is sensitized to species-specific antigens and has a pool of memory cells, which are restimulated and reexpanded when the target antigens, "autoantigens," are presented to the recipient's immune system by either the recipient's APCs repopulating the grafted liver or by the donor's APCs sharing histocompatibility antigens with the recipient.

In contrast, akin to autoimmune liver disease outside transplantation, the pathogenesis of posttransplant *de novo* AIH remains to be defined. There are several nonmutually exclusive explanations: in addition to release of autoantigens from damaged tissue, a possible mechanism is molecular mimicry, whereby exposure to viruses sharing amino acid sequences with autoantigens leads to crossreactive immunity (63). Viral infections, which are frequent post transplant, may also lead to autoimmunity through other mechanisms, including polyclonal stimulation, enhancement and induction of membrane expression of MHC class I and II antigens, or interference with immunoregulatory cells. Another possible mechanism is suggested by animal experiments showing that the use of calcineurin inhibitors predisposes to autoimmunity and autoimmune disease, possibly by interfering with the maturation of T lymphocytes or with the function of regulatory T-cells, with consequent emergence and activation of autoaggressive T cell clones. Lastly, it has been reported that an antibody directed to glutathione-S-transferase T1 is present in patients who develop *de novo* immune-mediated hepatitis (66). Since the gene encoding this protein is defective in a fifth of caucasoid subjects and the encoded enzyme is absent in some of the reported patients, it is possible to speculate that graft dysfunction results from recognition as foreign of glutathione S-transferase T1 acquired with the graft.

## CONCLUDING REMARKS AND OPEN QUESTIONS

Before the recognition of its association with autoimmunity and its response to immunosuppressive treatment, AIH had a poor prognosis. Today, prognosis is excellent, with symptom-free long-term survival in most patients. Over the past 50 yr, several pathogenic aspects of AIH have been elucidated, including predisposing genetic factors and disease-specific humoral and cellular immune responses. Tasks for the future include a better understanding of the pathogenesis of AIH, ideally through the development of animal models faithfully reproducing the human disease, and the establishment of novel treatments aimed at specifically arresting liver autoaggression or at reinstating tolerance to liver antigens.

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# 21 Unique Aspects of Autoimmune Hepatitis in Children

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## KEY POINTS

- There are two main types of autoimmune liver disease in childhood: autoimmune hepatitis (AIH) and AIH/sclerosing cholangitis overlap syndrome (autoimmune sclerosing cholangitis [ASC]).
- AIH is divided into type 1, positive for antinuclear (ANA) and/or anti-smooth muscle (SMA) antibodies, and type 2, positive for anti-liver-kidney microsomal antibody type 1 (LKM-1).
- Most patients with ASC are positive for ANA and/or SMA.
- Antineutrophil cytoplasm antibodies (ANCA) are positive in a similar proportion of children with ASC and AIH type 1 but are usually negative in AIH type 2.
- In at least 20% of patients with ASC, the diagnosis can be achieved only if a cholangiography is performed, because the histological picture is identical to that of AIH.
- Immunofluorescence titers of 1:20 or more of ANA and SMA and 1:10 or more of anti-LKM-1 antibodies are significant in pediatrics, because autoantibodies are rare in healthy children.
- The presence of antibody to soluble liver antigen (SLA) is associated with worse disease severity in all types of autoimmune liver disease.
- Both AIH and ASC respond to treatment with prednisolone + azathioprine, but bile duct damage in ASC may progress despite treatment.
- Autoantibody immunofluorescence titer and immunoglobulin G (IgG) levels are good markers of disease activity and can be used to monitor response to treatment.
- Twenty percent of children with AIH type 1 or ASC, but none with AIH type 2, can eventually stop treatment with no relapse.

## INTRODUCTION

Autoimmune hepatitis (AIH) in childhood is an inflammatory liver disease characterized histologically by a dense portal tract mononuclear cell infiltrate and serologically by the presence of non-organ- and liver-specific autoantibodies and increased

levels of IgG, in the absence of a known etiology. AIH usually responds to immunosuppressive treatment, which should be instituted as soon as a diagnosis is made. In pediatrics, as well as in young adults, AIH often presents acutely and has a more aggressive course than in older patients. In children there are two liver disorders in which the liver damage is likely to arise from an autoimmune attack: classical AIH and AIH/sclerosing cholangitis overlap syndrome (ASC). A possible autoimmune pathogenesis has also been postulated for the so-called post liver transplant *de novo* AIH, a condition originally described in children and later confirmed in adults.

According to data collected at the King's College Hospital tertiary center, there is an increase in the yearly incidence of AIH and ASC in childhood, although referral bias may play a role. Thus, in the 1990s, these conditions were diagnosed in 2.3% of about 400 children older than 4 mo referred during 1 yr, whereas in the 2000s their incidence has increased to 12%.

## AUTOIMMUNE HEPATITIS

### CLINICAL FEATURES

Two types of childhood AIH are recognized: AIH type 1 is characterized by the presence of smooth muscle (SMA) and/or antinuclear (ANA) antibodies; AIH type 2 is positive for anti-liver-kidney microsomal type 1 (anti-LKM-1) antibodies (1). Type 1 AIH represents two-thirds of the cases. Type 2 AIH characteristically affects children or young adults. Severity of disease is similar in the two types (1). In both there is a predominance of girls (75–80%). Anti-LKM-1-positive patients are younger and have a greater tendency to present with acute liver failure, but the duration of symptoms before diagnosis and the frequency of hepatosplenomegaly are similar in the two groups. Both have a high frequency of associated autoimmune disorders (about 20%) and a family history of autoimmune disease (40%). Associated autoimmune disorders include thyroiditis, inflammatory bowel disease, vitiligo, insulin-dependent diabetes, and nephrotic syndrome in both types (1). Type 2 AIH can be associated with autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED), an autosomal recessive genetic disorder in which liver disease is reportedly present in about 20% of the cases (2).

There are three clinical patterns of disease (1):

1. In at least 40% of patients, the presentation is indistinguishable from that of an acute viral hepatitis (nonspecific symptoms of malaise, nausea/vomiting, anorexia, and abdominal pain, followed by jaundice, dark urine, and pale stools). Some children, particularly those who are anti-LKM-1 positive, develop acute hepatic failure with grade II to IV hepatic encephalopathy from 2 to 8 wk after onset of symptoms.
2. In 25 to 40% of patients, the onset is insidious, with an illness characterized by progressive fatigue, relapsing jaundice, headache, anorexia, and weight loss, lasting from several months and even years before diagnosis.
3. In about 10% of patients, there is no history of jaundice, and the diagnosis follows presentation with complications of portal hypertension, such as splenomegaly, hematemesis from esophageal varices, bleeding diathesis, chronic diarrhea, and weight loss.

The mode of presentation of AIH in childhood is therefore variable, and the disease should be suspected and excluded in all children presenting with symptoms and signs of prolonged or severe liver disease. The course of the disease can be fluctuating, with flares and spontaneous remissions, a pattern that may result in delayed referral and diagnosis. Most of the children, however, have clinical signs on physical examination of an underlying chronic liver disease, i.e., cutaneous stigmata (spider nevi, palmar erythema, leukonychia, striae), firm liver, and splenomegaly; at ultrasound the liver parenchyma is often nodular and heterogenous.

### DIAGNOSIS AND LABORATORY FINDINGS

Diagnosis of AIH is based on a series of positive and negative criteria defined by the International Autoimmune Hepatitis Group (IAHG) (3,4). Although these criteria have been produced mainly for research purposes, they may also be useful in clinical practice. Liver biopsy is necessary to establish the diagnosis of AIH, the typical histological picture including a dense mononuclear and plasma cell infiltration of the portal areas, which expands into the liver lobule; destruction of hepatocytes at the periphery of the lobule with erosion of the limiting plate (*interface hepatitis*); connective tissue collapse resulting from hepatocyte death and expanding from the portal area into the lobule (*bridging collapse*); and hepatic regeneration with hepatocyte "rosette" formation. In addition to the typical histology, other positive criteria include elevated serum transaminase and IgG/ $\gamma$ -globulin levels and the presence of ANA, SMA, or anti-LKM-1. Negative criteria relevant to the pediatric age are evidence of infection with hepatitis B or C virus, Wilson's disease, or drug or alcohol consumption.

**Autoantibodies** A key criterion for the diagnosis of AIH is detection by indirect immunofluorescence of ANA, SMA, or anti-LKM-1. Autoantibody detection not only assists in the diagnosis but also allows, as mentioned above, differentiation of AIH into type 1 and type 2. ANA/SMA and anti-LKM-1 are practically mutually exclusive; in those rare instances in which they are present simultaneously, the child is classified as having AIH type 2.

Recognition and interpretation of the immunofluorescence patterns is not always straightforward (5). The operator dependency of the technique and the relative rarity of AIH explain the not infrequent occurrence of errors in reporting, particularly of less frequent specificities such as anti-LKM-1. Problems do exist between laboratory reporting and clinical interpretation of the results, which are partly dependent on clinicians' unfamiliarity with the disease spectrum of AIH, but also partly dependent on insufficient standardization of the tests. This problem is being addressed by the Autoimmune Serology Committee of the IAHG (5).

The basic technique for the routine testing of autoantibodies relevant to AIH is indirect immunofluorescence on a freshly prepared rodent substrate that should include kidney, liver, and stomach to allow the detection of ANA, SMA, anti-LKM-1, and anti-liver cytosol type 1 (anti-LC-1, *see* a few paragraphs below), as well as antimitochondrial antibody (AMA), the serological hallmark of primary biliary cirrhosis, a disease affecting adults almost exclusively. Since a high proportion of healthy adults may show ANA or SMA reactivity at the conventional starting serum dilution of 1:10, the arbitrary dilution of 1:40 is considered clinically significant by the IAHG in the adult population. In contrast, in healthy children autoantibody reactivity is infrequent, so that titers of 1:20 for ANA and SMA and 1:10 for anti-LKM-1 are clinically relevant. Hence, the laboratory should report any level of positivity from 1:10, and the attending physician should interpret the result within the clinical context and the age of the patient.

ANA is detectable as a nuclear staining in kidney, stomach, and liver. Its pattern can be homogeneous, or coarsely, or finely speckled. In most cases of AIH, but not all, the pattern is homogeneous. For a clearer and easier definition of the nuclear pattern, HEp2 cells, which have prominent nuclei, can be used. These cells, however, should not be used for screening purposes, because nuclear reactivity to HEp2 cells is frequent at low serum dilution (1:40) in the normal population. ANA reactivity is not specific to AIH.

SMA is detected on rodent kidney, stomach, and liver. On the renal substrate, it is possible to visualize a V (vessels), G (glomeruli), and T (tubules) staining. VG and VGT patterns are the most frequently detected in AIH (6). The VGT pattern corresponds to the so-called F actin or microfilament (MF) pattern observed using cultured fibroblasts as substrate. Although "anti-actin" reactivity is present in most patients with AIH type 1, some 20% of SMA-positive AIH type 1 patients do not have the F-actin/VGT pattern. The absence, therefore, of anti-actin SMA does not exclude the diagnosis of AIH (7).

Anti-LKM-1 stains the liver cell cytoplasm and the P3 portion of the renal tubules brightly but does not stain gastric parietal cells. Anti-LKM-1 is often confused with AMA, since both autoantibodies stain liver and kidney, although AMA stains the liver more faintly and the renal tubules more diffusely, with an accentuation of the small distal ones; in contrast to anti-LKM-1, it also stains the gastric parietal cells. In the context of childhood AIH, patients reported to be AMA positive are in reality almost invariably positive for anti-LKM-1, AMA-positive AIH

**Table 1**  
**Clinical, Laboratory, and Histological Features at Presentation of Autoimmune Hepatitis Type 1, Autoimmune Hepatitis Type 2, and Autoimmune Sclerosing Cholangitis**

Feature	Type 1 AIH	Type 2 AIH	ASC
Median age in years	11	7	12
Females (%)	75	75	55
Mode of presentation (%)			
Acute hepatitis	47	40	37
Acute liver failure	3	25	0
Insidious onset	38	25	37
Complication of chronic liver disease	12	10	26
Associated immune diseases (%)	22	20	48
Inflammatory bowel disease (%)	20	12	44
Family history of autoimmune disease (%)	43	40	37
Abnormal cholangiogram (%)	0	0	100
ANA/SMA (%)	100	25	96
Anti-LKM-1 (%)	0	100	4
p-ANCA (%)	45	11	74
Anti-SLA (%) <sup>a</sup>	58	58	41
Increased IgG level (%)	84	75	89
Partial IgA deficiency (%)	9	45	5
Low C4 level (%)	89	83	70
Increased frequency of HLA			
<i>DRB1*0301</i>	Yes	No <sup>b</sup>	No
<i>DRB1*0701</i>	No	Yes	No
<i>DRB1*1301</i>	No	No	Yes
Interface hepatitis (%)	66	72	35
Biliary features (%)	28	6	31
Cirrhosis (%)	69	38	15
Remission after immunosuppressive treatment (%)	97	87	89

Abbreviations: AIH, autoimmune hepatitis; ASC, autoimmune sclerosing cholangitis; ANA, antinuclear antibodies; SMA, smooth muscle antibody; LKM-1, liver-kidney microsomal type 1 antibody; p-ANCA, perinuclear antineutrophil cytoplasmic antibody; SLA, soluble liver antigen; IgG, immunoglobulin G; IgA, immunoglobulin A; C4, C4 component of complement; HLA, human leukocyte antigen.

<sup>a</sup>Measured by radioligand assay.

<sup>b</sup>But increased in HLA *DRB1\*0701* negative patients.

Data from refs. 1 and 17.

being extremely rare (8). Identification of the molecular targets of anti-LKM-1, i.e., cytochrome P4502D6, and of AMA, i.e., enzymes of the 2-oxo-acid dehydrogenase complexes, has led to the establishment of commercial immunoassays based on the use of recombinant or purified antigens (14), which can resolve any doubts remaining after immunofluorescence examination.

Other autoantibodies less commonly tested but of diagnostic importance in pediatric AIH include those to LC-1, antineutrophil cytoplasm (ANCA), and antisoluble liver antigen (SLA). Anti-LC-1, which can be present on its own, but frequently occurs in association with anti-LKM-1, is an additional marker for AIH type 2 and targets formimino-transferase cyclodeaminase (FTCD) (9). In AIH type 1, as well as in inflammatory bowel disease and sclerosing cholangitis, ANCA is frequently found and targets a peripheral nuclear antigen (hence the suggested name of p-ANNA, i.e., peripheral antinuclear neutrophil antibody). P-ANNA is virtually absent in type 2 AIH (7). Anti-SLA, which was originally described as the hallmark of a third type of AIH (10), is also found in some 50% of pediatric patients with type 1 and type 2 AIH, in whom it defines a more severe course (11).

A small proportion of children with AIH have undetectable autoantibodies. The prevalence and clinical characteristics of

this rare seronegative form of AIH, which responds to immunosuppression similarly to the seropositive forms, remain to be defined.

**Comparison Between Type 1 and Type 2 AIH** Clinical, laboratory, and histological features of type 1 and 2 AIH are summarized in Table 1. In Northern Europe, type 1 AIH is associated with the possession of human leukocyte antigen (HLA) *DRB1\*03* (1,2), whereas type 2 AIH is associated with *DRB1\*07* (13). In South America, possession of the HLA *DRB1\*1301* allele, which predisposes to pediatric AIH-1 in that population, is also associated with persistent infection with the endemic hepatitis A virus (14,15).

Pediatric patients with AIH, whether anti-LKM-1 or ANA/SMA positive, have isolated partial deficiency of the HLA class III complement component C4, which is genetically determined (16).

Anti-LKM-1-positive patients have higher levels of bilirubin and transaminases at presentation than those who are ANA/SMA positive and present significantly more frequently with fulminant hepatic failure (1). Excluding children with the fulminant presentation, a severely impaired hepatic synthetic function, as assessed by the presence of both prolonged prothrombin time and

hypoalbuminemia, is more common in ANA/SMA-positive than in anti-LKM-1-positive patients. The vast majority of patients have increased levels of IgG, but some 20% do not, indicating that normal IgG values do not exclude the diagnosis of AIH. Partial IgA deficiency is significantly more common in LKM1-positive than in ANA/SMA-positive patients.

The severity of interface hepatitis at diagnosis is similar in both types, but cirrhosis on initial biopsy is more frequent in type 1 than in type 2 AIH, suggesting a more chronic course of disease in the former. Of note is that most patients already cirrhotic at diagnosis present with a clinical picture reminiscent of that of prolonged acute virus-like hepatitis. Multiacinar or panacinar collapse, which suggests an acute liver injury, is more frequently seen in type 2 AIH. The question of whether the acute presentation in these patients represents a sudden deterioration of an underlying unrecognized chronic process or a genuinely acute liver damage remains open. Progression to cirrhosis during treatment is more frequent in type 1 AIH. As mentioned above, in both, a more severe disease and a higher tendency to relapse is associated with the possession of antibodies to SLA, which are present in about half of the patients with AIH type 1 or 2 at diagnosis (11).

**Differential Diagnosis** Since positive autoimmune serology can be present in conditions other than AIH, in particular (17) ASC (*see* that section below), chronic hepatitis B (18) or C (19) virus infections, and Wilson's disease (20), all these disorders must be considered in the differential diagnosis and excluded. ASC, which is described in the section of that name below, shares the same serological profile of type 1 AIH but has typical bile duct lesions on cholangiography. Up to 50% of children with hepatitis B and C are positive for ANA and/or SMA, usually at low titers, and up to 10% of patients with chronic hepatitis C have anti-LKM-1 antibodies. In these patients the histology can also mimic that of AIH, although usually the degree of inflammation is milder. Detection of the typical viral markers allows a correct diagnosis. ANA, and at times SMA, can be present in Wilson's disease, in association with high IgG and an inflammatory liver histology, which can make the differential diagnosis with AIH type 1 difficult. Urinary, serum, and liver tissue copper studies and search for Kayser Fleischer rings should be performed in all cases.

**Autoimmune Polyendocrinopathy-Candidiasis-Ectodermal Dystrophy (APECED)** APECED is a monogenic disorder (21,22) with a variable phenotype. About 20% of the cases develop AIH that resembles AIH type 2 (2). This condition, also known as autoimmune polyendocrine syndrome 1, is an autosomal recessive disorder caused by homozygous mutations in the *AIRE1* gene and characterized by a variety of organ-specific autoimmune diseases, the most common of which are hypoparathyroidism and primary adrenocortical failure, accompanied by chronic mucocutaneous candidiasis.

### ETIOLOGY AND PATHOGENESIS

The etiology of AIH is unknown, although both genetic and environmental factors are involved in its expression. Etiological hypotheses and possible mechanisms leading to

the liver autoimmune attack are described in Chapter 20 (Autoimmune Hepatitis).

### MANAGEMENT AND PROGNOSIS

AIH is exquisitely responsive to immunosuppression. The rapidity and degree of response depends on the disease severity at presentation. All types of presentations, apart from fulminant hepatic failure with encephalopathy, respond to standard treatment with prednisolone with or without azathioprine.

Standard treatment for AIH consists of prednisolone 2 mg/kg/d (maximum 60 mg/d), which is gradually decreased over a period of 4 to 8 wk with progressive normalization of the transaminases, and then the patient is maintained on the minimal dose able to sustain normal transaminase levels, usually 2.5 mg/d or 5 mg/d depending on the age (1,23). During the first 6 to 8 wk of treatment, liver function tests should be checked weekly to allow frequent fine-tuning, avoiding severe steroid side effects. If progressive normalization of the liver function tests is not obtained over this period of time or if too high a dose of prednisolone is required to maintain normal transaminases, azathioprine is added at a starting dose of 0.5 mg/kg/d, which, in the absence of signs of toxicity, is increased up to a maximum of 2 to 2.5 mg/kg/d until biochemical control is achieved. Azathioprine is not recommended as first-line treatment because of its hepatotoxicity in severely jaundiced patients, but 85% of the patients will eventually require azathioprine addition. A preliminary report in a cohort of 30 children with AIH suggests that measurements of the azathioprine metabolites 6-thioguanine and 6-methylmercaptopurine are useful in identifying drug toxicity and nonadherence and in achieving a level of 6-thioguanine considered therapeutic for inflammatory bowel disease (24), although the ideal therapeutic level for AIH has not been determined.

Although an 80% decrease in initial transaminase levels is obtained within 6 wk from starting treatment in most patients, complete normalization of liver function may take several months. In the King's College Hospital series, normalization of transaminase levels occurred at median of 6 mo in ANA/SMA-positive children and 9 mo in LKM-1-positive children (1). Relapse while on treatment is common, occurring in about 40% of the patients and requiring a temporary increase in steroid dose. The risk of relapse is higher if steroids are administered on an alternate-day schedule, often instituted in the belief that it has a less negative effect on the child's growth. Small daily doses are more effective in maintaining disease control and minimizing the need for high-dose steroid pulses during relapses, resulting in more severe side effects.

Cessation of treatment is considered if a liver biopsy shows minimal or no inflammatory changes after at least 1 yr of normal liver function tests. However, it is advisable not to attempt to withdraw treatment within 2 yr from diagnosis or during or immediately before puberty, when relapses are more common. An important role may be played by nonadherence, which is frequently underestimated in teenagers. In the King's College Hospital experience, successful long-term withdrawal of treatment was achieved in 20% of patients with AIH type 1 but in none with AIH type 2 (1).

In pediatrics, an important role in monitoring the response to treatment is the measurement of autoantibody titers and IgG levels, the fluctuation of which is correlated with disease activity (25).

Despite the efficacy of standard immunosuppressive treatment, severe hepatic decompensation may develop even after many years of apparently good biochemical control, leading to transplantation 10 to 15 yr after diagnosis in 10% of the patients. Overall, in the King's College Hospital series, over 97% of the patients treated with standard immunosuppression were alive between 0.3 and 19 yr (median 5 yr) after diagnosis, including 8% after liver transplant. Side effects of steroid treatment were mild, the only serious complication being psychosis during induction of remission in 4%, which resolved after prednisolone withdrawal. All patients developed a transient increase in appetite and mild cushingoid features during the first few weeks of treatment. After 5 yr of treatment, 56% of the patients maintained the baseline centile for height or went up across a centile line, 38% dropped across one centile line, and only 6% dropped across two centile lines (26).

Sustained remission, achieved with prednisolone and azathioprine, can be maintained with azathioprine alone in some children with AIH type 1, akin to the experience in adults (27), but not in AIH type 2.

To avoid high-dose steroid side effects, Alvarez et al. have induced remission in 71% of treatment naïve children with AIH using cyclosporine A alone for 6 mo, followed by maintenance with low-dose prednisone and azathioprine (28). However, whether this mode of induction has any advantage over the standard treatment remains to be evaluated in controlled studies to be conducted in specialized centers on a large number of patients stratified for disease severity.

In those patients (up to 10%) in whom standard immunosuppression is unable to induce stable remission or who are intolerant to azathioprine, mycophenolate mofetil at a dose of 20 mg/kg twice daily can be successfully used (26). In case of persistent lack of response or of intolerance to mycophenolate mofetil (headache, diarrhea, nausea, dizziness, hair loss, and neutropenia), the use of calcineurin inhibitors (cyclosporine A or tacrolimus) should be considered.

Children who present with acute hepatic failure pose a particularly difficult therapeutic problem. If not encephalopathic, they usually benefit from conventional immunosuppressive therapy, but only one of the six children with acute liver failure and encephalopathy in the King's College Hospital series responded to immunosuppression and survived without transplant (1).

### AUTOIMMUNE HEPATITIS/SCLEROSING CHOLANGITIS OVERLAP SYNDROME

Autoimmune hepatitis/sclerosing cholangitis overlap syndrome (ASC) has the same prevalence as AIH type 1 in childhood (17). This has been shown in a prospective study conducted over a period of 16 yr, in which all children with serological (i.e., positive autoantibodies, high IgG levels) and histological (i.e., interface hepatitis; Fig. 1A) features of

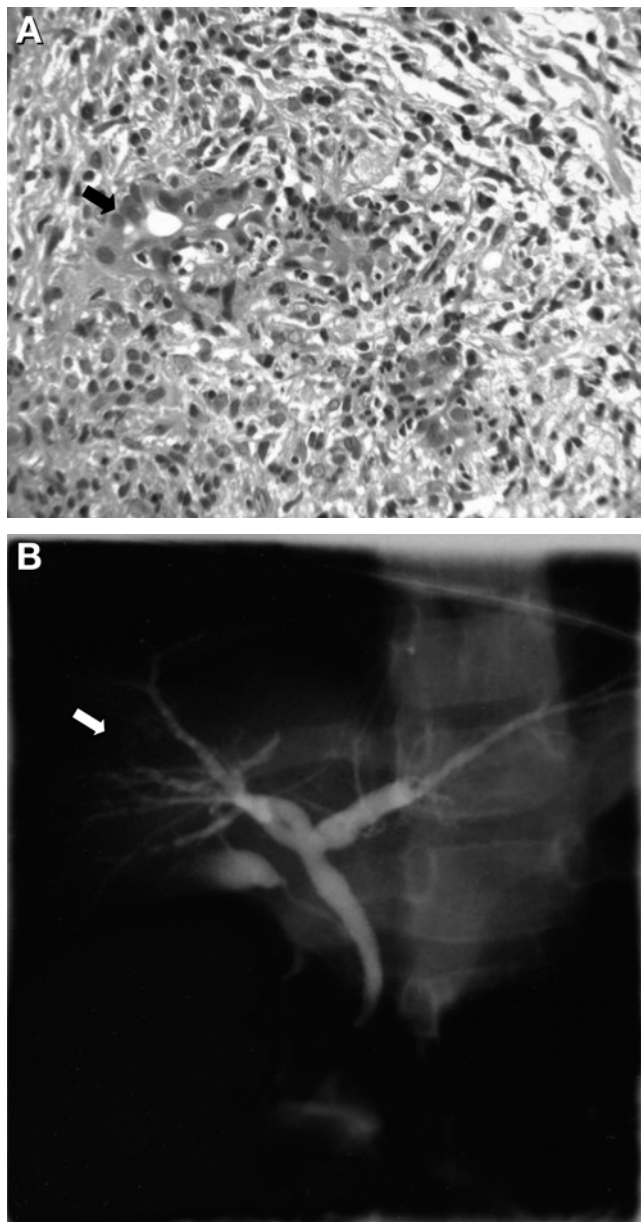
autoimmune liver disease underwent a cholangiogram at the time of presentation. Approximately 50% of these patients had alterations of the bile ducts characteristic of sclerosing cholangitis, although generally less advanced than those observed in adult primary sclerosing cholangitis (Fig. 1B). One-fourth of the children with ASC, despite abnormal cholangiograms, had no histological features suggesting bile duct involvement, and the diagnosis of sclerosing cholangitis was only possible because of the cholangiographic studies. Virtually all patients were seropositive for ANA and/or SMA. Fifty-five percent were girls, and the mode of presentation was similar to that of typical AIH. Inflammatory bowel disease was present in about 45% of children with ASC compared with about 20% of those with typical AIH, and 90% of children with ASC had greatly increased serum IgG levels. At the time of presentation, standard liver function tests did not help in discriminating between AIH and ASCs, although the alkaline phosphatase/aspartate amino transferase ratio was significantly higher in ASC (Table 2). p-ANNAs were present in 74% of patients with ASC compared with 45% of patients with AIH type 1 and 11% of those with AIH type 2. Susceptibility to ASC in children is conferred by the possession of HLA *DRB1\*1301(29)*. Clinical, laboratory, and histological features of type 1 and 2 AIH and ASC are compared in Table 1.

Children with ASC respond to the same immunosuppressive schedule described above for AIH (17), liver test abnormalities resolving within a few months after starting treatment in most patients. Steroids and azathioprine, however, although beneficial in abating the parenchymal inflammatory lesion, appear to be less effective in controlling the bile duct disease. Following favorable reports in adult primary sclerosing cholangitis (30,31), ursodeoxycholic acid is added at a dose of 20 to 30 mg/kg/d, although there is no information as to whether it is helpful in arresting the progression of ASC. Akin to AIH, measurement of autoantibody titers and IgG levels is useful in monitoring disease activity and response to treatment (54). The medium-term prognosis is good, with a reported 7-yr survival of 100%, although 15% of the patients required liver transplant during this period of follow-up (17).

Evolution from AIH to ASC has been documented, suggesting that AIH and ASC are part of the same pathogenic process (17).

### DE NOVO AIH AFTER LIVER TRANSPLANT

In the late 1990s, it was observed that AIH can arise *de novo* after liver transplantation in children who had not been transplanted for autoimmune liver disease (32). Characteristic of this condition is a histological picture of interface hepatitis and multilobular collapse associated with increased IgG levels and positive autoantibodies. These include ANA, SMA, and classical anti-LKM-1, but also atypical anti-LKM-1, staining the renal tubules but not the liver. After this original report, *de novo* AIH after liver transplant has been confirmed by several studies in both adult and pediatric patients (33,34). Importantly, treatment with prednisolone and azathioprine using the same schedule for classical AIH, concomitant with reduction in the calcineurin inhibitor dose, is highly effective in *de novo* AIH, leading to excellent graft and patient survival. It is of interest that these



**Fig. 1.** Liver histology and cholangiogram from a child with autoimmune sclerosing cholangitis. (A) Dense portal lymphocyte and plasma cell infiltrate, extending to and disrupting the parenchymal limiting plate (interface hepatitis). The arrow shows a bile duct infiltrated by inflammatory cells. Hematoxylin & eosin staining. (B) Endoscopic cholangiography showing multiple strictures affecting mainly the right intrahepatic bile duct system (arrow).

patients do not respond satisfactorily to standard antirejection treatment, making it essential to reach an early diagnosis to avoid graft loss.

The possible pathogenesis of *de novo* AIH is discussed in Chapter 20 (Autoimmune Hepatitis).

## CONCLUDING REMARKS AND OPEN QUESTIONS

Over the past two decades, there has been a sharp increase in the incidence of the diagnosis of AIH in children. Whether this is

**Table 2**  
Laboratory Parameters at Presentation in Children With Autoimmune Hepatitis (AIH) and Autoimmune Sclerosing Cholangitis (ASC)<sup>a</sup>

	AIH	ASC
Bilirubin (nv < 20 mmol/L)	35 (4–306)	20 (4–179)
Albumin (nv > 35 g/L)	35 (25–47)	39 (27–54)
AST (nv < 50 IU/L)	333 (24–4830)	102 (18–1215)
INR (< 1.2)	1.2 (0.96–2.5)	1.1 (0.9–1.6)
GGT (nv < 50 IU/L)	76 (29–383)	129 (13–948)
AP (nv < 350 IU/L)	356 (131–878)	303 (104–1710)
AP/AST ratio	1.14 (0.05–14.75)	3.96 (0.20–14.20)

Abbreviations: AST, aspartate aminotransferase; INR, international normalized prothrombin ratio; GGT,  $\gamma$ -glutamyl transpeptidase; AP, alkaline phosphatase; nv, normal values.

<sup>a</sup>Values are given as medians (ranges).

Data from ref. 17.

owing to a real increase in frequency or to an increased awareness of the disease remains to be clarified. If diagnosed and treated early, AIH has an excellent prognosis; only about 10% of the children who achieve remission with immunosuppression require liver transplantation 10 to 20 yr after presentation. However, currently available immunosuppression is not specific and has unpleasant side effects. It is hoped that a better understanding of the pathogenic mechanisms leading to AIH will allow a targeted and less toxic therapeutic approach in the near future.

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# 22 Overlap Syndromes of Autoimmune Hepatitis With Primary Biliary Cirrhosis and Primary Sclerosing Cholangitis

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ULRICH BEUERS AND CHRISTIAN RUST

## KEY POINTS

- In hepatology, overlap syndromes represent variant forms of the classical autoimmune hepatopathies, autoimmune hepatitis (AIH), primary biliary cirrhosis (PBC), and primary sclerosing cholangitis (PSC).
- Overlap syndromes are ill defined. They present with both hepatitic and cholestatic biochemical and histological features of AIH, PBC, and/or PSC and mostly show a progressive course.
- AIH-PBC overlap syndromes have been described in nearly 10% of patients with AIH or PBC.
- AIH-PSC overlap syndromes (*autoimmune sclerosing cholangitis* [ASC]) have been observed in 6 to 8% of patients with AIH or PSC and are mainly diagnosed in children and adolescents.
- Autoimmune cholangitis (AIC) or AMA-negative PBC shares many features with PBC and is not regarded as an overlap syndrome but as an outlier syndrome.
- Medical treatment of AIH-PBC and AIH-PSC overlap syndromes is empiric and may include ursodeoxycholic acid and immunosuppressive therapy. In AIC, ursodeoxycholic acid is regarded as an adequate treatment option. Liver transplantation is the treatment of choice for end-stage disease.
- A deeper insight into the pathogenesis of these variant autoimmune liver diseases is needed for the development of more adequate therapies.

## INTRODUCTION

The term “overlap syndrome” has been introduced to the field of hepatology to describe variant forms of autoimmune hepatitis (AIH) which present with the characteristics of AIH on the one hand and primary biliary cirrhosis (PBC) or primary sclerosing cholangitis (PSC) on the other hand. There is still controversy over whether these “overlap syndromes” form distinct entities or

are only variants of the major autoimmune hepatopathies. Standardization of diagnostic criteria for overlap syndromes has not been achieved so far, and misuse of the term “overlap syndrome” is common in clinical practice (1).

Diagnostic criteria of AIH have been well defined by an international group of experts. A scoring system comprising characteristic clinical, biochemical, and histologic features of AIH provides support for diagnosing AIH (2). Criteria for the diagnosis of PBC and PSC are less well defined. The presence of antimitochondrial antibodies directed against the E2 subunit of the pyruvate dehydrogenase complex (AMA-M2s), a cholestatic serum enzyme pattern, and a “florid bile duct lesion” are hallmarks of PBC, whereas typical cholangiographic findings of bile duct stenoses and dilations, a cholestatic serum enzyme pattern, a concomitant inflammatory bowel disease, and the presence of atypical perinuclear antineutrophil cytoplasmic antibodies (p-ANCAs) represent typical findings of PSC (Table 1).

A meticulous literature review revealed that variant forms of autoimmune hepatitis are not uncommon and form a considerable fraction of autoimmune liver disease (3) (Fig. 1). Overlap syndromes of AIH and PBC, as well as AIH and PSC have been described. In addition, other variants of autoimmune liver disease have been reported. These have been termed outlier syndromes (3) and include autoimmune cholangitis (AIC; AMA-negative PBC) and cryptogenic chronic hepatitis. Overlap of AIC and AIH has also been reported (Table 2).

The term “overlap syndrome” should be avoided when pure comorbidity, e.g., acute viral hepatitis in a patient with AIH or chronic hepatitis C in a patient with PBC exists. Single cases have been described in which the diagnosis of autoimmune liver disease changed during the long-term course, and transitions from PBC to AIH (4,5) as well as from AIH to PSC (6) have been described. These cases could also be viewed separately from overlap syndromes (Table 2).

So far, no consensus has been reached on the definition of overlap and outlier syndromes in hepatology. The present

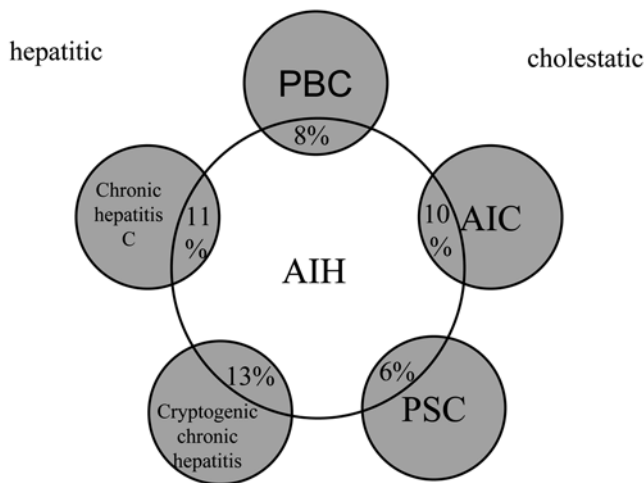


**Table 1**  
**Characteristic Features of Autoimmune Liver Diseases**

Feature	AIH	PBC	PSC	AIC
Female/Male	4:1	9:1	1:2	9:1
AP (×N)/ALT (×N)	<2	>0.3	>0.3	>0.3
Ig elevation	IgG	IgM	IgG, IgM	IgM
Autoantibodies	ANA, anti-SMA, LKM, SLA, p-ANCA	AMA, AMA-M2	p-ANCA	ANA, ASMA
HLA association	A3, B8, DR3, DR4	DR8	DR52	B8, DR3, DR4
Histology	Lymphocytic interface hepatitis (moderate/severe)	Florid bile duct lesion	Fibrosing bile duct lesion	Florid bile duct lesion
Diagnosis	AIH score > 15	AMA-M2, cholestatic serum enzyme pattern, compatible histology	Stenoses/dilations of bile ducts by cholangiography, cholestatic serum enzyme pattern, inflammatory bowel disease, p-ANCA	Cholestatic serum enzyme pattern, AMA <sup>-</sup> , ANA <sup>+</sup> or ASMA <sup>+</sup> , histology compatible with PBC
First-line medical therapy	Corticosteroids + azathioprine	UDCA	UDCA	UDCA

Abbreviations: ALT, alanine aminotransferase; AMA, antimitochondrial antibody; ANA, antinuclear antibody; AP, alkaline phosphatase; LKM, liver-kidney microsomal; N, upper limit of normal; P-ANCA, perinuclear antineutrophil cytoplasmic antibody; SLA, soluble liver antigen; SMA, smooth muscle antibody; UDCA, ursodeoxycholic acid.

“Overlap syndromes” show characteristics of autoimmune hepatitis (AIH) on the one hand, and primary biliary cirrhosis (PBC), primary sclerosing cholangitis (PSC), or autoimmune cholangitis (AIC) on the other hand.



**Fig. 1.** Frequency of serological and histologic features shared by autoimmune hepatitis (AIH) and other chronic liver diseases. Eight percentage of AIH patients were positive for AMA-M2, specific for primary biliary cirrhosis (PBC), 6% had histologic lesions suggestive of primary sclerosing cholangitis (PSC), 13% had all features of AIH but lacked autoantibodies, 11% were positive for hepatitis C virus RNA, and 10% shared features of autoimmune cholangitis (AIC). (Modified from ref. 3.)

chapter summarizes current views on overlap of AIH and PBC, AIH and PSC, and AIH and AIC. The outlier syndrome AIC is also discussed, although it does not, strictly speaking, fulfill the criteria of overlap syndromes.

**Table 2**  
**Variants of Autoimmune Liver Diseases**

<b>Overlap syndromes</b>
AIH - PBC
AIH - PSC
AIH - AIC
<b>Outlier syndrome</b>
AIC
<b>Change of diagnosis of autoimmune liver disease</b>
PBC ↔ AIH
AIH ↔ PSC

Abbreviations: AIC, autoimmune cholangitis; AIA, autoimmune hepatitis; PBC, primary biliary cirrhosis; PSC, primary sclerosing cholangitis.

**AIH-PBC OVERLAP SYNDROME**

In clinical practice, the two major immune-mediated hepatopathies, PBC and AIH, can easily be differentiated by symptoms, biochemical tests, and histological findings (see previous chapters). Typical features are summarized in Table 1. Although female sex predominates in both AIH (80%) and PBC (90%), serum liver tests in AIH typically show a hepatitic feature (alkaline phosphatase × upper limit of normal [AP (× N)]/alanine aminotransferase [ALT (× N)] < 2), whereas such tests in PBC are characterized by predominant elevation of AP and γ-glutamyl transferase (γ-GT) and only mild elevation of serum transaminases. In addition, IgG is the predominant immunoglobulin in serum of AIH patients, whereas IgM is elevated in most patients with PBC. In the 1970s, the first cases of AIH-PBC overlap syndrome were reported (7,8) but this

**Table 3**  
**Characteristic Features of AIH-PBC Overlap Syndrome**

Parameter	Findings
Serum tests	ALT, AST, AP, $\gamma$ -GT elevated IgG moderately elevated, IgM elevated
Autoantibodies	AMA-M2 + ANA and/or anti-SMA + ( $>50\%$ ); SLA +
Histology	Moderate to severe interface hepatitis (predominant features of AIH)
HLA type	HLA DR3 or DR4 positive

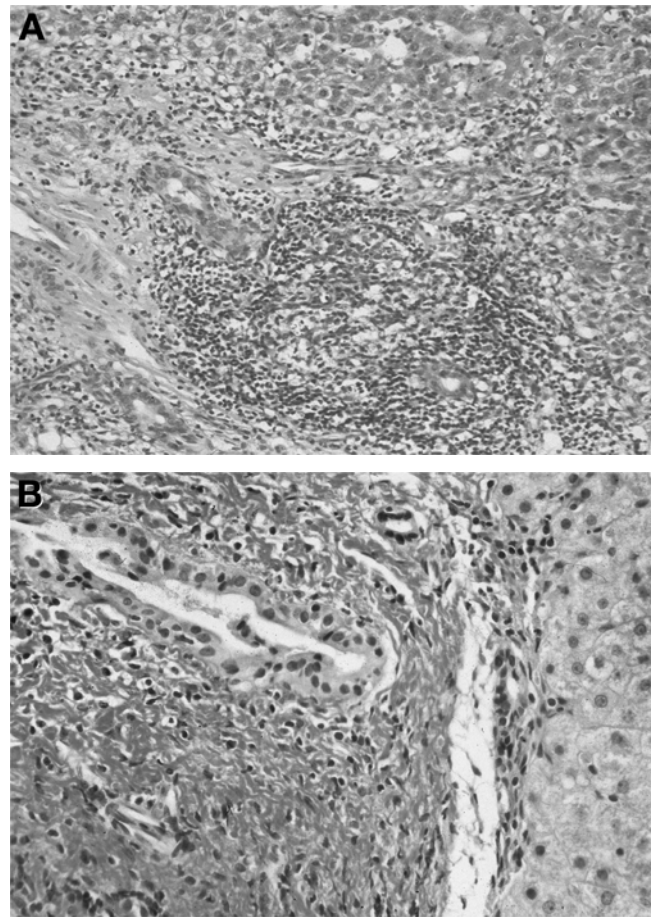
Abbreviations: ALT, alanine aminotransferase; ANA, antinuclear antibody; AP, alkaline phosphatase; AST, aspartate aminotransferases;  $\gamma$ -GT,  $\gamma$ -glutamyl transferase; SLA, soluble liver antigen; SMA, smooth muscle antibody.

entity was assumed to be exceedingly rare. In two careful analyses, however, AIH-PBC overlap was found in 8% of 199 patients with AIH ( $n = 162$ ) or PBC ( $n = 37$ ) (3) and in 9% of 130 patients with PBC (5). In the latter study, an AIH-PBC overlap syndrome had to fulfill two or three criteria of PBC: (1)  $AP > 2 \times N$  or  $\gamma$ -GT  $> 5 \times N$ ; (2) AMA positivity; and (3) florid bile duct lesions; as well as AIH: (1)  $ALT > 5 \times N$ ; (2)  $IgG > 2 \times N$  or anti-smooth muscle antibody (SMA) positivity; and (3) moderate or severe periportal lymphocytic piecemeal necrosis). Flares of hepatitis were reported in one patient under ursodeoxycholic acid (UDCA) treatment (5). Characteristic features of patients with AIH-PBC overlap syndrome are summarized in Table 3 and include elevation of serum transaminases, markers of cholestasis, immunoglobulins M and G, the presence of AMA-M2, and histological findings compatible with AIH.

Interestingly, AIH can develop in patients with long-standing PBC. In a retrospective analysis, this consecutive form developed in 4.3% of a cohort of 282 patients with PBC in a single center after up to 13 yr of follow-up (9). Most patients required additional treatment with an immunosuppressive regimen in addition to UDCA, emphasizing the need for an early diagnosis of this condition.

A closer analysis in a German population revealed that patients with AIH-PBC overlap (compared with AIH and PBC patients;  $n = 20$  each) present with typical features of PBC (anti-M2-positive antimitochondrial antibodies and/or bile duct destruction characteristic of PBC) but a more hepatic picture and a good response to corticosteroid treatment (10). Intriguingly, patients with features of AIH-PBC overlap showed a genetic susceptibility typical of AIH, with a histocompatibility leukocyte antigen (HLA) type B8, DR3, or DR4 (AIH: 18/20; overlap: 17/20; PBC: 4/20). Thus, it was speculated that these patients are PBC patients who develop a more hepatic course of their cholestatic disease owing to their genetic susceptibility characteristic of AIH. Consequently, it was suggested that the term "PBC, hepatic form" be introduced (10).

It is important to note that the presence of antinuclear antibodies in patients with PBC is not a marker of AIH-PBC overlap but is found at considerable rates in PBC patients



**Fig. 2.** (A) A 26-yr-old woman presented with jaundice that had begun 2 wk previously, fatigue, pruritus, and elevated serum liver tests (bilirubin 34.2 mg/dL, ALT 672 U/L (normal  $< 20$ ), AST 925 U/L (normal  $< 16$ ), AP 377 U/L (normal  $< 190$ ), and  $\gamma$ -GT 14 U/L (normal  $< 19$ ). AMA, AMA-M2, and SLA were positive.  $\gamma$ -Globulins and IgM were elevated. Liver histology revealed portal and periportal lympho- and plasmacellular infiltration, bile ducts with degenerative changes, piecemeal necrosis, and lobular inflammation. A diagnosis of AIH-PBC overlap syndrome was made. The patient discontinued medical treatment against medical advice after dismissal when she felt well. She did not present for follow-up visits. (Hematoxylin & eosin, original magnification  $\times 20$ ) (B) After 6 yr, the patient presented for the second time. She reported well-being and had no complaints. Serum liver tests were abnormal (bilirubin 1.74 mg/dL, ALT 62 U/L, AST 65 U/L, AP 357 U/L, and  $\gamma$ -GT 153 U/L. AMA, AMA-M2, and SLA were strongly positive, and  $\gamma$ -globulins, IgG, and IgM were all markedly elevated. Liver histology revealed predominantly portal inflammation, ductopenia, degenerative alterations of bile ducts, and complete cirrhosis of the liver. Medical treatment with UDCA (14 mg/kg/d) was started, and immunosuppressive therapy with azathioprine and prednisolone in addition to UDCA was recommended. (Elastica-van Giessen, original magnification  $\times 38$ ; Courtesy of Professor J. Müller-Höcker.)

without any further signs of AIH (11). In contrast, in a subgroup of patients with PBC, the presence of soluble liver antigen (SLA) autoantibodies was a marker of AIH-PBC overlap (12) (Fig. 2).

**Table 4**  
**Comparison of Children With ASC (AIH-PSC Overlap Syndrome) and Those With AIH or PSC Seen in the Same Time Interval at One Single Unit**

Parameter	ASC (n = 27)	AIH (n = 28)	PSC (n = 9)
Age at diagnosis (yr)	11.8 (2.3–16)	10.5 (2.2–14)	6.6 (2–14.5)
Mode of presentation			
Prolonged acute hepatitis (%)	37	50	11
No history of jaundice (%)	37	32	89
Signs at diagnosis			
Jaundice (%)	56	68	(11)
Hepatomegaly (%)	56	68	78
Splenomegaly (%)	52	61	67
Symptoms at diagnosis			
Diarrhea (%)	37	29	67
Abdominal pain (%)	30	29	33
Associated disorders			
Autoimmune disorders (%)	48	39	44
Inflammatory bowel disease (%)*	44	18	33
Laboratory features			
Total bilirubin (× N)*	1.0 (0.2-8.9)	1.8 (0.2-15.3)	0.8 (0.3-1.3)
γ-GT (× N)	2.6 (0.3-19.0)	1.5 (0.6-7.7)	2.8 (0.5-13.8)
AST (× N)*	2.0 (0.4-24.3)	6.7 (0.5-96.6)	1.8 (0.5-15.2)
AP (× N)	0.9 (0.3-4.9)	1.0 (0.4-2.5)	1.4 (0.1-2.0)
AP (× N)/AST (× N)*	0.7 (0.1-2.4)	0.2 (0.1-2.5)	0.9 (0.2-1.7)
ANCA-positive (%)*	74	36	44
Histological features			
Moderate/severe inflammation of			
Portal tract (%)	58	92	No data available
Periportal area (%)	35	58	
Lobules (%)	31	61	

Abbreviations: ANCA, antineutrophil cytoplasmic antibody; AP, alkaline phosphatase; AST, aspartate aminotransferase; γ-GT, γ-glutamyl transferase.

\*Ranges are in parentheses. Laboratory data adapted to the upper limit of normal (N).

\*  $p < 0.05$  between ASC and AIH.

Data from ref. 6.

## THERAPY

The low prevalence of AIH-PBC overlap syndrome has made it impossible to perform controlled therapeutic trials in these patients. Thus, therapeutic recommendations rely on experience in the treatment of either AIH or PBC (for details, see previous chapters). It appears useful to start with UDCA treatment (13–15 mg/kg/d) according to the premise “at least do not harm” (13) and, if this therapy does not induce an adequate biochemical response, to add a glucocorticosteroid at tolerable doses (e.g., prednisone 10–15 mg/kg d) (13,14).

In a recent retrospective study, which included 17 patients with a well-defined AIH-PBC overlap syndrome and a median follow-up of 7.5 yr, liver fibrosis rapidly progressed in most patients receiving only UDCA (15). In contrast, progression of liver fibrosis was not observed in patients treated with a combination of UDCA and corticosteroids. Thus, a combined therapy might be the best therapeutic option in patients with well-defined AIH-PBC overlap syndrome. The role of other immunosuppressive agents, e.g., azathioprine (1–1.5 mg/kg/d), in the management of patients with AIH-PBC overlap syndrome has not been determined, but its successful use in AIH makes this immunosuppressant an attractive alternative/addition to

corticosteroids when long-term immunosuppressive therapy is needed (14).

Corticosteroid-resistant patients with AIH-PBC overlap syndrome may exist, and intermediate treatment with other immunosuppressants such as cyclosporine A has been considered (16).

## AIH-PSC OVERLAP SYNDROME

Overlap of AIH and PSC has been described anecdotally in a number of reports during the last decades, in both children and adults (17–24), and is today generally assumed to exist in a considerable portion of mainly young patients with autoimmune liver disease. Indeed, diagnosis of an overlap syndrome was established in 8% of 113 PSC patients evaluated retrospectively using the modified AIH score (25). However, in a second cohort of 211 PSC patients, only 1.4% fulfilled the criteria for AIH-PSC overlap (26). Differences in (1) age of the study populations, (2) autoantibodies taken into consideration, and (3) degree of completeness of analyzed data may have contributed to these variant results (27).

The most intriguing report on AIH-PSC overlap syndromes has come from King’s College Hospital in London (6). In a

16-yr prospective study, the authors followed a group of 55 children who showed clinical, biochemical, and histological signs of AIH. Among these children, 27 were diagnosed as having sclerosing cholangitis on the basis of cholangiographic findings. As these children otherwise showed signs of AIH, the term *autoimmune sclerosing cholangitis* (ASC) was proposed for this AIH-PSC overlap syndrome. Characteristic features of these patients are summarized in Table 4 in comparison with children who have AIH and PSC. These features show that patients with AIH and ASC present with similar signs and symptoms, but patients with ASC more commonly suffer from inflammatory bowel disease and are more often positive for ANCA in serum than those with AIH. Serum transaminases tend to be higher in AIH, but serum alkaline phosphatase, although mostly elevated in PSC, may be normal in both diseases. Thus, the findings reported suggest that AIH and ASC belong to the same disease process and that they also overlap with PSC. It may, therefore, be speculated that ASC represents a variant of AIH and PSC rather than a distinct disease entity different from these disorders.

### THERAPY

UDCA is widely used in the treatment of PSC, although long-term efficacy remains unproved so far (28–32). In ASC, UDCA therapy has accordingly been performed, but beneficial effects have been documented only incompletely. UDCA has been used in combination with immunosuppressive regimens in ASC (6). A response to immunosuppressive therapy has been documented. Thus, UDCA in combination with an immunosuppressive regimen may be an adequate medical treatment for most patients with ASC.

### AUTOIMMUNE CHOLANGITIS

AIC (synonymous with AMA-negative PBC) was defined as an idiopathic disorder of unknown cause that shares many features with PBC including the female preponderance, typical symptoms of fatigue and pruritus, cholestatic serum enzyme pattern, and characteristic “florid lesions” of small ductules leading to fibrosis and cirrhosis of the liver in the long term (33). However, AIC, by definition, is AMA negative and typically presents with antinuclear antibodies (ANAs) with or without SMAs. AIC lacks uniform diagnostic criteria. The first report of patients with AIC was published in 1987 (34) when three women with “immunocholangitis” were described who presented with typical signs and symptoms of PBC but were AMA negative and ANA positive and responded to immunosuppressive therapy. Since this first report, there has been debate whether PBC and AIC are separate entities (35,36) or variants of one single disease differing only in their pattern of associated autoantibodies (37–41).

A recent study supports the latter view: 30 patients who fulfilled criteria for the diagnosis of PBC but who tested negative for AMA by immunofluorescence (thus these patients would be classified as AMA-negative PBC, or AIC) were then screened with a newly developed recombinant enzyme-linked immunosorbent assay (ELISA) that detected autoantibodies directed against human E2 members of the 2-oxo-acid

dehydrogenase complex family. Twenty-two of the 30 patients (73%) tested positive for this new AMA-M2 recombinant assay, whereas none of 316 controls were reactive (42). Thus, these data suggest that most “AIC patients” suffer from PBC. The development of sensitive AMA-M2 assays appears crucial for making an exact diagnosis in these cases.

AIC has also been discussed as a variant of AIH (3,43), a hybrid of PBC and AIH (44), and a result of consecutive occurrence of PBC and AIH (4).

Autoantibodies to carbonic anhydrase have been identified in patients with AIC and were proposed as markers of differentiation between AIC and PBC (45). However, the specificity of these markers for AIC was later questioned (46).

Antilactoferrin antibodies were also identified in patients with AIC, and lactoferrin was discussed as a potential target antigen in AIC (47). However, antilactoferrin antibodies were recently identified in AIH (25%), PBC (25%), PSC (29%), and AIC (35%) at similar rates and are thus not specific (48). A comparative study of antibody expression in PBC and AIC using phage display strongly supported the view that similar autoimmune targeting directed toward the E2 subunit of the mitochondrial pyruvate dehydrogenase complex (PDC-E2) occurs in PBC and AIC (49).

Clinical assessment of a cohort of patients with AIC (defined by [1] ANA and/or SMA seropositivity and/or hypergammaglobulinemia; [2] absence of AMA by immunofluorescence; [3] biochemical and/or histological features of cholestatic and hepatocellular injury; and [4] exclusion of chronic viral, metabolic, or toxic liver disease) revealed higher serum levels of AST and lower levels of IgM than in PBC patients (50). AIC was distinguished from PSC by a female preponderance, lower levels of alkaline phosphatase, higher frequency of autoantibodies, HLA associations similar to AIH, and absence of inflammatory bowel disease (50). It was concluded that AIC may comprise various forms of diverse conditions including atypical PBC, small duct PSC, idiopathic adulthood ductopenia, and transitional stages of the classic diseases, or a separate entity with varying manifestations (50).

### THERAPY

No controlled trials have been performed in patients with AIC. Treatment with UDCA at doses identical to those administered in PBC (13–15 mg/kg/d) appears justified. However, results were poor in part, as were those after treatment with corticosteroids (50).

### AIH-AIC OVERLAP SYNDROME

Concomitant features of AIH and AIC have been reported. A recent case report described a woman with mixed biochemical and histological features of AIH and AIC including hepatitic and cholestatic biochemical changes, interface hepatitis, and bile duct lesions with portal granulomata and bile duct proliferation. The patient was AMA negative. She responded to combined treatment with UDCA, prednisone, and azathioprine (51).

Histologic specimens of patients with AIH were studied for the presence of bile duct injury, which is regarded as atypical

of AIH (2). Surprisingly, 24% of patients showed features of bile duct injury, although they otherwise fulfilled all criteria for the diagnosis of AIH (52). Thus, the diagnosis of an AIH-AIC overlap syndrome cannot be based on the presence of bile duct injury only (except for granulomatous lesions, which are not described in AIH) when other features are compatible with the diagnosis of AIH.

## CONCLUDING REMARKS

Autoimmune hepatopathies and their variant forms are still incompletely defined. Clear-cut diagnostic criteria are only available for AIH (2), but not for the other major entities, PBC and PSC, although the presence of AMA-M2 and typical cholangiographic features, respectively, are well-accepted hallmarks of these disorders. Therefore, definition of variant forms of the major autoimmune hepatopathies is even more difficult. Treatment of variant forms is not validated and is based on therapy of their parent disorders (AIH, PBC, and PSC). UDCA and immunosuppressive drugs are cornerstones for the actual therapy of variants of autoimmune liver disease. A deeper insight into the pathogenesis of these autoimmune liver diseases is needed for the development of more adequate treatment options.

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# 23 Animal Models of Autoimmune Liver Diseases

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MARKUS BIBURGER AND GISA TIEGS

## KEY POINTS

- The term *autoimmune liver diseases* comprises three types of putative autoimmune disorders, namely, autoimmune hepatitis (AIH), primary biliary cirrhosis (PBC), and primary sclerosing cholangitis (PSC), along with their overlap syndromes. The etiology of these diseases is unresolved.
- Existence of a genetic predisposition, presence of autoantibodies, and association with other autoimmune diseases substantiate the classification of these diseases as autoimmune disorders. However, this classification is still under discussion, especially for PSC.
- Many important insights into the pathogenesis and mechanisms of liver injury in these diseases have been gained from studies in patients, but for several reasons (e.g., the relatively long time between the initial triggering events that cause the onset of these disorders and presentation of patients with clinical symptoms) our understanding of the pathogenesis is incomplete.
- Animal models allow the analysis of singular aspects or complex immunopathogenic mechanisms of disease initiation and progression; however, there is no animal model that perfectly emulates all aspects of any of these disorders.
- Animal models for AIH, inflammation, and liver injury that are reminiscent of several aspects of autoimmune diseases can be induced either by immunization with liver homogenate or DNA encoding human antigens of AIH-associated autoantibodies or by injection of T-cell mitogens like ConA or NKT-cell activating  $\alpha$ -galactosylceramide. The environmental toxicant trichloroethylene induces autoimmune hepatitis by acceleration of the innate response in autoimmune-prone mice.
- Models addressing disturbances of self-tolerance in AIH were established with neonatally thymectomized mice as well as knockout mice lacking TGF- $\beta$  or the autoimmune regulator AIRE.
- Animal models based on immunization with carbonic anhydrase-II (CA-II) or the E2 component of pyruvate-dehydro-

genase complex (PDC-E2), antibodies to which have been reported in patients with PBC, as well as immunization with isolated biliary epithelial cells, were established for an increased comprehension of PBC. In PBC, activated cross-reactive B cells may support priming of self-antigen-specific T cells and thereby contribute to pathogenesis.

- In animal models for sclerosing cholangitis, experimental injury of cells of the biliary ducts or hepatic arteries, e.g., induced by toxins or haptens, reveal insight into aspects of bile duct damage. Models based on induction of small bowel bacterial overgrowth or experimental colitis highlight the likely contribution of bacterial cell wall products and consequent induction of immunological responses, especially of the innate immune system, in PSC.

## INTRODUCTION

### AUTOIMMUNE LIVER DISEASES IN HUMANS

The term *autoimmune liver diseases* in its common use comprises three types of disorders, namely, autoimmune hepatitis (AIH), primary biliary cirrhosis (PBC), and primary sclerosing cholangitis (PSC), as well as their overlap syndromes. Previous reports (1,2) indicated an incidence (identified patients) of 1 to 2:10.000 for AIH, 1:10.000 for PBC, and 0.5:10.000 for PSC. However, there is a pronounced probability for significantly higher case numbers of unidentified autoimmune liver diseases owing to low severity of symptoms in the early stages, especially for PBC and PSC and also most of AIH cases. There is a predisposition of women to the development of AIH (3–4:1 female/male patients) and PBC (9:1), whereas PSC predominantly affects men (1:2).

Because AIH (1) is significantly associated with an increased incidence for other autoimmune-diseases, (2) is frequently accompanied by circulating autoantibodies, and (3) responds well to immuno suppressive treatment, have AIH is classified as an autoimmune-disorder beyond dispute. Some of these aspects, such as association with other autoimmune diseases, are also relevant for PBC and PSC, and the frequent presence of autoantibodies has been affirmed at least for PBC, thereby legitimizing the notion that these disorders are regarded as autoimmune diseases as well.

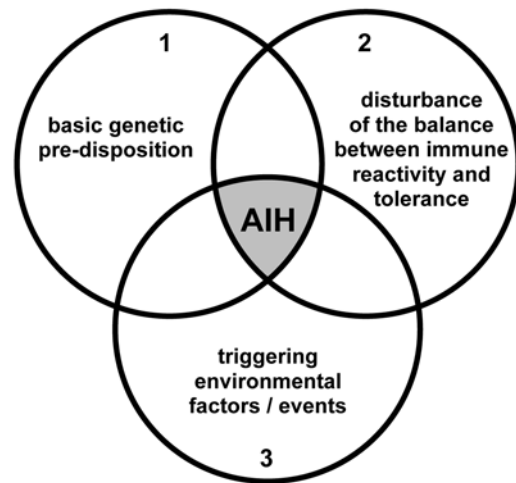
## AUTOIMMUNE HEPATITIS

AIH is an idiopathic disorder that leads to cirrhosis. Most strikingly, AIH is characterized by hypergammaglobulinemia, high titers of a wide range of circulating autoantibodies, a genetic predisposition, and a striking response to immunosuppressive therapy (reviewed in refs. 3 and 4). Besides the gender-associated prevalence mentioned above, there is also a genetic predisposition with respect to certain haplotypes of human leukocyte antigens since most patients are positive for HLA-DR3, HLA-DR4, and HLA-B8. Also, a silent gene at the C4A locus with consequent partial deficiency of the complement component C4 is associated with AIH (4).

In the vast majority of cases, antibodies against autoantigens are found in the blood and are used for classification and diagnosis of AIH. These characteristic autoantibodies are antinuclear antibody (ANA), smooth muscle antibody (SMA), and liver-kidney microsomal (LKM) antibodies, which mainly are directed against cytochrome P450 (CYP) 2D6 and UDP-glucuronosyl-transferases, as well as antimitochondrial antibody (AMA) and antibodies that recognize a soluble liver antigen (SLA) or the liver-pancreas (LP) antigen (4,5). Moreover, circulating T lymphocytes and T-cell clones of liver biopsies of patients with AIH were shown to recognize the human asialoglycoprotein receptor (hASGPR) (6), and circulating anti-ASGPR autoantibodies were closely associated with AIH (7). It has been demonstrated that most of the T cells that infiltrate liver tissue are of the CD4<sup>+</sup>/CD8<sup>-</sup> phenotype (6,8). Interestingly, CD4<sup>+</sup> T-cell clones expanded from blood or liver tissue of AIH patients that recognize major epitopes of the LKM-1 antigen reacted in a HLA class II-restricted manner and secreted large amounts of interferon- $\gamma$  (IFN- $\gamma$ ), also providing evidence for a significant Th1 response in AIH (9). In contrast to the predominance of CD4<sup>+</sup>/CD8<sup>-</sup> and the occurrence of CD8<sup>+</sup>/CD4<sup>-</sup> T cells in liver tissue of patients with autoimmune liver disease, natural killer (NK) cells seem to be infrequent (8).

Another feature of AIH is the development of an antigen-specific and-nonspecific immunosuppressive state in the phase of spontaneous remission (10). As just mentioned, chronic AIH responds to immunosuppressive treatment. Actually, this was the first chronic liver disease in which medical therapy was associated with prolonged survival. The standard treatment of AIH is monotherapy with prednisone or combination therapy with prednisone and azathioprine. If this treatment fails or causes drug intolerance, alternative immunosuppressive therapies with second-generation corticosteroids, calcineurin inhibitors, or others are possible (5).

There is an ongoing discussion of whether AIH might not be a single disorder but rather comprise a heterogeneous group of disease entities, probably also with different etiologies. Current opinion suggests that AIH develops on the basis of genetic predisposition like sex and the presence of certain HLA haplotypes, under the influence of one or probably several environmental factors like infectious agents or toxic substances (3). Indeed, several drugs or chemicals were shown to induce hepatitis with autoimmune involvement, e.g., tienilic acid,



**Fig. 1.** The cooperation of several factors may be necessary to culminate in the onset of autoimmune hepatitis (AIH).

dihydralazine, and halothane (4). Moreover, AIH is frequently associated with other autoimmune disorders including autoimmune thyroid disease, synovitis, ulcerative colitis, and also other autoimmune liver diseases, i.e., PBC and PSC (5). This indicates that basic genetic aberrations may cause impairment of the immune-modulatory systems that in healthy individuals maintain self-tolerance even under conditions of pronounced immune activation by environmental factors (Fig. 1). This detrimental cooperation of three types of factors, i.e., basic genetic predisposition, a disorder disrupting the balance between immune reactivity and tolerance, and finally environmental factors may result in the onset of autoimmune hepatitis (Fig. 1) and may (perhaps in a modification of this scheme), also account for PBC and PSC.

## PRIMARY BILIARY CIRRHOSIS

PBC is an autoimmune disease of the liver marked by the slow progressive destruction of the small septal and intrahepatic bile ducts, associated with an infiltration of plasma cells and lymphocytes in the portal tracts. Damage of these bile ducts leads to cholestasis, which, over the years causes fibrosis, cirrhosis, and ultimately liver failure. For differential diagnostics, circulating AMAs are considered an important characteristic of PBC, since they are present in 95% of patients with this disease. These AMA autoantibodies have also been suggested to play a pathogenic role in PBC. In addition, the infiltration of autoreactive T cells—highly enriched with CD8<sup>+</sup> T cells compared with peripheral blood—into the liver suggests a functional role of T cells in the pathology of PBC. Like AIH, susceptibility to PBC also appears to be linked to genetic factors. An increased risk for PBC development has been reported to be associated with HLA haplotypes like DR8, DR3, and possibly DR4 in Caucasians and DR2 in Japanese patients. In addition, tumor necrosis factor (TNF) polymorphisms (11) and cytotoxic T-lymphocyte antigen (CTLA-4) exon-1 polymorphism (12) have been linked to susceptibility to PBC development.



### PRIMARY SCLEROSING CHOLANGITIS

PSC is a chronic cholestatic liver disease characterized by periductal inflammation of both intrahepatic and extrahepatic bile ducts. Progressive loss of bile ducts impairs bile flow, ultimately resulting in liver cirrhosis. One hallmark of this disease is the concomitance of inflammatory bowel disease, which is diagnosed in 71% of PSC patients (13).

Association with different HLA haplotypes has been reported, indicating genetic predisposition for disease susceptibility. However, in contrast to other autoimmune liver diseases, PSC predominantly affects males, and disease-specific autoantibodies are absent. In fact, there is an association of PSC with the occurrence of perinuclear antineutrophil cytoplasmic antibodies (p-ANCAs). P-ANCAs are found in nearly 9 of 10 patients with PSC. However, these antibodies are not disease specific, since they are also often found in AIH type 1 patients and those with ulcerative colitis, as well as in some PBC patients. Therefore, the assessment of PSC as an autoimmune disease is still under discussion. Also, alternative processes without relation to autoimmunity (like the presence of some tumor entities, toxins affecting the biliary system, or arterial ischemia) induce symptoms reminiscent of PSC.

### ANIMAL MODELS OF AUTOIMMUNE LIVER DISEASES

According to the dimensions of this field of research, the number of animal models that address important questions regarding mechanisms of onset and progression of autoimmune liver diseases exceeds the number of models that can be described here. Thus, unfortunately, many excellent reports could not be included in this article.

#### ANIMAL MODELS OF AIH

Although immunosuppressive treatment shows high survival rates, permanent remission is low, and glucocorticoids as well as azathioprine may cause severe adverse reactions (5). Therefore, experimental models of AIH were established to investigate the underlying immunological mechanisms and to test the efficacy of new immunosuppressive drugs.

**Experimental AIH** As early as 1974, Hopf and Meyer zum Buschenfelde demonstrated the induction of chronic hepatitis and immunoglobulin-binding cell-surface membranes of hepatocytes upon repeated immunization of rabbits with crude liver antigen preparations (14,15). These preparations, referred to as liver-specific lipoprotein (LSP), contained plasma membrane antigens isolated from whole-liver homogenates and thus contained some liver-specific antigens. Repeated immunization of SMA mice with whole-liver homogenates or LSP caused development of liver-specific antibodies, chronic portal infiltration containing lymphocytes and plasma cells, and in some mice moderate to severe interface hepatitis. In addition, transfer of spleen cells from diseased mice induced hepatic inflammation in nonimmunized mice (16).

In 1990 Lohse et al. described an experimental autoimmune hepatitis (EAH) model that was inducible by intraperitoneal immunization of C57BL/6, Balb/c, and C3H/He-mice as well as Lewis rats with syngeneic soluble liver antigens, i.e., liver

homogenate centrifuged at 100,000g (S-100) in complete Freund's adjuvant (17). EAH exhibited a chronic time course, it could be passively transferred with concanavalin A (ConA)-activated splenocytes, and a specific proliferative response to S-100 could be demonstrated in spleen cells from EAH mice. Autoantibody production was demonstrated in this model but seemed not to play a critical role for disease induction and did not recognize autoantigens that are characteristic of AIH in humans (18). Also, two other criteria of AIH could be demonstrated in this model, i.e., induction of an immunosuppressive state to specific and unrelated antigens (19) and different susceptibility of various mouse strains that may be regarded as an exemplary analogy to genetically determined differences in predispositions to AIH development in humans: C57BL/6 mice, were found to be more susceptible than Balb/c and C3H/He mice, and no onset of EAH was found in Lewis rats (17). Interestingly, a similar graduation of disease susceptibility for these mouse strains had been found in earlier experiments by Mori et al. in which immunization of the same three strains of mice with syngeneic crude liver proteins produced prominent liver changes histologically mimicking human hepatitis in the livers of C57BL/6 mice. In addition, autoantibody against LSP was found in the serum of immunized C57BL/6 mice but not in the sera of other strains after immunization.

#### Trichloroethylene-Promoted Hepatitis in MRL Mice

Recently, a CD4<sup>+</sup> T-cell-dependent AIH has been described in genetically predisposed mice, i.e., in the autoimmune prone MRL<sup>+/+</sup> strain, upon injection of the environmental toxicant trichloroethylene (20). Here, trichloroethylene accelerated the innate autoimmune response in these mice. Autoimmune hepatitis in this model was associated with a significant increase in serum ANA levels and a dose-related increase in the percentage of activated CD4<sup>+</sup> T cells in both spleens and lymph nodes of mice, which produced inflammatory or Th1 cytokines. Following trichloroethylene treatment, a significant increase in portal mononuclear infiltration was also detected. The alterations in CD4<sup>+</sup> T-cell response depended on hepatic metabolism of the toxicant (21). This model may be regarded as an example for the hypothesis of induction of AIH in genetically susceptible individuals probably already suffering from underlying diseases.

**The Concanavalin A Model** Con A is a mitogenic plant lectin that is widely used to activate T lymphocytes in vitro. It binds mannose residues of many different glycoproteins and thus pan-activates lymphocyte populations largely irrespective of their antigen specificity. Upon injection into mice (22) or rats (23), ConA induces acute (22,23) or chronic (24) inflammatory liver injury. The acute inflammatory liver injury induced in mice by a single intravenous ConA injection in doses of 10 to 20 mg/kg (25) has been shown to depend on the activation of T cells and macrophages (25) and is associated with the release of transaminases and intrahepatic DNA fragmentation within 8 h (25,26). After 24 h transaminase levels start to decline, and liver regeneration becomes evident (27,28).

The cytokines that mediate ConA-induced hepatitis have been clearly identified to be TNF- $\alpha$  in its soluble and membrane-bound

precursor form (29–35), IFN- $\gamma$  (36–39), as well as the IFN- $\gamma$  inducing cytokines interleukin-12(IL-12) (40) and IL-18 (35), and macrophage inflammatory protein-2 (MIP-2) (41), whereas IL-6 family members, e.g., IL-6 (29,37,42) and IL-11 (43), as well as IL-10 (44–46), seem to be protective. Moreover, nitric oxide (NO), derived from inducible nitric oxide synthase (iNOS), was shown to mediate ConA-induced liver injury (47,48). The intrahepatic macrophages, i.e., the Kupffer cells, were determined to contribute to ConA-induced hepatitis by providing the central mediator TNF- $\alpha$  (49), which induces caspase-3- and caspase-8-independent and probably c-Jun N-terminal kinase (JNK)-dependent liver cell damage (50,51). The T cells that mediate ConA hepatitis probably belong to a T-lymphocyte subset that is part of the innate immune response, i.e., the natural killer T (NKT) cells, which were suggested to contribute to liver damage by a Fas-mediated mechanism in this model (52–54). NKT cells represent a subpopulation of mature T cells that express NK surface markers such as NK1.1, IL-2R $\beta$ , (and to some extent Ly49A and Ly49C) and reveal the Thy1<sup>high</sup>, CD44<sup>high</sup>, CD45RB<sup>high</sup> phenotype of activated T cells (defined in C57BL/6 mice; see ref. 55). Most murine NKT cells bear a T-cell receptor (TCR) with invariant V $\alpha$ 14-J $\alpha$ 281 TCR $\alpha$  chain and a restricted TCR $\beta$  repertoire, recognizing glycolipids presented by the MHC class I-like molecule CD1d, which is essential for development of invariant NKT cells of thymic origin. Liver and thymus are enriched with CD1d-restricted NKT cells, most of which are CD4<sup>+</sup> and to a lesser extent double negative. Several experimental results suggest that NKT cells are key effector cells in ConA-mediated liver damage including the fact that absence of NKT cells by depletion (52). Or knockout of the V $\alpha$ 14-J $\alpha$ 281 TCR $\alpha$  chain (53), or of CD1d (54) mediates resistance to ConA-induced hepatitis. ConA susceptibility can be restored in V $\alpha$ 14 knockout mice and CD1<sup>-/-</sup> mice by adoptive transfer of functional NKT cells (53,54). Also, V $\alpha$ 14/V $\beta$ 8.2-transgenic RAG<sup>-/-</sup> mice are susceptible to ConA (53), in these mice NKT cell populations are intact, whereas T cells, B, cells and NK cells are missing.

It has been suggested that ConA hepatitis represents a model of AIH, since it matches several criteria of AIH and EAH (Table 1). Although ConA is definitely not an autoantigen, and autoantibody production has never been reported, ConA hepatitis depends on the activation of thymus-dependent T lymphocytes, in particular on CD4<sup>+</sup> T cells (25), which are the predominant infiltrating T-cell subpopulation (29,56), and Th1 (IL-2, IFN- $\gamma$ , and TNF- $\alpha$ ) as well as Th2 cytokines (IL-4 and IL-10) are detectable in plasma of injured animals (25,30,36,57). Especially in a model of chronic ConA hepatitis, induced by repeated ConA injections, the initial Th1 response shifts to a Th2 and profibrogenic cytokine response, with IL-10, IL-4, and transforming growth factor- $\beta$  (TGF- $\beta$ ) being the main cytokines expressed in liver tissue (58). Moreover, it has been shown that ConA hepatitis can be transferred with liver-infiltrating mononuclear cells from ConA-treated Balb/c mice to ConA-treated athymic nude mice (56), which are otherwise protected from ConA-induced liver injury

**Table 1**  
**Conformance to Criteria of Autoimmunity in Three Murine Models of Immune-Mediated Hepatitis**

Criteria of autoimmunity	Hepatitis model		
	EAH	ConA	$\alpha$ -GalCer
Antigen-specific T cell response	Yes	No	Yes (glycolipid)
Predominance of CD4 <sup>+</sup> T cells	Yes	Yes	Yes
Passive transfer of disease possible	Yes	Yes	n.t.
Genetic prevalence (strain differences)	Yes	Yes	Yes
Specific production of autoantibodies	Yes (n.r.)	n.t.	Questionable
Immunosuppression in state of remission	Yes	Yes	Yes
Response to immunosuppressive drugs	Yes	Yes	Yes

Abbreviations: n.t., not tested; n.r., not relevant for disease;  $\alpha$ -GalCer,  $\alpha$ -galactosylceramide, ConA, concanavalinA, EAH, experimental determined hepatitis.

(25,56). Also, strain differences with respect to susceptibility have been observed (22,59), with C57BL/6 mice being the most susceptible of the strains tested.

Immunosuppression in a state of remission/regeneration, i.e., 48 h after ConA administration (27,60), with respect to thymus-dependent antibody response, as seems to occur in AIH and EAH, has been reported (60–62), and, 1wk after a first ConA treatment, mice develop tolerance against immune-mediated liver injury upon ConA rechallenge (63). Last but not least, ConA hepatitis highly responds to immunosuppressive treatment such as glucocorticoids, cyclosporin A, and tacrolimus (25,29), and a plethora of new antiinflammatory and immunosuppressive drugs has been tested in this model (33,47,48, 57,64–74). When the data are taken together, ConA hepatitis matches several criteria of AIH, but it is not a model of AIH in the strict sense because (in contrast to EAH, which is inducible by (auto)antigens in syngeneic liver homogenate one remarkable hallmark of autoimmunity, i.e., the existence of an autoantigen, is missing. However, ConA seems to mimic immune reactions that may also be evoked by an autoantigen.

**The  $\alpha$ -Galactosylceramide Model** In 1995, Kobayashi et al. described the immunostimulating properties of  $\alpha$ -galactosylceramide ( $\alpha$ -GalCer, or KRN7000), a novel synthetic compound corresponding to a glycolipid derived from a marine sponge (75). Although this compound had originally been developed and proved to be effective as an antitumoral agent in animal cancer models, it was also found to induce moderate liver injury in mice (76,77). Detectable serum transaminase activities upon  $\alpha$ -GalCer injection into mice is preceded by rapid and pronounced expression of a number of cytokines including IL-2, IL-4, IL-6, TNF- $\alpha$  and IFN- $\gamma$ , as found in plasma and mRNA levels in liver tissue (78). This two-edged cytokine response, with expression of both the Th1 and Th2

cytokines IFN- $\gamma$  and IL-4, is a well-known effect of NKT cell activation. Through the use of neutralizing antibodies, TNF- $\alpha$  was identified as an important mediator for hepatic injury in this model that increased Fas ligand expression on NKT cells (78,79), whereas IFN- $\gamma$  did not account for hepatitis induction (78,80).

In contrast to ConA hepatitis, which is probably not induced by an (auto)antigen and thus lacks one central hallmark of autoimmunity,  $\alpha$ -GalCer can be considered a surrogate antigen for natural autoantigens that may be presented to NKT cells by CD1d (Table 1). The HLA-associated differences in genetic prevalence of AIH development in humans might be regarded as a hint for the importance of MHC-mediated antigen presentation, at least in some forms of AIH.  $\alpha$ -GalCer is strictly dependent on presentation of the MHC-homologous CD1d molecule and exerts its activating function via antigen-specific TCR recognition. The criteria of a prevalent role of CD4<sup>+</sup> lymphocytes in pathogenesis is also seen in  $\alpha$ -GalCer hepatitis, since intrahepatic NKT cells, activated by this surrogate antigen, are predominantly CD4<sup>+</sup> or, to a lesser extent, double negative. Passive transfer of disease with mononuclear cells from the liver has been shown for EAH and for ConA hepatitis but not yet for  $\alpha$ -GalCer hepatitis. Strain differences, which may reflect the genetic prevalences that are typical of autoimmune disorders are also present in  $\alpha$ -GalCer hepatitis, in which C57BL/6 mice reveal a higher susceptibility to  $\alpha$ -GalCer-induced liver injury than Balb/c mice (78). In the latter strain, also both  $\alpha$ -GalCer-induced TNF- $\alpha$  expression and FasL expression on NKT cells are less pronounced than in C57BL/6 mice (78). Specific production of autoantibodies, a characteristic of humoral autoimmunity, has not been found in  $\alpha$ -GalCer hepatitis. However, it is worth mentioning, that  $\alpha$ -GalCer-induced autoantibody production has been observed in a murine model of lupus, another autoimmune disorder (81).

Induction of an immunosuppressive state in the phase of remission is another feature of AIH.  $\alpha$ -GalCer treatment also induced a state of immunosuppression, since NKT cells from mice pre-challenged with  $\alpha$ -GalCer in vivo showed little cytokine production and reduced proliferation in vitro (82) and,  $\alpha$ -GalCer pretreatment of C57BL/6 mice results in tolerance against NKT-mediated liver injury upon  $\alpha$ -GalCer rechallenge (83). Since anti-TNF- $\alpha$  treatment—which protects from  $\alpha$ -GalCer-induced liver injury, as described just above (78,79) can be regarded as an antiinflammatory approach, it can be stated that  $\alpha$ -GalCer hepatitis responds to immunosuppressive treatment as well and thus fulfills another hallmark of AIH. As mentioned above,  $\alpha$ -GalCer can be considered a surrogate for a physiologic antigen and—upon presentation to antigen-specific TCRs—induces onset of liver injury in a manner that might bear resemblance to activation of autoreactive T cells in AIH. Thus,  $\alpha$ -GalCer-mediated liver injury may be regarded as a murine model for AIH (Table 1).

**AIH Induction by DNA Immunization** Recently, Lapierre et al. presented a murine model for type 2 AIH characterized by the presence of anti-LKM-1 and anti-liver cytosol type 1 (anti-LC-1) autoantibodies in humans. Based on their

previous findings that the targets of LKM-1 and LC-1 antibodies are cytochrome P450 2D6 (CYP2D6), and formiminotransferase cyclodeaminase (FTCD), respectively, they developed a model of AIH induction by xenoimmunization with these human antigens (84). DNA immunization of C57BL/6 female mice with a pCMV plasmid containing the extracellular region of mouse CTLA-4 (as a secretory signal and immunological modulator) and the antigenic regions of human CYP2D6 and human FTCD caused elevated alanine aminotransferase (ALT) levels, with peaks at 4 and 7 mo post injection as well as periportal, portal, and intralobular liver inflammatory infiltrates causing intrahepatic accumulation of mainly CD4<sup>+</sup> lymphocytes, but also CD8<sup>+</sup> and B lymphocytes. In addition, immunized mice not only developed antibodies against the xeno antigens but also developed autoantibodies against mouse antigens (anti-mouse LKM-1 and anti-mouse LC-1). Thus, DNA immunization against human autoantigens is able to break tolerance in mice and to induce an autoimmune liver disease.

The authors interpret the liver injury in this model to be caused by molecular mimicry between foreign and self-antigens. Such molecular mimicry had been analyzed by the same group by investigating the potential of infectious agents to initiate autoreactivity through molecular mimicry. In these experiments, transgenic mice expressing lymphocytic choriomeningitis virus nucleoprotein (NP) developed liver injury when vaccinated with plasmids expressing NP as an intracellular or secretory protein (85). CTLs were found to be activated in peripheral lymphoid organs by DNA vaccination; they then migrated to the periportal and lobular areas of the liver, where their presence was associated with a significant degree of cytolysis. This suggests that local injury may not be essential to initiate autoreactivity and onset of AIH.

In a follow-up study to the CYP2D6/FTCD xeno-immunization mentioned above, this model was extended to different mouse strains, i.e., C57BL/6, 129/Sv, and BALB/c, to assess the potential contribution of MHC and non-MHC genes, taking advantage of their different genetic configuration with regard to MHC and non-MHC genes (86). All mice revealed increased IgG levels. However, whereas C57BL/6 mice (MHC: H-2<sup>b</sup>) showed elevated serum ALT levels, autoantibodies, antigen-specific CTCS, and lobular and periportal inflammatory infiltrate upon DNA immunization, 129/Sv (MHC: H-2b) mice showed slightly elevated ALT levels and sparse liver lobular infiltrate and CTCS and BALB/c (MHC: H-2<sup>d</sup>) mice did not develop liver inflammation. This suggests that a class II MHC haplotype (H-2<sup>b</sup>) is permissive but not sufficient for the development of outright AIH in this model.

**Transgenic Models** Like models of AIH induction by DNA immunization, several models using transgenic mice have also revealed important insights into aspects of AIH induction by breaking of tolerance, and immune activation at extrahepatic sites, and bystander killing of hepatocytes.

Upon adoptive transfer of H-2 Kb-specific TCR transgenic T cells into transgenic 178.3 mice ubiquitously expressing H-2 Kb antigen, Bowen et al. detected rapid and selective accumulation of transgenic CD8<sup>+</sup> T cells in the liver of intact

recipients despite ubiquitous expression of the respective antigens (87). T cells retained in the liver underwent activation and induced transient hepatitis. Similar results were obtained if bone marrow chimeras in which 178.8-derived bone marrow was used for reconstitution of non-transgenic mice. Since in the livers of these bone marrow chimeras not hepatocytes but only intrahepatic bone marrow-derived cells express the antigen, this suggests that intrahepatic accumulation, activation of T cells, and subsequent hepatitis do not depend on antigen expression by hepatocytes. This “bystander hepatitis” was found to be dependent on TNF- $\alpha$  and IFN- $\gamma$ .

Voehinger et al. injected TCR-transgenic T cells that recognize the CTL epitope GP33 of the lymphocytic choriomeningitis virus glycoprotein into ALB1 transgenic mice, which express this antigen nearly exclusively in the liver under control of the mouse albumin promoter (88). However, TCR-transgenic cells ignored the GP33 transgene expressed in hepatocytes of the ALB1 mice. This ignorance of adoptively transferred TCR-transgenic cells in ALB1 mice was broken if the recipient mice were infected with lymphocytic choriomeningitis virus. In this setting injected GP-33-specific TCR-transgenic T cells induced hepatitis in ALB1, but not in control mice.

Taken together, the reports using DNA immunization or transgenic animals suggest that T cells recognizing antigens expressed by hepatocytes will not induce hepatitis and hepatocyte damage as long as no other additional events occur that will abrogate tolerance to these antigens. In addition, activation of CTLs does not have to take place in the liver itself but can be triggered in extrahepatic peripheral lymphoid organs, with hepatitis being induced by activated CTLs that subsequently migrate to the liver. Moreover, activated CTLs may also cause “innocent bystander” damage of hepatocytes upon accumulation in the liver, even if the antigens recognized by these CTLs are not expressed on hepatocytes.

**Models with Physiologically/Genetically Modified Mice Drug/Immunization-Induced Hepatitis Models** In the models just mentioned above autoimmune(-like) hepatitis is induced by triggering of immune-activating processes (be they antigens or pharmacologic substances).

These models are valuable for improved comprehension of the active immunological mechanisms that induce and promote liver injury and support the hypothesis of an increased risk for induction of AIH in individuals owing to a primary genetic predisposition. In many of these models, there is a clear association of the genetic background, i.e., the respective mouse strain, with susceptibility to onset and/or severity of hepatitis. However, with the exception of the trichloroethylene-promoted hepatitis in autoimmune-prone MRL mice, none of these models provides a hint for the involvement of actual defects that may cause the disruption in balance between immune reactivity and tolerance and thus facilitate the loss of self-tolerance. Animal models in which autoimmune hepatitis occurs owing to defined alterations may shed light on this latter aspect.

**Models with Physiologically/Genetically Modified Mice Neonatal Thymectomy** In 1987 Watanabe et al. found that immunization of A/J mice with syngeneic crude liver proteins induces pathologic liver changes that are more pronounced in thymectomized mice than in the nonthymectomized controls (89). Both groups revealed anti-LSP autoantibodies, but their levels were higher in the thymectomized mice. In adoptive transfer experiments, spleen cells of neonatally thymectomized mice showed reduced suppressor activity to LSP compared with control mice. The authors suggested that neonatal thymectomy abolishes tolerance to LSP owing to suppressed thymic suppressor activity toward autoantigen and that this may result in aggravated liver damage.

In neonatally thymectomized mice, a regulatory factor might be missing that modulates development of autoantibodies, since a monoclonal antibody named LSA-1 could be derived from splenic B cells of a thymectomized Balb/c mouse. LSA-1 reacted with LSP and hepatocyte surface membranes (90). LSA-1 recognized an antigen that is at least to some degree liver specific, facilitated complement-mediated lysis of hepatocytes in vitro, and induced liver injury upon intravenous injection. This suggested a potential role for autoreactive antibodies in necrotic damage of hepatocytes in AIH patients.

However, the time point of thymectomy appears crucial in corresponding AIH models, since Myozaki et al. reported AIH and occurrence of liver-specific autoantibodies in C3H/HeN mice that were thymectomized 2 d after birth but not in those that underwent thymectomy on d 7 after birth. Some of the early thymectomized mice but none of the other group revealed hepatic inflammation and mononuclear cell infiltration in the portal area. Serum levels of autoantibody level to crude liver proteins in mice with hepatitis was higher than these in mice without hepatitis and reacted with several antigens in liver protein preparations.

**Knockout of Autoimmune Regulator Type 1** A model for defects in immunoregulatory genes may be deficiency of the gene for autoimmune regulator type 1 (AIRE-1). Mutations in the autoimmune regulator (*aire*) gene cause the recessively inherited disorder called autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) or autoimmune polyendocrinopathy syndrome type 1 (APS-1). Patients with this disease suffer from a number of organ-specific autoimmune diseases that can also be associated with AIH. AIRE is involved in the expression of ectopic proteins by medullary thymic epithelial cells. This allows the development of central tolerance and contributes to the prevention of organ-specific autoimmunity (reviewed in refs. 91–93). One characteristic of APS-1 is a high variability in the number and character of the disease components in the corresponding patients.

Recently, Jiang et al. (94) analyzed the effect of loss of function mutations in the AIRE gene, which causes a defect in the clonal deletion of autoreactive thymocytes in several mouse strains. Therefore, this AIRE knockout mutation was back-crossed to mice of diverse genetic backgrounds.

In addition to disease development in other organs, AIRE deficiency also caused autoimmune liver damage in 40% of Balb/c mice and more than 80% of NOD and SJL mice, but not in C57BL/6 mice. This clearly indicates an important role of AIRE in suppression of autoimmune hepatitis but with strong dependence on the genetic background. However, this genetic background differently affects the relevance of AIRE deficiency for development of autoimmunity in the different organs, since in all four mouse strains, e.g., also in C57BL/6 mice, AIRE deficiency causes inflammation in salivary glands, lung, and prostate. In AIRE-deficient mice autoantibodies were also found with the distribution of their reactivities showing clear strain distinctions that correlated, albeit not completely, with strain-specific patterns of organ infiltration. This influence of the respective genetic background perfectly reflects the variability between individual patients with AIRE impairment.

The authors extended the analysis of AIRE deficiency in mice by crossing these animals with mice carrying an additional defect in central tolerance induction, i.e., deficiency of transcription factor Foxp3, which is required for the generation and activity of the CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells (95). The double-deficient mice had fulminant autoimmunity in very early life and also a markedly shortened life-span in comparison with single-deficient littermates (C57BL/6 background). They showed massive lymphoproliferation and exacerbated inflammatory damage of the liver and also in the lungs. In both Foxp3 deficient and Foxp3-AIRE double-deficient mice, the hepatic portal areas were heavily surrounded by polymorphic mononuclear cells and lymphocytes. Whereas most hepatocytes in Foxp3 single-deficient animals appeared intact, up to 50% of those in their double-deficient littermates were necrotic. Similar disease exacerbation was found upon combination of the *foxP3* null mutation with the genetic background non-obese diabetic (NOD) mice. This indicates that successive undermining of immunologic self-tolerance can exacerbate the severity of autoimmune diseases including AIH.

**TGF- $\beta$ 1 Knockout in Balb/c Mice** Gorham et al. described a mouse model characterized by spontaneous development of necroinflammatory hepatitis in Balb/c mice deficient in the immunomodulatory cytokine TGF- $\beta$ 1 (96). In this model, TGF- $\beta$ 1<sup>-/-</sup> mice were extensively back-bred to the BALB/c background. TGF- $\beta$ 1 is a pleiotropic cytokine that exhibits a variety of antiinflammatory activities and inhibits the development of autoimmune disease in several model systems. TGF- $\beta$ 1 is required for normal immune homeostasis and prevention of autoimmunity, since TGF- $\beta$ 1-deficient mice develop inflammatory lesions involving several organs, i.e., mainly the heart and lungs. The BALB/c background dramatically modified the phenotype of TGF- $\beta$ 1<sup>-/-</sup> mice, with these mice developing lethal necroinflammatory hepatitis on this genetic background (96). Livers of these mice contained large numbers of activated CD4<sup>+</sup> T cells with a Th1 phenotype, as characterized by their production of large quantities of IFN- $\gamma$  but little IL-4. Necroinflammatory liver disease in this model

is CD4<sup>+</sup> T-cell dependent (97). Cross-breeding with IFN- $\gamma$  knockout mice inhibited development of necroinflammatory hepatitis, thereby revealing an indispensable role of this cytokine in the Balb/c TGF- $\beta$ 1<sup>-/-</sup> model.

Interestingly, TGF- $\beta$ 1 deficiency only on the BALB/c background, but not back-crossed to another background (129/CF-1 hybrid) or in TGF- $\beta$ 1<sup>-/-</sup> “donor” mice on C57BL/6 background, uniformly induced an aggressive necroinflammatory hepatitis. This shows that the BALB/c background confers a different organotropism on the inflammatory phenotype associated with the TGF- $\beta$ 1<sup>-/-</sup> defect. In conclusion, this model represents a murine model of spontaneously developing hepatitis that is restricted by genetic background, associated with an immunoregulatory defect and is dependent on the Th1 cytokine IFN- $\gamma$ . Thus, the Balb/c TGF- $\beta$ 1<sup>-/-</sup> model recapitulates central aspects of AIH.

Since TGF- $\beta$ 1<sup>-/-</sup> globally raises the threshold for T-cell activation, one might expect that in Balb/c TGF- $\beta$ 1<sup>-/-</sup> knockout mice, T cells might become aberrantly activated and induce hepatitis in response to signals that ordinarily were not sufficient for T-cell activation. Thus, pathology would be expected to be largely antigen independent, and CD4<sup>+</sup> T cells, regardless of antigen specificity, would become activated in TGF- $\beta$ 1<sup>-/-</sup> mice, with subsequent organ pathology. However, in recent follow-up studies, Gorham and co-workers found that restriction of the CD4<sup>+</sup> TCR repertoire by crossing BALB/c-TGF- $\beta$ 1<sup>-/-</sup> mice with DO11.10 TCR transgenic mice prevents immune pathology (98). This indicates that immune pathology in TGF- $\beta$ 1<sup>-/-</sup> mice is not caused by extensive loss of T-cell regulation but is to an important extent self-antigen triggered. It is worth recapitulating that in most of the AIH models mentioned above in which strain differences were assessed, Balb/c mice belonged to the less susceptible strains for development of AIH. In contrast, necroinflammatory hepatitis by TGF- $\beta$ 1 deficiency is restricted on this background. A summary of these results reveals that no strain can principally be regarded as “liver autoimmunity prone” and that complex influences of MHC and non-MHC genes are responsible for susceptibility to a liver autoimmune inflammation.

#### ANIMAL MODELS OF PRIMARY BILIARY CIRRHOSIS

Studies in patients have provided enormous insights into the pathological processes in PBC, but because of the long time between the key steps in the breakdown of immunological tolerance and presentation of patients, only limited information regarding the mechanism of tolerance breakdown is available from these studies. Therefore animal models have been developed to analyze mechanisms of tolerance breakdown. In addition, researchers tried to clarify the role of AMAS, which are a characteristic of PBC, using animal models, AMA, may precede the clinical, biochemical, and histological features of PBC, suggesting that these antibodies might play a important role in PBC pathogenesis. Biliary epithelial cells (BECS) in PBC, but not in other liver diseases and normal liver, have AMA- recognizable epitopes present on their plasma membrane.

### Immunization With Isolated Biliary Epithelial Cells

Using neonatally thymectomized mice, which are prone to organ-specific autoimmune diseases, Kobashi et al. induced mononuclear cell infiltration consisting of lymphocytes, plasma cells, and macrophages around bile ducts upon repeated immunization with porcine intrahepatic bile duct epithelial cells in Freund's adjuvant (99). Such infiltration was largely absent in neonatally thymectomized control mice that had been immunized with porcine gallbladder epithelial cells, porcine splenocytes, or Freund's adjuvant only, as well as in non-thymectomized mice immunized either with the porcine bile duct epithelial cells or with Freund's adjuvant only. Bile ducts of responsive mice revealed degenerative changes.

Upon immunization of Wistar/HD rats with purified hyperplastic cholangiocytes isolated after bile duct ligation from either syngeneic Wistar or allogeneic Fischer 344 rats, Uedo et al. found histologic evidence of nonsuppurative cholangitis without inflammation in other organs (100). Control rats that had been immunized with bovine serum albumin (BSA) or hepatocytes showed no cholangitis. Portal tract infiltrates around bile ducts consisted of CD3-positive lymphocytes, whereas B cells and exogenous monocytes/macrophages were essentially absent. Nonsuppurative cholangitis could be induced in naïve recipients by transfer of unfractionated ConA-stimulated spleen cells from cholangiocyte-immunized rats but not with cells from BSA-immunized rats. In addition, the authors reported a specific antibody response against cholangiocyte proteins in sera of cholangiocyte-immunized rats.

**Immunization With Carbonic Anhydrase-II** Carbonic anhydrase-II (CA-II), an enzyme that catalyzes hydration of carbon dioxide to bicarbonate and hydrogen ions, antibodies to which have been reported in patients with PBC independent of the presence of AMA, is exclusively expressed in biliary endothelial cells of the liver. Upon repeated intraperitoneal immunization of Balb/c and DBA-IJ mice with CA-II in the absence of adjuvant, Ueno et al. found inflammation that was restricted exclusively to the liver and was associated with T-cell infiltration (predominantly CD4<sup>+</sup>) around bile ducts and lymphocyte invasion between cholangiocytes (101). CA-II immunization significantly increased the frequency of cholangitis in both mouse strains in comparison with to respective control mice immunized BSA. Balb/c mice were more susceptible to disease onset than DBA-IJ mice. Adoptive transfer of spleen cells from CA-II-immunized Balb/c mice resulted in cholangitis in two of three Balb/c recipients.

**Immunization With the E2 Component of Pyruvate-Dehydrogenase Complex** The AMAs are directed at members of the 2-oxo-acid dehydrogenase components of multienzyme complexes in particular, the E2 and E3 binding protein (E3BP) components of the pyruvate dehydrogenase complex (PDC) (102). Autoantibody and autoreactive T-cell responses specific for the self-antigen PDC are almost ubiquitous in PBC patients.

In early immunization experiments, Krams et al. had demonstrated antibody generation to PDC-E2 in BALB/c, AKR/J, C3H/J, and CBA/HeJ mice as well as rats, guinea pigs, rabbits, and rhesus monkeys upon immunization with purified

recombinant human PDC-E2 with species and strain differences appearing in response titers (103). However, these antibody responses were not associated with bile duct lesions. In the light of the results of Jones et al. described just below, this lack of PBC development in spite of antibody generation might be caused by the use of foreign (human) PBC.

To characterize mechanisms of breakdown of tolerance to self-PDC, Jones et al. described a model using SJL/J mice (104), which, comparable to healthy humans, are fully tolerant of self-PDC under normal conditions, mounting neither antibody nor T-cell responses following sensitization with the self-antigen. However, upon immunization with non-self PDC, i.e., in this case bovine PDC (95% sequence identity with the murine homologs), SJL/J mice revealed pronounced B- and T cell responses within several weeks. Sensitized mice produced IgG antibodies that were reactive with both foreign and self-PDC, but splenic T cells from these mice responded to stimulation with foreign PDC but not self-PDC. This suggests that the initial T-cell response following sensitization with non-self-PDC is to non-conserved epitopes, whereas the resulting B-cell response is directed at conserved epitopes. Sensitization with either foreign or self-PDC only caused mild bile duct lesions deficient in CD8<sup>+</sup> T cells.

However, coimmunization with mixed self-PDC and foreign PDC induced the breakdown of self-tolerance, which led to generation of both antibody and T-cell responses to self-PDC. Breakdown of T-cell tolerance was shown by the significant lymphoproliferation and IFN production observed after *in vitro* stimulation of splenic mononuclear cells with self-PDC. Upon this immunization with mixed self-PDC and foreign PDC, bile duct lesions were significantly larger and heavily infiltrated by CD8<sup>+</sup> T cells. Liver-infiltrating T cells derived from mice cosensitized with self-PDC and foreign PDC but not those derived from control animals showed reactivity with self-PDC, suggesting a possible role for autoreactive PDC-specific T-cell responses in pathogenesis. The authors hypothesize that B-cell crossreactivity between foreign and self-PDC enhances the potential for breakdown of T-cell self-tolerance by allowing efficient presentation of self-antigens, either upon uptake of self-antigen by crossreactive B cells after it has been bound by cell-surface immunoglobulin and subsequent efficient presentation of self-epitopes to and priming of potentially autoreactive T cells or upon uptake of self-antigen/IgG-immune complexes by dendritic cells and subsequent T-cell priming.

These results support the hypothesis that breakdown to self-PDC in patients might be caused by immunological coexposure of self-PDC and foreign PDC such as may occur during bacterial infection of patients. To test the validity of this interpretation the authors exposed naïve female SJL/J mice to self-PDC alone and in the presence of purified splenic B cells from animals primed with foreign PDC (or controls) or purified immunoglobulin G from the same animals (105). Breakdown of T-cell tolerance to self-PDC, as assessed by splenic T-cell proliferative response to antigen, was seen in most animals receiving self-PDC together with purified PDC-reactive B cells, but not in animals receiving self-PDC with purified anti-PDC IgG or with B cells from animals sensitized with an irrelevant antigen. This supports the hypothesis that

breakdown of T-cell tolerance to the highly conserved self-antigen PDC may be mediated by high-level presentation of self-derived epitopes by activated crossreactive B cells.

Recently, Palmer et al. provided evidence that exposure to covalently modified self-PDC can, in the correct proimmune environment, replicate the full breakdown of B-cell and T-cell immune tolerance to PDC seen in PBC (106). Immunization of SJL/J mice with a covalently modified (biotinylated) preparation of self murine PDC elicited high-titer, high-affinity antibody responses reactive with both the modified and the native immunogen. In addition, significant MHC class II-restricted splenic T-cell responses to native PDC preparation were found in animals immunized with the biotinylated immunogen. One potential etiological pathway in PBC could thus be the breakdown of tolerance to self-PDC after exposure to self-antigen that has been covalently modified in the metabolically active environment of the liver. In addition to bacterial infection, with immune responses to bacterial PDC as described above, this could be a second mechanism by which PDC tolerance could be overcome and lead to PBC induction caused by self-antigen-specific T cells upon priming with the help of activated crossreactive B cells.

#### ANIMAL MODELS OF PRIMARY SCLEROSING CHOLANGITIS

As for the other autoimmune liver diseases, there is no perfect animal model for PSC that can fully reproduce human PSC cholangitis. However, animal models have addressed several potential triggering events and mechanisms of disease progression. Such models include those involving injury to epithelial or endothelial cells of the bile system, toxic, infectious, or intraluminal injury of the biliary tract, as and bacterial cell wall constituents or colitis.

Biliary epithelial cells (BECs) have been regarded as targets of the immune response in vanishing bile duct syndromes (107). However, BECs express neither class II HLA nor intercellular adhesion molecule-1 (ICAM-1). This rather disqualifies these cells as professional antigen presenters and CTL targets, and it is unlikely that these cells are the primary targets in PSC. In addition, lesions that are characteristic of PBC and would be expected upon immunological damage are rarely found in PSC. Thus, in this regard experimental induction of graft-vs-host diseases (GVHDs) in which nonsuppurative destructive cholangitis is typical, reveals a significant deviation from human PSC. However, upon induction of experimental GVHD across minor histocompatibility antigens by injecting spleen and bone marrow cells (9:1) of congenic B10.D2 mice into sublethally irradiated BALB/c mice, Nonomura et al. observed distinct ductal and periductal fibrosis of both intrahepatic and extrahepatic bile ducts after 2–3 mo. This was preceded by nonsuppurative cholangitis with accumulation of inflammatory cells, mainly lymphocytes, around the bile ducts and infiltration of the duct epithelial layer and with degenerative changes in the epithelial cells.

Using *Mdr2*<sup>-/-</sup> mice, which carry a targeted disruption of the multidrug resistance gene and were found to develop macroscopic and microscopic features reminiscent of primary and secondary sclerosing cholangitis in humans (like extra- and intrahepatic

biliary strictures and dilations, onion skin-type periductal fibrosis, and focal fibroobliteration of bile ducts), Fickert et al. addressed the mechanisms leading to bile duct damage (108). They found that sclerosing cholangitis in these *Mdr2*<sup>-/-</sup> mice is a multistep process, with bile acid leaking back into portal tracts via the disrupted tight junctions and basement membranes of bile ducts, thereby inducing portal inflammatory infiltration and activation of proinflammatory and profibrogenic cytokine responses. This results in activation of periductal myofibroblasts and fibrosis, which detaches bile duct epithelial cells from the peribiliary plexus and in the end causes atrophy and death of the bile duct epithelium.

Intraarterial infusion of floxuridine into either dogs or rhesus monkeys (109) or infusion of ethanol into hepatic arteries of rhesus monkeys (110), which causes breakdown of the structural integrity of arteries and capillaries, caused fibrous inflammation and diffuse stricturing of intrahepatic biliary ducts. These effects resemble those of floxuridine injection in chemotherapy, which has caused fibrous inflammation and diffuse, focal strictures of intrahepatic and/or extrahepatic bile ducts strongly reminiscent of PSC and PSC-like symptoms in Kussmaul-Maier disease (also termed polyarteritis nodosa or periarteritis nodosa), an serious immune-mediated blood vessel disease in which small and medium-sized arteries become swollen and damaged. These data indicate that arterial damage can lead to the onset of symptoms typical of sclerosing cholangitis in both humans and animal models.

2,4,6-Trinitrobenzenesulphonic acid (TNBS), a chemical hapten, can elicit an immune response to a carrier protein upon binding to such a protein. TNBS induced PSC-reminiscent symptoms upon injection into portal veins (111) or bile ducts (112–114) of rats. Upon portal-venous infusion of TNBS in female Lewis rats, Orth et al. found inflammation of the portal tracts associated with transient infiltration of macrophages and subsequently CD3<sup>+</sup> lymphocytes (111). Increased levels of alkaline phosphatase, aspartate aminotransferase, and bilirubin as well as bile duct proliferation occurred in a dose-dependent manner. Additional results were increased MHC class II expression on BECs and the occurrence of ANCA, which are characteristic of PSC and are found in up to 88% of PSC patients. ANCA in this model were specific for catalase, myeloperoxidase, and actin.

As reported by Mourelle et al. a single intracholedochal injection of TNBS resulted in significant increases in all serum markers (aspartate aminotransferase, alkaline phosphatase, and bilirubin), as well as inflammatory cell infiltrates in portal areas and around bile ducts, indicating pericholangitis (112). In some rats, dilation of extrahepatic biliary ducts associated with ductal proliferation and thin porto-portal fibrotic septa was observed. In a control group that had received bile duct ligation, ductal proliferation and fibrosis was induced in all cases also, but without prominent pericholangitis.

Orth et al. described a model of TNBS injection into bile ducts that had been dilated owing to a mild stenosis in 8-wk-old female Lewis rats with resulting chronic fibrosing cholangitis in TNBS-treated rats (113). These authors again found elevated

alkaline phosphatase levels, and inflammatory infiltrates, as well as MHC class II antigen upregulation and distortion, irregularities, beading, and strictures of the bile ducts by retrograde cholangiography. Spontaneous IFN- $\gamma$ , TNF- $\alpha$  and IL-10 production of liver mononuclear cells was increased. In addition, between 1 and 12 wk after TNBS injection, ANCAS with specificity against myeloperoxidase, catalase, and actin were found in this model. However, in a follow-up analysis characterizing the long-term outcome of TNBS-induced cholangitis in this model, after 8 and 12 mo the authors found no evidence of cholangitis in serum chemistry or histology or retrograde cholangiography of TNBS-treated rats even with 75% of these animals being positive for ANCAS (114). This was interpreted by the authors as a hint that a single initial insult was insufficient to trigger long-term chronic progressive inflammation, and that perpetuation of inflammation might require additional stimuli.

Toxic substances like  $\alpha$ -naphthylisothiocyanate (ANIT), applied orally (115) or formalin-injected into livers (116) or extrahepatic bile ducts (117) of rats, can induce symptoms such as inflammation in portal, periportal, and/or peribiliary areas, portal fibrosis, or peribiliary sclerosis. In a model of ANIT administration, aberrant expression of MHC class II molecules by BECS, progressively escalating Th1 cytokine expression, and increased alanine aminotransferase and bilirubin levels were also described. Tjandra et al. also used a model of oral ANIT application to characterize potential reasons for the poor response of PSC to steroid treatment. They found that in hepatic T lymphocytes of ANIT-fed rats, mRNA and protein levels of glucocorticoid receptor were significantly reduced in comparison with controls, whereas glucocorticoid receptor mRNA and protein expression in splenic and circulating T lymphocytes was similar in both groups (118). This reduced glucocorticoid receptor expression by liver T cells was associated with reduced sensitivity of hepatic CD4<sup>+</sup> T cells to inhibitory effects of dexamethasone.

Another characteristic feature of PSC is the association with concomitant inflammatory bowel disease in most patients. Thus, several researchers addressed potential correlations of intestinals and biliary hepatic disorders.

Since PSC frequently occurs in association with ulcerative colitis, Tjandra et al. hypothesized that colitis might predispose patients to bile ductular injury (119). They tested the susceptibility of Sprague-Dawley rats with experimental colitis to toxin-induced cholangitis by intracolonic application of TNBS or ethanol vehicle rats followed by subsequent administration of the BEC toxin ANIT or vehicle. After 7 d, the authors found that the portal inflammation centered on damaged bile ducts found in ANIT-treated noncolitic rats was markedly attenuated in ANIT-treated colitic rats. This effect was associated with twofold higher levels of hepatic IL-10 mRNA in colitic compared with noncolitic rats, whereas TNF- $\alpha$  levels were not influenced.

However, upon creation of jejunal self-filling blind loops, Lichtman et al. found small bowel bacterial overgrowth (120) that resulted in weight loss, hepatomegaly, and hepatic inflammation in Lewis rats and (with a delay) also in Wistar rats (12 wk in contrast to 4 wk after surgery for Lewis rats). However, rats

with self-emptying blind loops, having only slightly increased bacterial counts, did not develop hepatic injury. Hepatic injury in these rats involved bile duct proliferation, fibrosis, and acute and chronic periportal and focal parenchymal inflammation (120). In addition, these rats had extrahepatic ductal dilation and ectasia with irregular, beaded, rapidly tapering, and tortuous intrahepatic ducts (121). The delayed development of these symptoms in Wistar rats and the absence of hepatic injury in Buffalo rats (120) indicated a genetic difference in susceptibility to disease onset in this model. These results clearly indicated that experimental small bowel bacterial overgrowth causes hepatic inflammation with subsequent fibrosis in susceptible rat strains. This was further supported by the observation, that concomitant treatment with certain antibiotics in this model protected from development of disease symptoms and was curative upon subsequent treatment. Both metronidazole and tetracycline, which eliminated *Bacteroides* sp. from blind loops were effective, but polymyxin B and gentamicin, which did not affect *Bacteroides*, were not (122).

Since anaerobic cultures of blood, peritoneum, liver (120,122), and spleen (122) were negative in rats with small bowel bacterial overgrowth in all strains, the authors suggested that bacterial cell wall polymers or other bacterial toxins from the blind loop rather than bacterial infection of the liver itself caused the hepatic lesions observed. In fact, Lewis rats with small bowel bacterial overgrowth were not protected from liver injury by treatment with ursodeoxycholic acid, prednisone, methotrexate, and cyclosporin A, but hepatic injury was significantly prevented by mutanolysin (123). Mutanolysin is a muralytic enzyme that splits the  $\beta$ 1-4 *N*-acetylmuramyl-*N*-acetylglucosamine linkage of peptidoglycan-polysaccharide (PG-PS), a bacterial cell wall polymer with potent inflammatory and immunoregulatory properties. Other effects like diminution of total bacterial numbers within the loop, elimination of *Bacteroides* sp., or reduction in mucosal PG-PS transport were excluded. However, elevation of plasma anti-PG antibodies and TNF- $\alpha$  levels, which occurred in control rats upon small bowel bacterial overgrowth, were prevented by mutanolysin treatment. TNF- $\alpha$  production induced by small bowel bacterial overgrowth was at least in part mediated by the innate immune-response of Kupffer cells to PG-PS, since isolated Kupffer cells also secreted TNF- $\alpha$  upon PG-PS stimulation in vitro. This in vitro response was also diminished by mutanolysin. In summary, these data suggest that systemic uptake of PG-PS derived from endogenous enteric bacteria contributes to hepatobiliary injury upon small bowel bacterial overgrowth.

Using a mouse model of experimental colitis induced by oral administration of dextran sulfate sodium to CD1 mice, Numata et al. analyzed the potential association of cholangitis with experimental colitis (124). They found that hepatobiliary disorders were complicated in experimental colitis since about one-third of treated mice had inflammatory cell infiltration and focal necrosis in the liver. The CD4/CD8 ratio increased in the liver, but not in colon, and transient changes in the NKT cell population in both organs were reported, as well as a Th1 shift of cytokine production.



In a follow-up analysis, the authors characterized the immunopathogenic role of NKT cells in this model, using the NKT cell-activating glycolipid  $\alpha$ -GalCer. They found that repeated administration of  $\alpha$ -GalCer for 1 mo improved survival rate, weight gain, and inflammation score (125). This protective effect was associated with decreased IFN- $\gamma$  release by mononuclear cells from the liver, as well as reduction in the CD4/CD8 ratio and both NKT cell and NK cell numbers. Thus, by modification of the Th1/Th2 balance leading to a reduction in Th1 predominance and/or nonresponsiveness of the NKT cell population (known to be properties of repeated  $\alpha$ -GalCer administration),  $\alpha$ -GalCer may exert therapeutic effects.

In summary, these models persuasively suggest that cell wall components of certain bacteria may represent pathogenic stimuli that—in susceptible hosts, i.e., certain animal strains or individual patients with submissive genetic predisposition—might lead to the onset of hepatobiliary disorders like PSC, possibly via activation of the innate immune system.

### CONCLUDING REMARKS AND OPEN QUESTIONS

It is clear that none of the animal models described above includes all aspects of the respective disorders. This holds true for all three disorders comprised by the term autoimmune liver diseases, i.e., autoimmune hepatitis, primary biliary cirrhosis, and primary sclerosing cholangitis. Some of these models address the experimental induction of pathogenic processes resulting in disorders that resemble the clinical symptoms that prompt patients to seek medical help. Other models analyze experimentally induced—or in few cases spontaneous—onset of pathological processes that cause symptoms corresponding to characteristic but attendant symptoms of a particular disease, not relevant for actual liver damage (e.g., occurrence of typical but not disease-mediating autoantibodies). In some models, initial events can be characterized that cause one or a few symptoms reminiscent of clinically relevant disease-promoting symptoms in humans, whereas other symptoms are missing. Thus, for any of these diseases, the “perfect model” is missing, one that characterizes relevant genetic predisposition factors, triggering events that cause disease onset as well as disease progression by stepwise succession of disorders that induce one another and cooperate to culminate finally in all typical clinical symptoms. It is questionable whether modification of an existing animal model or an entirely new model will ever be able to fulfill these criteria, simply because of specific differences between animals and humans. However, one should keep in mind that individual patients also differ from one another to an extent that causes development of individual disease patterns.

In addition, a model that would fulfill the all these criteria would no longer fulfill the main criteria of being a model *per definitionem* at all: reduction and abstraction, i.e., a model will refer only to some aspects of the phenomenon in question, with neglect of several aspects in order to emphasize others.

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**ALCOHOLIC  
AND NONALCOHOLIC  
FATTY LIVER DISEASES**

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**IV**

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# 24 The Immune Response in the Pathogenesis of Alcoholic Liver Disease

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## KEY POINTS

- Clinical and histological characteristics of alcoholic liver disease (ALD) suggest the presence of altered immune reactivity.
- The association of autoantibodies and cytotoxic T lymphocytes in ALD suggest a loss of immune regulation leading to autoimmune responses.
- Covalent binding of alcohol metabolites to proteins (adducts) can induce an immune response that is specific for multiple epitopes.
- Both the presence of adducts in liver tissue and the detection of antibodies to adducts are observed in ALD.
- Protein-aldehyde adducts can induce an immune response to unmodified protein epitopes, which can result in autoimmune reactions.
- Animal models support the hypothesis that an immune response to these metabolically derived protein adducts play a role in the development and/or progression of ALD.
- Alcohol exposure alters both innate and adaptive immune responses in ways that enhance proinflammatory and profibrotic activity.
- Although many factors are involved in the immune abnormalities seen in ALD, endothelial cells, endotoxins, Kupffer cells, and monocytes all play initiating roles in the resultant inflammatory response.
- Cytokine/chemokine production is seen in ALD, and critical roles exist for TNF- $\alpha$ , IL-8, and IL-6.
- Humoral and genetic factors appear to modulate immune responses in ALD.
- Understanding the cellular and molecular mechanisms of abnormal immune responses in ALD should lead to new therapeutic approaches.

## INTRODUCTION

Many hypotheses have been put forward to explain ethanol-induced liver damage, including direct and indirect toxicity. Despite many years of intensive research, the molecular

mechanisms involved in the toxicity of this simple compound have still not been fully clarified. Initial studies investigated the direct toxic effects of ethanol on tissues, but it is now believed that the products of ethanol metabolism are the major factors causing alcoholic liver disease (ALD). The metabolic products and metabolic events induced by alcohol ingestion are varied in nature and vast in scope. Major research interest has focused on the effects of aldehyde production, oxygen radical formation, mitochondrial membrane permeability defects, endotoxins, and infectious agents as factors in hepatocellular dysfunction and cell death. At the same time, increasing evidence suggests that immune-mediated mechanisms are involved in ALD.

The association of excessive alcohol use with infections, inflammatory mediated liver disease, and increased cancer development have all suggested that alcohol may mediate clinical diseases by altering host immune function. Many clinical and experimental studies have clearly demonstrated that alcohol exposure results in abnormal immune responses (1–3). However, interpretation of these *in vivo* and *in vitro* human and animal studies has been difficult because of marked differences in alcohol administration and the *in vivo* model systems used. In addition, chronic alcohol abuse is associated with a variety of other abnormalities that can contribute to immune dysfunction, caused by malnutrition, vitamin deficiency, and advanced tissue damage such as liver cirrhosis. Although it is clear that patients with chronic alcohol abuse have multiple immune abnormalities, the direct role of these immune factors in subsequent clinical disease has often not been proved.

A novel hypothesis has suggested that ethanol induces new antigenic structures (neoantigens) that provoke an autoimmune response involved in the development and/or progression of ALD. Indeed, it has been shown in several laboratories that covalently modified proteins (adducted proteins) are formed in humans and laboratory animals as a result of the consumption of alcohol. Evidence has also accumulated to support the hypothesis that such modified proteins are immunogenic and may be involved in the pathology of ALD (4).

## CLINICAL EVIDENCE FOR IMMUNE-MEDIATED LIVER INJURY IN ALCOHOLIC LIVER DISEASE

Increasing interest has centered around the possible role of the immune system in the pathogenesis and perpetuation of ALD. This is because ALD has many clinical manifestations suggesting that immune mechanisms may be contributing to liver tissue damage (5). Indirect proof for this association can be found in the observations that liver injury can persist for some time after the withdrawal of alcohol and that some patients who quit drinking and experience complete histological recovery from ALD rapidly redevelop alcoholic hepatitis once they return to the consumption of alcohol (6). This rapid disease recurrence suggests an anamnestic type of response that is reminiscent of most immune reactions.

### INFLAMMATORY/IMMUNE NATURE OF HISTOPATHOLOGICAL CHANGES IN ALCOHOLIC LIVER DISEASE

Cirrhosis usually develops after recurrent episodes of alcoholic hepatitis and is considered the final stage of alcohol-induced hepatic injury. Hepatocellular injury and perhaps direct actions of aldehydes on collagen-forming non-parenchymal cells may induce a fibrogenic response (7,8). Additionally, the chronic consumption of alcohol impairs the ability of hepatocytes to proliferate in a response that is normally triggered by liver cell death in order to repair the damaged liver (9). Therefore, areas where the liver cells do not regenerate are typically small (i.e., micronodular) in actively drinking patients with cirrhosis. Once a patient quits drinking, the hepatocyte is released from the antiproliferative actions of alcohol, which often results in the development of macronodular cirrhosis (10).

The histopathology of ALD supports the hypothesis that alcohol-induced immune mechanisms contribute to the tissue dysfunction observed in chronic alcoholism. Three major histopathologic lesions have been associated with alcohol abuse: (1) alcoholic fatty liver (steatosis) (2) alcoholic hepatitis (steatonecrosis) and (3) alcoholic cirrhosis. Alcoholic fatty liver is a reproducible consequence of ethanol oxidation and results from the redox imbalance that follows ethanol oxidation (11). The excessive oxidative degradation of fatty acids results in the formation of triglycerides that accumulate as large droplets of fat in hepatocytes. There appears to be little evidence for a relationship of alcoholic fatty liver with the immune system.

The histopathology of alcoholic hepatitis is characterized by steatosis plus hepatocellular injury, accumulation of inflammatory cells, and usually some fibrosis. Some injured hepatocytes contain Mallory bodies comprised of eosinophilic, fibrillar material that is a condensation of cytoskeletal intermediary components. Although Mallory bodies are typical of alcoholic hepatitis, they are neither sensitive nor specific markers of ALD. However, they are a good example of the ability of alcohol to destroy normal mechanisms of intracellular trafficking, as well as cell to cell to matrix communication. The presence and sequestration of T lymphocytes in the liver

strongly suggests that immune mechanisms induce inflammation in the liver that leads to progressive fibrosis culminating in cirrhosis (12). T cells move to the liver during alcoholic hepatitis and are increased relative to the percentage of T cells found in peripheral blood. The colocalization of hepatocyte necrosis and lymphocytes strongly supports an immune contribution to the resulting tissue damage. Additionally, the lymphocytes are found to be mixed with granulocytes and Mallory bodies in the centrilobular zone and consist primarily of CD3-positive cells (both CD4<sup>+</sup> and CD8<sup>+</sup>), with a few B cells and no natural killer (NK) cells. In patients with alcoholic hepatitis, CD4<sup>+</sup> and CD8<sup>+</sup> cells occupy the portal tracts. However, CD8<sup>+</sup> cells are found localized to the edges of the portal tracts, whereas CD4<sup>+</sup> cells are in the center of the portal tracts. Both CD4<sup>+</sup> and CD8<sup>+</sup> cells colocalize in the periportal sinusoids (piecemeal or interface hepatitis) (13). Enhanced cell surface expression of MHC class I and II and upregulation of adhesion molecules (CD29, CD45RA, CD45RO) further support the concept that direct lymphocyte/hepatocyte contact is involved in ALD. Thus, these studies strongly support the conclusion that cytotoxic T cells play a role in the progression of alcoholic hepatitis.

Intense lobular infiltration with polymorphonuclear leukocytes separates alcoholic hepatitis from most other forms of hepatitis. The recognition that immune activation often releases cytokines acting as neutrophil chemotactic factors (14), coupled with data that correlate mortality in acute alcoholic hepatitis with circulating levels of proinflammatory cytokines, offers important insights into the pathogenesis of this lesion. Whether alcoholic hepatitis is an inevitable consequence of heavy alcohol use or is a prerequisite lesion for eventual alcoholic cirrhosis is not known. However, because this lesion is sometimes associated with a characteristic clinical syndrome (i.e., fever, leukocytosis, tender hepatomegaly, and jaundice), it has been the target of multiple therapeutic trials. Additionally, the cytokines involved suggest an immune-directed inflammatory process in the initiation and/or the progression of the histologic lesion. Certainly, this lesion is particularly consistent with the generation of an immune response.

Cirrhosis usually develops after recurrent episodes of alcoholic hepatitis and is considered the final stage of alcohol-induced hepatic injury. Hepatocellular injury and perhaps the direct actions of acetaldehyde on collagen-forming non-parenchymal cells induce a fibrogenic response (7,8). The chronic consumption of alcohol impairs the hepatocellular proliferative response that is normally triggered by liver cell death (9,15). Therefore, nodules of regenerating liver cells are typically small (i.e., micronodular) in actively drinking patients with cirrhosis. Abstinence releases the liver from the antiproliferative actions of alcohol and is often associated with the evolution of macronodular cirrhosis (10). Some of which appear to be liver specific, but others of which are shared with other organs, such as the kidney (16). Antibodies to LSP are detected in almost all patients with untreated chronic active hepatitis (thought to be an autoimmune disease) and in 27 to 29% (17) of ALD patients.

## TRANSPLANT REJECTION IN ALCOHOLIC LIVER DISEASE

Two clinical observations strengthen the hypothesis that immune mechanisms are involved in ALD. First, some patients with alcoholic hepatitis appear to respond to immunosuppressive therapy, indirectly suggesting an inflammatory response that may be mediated by immune effector mechanisms of tissue damage (24). Second, the rapid recurrence of hepatic fibrosis and/or cirrhosis in the transplanted liver following the resumption of alcohol ingestion again suggests that immune-mediated events have been initiated (6). This last observation suggests that nonhepatic host tissues play a pivotal role in initiating hepatocellular destruction of normal (nonhost) liver tissue.

## AUTOREACTIVITY IN ALCOHOLIC LIVER DISEASE

Early studies in ALD demonstrated elevated levels of nonspecific autoantibodies (antinuclear [ANA], antihistone, and other antibodies.) and polyclonal hypergammaglobulinemia. The detection of autoantibodies and cytotoxic responses to self-antigens in ALD clearly suggest that loss of self-tolerance and the resultant autoimmune responses are present in ALD. However, the first problem is to define a mechanism whereby autoantigens can be produced by alcohol ingestion. The hypothesis that antigen-specific immune responses directed at self liver proteins are present in ALD is supported by the number of organ-specific autoantibodies that have been reported in patients with ALD. Liver-specific protein (LSP) is a high-molecular-weight species-nonspecific lipoprotein associated with the plasma membrane of hepatocytes. It has a number of antigen specificities. Liver membrane antigen (LMA) antibodies react with an antigen that is distinct from LSP (17). IgG and IgA antibodies to LMAs have been demonstrated in around 10% of ALD patients with fatty liver, in 24% with alcoholic hepatitis, in 30% with active cirrhosis, and in 62% with inactive cirrhosis (18). Wiedmann and his colleagues found IgA anti-LMAs in 57% of patients with alcoholic hepatitis but did not find IgG antibodies (19).

The development of anti-cytochrome P450 (anti-CYP) autoreactivity is not uncommon in liver diseases. Anti-CYP autoantibodies have been observed in patients with hepatitis caused by dihydralazine (anti-CYP1A2), tienilic acid (anti-CYP2C9), or halothane (anti-CYP2E1), as well as during hypersensitivity reactions to aromatic anticonvulsants (anti-CYP3A). Additionally, CYP2D6 is a target for the anti-liver-kidney microsome type 1 (LKM-1) antibodies present in patients with type 2 autoimmune hepatitis and hepatitis C. Recently, Vidali et al. (20) demonstrated that the presence of anti-CYP2E1 autoantibodies during ALD is associated with advanced liver disease and suggested that CYP2E1 modification by hydroxyethyl radicals (HERs) are a trigger for anti-CYP2E1. Similar immunoglobulins and autoantibodies recognizing cytochrome P450III EI HER adducts have also been found in the blood of human alcoholics (21,22) and the level of antibody has correlated with the severity of alcoholic liver disease (21,23).

## AUTOIMMUNITY IN ALCOHOLIC LIVER DISEASE

### EARLY EVIDENCE FOR IMMUNE-DIRECTED HEPATOCELLULAR INJURY

Multiple studies have demonstrated that alcohol and/or its metabolites induce new antigenic structures on native proteins (neoantigens) that can initiate immune responses in experimental situations. Sorrell and Leevy first described the role that alcohol metabolites might play in inducing abnormal antiliver immune responses in patients with ALD (25). Additional reports have since suggested that patients with alcohol-associated liver disease mount unique antigen-driven immune responses that target the liver. Leevy et al. (26) and Zetterman et al. (5) documented a cell-mediated immune response to alcoholic hyalin in patients with alcoholic hepatitis. Johnson and Williams (27) showed that T lymphocytes of patients with ALD undergo blast transformation upon exposure to liver homogenates. Other studies have shown that lymphocytes from alcoholics can kill autologous hepatocytes *in vitro* (28). Furthermore, hyalin obtained from patients with ALD induces cytokine secretion when mixed with autologous immune cells (29). These studies have provided the impetus for the current efforts to identify the antigen(s) that might trigger the altered immune responses leading to recognition of the alcohol-exposed hepatocyte as non-self.

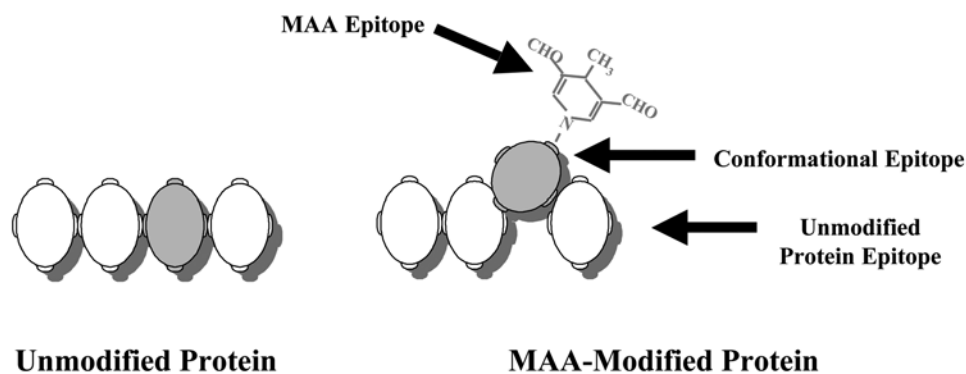
### NATURE OF ANTIGENS INVOLVED IN ALCOHOLIC LIVER DISEASE

The pioneering work of Tuma and Sorrell (30) proposed a mechanism that could contribute to the pathogenesis of ALD. Covalent binding of alcohol metabolites to proteins (adducts) are known to interfere with protein function, especially if there is a lysine residue in a functionally critical location, such as in tubulin and in lysine-dependent enzymes (30,31). These adducted proteins result in altered protein metabolism and cellular function. In addition, different investigative teams have clearly demonstrated that these adducted proteins form immune reactive proteins that act as true immunogens (32–35). A variety of protein adducts have been shown to be immunogenic and include proteins complexed with acetaldehyde, malondialdehyde, 4-hydroxynonenal, malondialdehyde-acetaldehyde hybrids, and HERs. The significance of the immune response to these adducted proteins is that antibodies to these chemically defined epitopes have been detected in the serum of both humans and animal models following chronic alcohol consumption (34–36). In addition, these protein adducts have been directly detected in liver tissue following alcohol consumption (33,37).

It has also been shown that these adducted proteins can induce specific immune responses, to the adduct, the adduct plus protein (conformational antigens), and the unmodified parts of the protein. Thus, it is not hard to speculate that these adducted proteins may be important in producing immune reactions resulting in tissue damage including ALD. A number of different adducts have been suggested to be involved in ALD, the most common of which are discussed below.

Acetaldehyde, the first metabolite of ethanol, forms adducts by binding to reactive lysine residues of proteins. However,





**Fig. 1.** Epitopes associated with malondialdehyde-acetaldehyde (MAA)-adduction of proteins. Unmodified proteins modified with MAA change the structural conformation of the proteins, resulting in the development of immune responses to at least three different epitopes. Antibodies and T cells can recognize: (1) the MAA epitope; (2) parts of the protein that remain unmodified (unmodified protein epitope); and (3) epitopes that result from the combination of the MAA adduct and the unmodified protein (conformational epitope).

the level of modification is dependent on the concentration of acetaldehyde and the proteins that are present. Indeed, the most prominent acetaldehyde adduct formed (*N*-ethyl lysine) occurs in the presence of strong reducing agents, but the presence of this adduct in patients or animals consuming ethanol is controversial (4,31). Stable cyclic imidazolidinone structures can also be detected when acetaldehyde combines with the free  $\alpha$ -amino group of the amino-terminal valine of hemoglobin in the absence of reducing agents, but the roles of these adducts in ALD have not been elucidated.

Aldehydic products of lipid peroxidation, such as malondialdehyde (MDA) and 4-hydroxynonenal (HNE), also form Schiff's base adducts with proteins. MDA is a highly reactive dialdehyde originating from the nonenzymatic lipid peroxidation that occurs during phagocytosis by monocytes and from arachidonic acid catabolism in thrombocytes. The free radical-mediated oxidation of long-chain polyunsaturated fatty acids leads to the production of 4-hydroxynonenal, which can react with the sulfhydryl groups of proteins. Oxidative modification of proteins with MDA and HNE have been demonstrated to occur *in vivo* on arterial vessel walls of atherosclerotic lesions. Similar epitopes have also been found from the liver specimens of patients with ALD and from animals with experimental iron overload (38).

Tuma and co-workers have shown the formation of hybrid adducts with acetaldehyde and MDA, designated MAA adducts, in livers of ethanol-fed rats (39,40). The uniqueness of this adduct is in the synergistic manner in which acetaldehyde and MDA react to form this highly stable adduct. An important aspect regarding the relevance and significance of MAA adducts, in addition to demonstrating their formation during ethanol consumption, is that they exhibit biological effects that may be relevant to their role in inducing liver damage and that include the induction of immune responses and cytokine/chemokine secretion, increased adhesion molecule expression, disruption of endocytosis and degradation, increased release of fibronectin, and stimulation of collagen secretion.

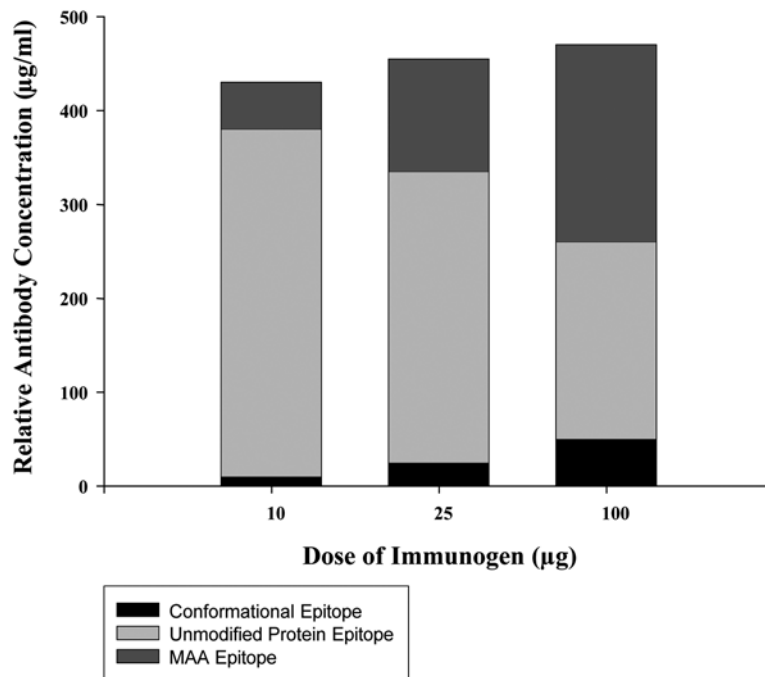
Although many protein adducts are immunogenic in the presence of potent adjuvants, MAA adducts are unique in that they have the ability to induce immune responses in the absence of adjuvants (38,41). Thus, MAA adduction represents an important mechanism by which T-cell and B-cell responses can be developed to soluble proteins *in vivo*. As discussed just below, MAA adducts have also been shown to induce antibody and T-cell responses to the several different epitopes on the adducted protein. Indeed, more recent studies have shown that reactivity to self-proteins can be initiated if first modified by MAA adducts. Therefore, these findings strongly suggest that MAA adducts could contribute to immune reactions that stimulate the production of antibodies and/or T cells against MAA adducts and/or self liver antigens (autoantigens), which would result in specific organ damage.

HERs, which are a reactive species resulting from the oxidation of ethanol in the presence of iron, have been described in the liver microsomes of ethanol-fed animals (42). No studies have been carried out on the structure of adducts formed by the reaction of exogenously generated HERs with proteins or peptides. However, it is known that modified proteins are formed *in vivo* as HER-modified proteins can be detected by enzyme-linked immunosorbent assay (ELISA) in the livers of ethanol-fed rats (38).

#### TYPES OF IMMUNE RESPONSES

Given that ethanol-induced metabolically derived adducts form on various proteins, there are a number of specific immune responses that can be directed at different and unique immune epitopes. An illustration of these immune targets is given in Fig. 1.

**Response to the Metabolite** Most of the original studies have suggested that immune responses to the specific metabolite (adduct) epitope were responsible for the specificity of liver damage in ALD. Since the metabolites of ethanol are produced in the liver and released to the surrounding cells, it has been suggested that the antibodies and T cells respond to these modified proteins and cause the specific damage to the liver. Although antibodies and T cell have been shown to react with



**Fig. 2.** Immunogenicity of soluble malondialdehyde-acetaldehyde (MAA)-modified (adducted) proteins. C57G1/6 mice were immunized with MAA-modified bovine serum albumin (BSA), or human serum albumin (HSA) without the use of adjuvant. Antibodies to unmodified protein epitopes (BSA), MAA epitopes (HSA-MAA), and total epitopes (BSA-MAA) were determined. “Conformational” epitopes were calculated as the difference between the activity on BSA-MAA minus the activity on BSA.

these adducts, it has also been shown that adducted proteins are cleared rapidly by cells of the reticuloendothelial system. Thus, it is highly likely that these adducts would not persist long enough to induce local antibody and/or T-cell responses. Also, with the exception of the MAA adduct, none of the other adducts have been shown to induce immune responses in the absence of adjuvants.

**Response to the Metabolite and Protein** An alternative mechanism would target specific epitopes formed by the physical aspects of the protein-adduct, as has been observed with the autoimmune hemolytic anemia associated with penicillin, in which the antibody reactivity is not to the red cell or to the penicillin but to the combination (conformational antigen or neoantigen). By this immune mechanism, liver damage would only occur when both the carrier protein and the metabolite are present and would explain the development and progression of ALD following prolonged ethanol consumption. Once the metabolite is removed, then the specificity would be removed, resulting in little or no tissue damage. However, upon resumption of alcohol consumption, the adducts would again complex with proteins, the memory cells of the immune system would be initiated, and the damage would be accelerated. Thus, each time the patient drank, the process would become more severe.

**Response to the Modified Self-Protein or the Induction of Autoimmunity** A potent mechanism of immune-mediated liver damage would be the induction of an immune response to unmodified liver cells or proteins. Recent studies have

demonstrated that immunization with soluble MAA-modified proteins results in both a humoral and cellular response to epitopes found on the unmodified protein structure. The initial studies were performed utilizing highly purified foreign albumin (bovine serum albumin [BSA]) modified *in vitro* with MAA. As illustrated in Fig. 2, immunization with low doses of BSA-MAA produced antibodies specific for: (1) the MAA epitope, (2) the unmodified BSA epitopes, and (3) the unique epitopes seen only on the intact BSA-MAA complexed structure (assumed to be conformational epitopes). The relative amount of protein VS MAA antibody response was dependent on the dose of the immunogen used, with low doses producing antibodies primarily to the unmodified protein structure. Similar results were obtained when syngeneic liver cytosol proteins were used as a soluble immunogen without adjuvant. These studies clearly demonstrate that it is possible to break tolerance to self-antigens by modifying the self protein with MAA, with a resultant autoimmune response. This unique mechanism of ethanol-induced loss of self-tolerance results in an increased number of autoreactive cells produced with each exposure of ethanol. Eventually, over time the response would be of such magnitude that the liver damage could occur.

#### ANIMAL MODELS SUPPORTING AN AUTOIMMUNE COMPONENT IN ALCOHOLIC LIVER DISEASE

If the metabolically derived protein adducts play a significant role in the development and/or progression of ALD, then it should be possible to administer the adducts to naive animals

**Table 1**  
**Immune Reactivity Against HSA-MAA in Patients**  
**With Liver Disease**

<i>Patient group</i>	<i>Relative IgG binding to HSA-MAA</i>
Alcoholic liver disease	5.2
Nonalcoholic liver disease	2.25
Heavy drinkers without liver disease	1.25
Healthy control subjects	1.0

Abbreviations: HAS-MAA, human serum albumin-malondialdehyde-acetaldehyde.  
 Adapted from ref. 46.

and initiate ALD. Immunization of animals with aldehyde-protein conjugates in the presence of adjuvants results in antibody responses to the aldehyde adduct regardless of the protein to which it was attached (43). In an experimental model of chronic oral alcohol consumption by guinea pigs, the investigators showed that the combination of repeated immunizations with nonreduced acetaldehyde adduct-modified foreign proteins and alcohol feeding resulted in hepatic fibrosis in the periportal and perivenular areas (44). Antibodies to acetaldehyde adducts were detected in the serum of all animals immunized with acetaldehyde-modified foreign proteins independent of exposure to alcohol. However, significantly increased levels of fibrosis were only observed in the immunized animals that had ingested alcohol. Thus it was suggested that following the chronic consumption of alcohol the immune response was directed against the adduct epitopes, initiating an inflammatory response leading to hepatic fibrosis.

In another study by Shimada et al. (45), it was shown that immune responses could be initiated in mice following immunization with mouse albumin (self-protein) modified with acetaldehyde under reducing conditions and following ethanol feeding. Interestingly, the T-cell proliferative response as measured by a stimulation index was increased in ethanol-fed mice immunized with mouse albumin modified with acetaldehyde under reducing conditions. However, no inflammatory cells or tissue damage were observed in the livers of these animals. Also, there were no differences in plasma activities of aspartate transaminase and alanine aminotransaminase between the groups of mice regardless of ethanol feeding or immunization. Although failing to produce clear hepatocyte dysfunction, these studies did demonstrate the induction of autoimmune responses in vivo. It is probable that the nature of the adduct chosen was not biologically relevant to the development of liver damage, that is, as noted above, the reduced adduct has not been found in the livers of rats or humans chronically consuming ethanol using a monoclonal antibody to *N*-ethyl lysine and may not represent a biologically significant chemical structure. The immune response to the reduced adduct could certainly be generated under the conditions utilized, and ethanol may alter this response, but responding to the reduced adduct would appear to have no role in the development of ALD in this model.

Some of the most compelling experimental evidence that these adducts may play a significant role in the development of ALD comes from recent reports by Thiele et al. (41) in

experiments with the MAA adduct. In these studies, MAA-modified self-proteins were capable of inducing immune responses in the absence of adjuvants to the adduct and the self-protein to which it had been adducted. Further studies showed that daily injections of MAA-modified self liver cytosols into mice results in the development of hepatocellular damage, as evidenced by increased levels of alanine aminotransferase, induction of smooth muscle actin, and histologic changes consistent with early ALD. These studies appear to be promising in the development of a model system that may help to elucidate the mechanism(s) by which these adducted proteins are initiating tissue damage. At a minimum, these animal models offer proof of concept regarding the ability of metabolically derived alcohol-dependent protein adducts to induce an autoimmune response directed at liver tissue.

#### CLINICAL CORRELATIONS

Although the biological effects of protein adduct-induced immune responses are intriguing in these in vitro and animal models, demonstrating their direct role in human ALD has not been adequately confirmed. However, the presence of antibodies to the MAA-adducted proteins does have both diagnostic and prognostic significance in human disease. Rolla and associates correlated the presence and titer of antibodies to MAA-modified human serum albumin (HSA-MAA) with both the diagnosis of ALD and the severity of the liver injury (46). As reviewed in Table 1, levels of circulating antibodies to HSA-MAA adducts were seen at significantly higher titers in patients with ALD compared with alcoholics without liver disease and nondrinking controls. Most interestingly, patients with more severe ALD (Maddrey's Discriminant Function [DF] greater than 60) had a twofold higher titer of anti-HSA-MAA antibodies than did ALD patients, with a less than 60 DF score. Supporting evidence for the use of antiadduct antibodies in human ALD was also reported by Albano et al., who investigated HERs (47).

#### NATURE OF THE IMMUNE RESPONSES IN ALCOHOLIC LIVER DISEASE

There are multiple potential effects of alcohol on the innate and adaptive immune responses. In addition to the induction of new antigenic structures on native proteins (neoantigens), alcohol and its metabolites appear to modulate existing immune responses to foreign antigens and selfantigens. In vitro studies show that alcohol alters inflammatory and immune

cytokines that are directly involved in cytolysis, fibrosis, and cellular regeneration.

### INNATE IMMUNE MECHANISMS

The innate immune system is involved in the initial and extremely rapid response of cells to dangerous stresses, such as pathogens, malignancy, or tissue injury. The Toll-like receptors (TLRs) are part of the pattern recognition receptor family and are expressed on many different cell types. Activation of these receptors can cause the stimulation of a number of different genes that control the expression of inflammatory cytokines/chemokines, which results in the recruitment of neutrophils and macrophages to the site of inflammation. Recent data have shown that the development of ALD follows a pattern that is highly characteristic of the innate immune response (48).

Endotoxins interact with components of the innate immune response and appear to play a major role in the development of ALD (49). It has been suggested that ethanol increases the translocation of endotoxin from the gut lumen to the portal circulation. Normally, Kupffer cells bind the lipopolysaccharide (LPS) component of endotoxin through CD14 (LPS binding protein) and the TLR4. The nonparenchymal cells of the liver have been shown to be activated by bacterial endotoxins such as LPS. This leads to the production of inflammatory and fibrogenic cytokines, reactive oxygen species, and the recruitment of various inflammatory cells into the liver (49). In addition, in rodent models fed a diet high in fat and ethanol, lesions develop that are very similar to those observed in human alcoholic hepatitis and lend support for a role of endotoxin, CD14, and TLR4 in the induction of liver damage. The eradication of Gram-negative bacteria by antibiotics prevents liver injury in some animal models. In other studies using CD14 and TLR4 knockout mice, alcohol-induced liver injury is attenuated (48).

Kupffer cells are a key component of innate immunity in the liver and are affected directly by alcohol, as initial alcohol consumption, tends to induce a tolerance to endotoxin. However, with further alcohol consumption, the Kupffer cells actually become more responsive as CD14 is induced by ethanol exposure. Also, Kupffer cells activated by endotoxin release a group of chemokines that cause an increased infiltration into the liver of both mononuclear cells and neutrophils. Thus, Kupffer cells first cause leukocyte infiltration by releasing C-X-C or  $\alpha$ -chemokines, followed by C-C or  $\beta$ -chemokines that induce monocyte infiltration.

Recent studies have suggested that apoptosis plays a major role in the development and/or progression of ALD. Many investigators have shown that apoptosis may be a result of occur after tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) causes the production by mitochondria of reactive oxygen species (50). This causes an increase in the mitochondrial membrane permeability which causes the pores to leak apoptosis-inducing factors (predominately cytochrome c), by which caspases are activated and the apoptotic cascade is initiated. Alternatively, TNF- $\alpha$  may initiate the death-inducing signaling complex, which is associated with the cytoplasmic portion of the TNF- $\alpha$  receptor and with caspase activation.

In addition to inducing apoptosis through the mitochondrial permeability transition pore opening, apoptosis can be induced by oxidative stress and is mediated through the Fas (CD95)-Fas ligand system (51). Both surface Fas protein expression and Fas ligand mRNA are increased in hepatocytes from patients with ALD. This dual expression would suggest that alcohol-induced liver injury to hepatocytes may be mediated by a paracrine or autocrine mechanism (fratricide).

Another aspect of innate immune responses is the activation of monocytes which has been well documented in patients with ALD. Many mechanisms exist, but the observed activation of monocytes, with the resultant production of proinflammatory cytokines plays a significant role not only in liver injury in ALD, but also in many systemic complications of ALD (i.e., fever, anorexia, and hypozincemia) (52).

### ADAPTIVE IMMUNE MECHANISMS IN ALCOHOLIC LIVER DISEASE

More and more data strongly suggest that the adaptive immune response plays an important role in the development of ALD. Evidence from humans with ALD and animal models supports a role for both the humoral and cell-mediated arms of the adaptive immune response in the pathogenesis of this disease.

**Humoral System** B cells in alcoholics without liver disease are found in normal or slightly reduced numbers. However, they are often significantly decreased in ALD even though they produce larger amounts of immunoglobulins. Both T and B cells display changes in their subset surface phenotypes, but these changes appear to be shorter lived in B cell than in T cells (53). Splenic B cells from ethanol-consuming animals show impaired proliferation in response to a T-cell-dependent antigen (sheep red blood cells) but normal proliferation to a T-cell-independent antigen (TNP-Ficoll), suggesting that B-cell functions are intact despite the exposure to ethanol (53). Intact T-cell-independent antibody responses are also detected in chronic alcoholics in the response to pneumococcal polysaccharide vaccination (54). In vitro studies demonstrate decreased interleukin-4 [IL-4]-induced B-cell proliferation and IL-4-induced Ig class switching, whereas IL-2-induced B-cell proliferation is not affected by ethanol (54). These T- and B-cell changes suggest the likelihood that there are alterations in the interactions between T lymphocyte subpopulations that are important for understanding the inappropriate immunoglobulin production and other defects of immune regulation in alcoholics.

**Cell-Mediated System** The consumption of ethanol significantly alters the lymphocyte cell numbers that can be isolated from the thymus and spleen. T-cell function and cytokine production are also abnormal. Studies showing varying levels of CD8<sup>+</sup> and CD4<sup>+</sup> T cells have been reported; such levels appear to reflect the degree of liver disease in the study populations. Additionally, there have been reports of altered expressions of various immunoregulatory molecules on the surface of the T lymphocytes in alcoholics (i.e., MHC class II and alteration of adhesion molecules) (53).

Chronic ethanol exposure results in a reduced antigen-specific T-cell response and impaired delayed-type hypersensitivity

reactions. Also, the distribution of and possible migration of circulating CD4<sup>+</sup> and CD8<sup>+</sup> T cell expression of L-selectin (CD62) appears to be modulated. In lymphocytes from patients with ALD, increased basal and stimulated expression of CD4, CD25, leukocyte function-associated antigen-1 (LFA-1), intracellular adhesion molecule-1 (ICAM-1) and LFA-3 markers, overexpression of activation markers, and TNF- $\alpha$  production are very similar to what has been observed following mitogen activation (53).

The allo-specific and mixed lymphocyte response of the responder cells from alcohol-consuming mice is significantly reduced, and exogenous IL-2 is not capable of reversing this suppression. It is now thought that alcohol intake decreases allostimulatory T-cell activation by decreasing accessory cell function. Increased IL-10 and IL-13, plus the reduced IFN- $\gamma$  production after acute alcohol use are more likely to contribute to both the reduced T-cell proliferation and the monocyte accessory cell function (53).

Most studies on the effects of alcohol on T cells are performed on cell populations derived from the peripheral blood or lymphoid tissues. However, the liver contains not only circulating lymphocytes but also liver-associated T cells. Although the function of these cells is not entirely clear, in patients with ALD these cells are characterized by an increase in the cytotoxic/suppressor T-cell subset (CD8<sup>+</sup>) and a decrease in the helper T-cell subset (CD4<sup>+</sup>). In both mice and humans, T cells are found in the liver sinusoids, indicating that the liver could be a site of extrathymic differentiation of these cells. In histological studies, sinusoidal lymphocytes are mostly in contact with Kupffer and endothelial cells. The ability of liver-associated T cells to adhere to normal liver tissue is higher than that of peripheral blood leukocytes, suggesting they have increased adhesion molecule expression. Indeed, quantitative immunohistochemistry has shown that liver-associated T cells are increased in patients with ALD and correlate with regenerating nodules, intralobular inflammation, central sclerosis, and abnormalities of Kupffer cells. Finally, data have shown that ethanol has a selective effect on the constitutive production of cytokines by liver-associated T cells. Therefore, these data strongly suggest that liver-associated T cells may be involved in the inflammatory process associated with ALD (53).

## INITIATING EVENTS

**Endothelial Cells** T cells home to the liver by binding different endothelium located in the portal tracts and the sinusoids. This binding is regulated by different cytokines/chemokines and their receptors. Thus, the increased infiltration of lymphocytes located at the limiting plate, rather than in the lobular parenchyma of ALD, can be explained by the response to these agents. When lymphocytic infiltrates are observed within the lobule, they are found in the centrilobular area associated with Mallory bodies and fibrosis. Importantly, the inflammatory infiltrate of lymphocytes is a common feature of many different chronic liver diseases, including ALD (12).

The effects of alcohol exposure on liver sinusoidal endothelial cells (SECs) provide insight into mechanisms associated with

the development of ALD. Liver SECs are one of the major populations of nonparenchymal cells and have been found to play an integral and active role in the development of various liver diseases. A major function of SECs is in host defense and homeostasis via their so-called scavenger function, whereby they recognize, internalize, and degrade a variety of extracellular matrix components and modified proteins. These "scavenger" receptors have been detected on a wide variety of cell types, including macrophages, fibroblasts, smooth muscle cells, and endothelial cells (55,56). The diversity of their biologic roles (57,58) stems from the unusually broad ligand specificity of this receptor type, (55,56), as they have been implicated in the endocytosis of a diverse array of ligands including chemically modified proteins, oxidized low density lipoprotein (LDL), polyribonucleotides, polysaccharides, anionic phospholipids, and other molecules, such as polyvinyl sulfate.

In fact, reports from our laboratory have shown that formaldehyde, acetaldehyde and MAA-modified proteins are removed by SECs (59). Thus, under normal conditions, MAA-modified proteins bind to scavenger receptors on SECs and are degraded. However, following chronic ethanol consumption, a 50 to 60% decrease in the degradation of these adducts has been shown (59). Additionally, MAA-adducted proteins can activate SECs in culture to upregulate adhesion molecules, including, ICAM-1, vascular cell adhesion molecule-1 (VCAM-1), platelet-endothelial cell adhesion molecule-1 (PECAM-1), P-selectin, and MHC class I and II. Thus, the altered regulation of leukocyte recruitment to the liver following alcohol exposure would enhance the inflammatory processes associated with tissue dysfunction.

Several investigators have demonstrated that antigen-specific T-cell activation can be generated by SECs (60). Unlike other vascular endothelial cells, SECs express MHC class II constitutively and thus can present antigen without prior stimulation (61). In addition, SECs express B7-1 (CD80) and B7-2 (CD86), as well as ICAM-1 (CD54), and will produce IL-1 (60). The dysregulation of this environment may play a significant role in the development of inflammation. In addition to upregulating adhesion molecules, MAA adducts induce the secretion of cytokines and chemokines such as TNF- $\alpha$ , monocyte chemoattractant protein-1 (MCP-1), and macrophage inflammatory protein-1 (MIP-2) (62). These studies suggest that following binding of MAA-modified proteins to the appropriate receptor SECs are induced to develop a pro-inflammatory response that could result in the infiltration of leukocytes into the liver.

Moreover, it has recently been shown that early injury to liver endothelial cells stimulates the production of cellular fibronectin, which initiates activation of quiescent stellate cells to become myofibroblasts (63), a key step in the fibrogenic process activated in the liver. Matrices deposited *in situ* by SECs from injured livers accelerated the conversion or "activation" of normal lipocytes to myofibroblast-like cells. Pretreatment of these matrices with monoclonal antibody to the EIIIA segment blocked this response. Also, recombinant fibronectin peptide containing the EIIIA segment was stimulatory to lipocytes in culture. These data strongly suggest that

the expression of EIIIA fibronectin by SECs is a critical early event in the liver's response to injury and that the EIIIA segment is biologically active, mediating the conversion of lipocytes to myofibroblasts (63).

**Lipopolysaccharide** One of the agents that has been strongly linked to the development of ALD is LPS, as the concentration of this bacterial cell wall material has been shown to increase in the blood following chronic ethanol consumption (64). This is thought to occur through increased gut mucosal permeability and decreased LPS clearance from the blood (65). Thurman et al. (66) have suggested a model of ALD wherein Kupffer cells are exposed to LPS, release cytokines and chemokines, and lead to inflammation and fibrosis. Support for this hypothesis can be found in the Tsukamoto and French model of ALD, in which there is a strong correlation between the degree of liver injury and endotoxemia (67). Interestingly, alcohol consumption increases the sensitivity of Kupffer cells to LPS. However, the mechanism for this has not been elucidated; current data support the hypothesis that endotoxin and mediators of endotoxin-induced cellular activation such as LPS binding protein (LBP), CD14, and TLR4 play a role in the pathogenesis of liver injury in ALD.

**Kupffer Cells** Hepatic Kupffer cells are the central phagocytic and immune regulating cells of the liver, and they play a pivotal role in both the development and progression of hepatic inflammation and fibrosis. The specific effects of alcohol on Kupffer cell function related to ALD are detailed throughout this review. In summary, both in vivo and in vitro studies have clearly demonstrated that alcohol alters Kupffer cell sensitivity to endotoxin, alters the production of pro-inflammatory and profibrotic cytokines/chemokines, and affects antigen processing and presentation.

**Monocytes** Monocytes play a critical role in the initiation and regulation of both inflammatory and immune responses. The inflammatory response in a normal host is suppressed by immunoinhibitory cytokines, which are normally initiated late in the inflammatory cascade. The most potent immunoinhibitory cytokines are transforming growth factor- $\beta$  (TGF- $\beta$ ) and IL-10, which are produced by both monocytes and T lymphocytes. IL-10 is a typical Th2 cytokine that promotes humoral immune responses and inhibits cellular immune response by shutting off the Th1 cytokines, antigen-specific T-cell proliferation, and inflammatory cytokine levels. Acute ethanol treatment results in increased secretion of IL-10 by human monocytes produced in vitro. These data suggest that one of the mechanisms by which ethanol use may disturb cellular immune responses is by increasing the IL-10 levels (54).

TGF- $\beta$  is another monocyte-produced anti-inflammatory cytokine that inhibits antigen-specific T-cell proliferation. Alcohol can induce TGF- $\beta$  production in monocytes and augment TGF- $\beta$  production in response to bacterial challenge in vitro. Ethanol-induced elevation in TGF- $\beta$  may have multiple implications for the immune system, including inhibition of inflammatory cytokine production by monocytes and other cells, inhibition of T-cell proliferation, and augmentation of Th2-type immune responses (54).

Therefore, these data would suggest that the response of monocytes and other cells can be modulated by ethanol as a result of the preferential expression of anti-inflammatory cytokines under various conditions. This could explain the differences observed in immune responsiveness following chronic, acute, or moderate alcohol consumption.

#### ROLE OF CYTOKINES/CHEMOKINES

Many of the features of ALD are associated with fever, malaise, anorexia, and leukocytosis, which are classic clinical manifestations of abnormal cytokine production (68). Models of alcohol-related liver injury have also been linked to the increased production of cytokines, which have caused chronic ethanol-exposed rodents to become more susceptible to endotoxin-mediated liver injury (69). Although many specific cytokines/chemokines have been studied, major interest has centered on TNF- $\alpha$ , IL-8, and IL-6.

**TNF- $\alpha$**  Many studies have shown that rodents fed ethanol have an enhanced response to endotoxin-induced TNF- $\alpha$  production (70). On the basis of these and other experiments, it has been suggested that high concentrations of TNF- $\alpha$  in serum are associated with liver injury and that these high levels of TNF- $\alpha$  are induced by the combination of ethanol and LPS. Isolation of nonparenchymal cells shows that Kupffer cells and endothelial cells are both responsible for the ethanol-induced increased synthesis of this cytokine (71,72). Multiple other studies have strongly suggested a pathophysiological role for TNF- $\alpha$  in ALD in both animal models and humans. Alcohol can also alter TNF- $\alpha$  production by diminishing prostaglandin E2 production and reducing glutathione levels. Glutathione, which is abnormally low in alcoholism, is an important regulator of TNF- $\alpha$  production or release (52). This observation provides therapeutic opportunities for patients with ALD.

Recently, Thiele et al. (71) have shown that acetaldehyde and MAA adducts will bind to the "scavenger" receptor on liver endothelial cells. Following binding, there is an increased secretion of TNF- $\alpha$  that is two to three times higher than LPS stimulation can achieve on its own. Thus, it was possible to show a synergistic effect between MAA adducts and LPS, demonstrating a new mechanism by which these two products may interact to induce TNF- $\alpha$  production leading to ALD. The mechanisms for TNF- $\alpha$ -induced liver damage have not been clearly defined, but may invoke both necrotic and apoptotic pathways (50).

The clinical relevance of TNF- $\alpha$  was shown by Felver and associates (73). Serum concentrations of TNF- $\alpha$  were increased in patients with alcoholic hepatitis, and during a 2-yr follow-up period, the patients who died had higher concentrations of TNF- $\alpha$ , than surviving patients did (73).

**IL-8** Rat hepatocytes, when exposed to ethanol, release a chemotactic factor whose activity is abolished by antiserum against rat IL-8. This cytokine has been shown to cause neutrophilia, enhance the release of lysosomal enzymes, and increase the expression of adhesion molecules on granulocytes (74). Reports have shown that levels of IL-8 are increased in patients with alcoholic hepatitis and in alcohol-dependent

patients without liver injury and that tissue levels of IL-8 appear to correlate with neutrophil infiltration (75,76). These data suggest that the accumulation and activation of neutrophils by IL-8 may be associated with liver injury in alcoholics.

**IL-6** Serum concentrations of IL-6, a cytokine responsible for much of the hepatic acute-phase response, are increased in patients with ALD. In patients with alcoholic hepatitis, concentrations of IL-6 in plasma correlate with biochemical and clinical features of the disease, whereas a decrease in levels of IL-6 correlates with clinical improvement (76). The pathogenic mechanisms leading to increased production of cytokines in ALD are not completely understood. It has been suggested that the stimuli for production of cytokines by macrophages and other types of cells in the liver include endotoxin, prostanoids, glutathione, and various types of adducts (52).

### FACTORS THAT MODULATE IMMUNE RESPONSES IN ALCOHOLIC LIVER DISEASE

The development of the autoantibodies and autoreactive T cells as outlined above is a strong suggestion that one of the mechanisms involved in the development and/or progression of ALD includes autoimmune responses. Every autoimmune disease has other components that are necessary for the development of that disease including hormonal, genetic, and environmental factors. ALD has been shown to correlate with a number of these factors.

#### GENETICS

The liver histology (percutaneous biopsy) in alcoholics has generally shown that a greater total alcohol consumption over the years of drinking is associated with a higher incidence of cirrhosis (77). However, the incidence of cirrhosis in alcoholics remains surprisingly low, and a simple dose-response relationship between alcohol intake and degree of liver damage does not exist. Studies have suggested that additional genetic or host-related environmental factors must influence susceptibility to the development of liver damage, and this is consistent with autoimmune disease.

The major genetic marker for susceptibility to any autoimmune disease has been the HLA or MHC genotype (78). There are several studies indicating that susceptibility to alcohol-induced liver injury is associated with the different alleles of the HLA-B locus. In one study, in a group of British Caucasian patients, there was an increased incidence of HLA-B8 in patients with alcohol-induced cirrhosis. Morgan et al. (79) confirmed this observation when they detected an increased incidence of HLA-B8 in British patients with alcohol-induced hepatitis but not in patients with inactive cirrhosis or steatosis. In another study using Chilean patients, an association of HLA-B13, with alcohol-induced cirrhosis was observed (80). It was concluded that this association with HLA-B13 indicated a genetically determined increased susceptibility to liver damage by alcohol. An association of alcohol-induced cirrhosis with allele BW40 has also been described in Scandinavian patients, and a similar conclusion was reached. However, other workers have been unable to establish that HLA B locus alleles are associated with ALD.

Most of the above studies utilized serological analysis as the method for HLA genotyping. However, this methodology is not specific enough to determine the precise structural identity of MHC molecules in unrelated individuals, who may have inherited closely related but distinct genes. There are now many known sequence variants of most serologically defined MHC alleles, which are quite similar to each other but differ by one or a few amino acids. Thus, traditional genetic studies done on ALD patients may have overlooked the presence of unique MHC alleles involved in disease susceptibility or progression. Newer molecular approaches may provide more detailed understanding of the genetic susceptibility in ALD, and any relationship to autoimmune responses.

#### HORMONES

The effects of hormonal influence on immune responses and autoimmunity are well-described. There is now considerable evidence that females are more susceptible to alcohol-induced liver injury (81). These reports have shown that in women, smaller amounts of alcohol (on a kilogram of alcohol per kilogram body weight basis) produce hepatitis after a shorter period of abuse and that alcoholic hepatitis may progress to cirrhosis more rapidly in women compared with men. Krasner et al. (81) reported a poorer prognosis in women with ALD, but this has not been confirmed by other workers (79). These positive associations are of interest in view of the fact, that in general, the prevalence of autoimmune diseases and of circulating autoantibodies is more common in women than in men.

From this information, it is reasonable to hypothesize that genetic and hormonal factors are involved in the development of ALD. It is also possible that the genetic and hormonal influences may work through exaggerated immune and inflammatory responses that occur following a defect in the regulatory balance of the immune system. However, this possibility remains a hypothesis under active clinical and experimental investigation.

#### ENVIRONMENTAL FACTORS

It is well known that the clinical expression of autoimmune disease often requires cofactors associated with the environment in order to initiate the tissue damage. Environmental factors include infectious agents, chemicals, mitogens, and so on. These factors do not normally cause abnormal immune responses on their own, but in conjunction with the underlying autoreactivity outlined above they may contribute to the development and/or progression of the disease.

**Hepatitis C** The effect of alcohol consumption on the clinical progression of chronic hepatitis C infection leading to progressive liver damage is well known (82). Although individuals who are both chronic alcohol abusers and positive for hepatitis C infections have additive effects in the development of liver disease, it is unclear whether these are two independent processes or whether both conditions may independently affect the immune system, to produce progressive tissue damage.

The pathogenesis of the accelerated hepatic injury described in coexisting hepatitis c virus (HCV) and alcoholism is not fully understood but is likely multifactorial. Liver biopsies in

HCV-infected patients who drink alcohol typically reveal a pattern of hepatic injury consistent with chronic viral hepatitis, suggesting that alcohol somehow potentiates the effects of HCV, rather than causing traditional alcohol-related liver injury (83). Alcohol use has long been associated with immune dysfunction, which may impact on the immune control of HCV. Alcohol also appears to have effects on HCV replication and/or clearance and may impact on the evolution of HCV quasispecies, presumably through its effects on the immune system. Alcohol use has been associated with functional impairment of granulocytes, macrophages, and lymphocytes (84) and has been reported to affect the humoral immune response to various viral antigens, including hepatitis A vaccine, hepatitis B vaccine, and immunogenic HIV peptides. Although malnutrition in ALD may contribute significantly to alcohol-related immune dysfunction, there is evidence that acute alcohol ingestion is associated with reduced T-cell proliferative responses (82). This decreased proliferation appears to be mediated by decreased monocyte accessory cell function and was associated with increased IL-10 and IL-13 levels and decreased IFN- $\gamma$  levels. The latter finding is suggestive of a Th2 cytokine predominance, which may play a role in HCV persistence. These data strongly suggest that alcohol may potentiate HCV liver disease by impairing the host HCV-specific immune response, once again resulting in the increased presence of the virus and the potential of prolonged tissue damage.

**Other Organisms** Besides bacterial endotoxins, a number of other infectious agents have been strongly associated as cofactors for ALD. Such organisms as hepatitis B virus, cytomegalovirus, or *Listeria monocytogenes* have all been shown to have immunopathologic effects on the liver following chronic ethanol consumption. It has been suggested that immunosuppression by alcohol results in a decreased ability of the host to clear these agents. Thus, a proinflammatory response mediated by macrophages, neutrophils, and cytokines is initiated that results in nonspecific tissue damage. Additionally, since many of these infectious agents infect the hepatocytes themselves, an antigen-specific CD8<sup>+</sup> cytotoxic T-cell response may result in increased tissue damage. Thus, these agents, in conjunction with alcohol consumption, alter the normal immune response in the liver and potentiate the tissue damage.

## CONCLUDING REMARKS AND OPEN QUESTIONS

Although the exact pathophysiology of ALD remains unsolved, there is substantial evidence that altered immune reactivity occurs as a consequence of alcohol ingestion in both humans and animal models. Clinically, the association of circulating autoantibodies, hypergammaglobulinemia (85), antibodies to unique hepatic proteins (25–27), and cytotoxic lymphocytes reacting against autologous hepatocytes (28) strongly suggests altered immune regulation.

Experimentally, the significant immune responses generated that specifically recognize proteins modified by metabolites of alcohol also point to the important role that immune reactions may play in inducing and/or sustaining an inflammatory cascade and tissue damage in the liver.

Although it appears to be well established that protein adducts can form *in vivo* after alcohol consumption and that such adducts can induce specific immune responses, many important questions must be answered before the direct role of adducts as causal factors in ALD can be established. It remains to be shown that immunological factors are directly responsible for liver necrosis, inflammation, or fibrosis. Although multiple studies suggest this association, there are still inadequate data to affirm direct relationships among adduct formation, antigen-specific immune responses, and liver tissue damage. This is particularly problematic in that no spontaneous models of alcohol ingestion leading to immune-mediated hepatocyte dysfunction have been described.

Although ethanol and its metabolites are too small to act as immunogens, acting as haptens, they could produce changes in the membranes of hepatocytes (28). Evidence in favor of this is provided by the demonstration of antibodies to liver membrane antigens reactive with ethanol-altered hepatocytes (16,17,21,22) and the presence of circulating cytotoxic lymphocytes reactive with autologous hepatocytes (28). Certainly, the histologic appearance of ALD is also suggestive of a chronic active hepatitis-like inflammatory disease. The increasing abnormalities reported when alcohol metabolites complex (adduct) with cellular proteins further suggest that immune responses to modified antigens (neoantigens) occur as a consequence of increased alcohol ingestion (21,22,32,34,35,40–44). However, the role of liver membrane antigens and/or neoantigens in ALD will remain uncertain until these antigens and their specificities are fully characterized.

The evidence that immune mechanisms have a pathogenic role in ALD is increasing but remains circumstantial. Thus several critical questions remain:

1. Are neoantigens or the true self-antigens the major targets of alcohol-induced immune responses?
2. Is the presence of autoreactivity in ALD a primary pathophysiological event or an epiphenomenon manifested as a secondary response to liver injury?
3. What is the relative contribution of immune-mediated cytokine production to the magnitude of the liver injury?
4. What immunosuppressive approaches may be beneficial in devising new therapeutic strategies in ALD?

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# 25 Immunomodulation Therapy for Alcoholic Hepatitis

## *Rationale and Efficacy*

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ROBERT O'SHEA AND ARTHUR J. MCCULLOUGH

### KEY POINTS

- Alcoholic hepatitis is a clinicopathologic entity resulting from direct and indirect mechanisms of hepatotoxicity.
- Patients with severe alcoholic hepatitis have a 30-d mortality of approx 65%.
- The severity of alcoholic hepatitis is defined by a Maddrey Discriminant Function of 32 or more or a MELD of 18 or more.
- Patients with severe alcoholic hepatitis have an over-aggressive immune system, a proinflammatory cytokine profile, and increased oxidative stress.
- Corticosteroids have proved to be effective therapy in severe alcoholic hepatitis by suppressing an overly stimulated innate immune system and consequently proinflammatory cytokines.
- The optimal duration of corticosteroid treatment needs to be reconsidered, and predictors of steroid treated responders need additional study.
- The efficacy of pentoxifylline needs to be confirmed and compared with that of steroids in severe alcoholic hepatitis. The combination of pentoxifylline and steroids may be more beneficial than either agent individually because of their different mechanisms of action.
- Although infliximab and etanercept may eventually be shown to be effective therapeutic agents in severe alcoholic hepatitis, their risk–benefit profile limits their clinical utility at the present time.
- Although based on a sound scientific rationale, antioxidants have not been shown to be efficacious in patients with severe alcoholic hepatitis. However, available studies have been flawed. Future studies need to consider different classes of antioxidants, and measures of oxidant stress need to be measured to gauge treatment effect.
- A number of new therapeutic agents, such as misoprostil and thalidomide, need to be studied in severe alcoholic hepatitis because of their lower cost and safety profile.

### INTRODUCTION

Alcohol is a widespread, socially-accepted hepatotoxin in most countries. Approximately two-thirds of the adult U.S. population drinks at least 18 drinks a year (1), and 7 to 10% of the US population meet the diagnostic criteria for alcohol abuse or alcoholism (2). In industrialized countries, up to 66% of all chronic liver disease is related to alcohol use (1,3). Alcohol accounts for 40 to 50% of all deaths owing to cirrhosis (1) and remains the most common cause of liver-related mortality (4). However, alcoholic liver disease (ALD) represents a spectrum of histologic changes and clinical outcomes, as shown in Fig. 1.

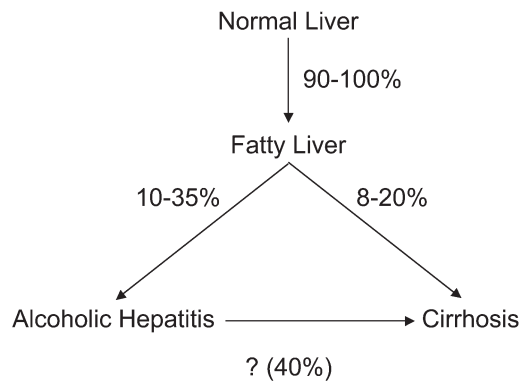
A normal liver may contain up to 5% of its volume as fat. It has been estimated that although 75 to 100% of heavy drinkers show evidence of fatty liver, only 8 to 20% of patients with fatty liver will develop cirrhosis, and only 10 to 35% will develop alcoholic hepatitis (5). The mortality rate of alcoholic fatty liver is insignificant unless it advances to cirrhosis (6,7). The age-adjusted death rate for alcoholic cirrhosis is 3.8 per 100,000 (1,8). The 5- and 10-yr survival rates for alcoholic cirrhosis without liver transplantation are 23 and 7%, respectively, which is significantly less than the rates for other forms of cirrhosis (Table 1).

Worldwide, alcoholic cirrhosis accounts for up to 50% of all cirrhosis-related deaths (9,10).

However, the topic of this discussion is alcoholic hepatitis, which has a mortality of up to 65% in hospitalized patients if untreated. The therapy for alcoholic hepatitis remains a much discussed and controversial area of clinical hepatology (11).

### PROGNOSIS

The mortality rate of hospitalized patients with alcoholic hepatitis varies widely. Based on clinical experience and many clinical trials, it is clear that patients with mild disease need not be treated with extraordinary measures. It is also likely that patients with severe disease *in extremis* may be too ill to respond to any form of therapy. Currently, alcoholic hepatitis is not a routine indication for orthotopic liver transplantation,



**Fig. 1.** Histological outcomes associated with heavy alcohol use.

**Table 1**  
Survival of Different Types of Cirrhosis

Etiology	No.	Survival (%)	
		5-yr	10-yr
Alcohol	82	24*	7*
Cryptogenic	13	33	20
HCV	62	38	24
HBV	42	48	20
Hemochromatosis	20	41	22
Autoimmune	16	46	23
PBC	36	56	39

Abbreviations: HCV, hepatitis C virus; HBV, hepatitis B virus; PBC, primary biliary cirrhosis.

\* $p < 0.05$  vs other forms of cirrhosis.

with particular cases considered on a case-by-case basis (12–14). Consequently, it is important to identify those patients who might benefit from aggressive intervention of treatment, as well as those for whom the therapeutic benefit–risk ratio is not favorable. In addition to allowing the clinician to tailor therapy according to disease severity in an individual patient, certain predictors of severity may allow for accurate evaluation of new therapies in patients with disease of similar severity (5).

### HEPATIC INFLAMMATION

Although the following may seem obvious, it is important for the clinician to determine whether the patient with alcoholic liver disease has inflammation as part of the diagnosis of alcoholic hepatitis. Histological findings have been shown to add to the discriminatory ability to predict outcomes in patients with alcoholic hepatitis. Hepatic inflammation, necrosis, and Mallory bodies are the most important prognostic factors, and help to differentiate patients at high risk of death from others without inflammatory changes (15).

In a study of 217 patients (140 cirrhotics and 77 noncirrhotics) with biopsy-proven ALD (15), the presence of hepatitis indicated a poor prognosis. Patients with cirrhosis and hepatitis had increased 1- and 5-yr mortality rates of 27 and 47% respectively, values higher than cirrhotic patients without hepatitis. This observation has been indirectly confirmed by a study that

found the presence of polymorphonuclear cells (PMNs) on liver biopsy to be a prognostic factor for early and late survival. The extent of infiltration with PMNs correlates with survival in patients treated with steroids (16). This may be due partly because PMNs are a source of hepatocyte growth factor in patients with severe alcoholic hepatitis (17). The degree of tissue cholestasis has also been shown to be prognostically important, with increasingly severe cholestasis a marker for poorer prognosis (18).

### RISK FACTORS

**Individual Risk Factors** A number of individual measures have been found that may play a role in the pathophysiology of alcoholic hepatitis (5,19). These include clinical features (presence of encephalopathy, new-onset ascites, and renal failure), demographics (age, female gender, and years of drinking), laboratory studies (hemoglobin level, vitamin B<sub>12</sub> levels, alkaline phosphatase, arterial ketone body ratio, prothrombin time, creatinine, bilirubin, change in bilirubin, and factor V levels), histologic features on liver biopsy (PMN count, extent of steatosis, cholestasis, and presence of megamitochondria), and markers of inflammation and cytokine activity (tumor necrosis factor- $\alpha$  [TNF- $\alpha$ ], interleukin [IL]-6, -8, and -10, C-reactive protein, presence of disseminated intravascular coagulation, serum endotoxin levels IL-5, and lipopolysaccharide [LPS] binding protein).

**Scoring Systems** Although several clinical scoring systems have been derived in patients with cirrhosis, relatively few have been specifically tested in alcoholic hepatitis. These indices have used a variety of factors to predict outcomes with varying success. They have included markers of hepatic metabolic activity, routinely collected biochemical parameters, clinical and demographic features, or scores based on hepatic histology. These severity of illness scores for ALD include the Child-Turcotte-Pugh (CPT) score (20), which is commonly used to estimate the severity of cirrhosis (20), the Combined Clinical Laboratory Index (CCLI) of the University of Toronto (21), the Maddrey Discriminant Function (MDF) (22), the Beclere model (16), the Mayo End-Stage Liver Disease (MELD), score (23–26), and, most recently, the Glasgow alcoholic hepatitis score (27) (Tables 2 and 3).

The MDF score was derived in clinical trials of patients with alcoholic hepatitis, and has since been widely applied clinically to the management of patients with this disease. It has been used to stratify patients' severity of illness for most of the research involving the use of steroids in patients with alcoholic hepatitis. In combination with the presence of encephalopathy, a "discriminant function score" of 32 or more is highly correlated with a more than 50% short-term mortality rate in patients with alcoholic hepatitis. Although the MDF is a continuous measure, its interpretation (using a threshold of less or more than 32) has converted it into an essentially categorical method of classification. It thus suffers from a related measurement problem, that is, once patients have exceeded that threshold, they cannot be further characterized without the use of an additional or alternative clinical prediction rule.

**Table 2**  
**Maddrey Discriminant Function Score for Alcoholic Hepatitis**

	<i>Discriminant function</i>	<i>Score indicating poor prognosis</i>
Initial	$(4.6 \times \text{prothrombin time [s]})$ serum bilirubin (mg/dL)	>93
Modified	$4.6 (\text{patient's prothrombin time} - \text{control time}) +$ serum bilirubin (mg/dL)	$\geq 32$

**Table 3**  
**Glasgow Alcoholic Hepatitis Score<sup>a</sup>**

<i>Measured parameter</i>	<i>Points</i>		
	<i>1</i>	<i>2</i>	<i>3</i>
Age	<50	$\geq 50$	—
WBCs (10 <sup>9</sup> /L)	<15	$\geq 15$	—
Urea (mmol/L)	<5	$\geq 5$	—
PT ratio/INR	<1.5	1.5–2.0	>2.0
Bilirubin ( $\mu\text{mol/L}$ )	<125	125–250	>250

Abbreviation: INR, international normalized ratio; PT, prothrombin time; WBCs, white blood cells.

<sup>a</sup>A score of  $\geq 9$  is associated with a poor prognosis.

Although many investigators have studied prognostic features, or derived predictive indices, relatively few of these indices have been independently validated. One review compared the CPT score and the Orrego score with the MDF in a Veterans Administration Cooperative AH Study, evaluating their ability to predict 30-d mortality (19). All correlated with survival (Fig. 2), but the less complex Maddrey criteria had the best correlation and the highest positive predictive value. Furthermore, the prognostic value of the MDF criteria has been confirmed prospectively.

More recently, investigators have applied the MELD score to predict the outcome in patients with alcoholic hepatitis. The MELD score was initially developed to predict outcomes in patients undergoing the transjugular intrahepatic portal-systemic shunt (TIPS) procedure and was later shown to predict outcome in patients awaiting liver transplant (23).

In a comparison of the MDF in patients with alcoholic hepatitis, the MELD score was shown to predict the outcome as well as the discriminant factor (24–26), as shown in Fig. 3. Using the usual cutoff ( $< 32$  or  $\geq 32$ ), vs MELD score of more than 18, the two indices had similar sensitivities, although the MELD score may have had a higher specificity. However, there has been no prospective confirmation of the utility of MELD in alcoholic hepatitis. In addition, there is no consensus as yet, regarding what MELD score should be used to predict severity of disease in alcoholic hepatitis (Table 4).

Dynamic models, which incorporate the changes in laboratory studies over time, have also been used to estimate the outcome in this patient group. Recently a French group identified the changes in bilirubin in the first week of hospitalization to be

significantly associated with outcome of patients with alcoholic hepatitis treated with prednisolone (28).

The discriminatory ability of the MDF score was also tested specifically against a model derived from a neural network using nine variables, including five laboratory features and four clinical variables: albumin, white count, creatinine, bilirubin, prothrombin time, along with the presence of encephalopathy, gastrointestinal bleeding, peritonitis, and ascites (29). Receiver operator characteristics (ROC) curve areas suggested that the neural network was significantly more sensitive; adding parameters from day 7 in the hospital suggested even greater ability to determine outcomes. This model, however, has not been widely applied, partly because of its mathematical and practical complexity. As a result, the MDF formula is still widely used by physicians to predict the outcome of patients with acute alcoholic hepatitis. However, it is likely that the MELD score may gain more acceptance in the future.

## PATHOPHYSIOLOGICALLY BASED TREATMENT

As shown in Table 5, alcohol exerts its hepatotoxicity by direct and indirect mechanisms of injury, which is balanced by the ability of the liver to regenerate.

However, since the focus of this discussion is treatment as it relates to immunosuppression and anticytokines and their associated oxidative stress, only the pathogenesis related to those therapies will be discussed (Fig. 4).

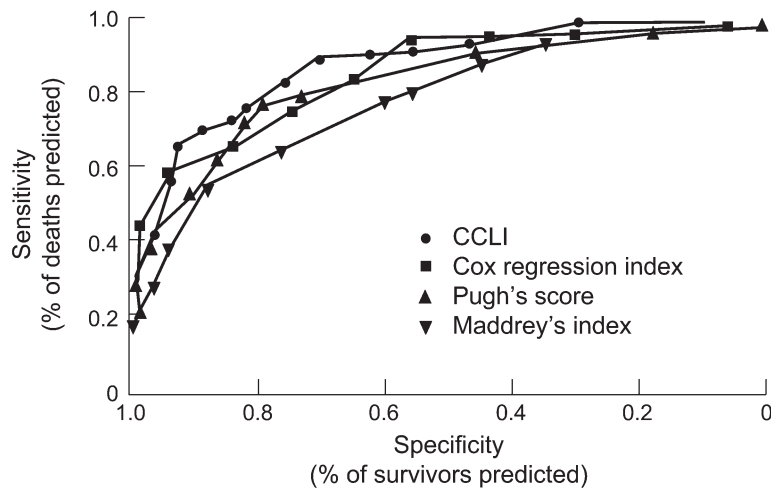
### CORTICOSTEROIDS

**Rationale** The specific rationale for the use of corticosteroids is to suppress an overly aggressive immune system, thought to be provoked by enhanced generation of neoantigens induced by acetaldehyde adducts (31). The seneoantigens include liver-specific lipoprotein, liver membrane antigen, Mallory bodies, epitopes of protein-aldehyde and acetaldehyde adducts (35), autoantibodies to P4502E1 and P4503A4 (36), and antibodies to liver antibodies to liver membrane antigen (37).

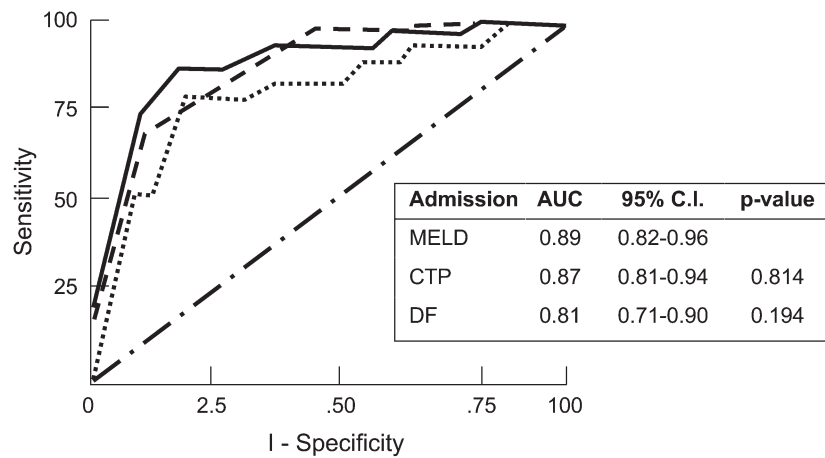
Recent studies suggest that there may be a genetic component to the overactive immune system in patients with alcohol-related injury. Polymorphisms for IL-10, which downregulates acute inflammation, could enhance susceptibility to ALD (38–40). Perhaps more interesting are the studies implicating a polymorphism encoding cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4) as a risk factor for ALD (41–43). CTLA-4 functions as a suppressor of T-cell-mediated immune responses, and the polymorphisms observed are associated with low CTLA-4 alleles, which, when combined with hydroxyethyl-modified antigens in serum, posed an additive risk of CYP2E1 antibody formation and ALD.

These findings emphasize the potential for intricate interactions among environmental factors, alcohol ingestion, and genetics in affecting immune-mediated injury in ALD.

Figure 5 displays the enhanced cytotoxicity of lymphocytes toward hepatocytes observed in patients with alcoholic hepatitis (44). Alcoholics have lower than normal numbers of all types of T cells (45). In addition, alcohol impedes the T cell's ability to multiply and exert an influence after activation (45).



**Fig. 2.** Comparison of different scoring systems using ROC curves to predict mortality in patients with alcoholic hepatitis. CCLI, composite clinical and laboratory index.



**Fig. 3.** ROC curves for Mayo End-Stage Liver Disease (Meld; -), Maddery Discriminant Function (MDF;...) and, Child-Turcotte-Pugh (CPT- - -)/ seers, with a diagonal reference line. AUC, area under the curve. (Data from ref. 26.)

**Table 4**  
Proposed Mayo End-Stage Liver Disease (MELD) Scores That Accurately Predict Disease Severity in Alcoholic Hepatitis

Study	Proposed score
Sheth et al. (24)	11
Dunn et al. (25)	21
Srikureji et al. (26)	18 <sup>a</sup> or 20 <sup>b</sup>

<sup>a</sup>MELD score on admission.

<sup>b</sup>MELD score 1 wk after admission.

Steroids also exert a direct antifibrotic effect by suppressing the expression of extracellular matrix proteins in the liver. Corticosteroids have established antiinflammatory effects that may directly impact on the pathophysiology of this disease. The role of gut-derived endotoxin in the hepatic damage mediated by stimulation of cytokines (IL-1, -6, and -8, TNF, and

transforming growth factor- $\beta$  [TGF- $\beta$ ]) has been recently emphasized in the pathophysiology of ALD (46–48). Because cytokine synthesis has been shown to be a highly regulated event, with an inhibitory feedback loop provided by glucocorticoids, the effect of steroids on alcoholic hepatitis may be partly related to their inhibition of cytokine production (49) (Fig. 6).

**Clinical Trials**

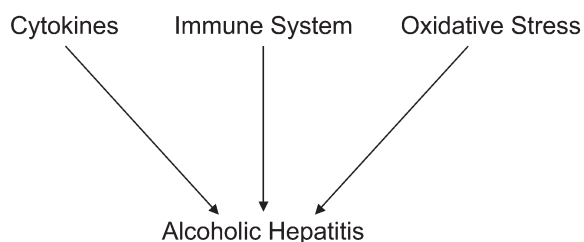
**Steroids** Corticosteroids have been used in the treatment of this disorder for seven decades and are thus the most extensively studied treatment modality. Their efficacy, however, remains controversial. As shown in Table 6, five randomized clinical trials suggested that corticosteroids reduce mortality compared with placebo, whereas eight others found no difference in outcomes (50–62).

Although the results are not consistent, multiple differences in trial design may explain the different outcomes. These

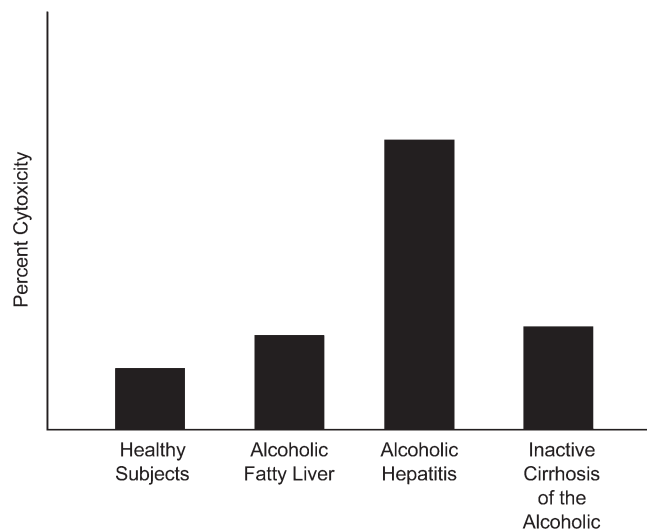
**Table 5**  
**Pathophysiology and Potential Therapies<sup>a</sup>**

<i>Direct injury</i>		<i>Indirect injury</i>	
<i>Proposed treatment</i>	<i>Mechanism</i>	<i>Mechanism</i>	<i>Proposed treatment</i>
Polyunsaturated lecithin (PUL) <sup>†</sup>	Membrane damage	“GUT”function Cytokines	Antibiotics, nutrition Anti-TNF- $\alpha$ Pentoxifylline* steroids $\geq$ interferon
S-adenosyl-L-methionine Propylthiouracil (PTU) <sup>+</sup>	Oxidative Hypermetabolism Regenerative capacity Nutrition <sup>+</sup> , oxandrolone <sup>+</sup> , hepatotropic agents	Immunological stress Fibrogenesis	Steroid* mechanism Colchicine <sup>†</sup> PUL <sup>+</sup>

<sup>a</sup>Therapies that have been investigated in randomized placebo-controlled trials and found to effective\*, ineffective<sup>†</sup>, or possibly effective<sup>+</sup>.

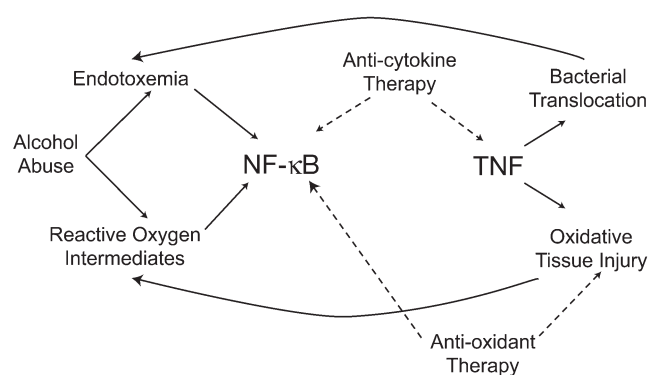


**Fig. 4.** The immune system, cytokines, and oxidative stress all play important roles in the pathogenesis of alcoholic hepatitis.



**Fig. 5.** Cytotoxic effects of lymphocytes on autologous liver cells from healthy subjects and from patients with alcoholic liver disease.

include differences in dose and duration of therapy, selection of patients (e.g., varying time intervals before randomization or inconsistent use of disease severity scoring), possible misclassification bias (e.g., differing percentages of patients who underwent liver biopsy to confirm the diagnosis), severity of illness, concomitant medical problems or medications, and



**Fig. 6.** The relationships among alcohol, endotoxemia, cytokines, and oxidative stress as well as potential sites for therapeutic intervention. NF- $\kappa$ , nuclear factor  $\kappa$ B; TNF, tumor necrosis factor.

undiagnosed chronic viral hepatitis infections. Despite these differences, three separate metaanalyses have found a benefit to the use of steroids (63–65). The results of the combined data from one of these meta-analyses (65) indicate that corticosteroids should perhaps be targeted to specific subsets of patients with severe disease. For example, steroid treatment provided protective efficacy in 27% of patients with hepatic encephalopathy, which increased to 40% among higher quality trials and in 51% of patients without gastrointestinal bleeding. Among subjects without hepatic encephalopathy, corticosteroids had no protective efficacy, and this lack of efficacy was consistent across all trial groups (Table 7).

In response to the metaanalysis suggesting a lack of efficacy (66), a reanalysis of pooled data from three placebo-controlled randomized trials, using the MDF as a measure of disease severity (67), concluded that treated patients had a significantly higher survival than patients given placebo: 84.6% vs 65% (Fig. 7). Extrapolating from this result, a number needed to treat of 5 (i.e., five patients treated to prevent one death) was calculated.

The efficacy of corticosteroids is substantiated by the fact that in the two prospective studies (61,62) that stratified patients according to disease severity quantified by the discriminant

**Table 6**  
Efficacy of Steroids in Clinical Trials

Author	Date	No. of patient	No. of deaths (%) [95% CI]		Relative risk (RR)
			Placebo	Steroid	
Porter et al. (51)	1971	20	7/9 77 [0.44–0.93]	6/11 55 [0.28–0.79]	1
Helman et al. (50)	1971	37	6/17 35 [0.14–0.62]	1/120 05 [0.0013–0.25]	0.143
Campra et al. (52)	1973	45	9/25 36 [0.2–0.56]	7/29 35 [0.18–0.57]	1
Blitzer et al. (53)	1977	33	5/16 31 [0.14–0.56]	6/12 50 [0.25–0.75]	1
Lesesne et al. (54)	1978	14	7/7 100 [0.63–1.0]	2/7 3 [0.09–0.65]	0.29
Shumaker et al. (56)	1978	27	7/15 47 [0.25–75]	6/12 50 [0.25–0.75]	1
Maddrely et al. (55)	1978	27	6/31 19 [0.09–0.36]	1/24 042 [0.009–0.20]	0.22
Depew et al. (51)	1980	28	7/13 54 [0.29–0.77]	8/15 53 [0.3–0.75]	1
Theodosi et al. (58)	1982	55	16/28 57 [0.39–0.74]	17/27 63 [[0.44–0.79]	1
Mendenhall et al. (59)	1984	178	50/88 57 [0.46–0.67]	55/90 61 [0.51–0.71]	1
Bories et al. (60)	1987	45	2/21 9 [0.029–0.29]	1/24 40 [0.0098–0.20]	1
Carithers et al. (61)	1989	66	11/31 36 [0.21–0.53]	2/35 057 [0.108–0.19]	0.16
Ramod et al. (62)	1992	61	16/29 55 [0.37–72]	4/32 13 [0.05–0.28]	0.23

**Table 7**  
Corticosteroids in Alcoholic Hepatitis—A Metaanalysis

Trial characteristic	No.	Risk ratio (RR) (95% CI)	Protective efficacy (1-RR)(%)
<b>Patients with hepatic encephalopathy</b>			
All trials	11	0.73 (0.58–0.92)	27%
GI bleeding excluded	7	0.49 (0.33–0.72)	51%
GI bleeding not excluded	4	1.06 (0.76–1.48)	NS
Quality score $\geq 4$	7	0.56 (0.38–0.83)	44%
Quality score $< 4$	3	1.05 (0.75–1.47)	NS
“Best estimate” <sup>a</sup>	5	0.64 (0.42–0.97)	36%
<b>Patients without hepatic encephalopathy</b>			
All trials	9	1.07 (0.68–1.71)	NS
GI bleeding excluded	5	1.01 (0.36–2.81)	
GI bleeding not excluded	4	1.21 (0.72–2.04)	NS
Quality score $\geq 4$	6	1.02 (0.47–2.26)	NS
Quality score $< 4$	3	1.18 (0.65–2.13)	NS
“Best estimate” <sup>a</sup>	4	1.01 (0.35–2.91)	NS

Abbreviation: No., number of trials; CI, confidence interval; RR, relative risk; NS, not significant.

<sup>a</sup>Best estimate: those trials with: (1) quality score 4; (2) baseline equivalence between groups; (3) exclusion of active gastrointestinal bleeding.

<sup>b</sup>If the 95%CI includes unity(1) then there is no significant therapeutic benefit (or protective efficacy) of corticosteroids for that subgroup.

function, both showed significant benefit in terms of 30-d hospital survival for patients with severe alcoholic hepatitis (Fig. 8). In addition, a follow-up study by Mathurin et al. (67) showed that steroids improved the survival at 1 yr but not 2 yr in these patients (Fig. 9).

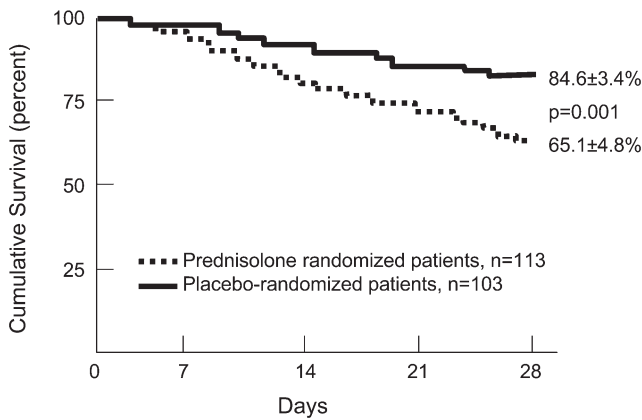
Nonetheless, many physicians still do not use corticosteroids for alcoholic hepatitis (68) even though the American College of Gastroenterology recommended in 1998 that corticosteroids should be used in the treatment of severe alcoholic hepatitis (69), as shown in Table 8.

There are a number of reasons for the reluctance of physicians to use corticosteroids. First, the largest controlled trial of corticosteroids in alcoholic hepatitis failed to show a benefit for this treatment (59). However, as shown in Fig. 10, when patients were stratified for disease severity by the MDF, corticosteroids were effective at an MDF between 35 and 54. The second reason is the personal experience of

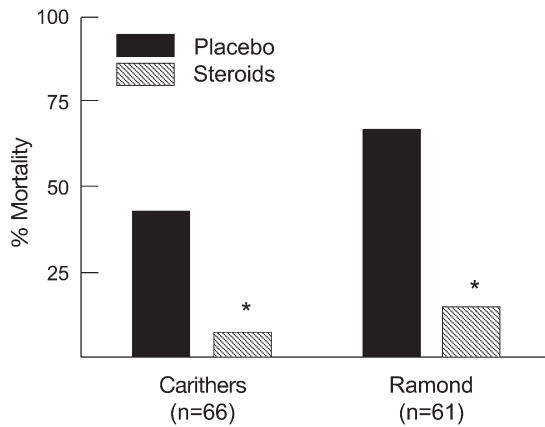
physicians that most alcoholic patients still die when treated with corticosteroids. This in fact is true, but it should be remembered that, as shown in Fig. 10, some patients are just too sick. The number can be calculated from Tables 6 and 7 and Fig. 8 and 10; the number of patients that need to be treated in order to see the benefit of corticosteroids is between five and seven. In addition, 25% of patients with the clinical diagnosis of alcoholic hepatitis fail to have that diagnosis on liver biopsy (70,71). Third, there are significant side effects, and, as discussed below, 4 wk of corticosteroids may be too long. Finally, many patients with severe alcoholic hepatitis have contraindications to the use of corticosteroids.

These combined data provide a number of tangible suggestions for patient management. First, only patients with severe disease (as defined by the presence of hepatic encephalopathy, the MDF, or possibly the MELD score) should be treated with corticosteroids. Second, as just noted, approx five to seven

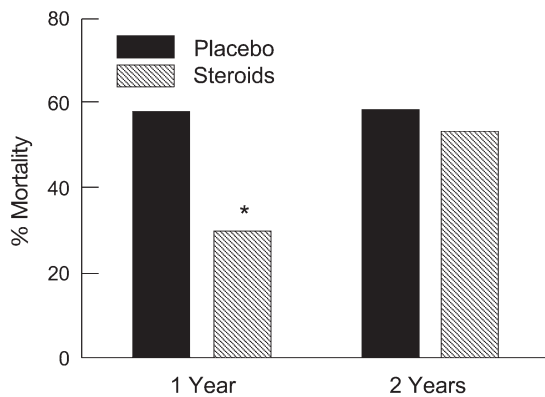




**Fig. 7.** Survival of patients with an MDF of or more 32 from three randomized controlled trials (59,61,62) (Data from ref. 67.)



**Fig. 8.** The effect of steroids vs placebo on survival in patients with alcoholic hepatitis (\*,  $p < 0.05$ ).

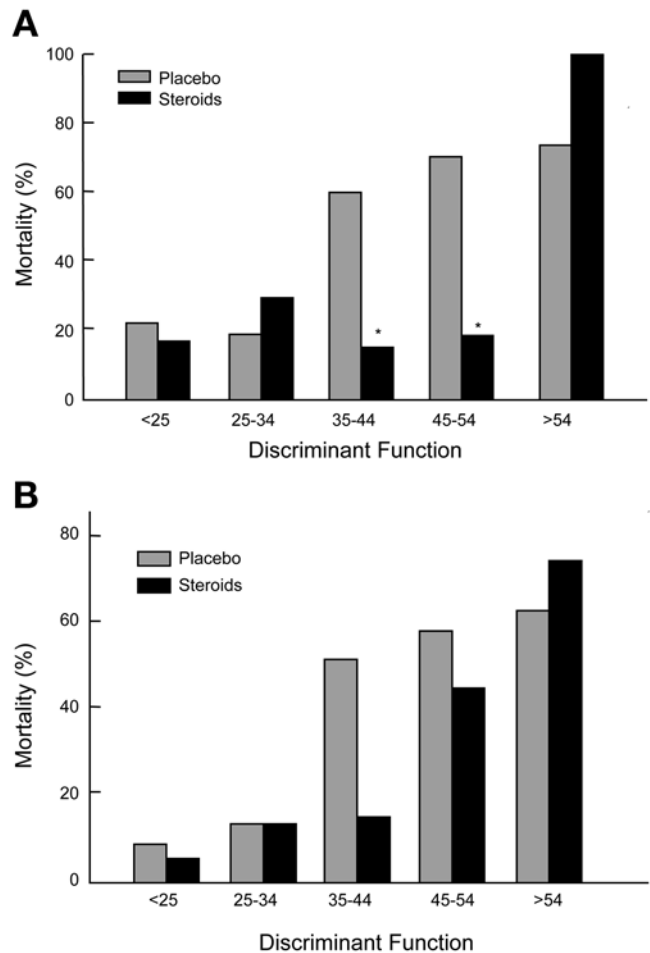


**Fig. 9.** Corticosteroids improved survival at 1 yr but not 2 yr.

patients need to be treated to avoid one death. This latter point emphasizes the importance of careful selection to avoid the side effects of corticosteroids in the other four to six patients who will derive no clinical benefit from corticosteroids. In general, this means excluding patients with active

**Table 8**  
**Alcoholic Hepatitis and Steroids: Why Do Hepatologists Disagree?**

1. Steroids were ineffective in the largest study  
Subsequent analysis showed them to be effective
2. Personal experience  
Number need to treat is 5–7  
Some patients just too sick  
Incorrect diagnosis
3. Side effects  
4 wk just too long
4. Contraindications  
GI bleeding, infection, pancreatitis



**Fig. 10.** Mortality of alcoholic hepatitis patients at 1 (A) and 6 (B) mo stratified by MDF (59). \* $p < 0.05$ .

infection and being certain of the diagnosis (liver biopsy may be necessary) because histologically confirmed alcoholic hepatitis may correlate poorly with the clinical impression of alcoholic hepatitis (70,71) and to as many as 28% of patients with a clinical picture of alcoholic hepatitis do not have histological features of alcoholic hepatitis on liver biopsy.

Third, based on pharmacologic considerations (prednisone is converted to the active form—prednisolone—in the liver) as well as the published clinical trial data, prednisolone (40 mg

**Table 9**  
**Comparative Benefits of Enteral Nutrition and Steroids**

Mortality	Enteral nutrition (n=35)	Steroids (N=36)
Mortality at 1 m (%)	31	25
Day of death (Median)	7	23
Mortality at 1 yr (%)	31	25

Data from ref. 73.

daily for 4 wk followed by a taper should be used in favor of prednisone (72). Fourth, although such treatment reduces mortality risk by 25%, there is still up to 44% mortality in patients receiving corticosteroids. Therefore, other therapies or combinations of therapies need to be considered. Consistent with this latter point, the use of corticosteroids for alcoholic hepatitis is infrequent (68) even though the American College of Gastroenterology recommended in 1988 that corticosteroids should be used in the treatment of severe alcoholic hepatitis (69).

**Steroids Plus Nutritional Supplementation** A number of early clinical trials have demonstrated that nutritional supplements (especially when positive nitrogen balance was achieved) were beneficial (5). One comparative trial (73) also reported enteral feedings to be as effective as corticosteroids, but each may be beneficial in different ways (Table 9). In the initial 10 d of treatment, steroids were more effective (presumably by decreasing the immune and inflammatory injury), whereas enteral nutrition was more effective after 10 d (presumably by improving gut function and hepatic regeneration).

This information provides the basis for hypothesizing that therapy for alcoholic hepatitis may be optimized by targeting both the time of intervention and the localization of the abnormality, as well as the different pathophysiologic consequences.

### ANTICYTOKINE THERAPY

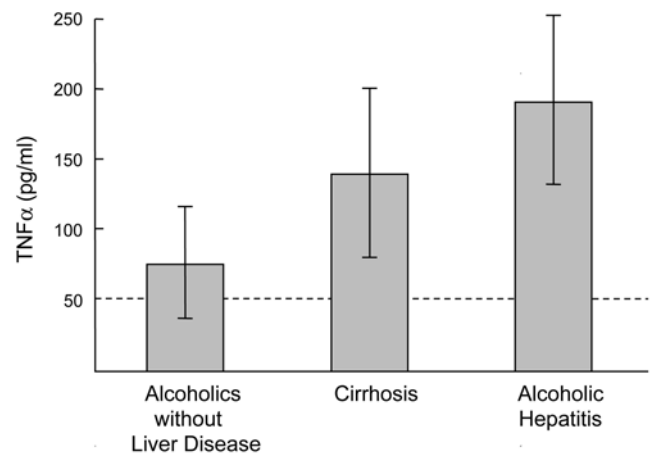
**Rationale** Since cytokines are essential to the processes of hepatocyte inflammation, death, and regeneration, it is not surprising that a great deal of work has focused on the markers of an abnormal cytokine milieu in these patients, as shown in Fig. 11 (74).

Serum and monocyte levels of TNF, as well as IL-1, IL-6, and IL-8, are elevated in alcoholic hepatitis (74–76). Animal models of alcohol-mediated injury indicate that TNF plays an important pathophysiologic role (77) (Fig. 12).

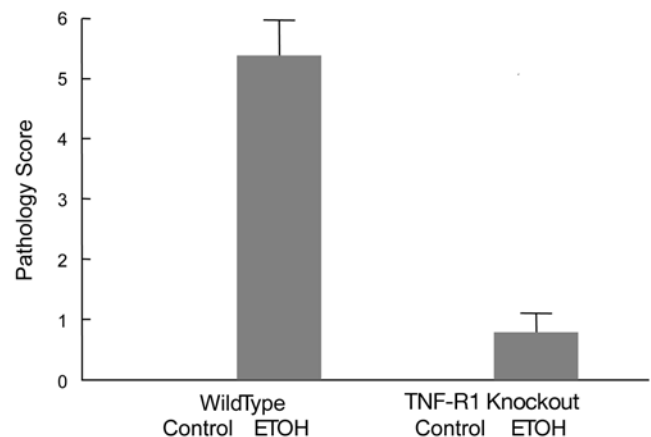
Therefore, as suggested in Fig. 6, therapy directed against alcohol- and endotoxin-induced cytokine production might be effective. Initial studies suggest that this is true.

TNF antibodies have been shown to prevent liver injury in a rat model (78). In humans, levels of soluble TNF receptors correlate linearly with an increased risk of mortality (79), and serum levels of TNF are high on admission and correlate with mortality (80,81). In addition, monocytes from patients with AH produce TNF- $\alpha$  at higher levels than controls in response to endotoxin.

**Pentoxifylline** Several recent studies have focused on the use of pentoxifylline (Fig. 13), a phosphodiesterase inhibitor initially used in the treatment of peripheral vascular disease



**Fig. 11.** Serum tumor necrosis factor (TNF) levels in patients with alcoholic liver disease.



**Fig. 12.** Alcohol diet increased hepatic injury in a control animal but not in a TNF-R1 knockout mouse.

based on its ability to increase erythrocyte flexibility, reduce blood viscosity, and inhibit platelet aggregation. Phosphodiesterase inhibition, however, has also been shown to have multiple effects on immune markers.

In particular, pentoxifylline has been shown to reduce the production of TNF- $\alpha$ , IL-5, IL-10, and IL-12. It also has been shown to decrease the transcription of IL-2 and TNF- $\alpha$  promoters in transiently transfected normal T cells, to inhibit the activation of nuclear factor- $\kappa$ B (NF- $\kappa$ B) and nuclear factor of activated T cells, and to stimulate activation of protein-1 and cAMP response element-binding proteins (82). In an animal model, it has been shown to reduce portal pressure in cirrhotics (83,84).

Based on these data, a clinical trial using pentoxifylline in 101 patients with severe alcoholic hepatitis was undertaken (85). Patients were randomized to receive either pentoxifylline 400 mg three times a day or placebo.

In-hospital mortality was significantly lower in pentoxifylline recipients compared with controls (24.5% vs 46.1% of patients)

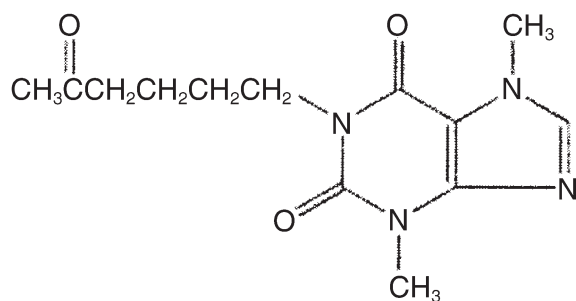


Fig. 13. Structure of pentoxifylline.

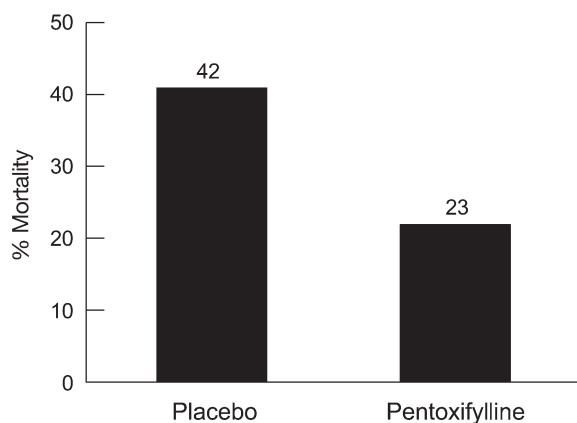


Fig. 14. Pentoxifylline decreased mortality in alcoholic hepatitis. (Data from ref. 85.)

(Fig. 14), yielding a relative risk of 0.59. Of the patients who died, hepatic failure with hepatorenal syndrome developed in significantly fewer pentoxifylline recipients, compared with controls (50% vs 91%). Last, new-onset renal impairment developed in significantly fewer pentoxifylline recipients, compared with controls; further progression in hepatorenal syndrome occurred in 4 of 18 patients in their respective treatment groups, yielding a relative risk of 0.3. The difference in mortality between the two groups suggests a number needed to treat of 4.7, which is almost identical to the number arrived at by Mathurin et al. (67) comparing the use of steroids with placebo. The mechanism whereby pentoxifylline decreased the development of hepatorenal syndrome is unclear, since, as shown in Fig. 15, improvement in the MDF was similar in the treated and control group.

Therefore the efficacy of pentoxifylline could be related to either direct effects on the liver (through any of the above possible mechanisms) or, alternatively, by a direct renal effect.

**Anti-TNF Treatment** These data, along with more recent studies on agents that inhibit particular cytokines, particularly TNF, have recently generated interest in this type of treatment (Fig. 16).

This may be relevant considering the presumed decreased risks of infection compared with steroids and the more specific antagonism of the inflammatory pathophysiologic pathway

in this disease, as discussed above. Two small uncontrolled pilot studies using infliximab (IgG-1 monoclonal antibody to TNF) suggested a benefit in alcoholic hepatitis (86,87). On the basis of these studies, a clinical trial using infliximab (10 mg/kg) in combination with prednisolone (40 mg/d) vs prednisolone alone was begun in France (88). A concern regarding the likelihood of infection using this form of treatment (89) was indeed verified. A total of 36 patients were randomized before the trial was stopped prematurely by the data safety monitoring board, based on a substantially higher death rate in the infliximab group (39% vs 11%) (Fig. 17).

Most of these deaths were related to a highly significant increase in the risk of infection in patients on active treatment compared with controls, who had been treated with prednisolone alone. However, this study was criticized because of the specifics of the study design (90) as well as the premise for the use of such therapy (91).

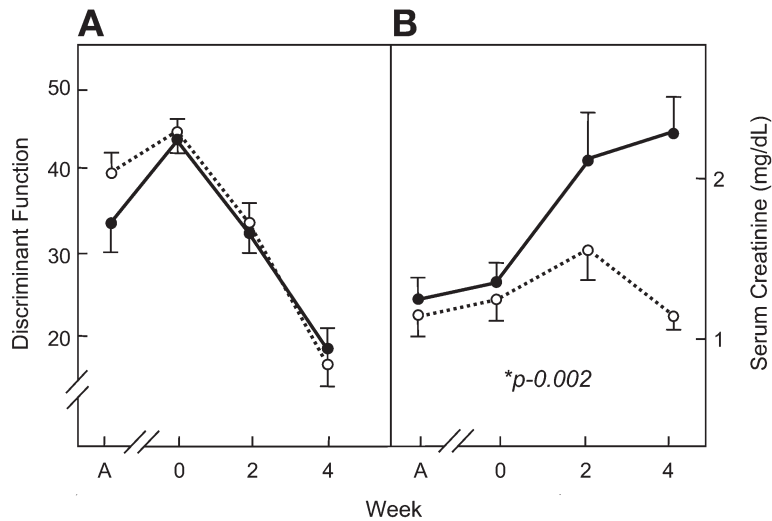
**Etanercept** Etanercept, a P75-soluble TNF receptor/FC fusion protein neutralizes soluble TNF and excludes an effect on membrane bound TNF. It has been used in a variety of rheumatologic disorders, including rheumatoid and psoriatic arthritis, as well as ankylosing spondylitis. The only published report on patients with liver disease involved 13 patients with moderate or severe alcoholic hepatitis who were treated for 2-wk (92). The 30-d survival rate for patients receiving etanercept was 92%. Adverse events (including infection, hepatorenal decompensation, and gastrointestinal bleeding) required premature discontinuation of etanercept in 23% of patients. Based on this study, a larger multicenter clinical trial is now under way.

Although these results are intriguing, the lack of a control arm, the inclusion of patients with relatively more moderate disease (making interpretation of survival statistics uncertain), and the high dropout rate temper the enthusiasm for the use of etanercept. Moreover, in light of these data from the studies of infliximab, the extent to which complete TNF inhibition (via antibody or receptor blockade) is useful, or how best to measure it, is unclear. The outcomes of further clinical trials are needed to answer these questions. In addition, questions have been raised regarding the extent to which TNF inhibition is useful in this disease, as TNF has been shown to be important in hepatic regeneration (93); therefore, one questions how much TNF inhibition may be helpful or whether TNF inhibition may become counterproductive.

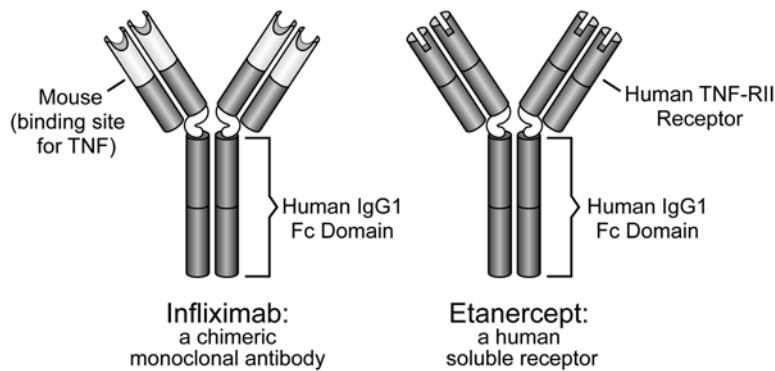
#### ANTIOXIDANTS

**Rationale** When taken in excess, alcohol causes oxidative stress (94), as shown in Fig. 18. This stress is derived from alcohol metabolism and the generation of superoxides, and induction of cytochrome P450 2E1 activity and the product of inducible nitric oxide synthetase (95–97). Alcohol-induced endothelial changes are also associated with oxidative stress and are rapidly reversed after withdrawal (98).

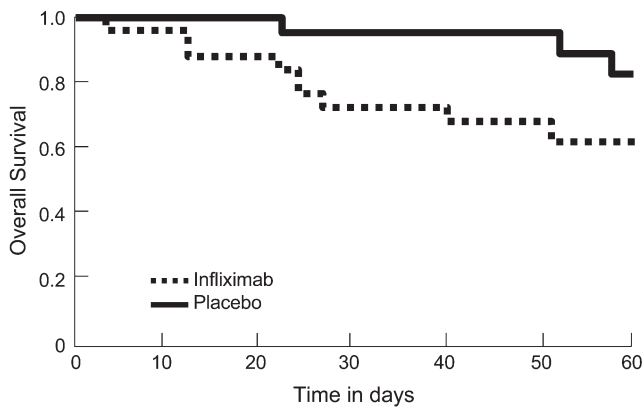
In vitro studies indicate that oxidative stress synthesizes lymphocytes causing TNF- $\alpha$  mediated cytotoxicity (95,98) that is mediated through cellular death domain pathways (99).



**Fig. 15.** Changes in MDF did not differ between the placebo and pentoxifylline treatment groups despite improved survival in the pentoxifylline group. (Data from ref. 85.)



**Fig. 16.** Structures of infiximab and etanercept. TNF, tumor necrosis factor.



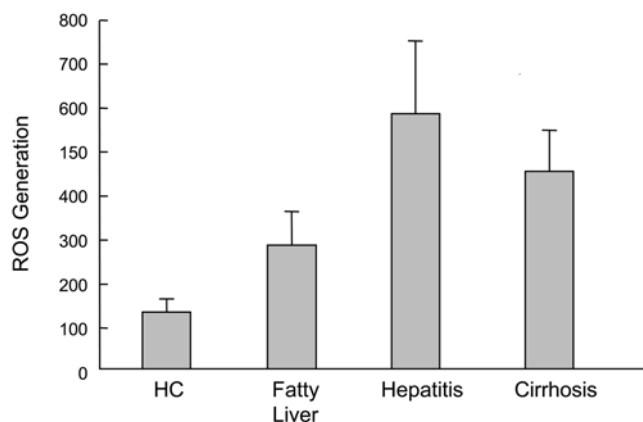
**Fig. 17.** Survival curves (Kaplan-Meier) demonstrated decreased survival in alcoholic hepatitis patients treated with infiximab. (Data from ref. 88.)

In addition, levels of vitamin E (100) are decreased, and mitochondrial glutathione is decreased by ethanol (101). These events may further lead to an imbalance between alcohol-induced oxidative stress and the endogenous components of cellular defense. Finally, antioxidants attenuate NF- $\kappa$ B activation and TNF- $\alpha$  production in alcoholic hepatitis patient monocytes and rat Kupffer cells in vitro (102). Therefore, as suggested in Fig. 7, therapy directed against oxidative stress may be beneficial in alcohol hepatitis. However, as yet this therapy has not shown benefit.

**Clinical trials**

**Vitamin E** Vitamin E when used alone was not shown to be significantly beneficial in either alcoholic hepatitis (103) or alcoholic cirrhosis (104). However, neither of these studies was optimally designed, and there are data suggesting that vitamin E when combined with other antioxidants may improve outcome in AH (105).

**S-Adenosyl-L-Methionine** S-adenosyl-L-methionine (SAME) is a naturally occurring molecule produced in vivo



**Fig. 18.** Release of reactive oxygen species (ROS) by nonstimulated neutrophils from controls and patients with increasing severity of alcoholic liver disease.

from methionine and adenosine triphosphate by the enzyme SAMe synthetase. It is an important compound in the synthesis of membrane phospholipids and also serves as a precursor for the production of glutathione, which, in turn, is a major physiologic defense mechanism against oxidative stress. Animal models have shown that glutathione depletion within hepatic mitochondria sensitizes the liver to alcohol-induced liver injury and that restoration of SAMe levels may protect the liver from alcohol liver injury (106). SAMe synthetase activity has been reported to be decreased in cirrhosis, and specific mechanisms include effects of production of TNF as well as levels of antiinflammatory cytokines (107). Subsequent studies have documented decreased levels of SAMe in patients with alcoholic hepatitis (108).

A trial of 62 patients with alcoholic cirrhosis treated with SAMe and followed for up to 2 yr was not able to detect a difference in overall mortality in treated patients vs controls. However, the subgroup with Child's A or B cirrhosis receiving supplementation showed a significant improvement in the rate of liver transplant or mortality (109). Although a systematic review failed to show any significant differences in outcomes in ALD patients treated with SAMe (110), the number of patients studied was low, and there is a pressing need for further trials in this area (111).

**Antioxidant Cocktail** A recent trial (112) compared a cocktail of eight different antioxidants with corticosteroids in alcoholic hepatitis. The antioxidants included in the cocktails were  $\beta$ -carotene, vitamin C, vitamin E, selenium, methionine, allopurinol, desferrioxamine, and N-acetylcysteine. As mentioned above, the rationale for this strategy is certainly justified (94). However, the limitations of the study have been discussed, and other antioxidants may be beneficial (113).

## POTENTIAL NEW THERAPIES

Thalidomide, misoprostol, adiponectin, and probiotics have all been shown in preliminary reports to have anti-cytokine properties (91,114–116). Emerging data suggest

**Table 10**  
**A Proposed Therapeutic Algorithm for Alcoholic Hepatitis<sup>a</sup>**

I. Perform liver biopsy if diagnosis is uncertain	
II. Determine disease severity <sup>b</sup>	
Low risk	High risk
1. Supportive care	1. Prednisolone Shorter duration (?)
2. Observation	2. Anticytokine therapy Pentoxifylline
	3. Nutritional supplements Nitrogen balance monitored
	4. SAMe
	5. Misoprostil
	6. Probiotics
	7. Thalidomide
	8. Adiponectin
	9. Anti-TNF therapy Etanercept/infliximab

Abbreviation: SAM, S-adenosyl-L-methionine; TNF, tumor necrosis factor.

<sup>a</sup>Therapies below the line remain experimental but deserve further study.

<sup>b</sup>High-risk patients are those with severe disease as defined by a discriminant function (Modified) of  $\geq 32$  or the presence of encephalopathy, or a Mayo End-Stage Liver Disease (MELD) score of  $\geq 18$ .

that a role exists for TNF- $\alpha$ -mediated apoptosis in alcoholic hepatitis (117), and therefore, use of such therapy with inhibition of apoptosis may be effective (118). Finally, aggressive new therapies to remove cytokines via leukocytapheresis (119) or other extracorporeal recirculating systems (120) deserve additional trials.

## CONCLUDING REMARKS

Table 10 provides a proposed management algorithm derived from available data and based on a number of hypotheses from a therapeutic optimist. Although this algorithm is speculative, its intent is to stimulate discussion and to emphasize several of the following points for both clinicians and clinical investigators.

1. Only patients with severe alcoholic hepatitis should be treated with more than general supportive therapy. Severity is defined by an MDF of 32 or more or a MELD score of 18 or more. The latter needs to be tested prospectively.
2. Nutritional supplements should be provided to patients with severe disease.
3. Although steroids have proved to be effective therapy in severe disease, the optimal duration of treatment needs to be reconsidered, and predictors of steroid-treated responders need additional study.
4. The efficacy of pentoxifylline needs to be confirmed and compared with steroids in severe disease.
5. Combination therapy with steroids and pentoxifylline may be more beneficial than either agent individually owing to their different mechanisms of efficacy.
6. Although infliximab and etanercept may eventually be shown to be effective therapeutic agents in severe alcoholic hepatitis, less expensive agents with better safety profiles should also be tested as anticytokines.

7. Future studies investigating antioxidants are needed to gauge efficacy by monitoring markers of oxidative stress. Antioxidants directed against the prooxidants inducible nitric oxide and myeloperoxidase need to be investigated in addition to the antioxidants currently being used.
8. Finally, the agents listed under Potential New Therapies above need to be investigated for efficacy and safety.

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# 26 Role of Immune Response in Nonalcoholic Fatty Liver Disease

*Evidence in Human and Animal Studies*

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## KEY POINTS

- The liver and adipose tissue are major sources of inflammatory mediators, termed adipocytokines, in humans and experimental animals.
- In both experimental animals and human subjects, nonalcoholic fatty liver disease (NAFLD) is a spectrum of liver damage that develops in the context of a chronic inflammatory state.
- In animal models of NAFLD, abnormalities of the hepatic innate immune system that lead to increased liver production of inflammatory cytokines contribute to this chronic inflammatory state.
- Hepatic depletion of NKT cell populations is one of the innate immune system defects in animal models of NAFLD.
- NAFLD-related decreases of liver NKT cells probably result from several mechanisms, including macrophage abnormalities, alterations in neurohumoral factors, and abnormal expression of CD1d on the surface of fatty hepatocytes.
- Hepatocyte endoplasmic reticulum (ER) stress appears to play a role in the decreased expression of CD1d on the cell membranes of fatty hepatocytes in animals with fatty livers.
- Various strategies that replenish NKT cell populations reduce inflammatory cytokine production and improve NAFLD in some animal models.
- Additional research is needed to determine whether similar immune abnormalities contribute to the pathogenesis of NAFLD.

## INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is a spectrum of hepatic pathology ranging from nonalcoholic fatty liver (NAFL; steatosis) at the most clinically benign end of the spectrum to cirrhosis at the opposite extreme, where most liver-related morbidity and mortality occur. Nonalcoholic steatohepatitis (NASH) is a lesion of intermediate severity. NASH is characterized by overt hepatocyte injury and death. It is

often accompanied by hepatic infiltration with inflammatory cells (1). Some individuals with NASH gradually accumulate hepatic fibrosis, and eventually develop cirrhosis (2). Over time, hepatocellular carcinomas emerge in a small proportion of individuals with NAFLD-related cirrhosis (3). The pathogenesis of NAFLD is the subject of much research because NAFLD is one of the most common causes of chronic liver disease, particularly in countries such as the United States that are in the midst of an obesity epidemic (4,5).

## ASSOCIATION WITH THE METABOLIC SYNDROME

Like obesity, NAFLD is strongly associated with the metabolic syndrome (6), a chronic inflammatory state. The metabolic syndrome is suspected in individuals who manifest two or more certain frequently associated disorders, namely, abdominal obesity, hyperglycemia, dyslipidemia (hypertriglyceridemia and low levels of serum high-density lipoproteins), and/or hypertension (7). The strength of the association between NAFLD and the metabolic syndrome has prompted recent recommendations that NAFLD be classified as a component of the metabolic syndrome (6). Recent evidence suggests that the metabolic syndrome is a chronic inflammatory state, characterized by the overproduction of proinflammatory factors relative to anti-inflammatory factors (8). Given the growing evidence that NAFLD develops in the context of this systemic, chronic inflammatory state, it is likely that immune responses play key roles in NAFLD pathogenesis. Conversely, fatty liver may also contribute to the inflammatory signaling that characterizes the metabolic syndrome.

## INFLAMMATORY MEDIATORS IN NAFLD

### TISSUE SOURCES (TABLE 1)

In individuals with NAFLD, major tissue sources of inflammatory mediators are adipose depots and liver (Table 1). In liver, multiple types of cells produce inflammatory mediators. Among the most studied include resident liver macrophages (Kupffer cells) and lymphocyte populations such as natural killer T (NKT) cells, which are particularly enriched in liver

Table 1

## Inflammatory Mediators in Nonalcoholic Fatty Liver Disease

Adipose tissue: produced by adipocytes and/or macrophages
Tumor necrosis factor- $\alpha$
Interleukin-6
Resistin
Adiponectin
Plasminogen activator inhibitor (PAI)-1
Angiotensinogen
Leptin
Fatty acids
Liver tissue: produced by hepatocytes, hepatic stellate cells, and/or immune cells
Tumor necrosis factor- $\alpha$
Interleukins-6, -10, -12, -15, and -18
Resistin
Adiponectin
Leptin

(9). However, emerging evidence demonstrates that hepatocytes and hepatic stellate cells are also important sources of immunomodulatory factors (10–14). In adipose tissue, inflammatory mediators (adipokines) are produced mostly by adipocytes and macrophages (15). Different adipose depots appear to have different propensities to produce inflammatory mediators—visceral fat is generally more proinflammatory than subcutaneous fat (16,17). The reasons for this are poorly understood but may include depot-dependent differences in adipocyte differentiation (18). Adipose-derived factors that tend to inhibit adipocyte maturation include resistin and plasminogen activator inhibitor (PAI)-1 (19,20). Immature adipocytes abundantly produce proinflammatory cytokines, such as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ). Production of antiinflammatory factors, such as adiponectin, is maximal in fully mature adipocytes (21).

#### ADIPOSE-DERIVED INFLAMMATORY MEDIATORS (ADIPOKINES) (TABLE 2)

Potentially pertinent adipokines in NAFLD pathogenesis include TNF- $\alpha$ , interleukin (IL)-6, resistin, adiponectin, leptin, and PAI-1 (Table 2). In addition to their ability to regulate adipocyte maturation, nutrient metabolism, and energy homeostasis, these factors are now known to modulate both hepatic injury and repair.

**TNF- $\alpha$**  TNF- $\alpha$  is a proapoptotic factor for many cell types, including hepatocytes (22). It also recruits inflammatory cells into tissues by upregulating adhesion molecules and chemokines. TNF- $\alpha$  activates intracellular stress-related kinases, such as inhibitor  $\kappa$ B kinase (IKK)- $\beta$ , Jun N-terminal kinase (JNK), and p38 Mitogen-activated protein kinase (MAPK), which are known to interrupt insulin-initiated signaling, leading to cellular insulin resistance (23–27).

**IL-6** IL-6 generally evokes hepatoprotective responses, including the production of hepatocyte growth factor by hepatic stellate cells (28). However, IL-6 also induces factors such as silencer of cytokine signaling (SOCS)-3 that interfere with insulin-mediated suppression of phosphoenol pyruvate carboxy

Table 2

## Adipocytokines and Nonalcoholic Fatty Liver Disease (NAFLD)

Mediator	Role in NAFLD
TNF- $\alpha$	Proapoptotic Proinflammatory Inhibits insulin actions
IL-6	Hepatic glucose output Peripheral insulin resistance
Resistin	Hepatic glucose output Proinflammatory
Adiponectin	Reduces steatosis Inhibits stellate cell activation
Leptin	Stellate cell activation/fibrosis
PAI-1	Stellate cell activation/fibrosis
Angiotensinogen	Stellate cell activation/fibrosis
Fatty acids	Proinflammatory

Abbreviations: IL-6, interleukin-6; PAI-1, plasminogen activator inhibitor-1; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ .

kinase (PEPCK) gene expression in hepatocytes (29). Sustained expression of PEPCK enhances postprandial hepatic gluconeogenesis, promoting hyperglycemia, which triggers compensatory hyperinsulinemia. IL-6 also interferes with muscle insulin sensitivity, thereby decreasing the efficiency of systemic glucose disposal. This exacerbates hyperglycemia, triggering further hyperinsulinemia and heterologous desensitization of insulin signaling in many cell types (11).

**Resistin** Resistin stimulates hepatic glucose output by inhibiting insulin-mediated suppression of gluconeogenesis. The mechanisms involved are thought to involve resistin-induced activation of transcriptional regulators, such as nuclear factor- $\kappa$ B (NF- $\kappa$ B) and SOCS-3, which upregulate hepatocyte production of TNF- $\alpha$ , and IL-6, and PEPCK, respectively (30,31).

**Adiponectin** Adiponectin antagonizes the effects of both TNF- $\alpha$  and resistin, perhaps in part by restoring the activity of AMP kinase (32,33). (Both TNF- $\alpha$  and resistin promote dephosphorylation and inactivation of AMP kinase [AMPK]). Activation of AMPK enhances lipid disposal by increasing mitochondrial  $\beta$ -oxidation of fatty acids, while inhibiting fatty acid biosynthesis (34). By reducing hepatocyte fatty acid stores, adiponectin reduces another important stimulus for hepatic cytokine production (*see Fatty Acids* below). Adiponectin also appears to inhibit myofibroblastic activation of hepatic stellate cells (HSCs). This limits HSC production of collagen matrix and reduces hepatic fibrosis (35).

**PAI-1 and Leptin** PAI-1 and leptin are other important regulators of HSCs. Both factors promote transition of quiescent HSCs to an activated, myofibroblastic phenotype, leading to liver fibrosis (36–38). PAI-1 also inhibits differentiation of preadipocytes into mature adipocytes, thereby modulating the profile of other adipocytokines that are produced by adipose depots (38).

**Fatty Acids** Adipose tissue is also an important source of fatty acids that are delivered to the liver for intermediary metabolism. Excessive accumulation of fatty acids within

hepatocytes is sufficient to trigger hepatic production of cytokines such as TNF- $\alpha$  and IL-6 that act both locally and at distant sites (e.g., skeletal muscle) to regulate insulin sensitivity (10).

#### LIVER-DERIVED INFLAMMATORY MEDIATORS

As mentioned earlier under Tissue Sources, multiple cell types residing in the liver produce factors that regulate inflammatory signaling, including liver epithelial cells (hepatocytes, cholangiocytes) and HSCs, as well as more typical immune cells. The latter include Kupffer cells (hepatic macrophages) and resident populations of lymphocytes, particularly NK T cells.

#### HEPATOCYTE-DERIVED FACTORS

**Cytokines** Liver epithelial cells have been generally underappreciated as sources of immunomodulatory factors. However, several recent lines of evidence demonstrate that such cells are likely to play important roles in orchestrating both local and systemic inflammatory responses. For example, studies of cultured hepatocytes demonstrate that these cells upregulate their production of TNF- $\alpha$  and IL-6 under conditions that promote accumulation of fatty acids (10). Other work that utilized transgenic mice with hepatocyte-specific activation of NF- $\kappa$ B signaling proved that the resultant increase in hepatocyte-derived IL-6 was sufficient to cause systemic insulin resistance (11). Hence, hepatic steatosis provokes hepatocyte secretion of inflammatory cytokines that modulate insulin sensitivity in hepatocytes themselves, as well as various other cells.

#### Immunomodulation via Lipid Antigen Presentation

Hepatocytes also express a major histocompatibility (MHC)-like molecule, CD1d, and thus are capable of presenting lipid antigens to the immune system (39,40). This process involves trafficking of CD1d among different endolysosomal compartments within hepatocytes, where poorly characterized “self” lipid antigens are loaded onto CD1d. Lipid-antigen-bearing CD1d molecules then traffic to hepatocyte plasma membranes, where they interact with T cell receptors (TCRs) on NKT cells. Interactions between CD1d-presented lipid antigens and TCRs on NKT cells appear to modulate NKT cell maturation and activation and thus are thought to influence autoimmunity and tumor surveillance (41). Thus, hepatocytes have the ability both to produce immunomodulatory cytokines, and to present lipid antigens to subpopulations of regulatory and cytotoxic T cells.

#### IMMUNE FUNCTIONS OF HEPATIC STELLATE CELLS

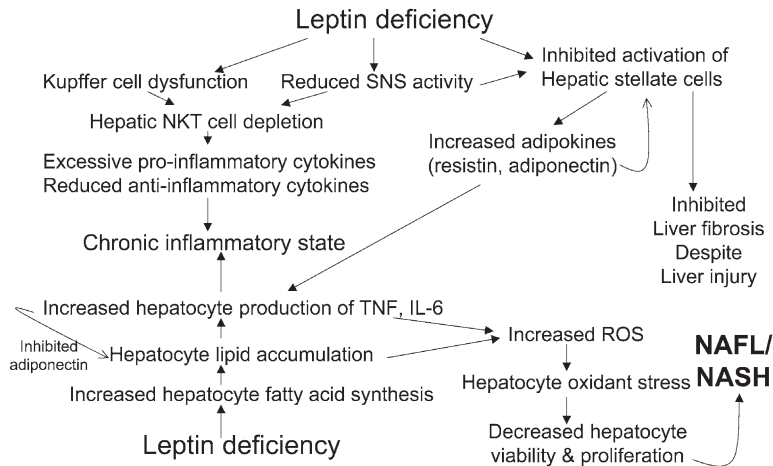
**Adipocytokine Production** HSCs also have important immune functions. Quiescent HSCs are adipocyte-like cells, and, as such, they are capable of producing various adipokines, including leptin, resistin, and adiponectin (13). These HSC-derived factors act locally to modulate the phenotypes of other liver cells that express receptors for these proteins. Both HSCs and hepatocytes, for example, express adiponectin receptors and thus are subject to autocrine-paracrine regulation by HSC-derived adiponectin. Adiponectin inhibits myofibroblastic differentiation of HSC (35), prevents lipid accumulation in hepatocytes, and promotes hepatocyte insulin sensitivity (33). HSCs, but not hepatocytes, express long forms of the leptin

receptor and thus are directly regulated by HSC-derived leptin. Leptin promotes proliferation, viability, and collagen matrix production by myofibroblastic HSCs (36,37). On the other hand, hepatocytes are well-recognized targets for resistin (42). Our group has preliminary evidence that resistin derived from HSC induces nuclear localization of NF- $\kappa$ B and expression of NF- $\kappa$ B-regulated target genes, including TNF- $\alpha$  and IL-6 in neighboring hepatocytes. Increases in hepatocyte IL-6 are accompanied by induction of SOCS-3 and PEPCK, actions that promote hepatocyte gluconeogenesis and increase hepatic glucose output. Thus, HSCs are emerging as important local sources of adipokines that regulate liver metabolism, inflammation, and fibrosis. In addition, HSCs are also capable of functioning as antigen-presenting cells to activate immune responses (43).

#### REGULATION OF INFLAMMATORY RESPONSES BY LIVER-ENRICHED POPULATIONS OF IMMUNE CELLS

**Hepatic Macrophages** There is little doubt that resident immune cells within the liver play important roles in tissue inflammatory responses. Compared with most other tissues, the liver is enriched with macrophages (9). Hepatic macrophages (termed Kupffer cells) express Toll-like receptors and function as key mediators of innate immune responses by producing soluble factors when activated by pathogen-associated molecules (PAMs), such as intestinal bacteria-derived lipopolysaccharide and peptidoglycans that bind to various Toll-like receptor family members (44–46). Kupffer cells produce factors such as TNF- $\alpha$ , IL-12, and IL-18 that enhance local inflammatory responses within the liver (47). In addition, they are an important source of various antiinflammatory factors, such as certain prostaglandins and IL-15 (47). Kupffer cells also express both classical MHC molecules and CD1d and thus function as antigen-presenting cells to activate immune responses to either peptide or lipid antigens (48,49).

**NKT Cells** The liver is also a preferred home for certain lymphocyte populations. In both mice and humans, liver lymphocyte populations are enriched with T cells bearing specialized TCRs that recognize lipids presented by CD1 (as opposed to more traditional peptide antigens presented by cell-surface MHC molecules) (50,51). Most CD1-restricted T cells coexpress relatively invariant TCR and surface markers for natural killer cells (NK in mice, CD161 in humans) and hence are termed invariant NKT (iNKT) cells. Recent studies used intravital microscopy in mice to demonstrate that NKT cells accumulate in the liver in large part because they express a specific chemokine receptor (CXCR6) that interacts with a chemokine (CXCL16) abundantly produced by hepatic sinusoidal endothelia (52). Within healthy livers, these NKT cells patrol the hepatic microenvironment and were visualized to “sample” CD1-presented antigens on hepatocytes several times each hour (52). At this point, relatively little is known about how various types of liver injury influence hepatocyte-NKT cell interactions. However, this might be important because, like NK cells, activated NKT cells can be directly cytotoxic, by Fas ligand (FasL), perforin, or granzyme-dependent mechanisms. In addition, they provide an immediate source of immuno-



**Fig. 1.** NAFLD pathogenesis in ob/ob mice. IL, interleukin; NAFL, nonalcoholic fatty liver; NASH, nonalcoholic steatohepatitis; NKT, natural killer T; ROS, reactive oxygen species; SNS, sympathetic nervous system; TNF, tumor necrosis factor.

regulatory cytokines, such as IL-4 and interferon- $\gamma$  (IFN- $\gamma$ ), which modulate cytokine production and activation by other cells engaged in innate and adaptive immune responses. As such, NKT cells also have properties of T-regulatory cells, modulating local production of pro- and antiinflammatory cytokines (53).

## ROLE OF IMMUNE RESPONSES IN NAFLD PATHOGENESIS: EVIDENCE FROM ANIMAL MODELS

### LESSONS FROM GENETIC MODELS OF OBESITY-ASSOCIATED NAFLD

Studies of experimental animals clearly demonstrate important roles for immunomodulatory cytokines in the pathogenesis of NAFLD. Genetically obese mice, particularly those with naturally occurring mutations in the *ob* gene that inhibits synthesis of the satiety factor (leptin), have been some of the most studied models of the metabolic syndrome (Fig. 1). Leptin-deficient ob/ob mice are obese and exhibit several other features of the metabolic syndrome, including insulin resistance, hyperglycemia, dyslipidemia, and NAFLD (54,55). Similar to many obese humans with the metabolic syndrome, ob/ob mice overproduce the proinflammatory cytokine TNF- $\alpha$ , relative to the TNF antagonist adiponectin (56). Various treatments that inhibit TNF- $\alpha$  (including neutralizing anti-TNF antibodies, insulin-sensitizing agents such as metformin, and recombinant adiponectin) improve NAFLD in ob/ob mice (56–58).

The underlying immune defect that causes the chronic inflammatory state of ob/ob mice is poorly understood. However, altered production of inflammatory mediators by Kupffer cells has been demonstrated, as has selective hepatic depletion of iNKT cell populations (59,60). Several factors may contribute to the latter, including excessive Kupffer cell production of factors such as IL-12 and IL-18 that inhibit iNKT cell viability concomitant with reduced production of IL-15, an iNKT cell viability factor, leptin deficiency-associated inhibition of sympathetic nervous system functions that promote

hepatic accumulation of iNKT cells, and reduced expression of CD1d on hepatocyte plasma membranes (60–62). Hepatic iNKT cell depletion in turn promotes Th-1 (proinflammatory) polarization of various cytokine-producing cells in the liver. The resultant chronic inflammatory state appears to promote NASH, because diverse strategies that replenish iNKT cells restore cytokine balance and reverse steatohepatitis in ob/ob mice (61–64). In ob/ob mice, deficiencies of leptin and norepinephrine also inhibit typical injury-related activation of HSCs (65,66). Hence, like many humans with chronic NASH, ob/ob mice do not develop cirrhosis (67).

Interestingly, by impeding the transition of quiescent HSCs into activated myofibroblastic cells, leptin deficiency preserves the adipocytic functions of quiescent HSCs. The latter includes production of another inflammatory factor, resistin (13). In addition to its ability to increase production of TNF- $\alpha$  and IL-6, resistin has been shown to antagonize the actions of adiponectin on AMP kinase, leading to reduced activity of this enzyme (30,68). Resistin is also a potent inducer of SOCS-3, which inhibits insulin signaling (31). In hepatocytes, the effects of resistin attenuate insulin-mediated suppression of gluconeogenesis, leading to enhanced hepatocyte glucose output (69). We recently showed that hepatic expression of resistin mRNA is increased in ob/ob livers and demonstrated that HSC-derived resistin contributes to insulin resistance by enhancing nuclear localization of NF- $\kappa$ B in hepatocytes and upregulating hepatocyte production of NF- $\kappa$ B target genes such as TNF- $\alpha$ , IL-1 $\beta$ , IL-6, and SOCS-3 that increase hepatic glucose output while inhibiting muscle insulin sensitivity. Thus, it is likely that multiple inflammatory mediators contribute to the complex phenotype of leptin-deficient ob/ob mice.

In other genetically obese mice with NAFLD and the metabolic syndrome, such as agouti (KK $\Delta$ y) mice, overproduction of TNF- $\alpha$  relative to adiponectin has also been demonstrated, and treatments that inhibit TNF activity, including supplemental adiponectin, improve NASH in these animals (70).

Production of proinflammatory cytokines, including TNF- $\alpha$ , is also increased relative to antiinflammatory cytokines, such as IL-10, in mice that develop steatohepatitis owing to a genetic deficiency of methionine adenosyl transferase (MAT)-1- $\alpha$ , the enzyme that catalyzes synthesis of *S*-adenosyl-L-methionine (SAME) from methionine in mature hepatocytes (71). Overproduction of inflammatory cytokines, including TNF- $\alpha$ , has also been noted in wild-type mice that develop obesity, NAFLD, and the metabolic syndrome when fed diets that are high in fat and/or sucrose (10,72). Mice that are genetically deficient in TNF receptor (TNFR)-1 are generally protected from the deleterious consequences of diet-induced obesity, confirming the significance of TNF-mediated, chronic inflammatory signaling in this process (10).

On the other hand, although expression of TNF- $\alpha$  is increased and production of adiponectin is decreased in wild-type mice that develop steatohepatitis when fed methionine choline-deficient (MCD) diets, neither disruption of the genes encoding TNF- $\alpha$  itself, nor TNFR1, protected mice from MCD diet-induced NAFLD (73). Similarly, Tsukamoto's group has reported that chronic intragastric administration of high-fat diets induces severe steatohepatitis in both wild-type and TNFR1-deficient mice (74). In the MCD diet model of NAFLD, other mediators appear to contribute to NF- $\kappa$ B activation in liver cells, because treatments that inhibit this process, such as adenovirus-mediated overexpression of a mutant, nondegradable I- $\kappa$ B, improve steatohepatitis (73). These findings demonstrate that other factors can replace TNF- $\alpha$  as a driving force for inflammatory signaling in liver and suggest that NAFLD can result from diverse inflammatory stimuli.

The MCD diet model of NAFLD is also instructive in another regard because MCD diet-fed mice develop severe NAFLD despite exhibiting enhanced sensitivity to insulin, rather than resistance to insulin, which is typical of many experimental animals and human subjects with NAFLD (75). Mice with hepatocyte-specific deletion of the insulin signaling inhibitor phosphatase and tensin homolog deleted on chromosome 10 (PTEN) also develop severe steatohepatitis in the context of enhanced hepatic insulin sensitivity (76) (Table 3). Interestingly, in both MCD diet-fed mice and PTEN-deficient mice, increased hepatic production of reactive oxygen species, features of severe NASH (i.e., ballooned hepatocytes with Mallory bodies, hepatic sinusoidal fibrosis), and hepatocellular carcinoma are common outcomes of chronic fatty liver disease (74,76). In the latter animals, PTEN deficiency results in hyperactivation of protein kinase B (PKB/Akt). This enhances phosphorylation of insulin receptor substrates that propagate insulin-initiated signals in hepatocytes (76). In many cell types, increased Akt activity is also protective against TNF-related apoptotic-inducing ligand (TRAIL)-mediated apoptosis. Akt inhibits apoptosis by phosphorylating key components of the cellular apoptotic regulatory circuit, as well as by activating NF- $\kappa$ B (77). Interestingly, Akt activation of NF- $\kappa$ B has also been shown to downregulate PTEN expression in many cells, and the resultant decrease in PTEN activity increases the cellular content of phosphorylated (i.e., bioactive) Akt (78). Hence,

**Table 3**  
NAFLD and Insulin Resistance

<i>Animal model</i>	<i>Insulin resistance</i>	<i>Liver histology</i>
ob/ob	Yes	NAFL/NASH
db/db(fa/fa)	Yes	NAFL/NASH
KKA <sup>y</sup>	Yes	NAFL/NASH
High fat/high sucrose diets	Yes	NAFL/NASH
MAT-1 $\alpha$ deficient	?	NAFL/NASH/HCC
MCD diet	No	NAFL/NASH/fibrosis/HCC
PTEN deficient	No	NAFL/NASH/fibrosis/HCC

Abbreviations: MAT, methionine adenosyl transferase; MCD, methionine choline deficient; PTEN, phosphatase and tensin homolog deleted on chromosome 10; NAFL, nonalcoholic fatty liver; NASH, nonalcoholic steato hepatitis; HCC, hepatocellular carcinoma.

**Table 4**  
Serum Adipocytokine Level and NAFLD Severity Correlations

<i>Adipocytokine</i>	<i>Correlation with NAFLD severity</i>
Leptin	No
Resistin	Yes
TNF- $\alpha$	Yes
Adiponectin	Yes (inverse)
PAI-1	Yes

Abbreviations: PAI-1, plasminogen activator inhibitor-1; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ .

induction of NF- $\kappa$ B during chronic inflammation may initiate a positive feedback mechanism that perpetuates Akt activity by suppressing PTEN function.

Since the predicted consequences of such Akt activation are insulin sensitivity and enhanced cell viability, it is unclear why progressive liver damage results from constitutive activation of Akt in the mature hepatocytes of PTEN-deficient mice. It also remains to be determined whether similar alterations in PTEN and Akt occur in other experimental models of "insulin sensitive" NAFLD, or in subgroups of NAFLD patients. Nevertheless, these results are important because they prove that, whereas insulin resistance is common in many experimental animals and humans with NAFLD, it is by no means required for either the initiation or progression of this type of liver damage.

## ROLE OF IMMUNE RESPONSES IN NAFLD PATHOGENESIS: HUMAN DATA

### IMPERFECT CORRELATION BETWEEN SERUM ADIPOKINE LEVELS AND LIVER DAMAGE (TABLE 4)

Studies of patients with NAFLD have benefited enormously from work in the aforementioned experimental animal models. Data gleaned from the animal studies have been most helpful in focusing attention on putative regulators of tissue insulin sensitivity, as well as hepatotoxicity, including TNF- $\alpha$ , IL-6, leptin, adiponectin, resistin, and PAI-1 (Table 4). As expected, much of the work in humans has sought a correlation among serum levels of the various adipokines, insulin resistance, and

the severity of liver damage (reviewed in ref. 2). Unfortunately, progress has been compromised by the lack of specific and sensitive biomarkers to reliably distinguish individuals with simple hepatic steatosis from those with NASH or cirrhosis, as well as difficulties in gauging hepatic (as opposed to systemic) insulin resistance with noninvasive testing. In addition, there is no guarantee that sporadic measures of adipokines in the systemic circulation accurately reflect adipokine levels (or activity) in the key target tissues in the metabolic syndrome.

**Leptin** Mindful of these caveats, consensus is emerging that serum levels of certain factors, such as leptin, are not generally useful for estimating either insulin sensitivity or liver damage in patients with the metabolic syndrome. The significance of hyperleptinemia is notoriously difficult to interpret because absolute deficiency of leptin (which occurs in ob/ob mice and in some humans with genetic obesity) clearly causes obesity, insulin resistance, and NAFLD (54,79). Nevertheless, when the leptin gene is intact, elevated leptin levels are an expected outcome of obesity (because leptin is produced by adipose tissue) (68). Indeed, hyperleptinemia has been noted in most obese patients with insulin resistance and NAFLD (2). The pathophysiological significance of this is unclear, however, because most obese individuals exhibit some degree of leptin resistance (80). Thus, it is difficult to know which (if any) obesity-related pathologies result from enhanced leptin signaling and which developed as a result of impaired leptin responses (i.e., leptin resistance). In any case, leptin levels have not been useful in differentiating individuals with steatosis from those with NASH or cirrhosis.

**Resistin** Elevated serum levels of resistin are also an expected consequence of excessive adiposity. Surprisingly, however, unlike serum leptin levels that generally fall with weight loss and increase with weight gain, serum levels of resistin do not consistently mirror changes in body mass index (BMI) (81). Recent evidence that resistin can be produced by cells that are abundant in other tissues, such as macrophages and HSCs, provides a potential explanation for those observations. In addition, emerging evidence that resistin and adiponectin exert opposing actions on key metabolic regulators, such as AMPK, suggest that tissue responses to resistin are likely to be influenced by many factors, including adiponectin. Nevertheless, at least one study demonstrated a significant positive correlation between serum resistin levels and the NAS histology score (which measures the severity of liver damage owing to NAFLD) (82). The relationship between resistin and liver damage persisted even after multiple regression analysis was done to control for potentially confounding effects of age, gender, BMI, and insulin resistance, suggesting that hyperresistinemia might help to differentiate individuals who develop NASH from those who have only hepatic steatosis.

**TNF- $\alpha$  and Adiponectin** As for resistin and adiponectin, disease-modifying cross-talk among various adipokines appears to be the norm, rather than the exception. In animal models of NAFLD, this has been consistently demonstrated for TNF- $\alpha$  and its antagonist, adiponectin. More specifically, agents that inhibit TNF- $\alpha$  generally increase serum adiponectin levels, and

those that work primarily by increasing adiponectin typically elicit secondary reductions in serum levels of TNF- $\alpha$  (or its surrogate markers, including soluble TNF receptors). A similar reciprocal relationship between adiponectin and TNF- $\alpha$  has been noted in humans (83). For example, in a recent study of NAFLD patients with either mild liver disease (i.e., steatosis) or more serious liver damage (i.e., NASH), the odds ratio for having NASH (as opposed to steatosis) significantly correlated either with decreased serum adiponectin or increased serum levels of TNF- $\alpha$  (84). Similarly, most treatments that have been reported to improve liver injury in patients with NAFLD, including diet and exercise, insulin-sensitizing drugs (e.g., metformin, thiazolidinediones), and pentoxifylline (which inhibits inflammatory cytokines), seem to increase serum adiponectin levels (2). Although commensurate reductions in serum TNF- $\alpha$  or IL-6 (a TNF-inducible cytokine) have not been demonstrated consistently in such studies, it is reasonable to infer that increased adiponectin inhibits the biological activity of TNF- $\alpha$  and resistin, proinflammatory factors that have been implicated in the pathogenesis of both insulin resistance and NAFLD.

**PAI-1 and Angiotensinogen** Both PAI-1 and angiotensinogen-mediated activation of angiotensin are known to promote fibrosis in experimental animals (20,85). These factors also appear to play a role in NAFLD pathogenesis/progression in humans. At least one study demonstrated a significant correlation between plasma levels of PAI-1 and the severity of hepatic steatosis (86). Another showed that treatment with the angiotensin receptor antagonist losartan improved liver histology in a small group of patients with NASH and hypertension (87).

#### DOUBTFUL CAUSE-EFFECT RELATIONSHIP BETWEEN INSULIN RESISTANCE AND NAFLD

Because NAFLD is often associated with insulin resistance, and TNF- $\alpha$  is clearly capable of inhibiting insulin signaling while adiponectin exerts opposing effects, the development of therapies for NAFLD has focused on improving insulin sensitivity. However, this approach might need to be reconsidered given the animal studies showing that at least some things that enhance insulin sensitivity (e.g., sustained inhibition of PTEN and activation of Akt in hepatocytes) actually have the opposite effect, i.e., they promote NASH and liver cancer (76). Given the apparent “disconnect” between hepatocyte insulin sensitivity and liver damage, it seems likely that the observed hepatic benefits of “insulin-sensitizing” agents might be more directly attributable to some of their other actions, such as their ability to block pro-inflammatory signaling. The latter is likely to be highly context dependent, and this might explain interindividual differences in the response to treatment. For example, peroxisome proliferator-activated receptor (PPAR- $\gamma$ ) agonists, such as TZDs, are predicted to enhance adipocyte differentiation, thereby increasing production of resistin, as well as adiponectin. Whether or not resistin provokes transcription of inflammatory cytokines (presumably a “bad” outcome) is likely to be influenced by the extent to which the agonists can activate PPAR- $\gamma$  in various target cells, thereby inhibiting the function of the inflammatory

*trans*-acting factor NF- $\kappa$ B p65. Regardless of any increase in TNF- $\alpha$  production that might occur, coincident cellular sensitivity to adiponectin might determine the ultimate effects of resistin on metabolism, energetics, and cell viability. Thus, in order to develop rationale approaches to prevent and reverse damage to the liver (and other tissues) that occurs in individuals with the metabolic syndrome, further research is needed to clarify the complex mechanisms that integrate metabolism with cell viability and tissue growth/repair.

## CONCLUDING REMARKS AND OPEN QUESTIONS

Nonalcoholic fatty liver disease is a spectrum of liver damage that often occurs in individuals with the metabolic syndrome. Studies in experimental animals and human demonstrate that the metabolic syndrome reflects a chronic inflammatory state and suggest that accompanying damage to tissues, such as the liver, results from relative overactivity of inflammatory mediators. It is becoming apparent that tissue damage can result both from direct noxious actions of these mediators on target cells and from the indirect consequences of adaptive maneuvers that attempt to preserve cell viability under chronic inflammatory pressure. Thus, heterogeneous mechanisms seem to generate overtly similar types of liver damage. Additional research is required to determine optimal therapies to prevent and improve NAFLD, but it seems likely that treatments may need to be tailored to match the particular mechanism(s) that drives liver damage in given individuals.

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# ACUTE LIVER FAILURE

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# 27 Mechanisms of Acute Liver Failure

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## KEY POINTS

- Acute liver failure is characterized by the sudden onset of liver failure in a patient without evidence of chronic liver disease.
- Four different mechanisms are mainly responsible: (1) Infectious (mostly viral), (2) drugs/toxins/chemicals, (3) cardiovascular, and (4) metabolic may trigger acute liver failure
- Viral hepatitis is one of the main causes of acute liver failure. Hepatitis B and non-A/non-C hepatitis are the most frequent forms.
- Suicidal acetaminophen ingestion is the most frequent cause of drug-induced liver failure.
- Three factors determine the prognosis of liver failure: (1) the metabolic consequences resulting from liver failure, (2) the release of mediators and toxic metabolites, and (3) the capacity of the remaining hepatocytes to restore liver mass.
- Clinically, two phases can be differentiated: the mechanisms that trigger liver failure and the clinical manifestations determining the outcome.
- Cerebral edema, infections, and renal failure are important clinical complications limiting the survival of the patients.
- Ammonia levels can be used for risk stratification in patients with acute liver failure and subsequent hepatic encephalopathy.
- Mild hypothermia might improve the outcome of patients with acute liver failure by reducing of intracranial pressure and improving disturbed autoregulation in cerebral blood flow.
- Cytokines are involved in the pathogenesis of acute liver failure as well as in controlling the balance between survival and proliferation of hepatocytes.

## INTRODUCTION

Acute liver failure is characterized by the sudden onset of liver failure in a patient without evidence of chronic liver disease. This definition is important, as it differentiates patients with acute liver failure from patients who suffer from liver failure owing to end-stage chronic liver disease.

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Clinically, patients present with severe liver failure (icterus and coagulation failure) and hepatic encephalopathy. The time between the first symptoms and the manifestation of hepatic encephalopathy has been shown to be crucial for the prognosis of these patients. Therefore several groups have included in their definition the time frame between the onset of symptoms and the start of encephalopathy. The most recent definition uses the term acute liver failure (ALF) as an umbrella and differentiates between three subgroups: hyperacute, acute, and subacute (Table 1). The time between first symptoms and encephalopathy in hyperacute ALF is 7 d, in acute ALF it is 8 to 28 d and in subacute ALF it is 5 to 26 wk (1,2).

## MECHANISMS OF DISEASE

Different causes may result in ALF. In principal four different classes can be differentiated: (1) infectious (mostly viral), (2) drugs/toxins/chemicals, (3) cardiovascular, and (4) metabolic (3) (Table 2).

There are obvious differences in the mechanisms that initially trigger liver failure. However, at the time of clinical presentation, in most cases a common final stage has been reached in ALF patients. At this stage, three main factors seem important in determining prognosis: (1) the metabolic consequences resulting from the loss of liver cell mass, (2) the release of mediators and toxic metabolites from liver tissue, and (3) the capacity of the remaining vital hepatocytes to restore liver mass (2,4).

Therefore in terms of the mechanisms that are important during ALF, two different phases of acute liver failure can be differentiated: the mechanisms that initially trigger liver failure and those that eventually determine outcome. (Of course this a somewhat artificial differentiation.) In the following discussion, the mechanisms/etiology leading to acute liver failure will be explored first and then the clinical factors influencing outcome.

## ETIOLOGY

### INFECTIOUS CAUSES

Viruses in particular are an essential cause of ALF and, depending on the geographical region, can comprise between 30 and 70% of all forms of ALF (2–4).

**Hepatitis A Virus** Only 0.1 to 0.4% of all infections with the hepatitis A virus (HAV) result in ALF. The proportion

**Table 1**  
**Subgroups of Acute Liver Failure**

	<i>Time between first symptoms and start of encephalopathy</i>
Hyperacute	< 7 d
Acute	8–28 d
Subacute	5–26 wk

**Table 2**  
**Causes of Acute Liver Failure**

<b>Infectious (viral)</b>
Hepatitis A
Hepatitis B
Hepatitis C
Hepatitis D
Hepatitis E
Hepatitis non-A/non-B
<b>Rare causes</b>
Herpes simplex virus
Herpes virus type 6
Varicellavirus
Cytomegalovirus
Epstein-Barr virus
Togavirus
Paramyxovirus
Parainfluenzavirus
<b>Drugs/toxins/chemicals</b>
Acetaminophen
<i>Amanita phalloides</i>
Halothane
Isoniazid
Sodium valprostate
Tetracycline
Nonsteroidal anti-inflammatory drugs (NSAIDs)
Pirprofen
Ketocanazole
<b>Cardiovascular</b>
Budd-Chiari syndrome
Hypotension (circulatory shock)
Heart failure (e.g., right ventricular)
Hyperthermia
Malignant tumors
Venoocclusive disease
Portal vein thrombosis
Sepsis
<b>Metabolic</b>
Wilson's disease
Reye's syndrome
Acute fatty liver of pregnancy (AFLP)
Hemolysis, elevated liver enzymes, low platelet count (HELLP) syndrome
Galactosemia
Hereditary fructose intolerance
Hereditary tyrosinemia

of patients with ALF is higher in older than in younger patients. This is of relevance, as over the last decades in Western countries HAV infection has occurred more frequently

in older patients, and thus the risk of ALF is increased in this population (5,6).

The pathogenesis of HAV-related ALF is not completely understood. Current studies indicate that a combination of a direct cytopathic effect of the virus and immune-mediated mechanisms results in liver destruction.

**Hepatitis B Virus** The risk of acute liver failure of all patients who are hospitalized because of an acute hepatitis B virus (HBV) infection is around 1% (7). Thus HBV—depending on the geographical region—is one of the leading causes of ALF.

In general, the virus itself is not cytopathic, but the immune response directed against the virus is essential (8). There are reports that certain viral strains with specific mutations might be important for the occurrence of ALF. However, these reports are mainly from specific cohorts and up to now have had no general implication for HBV-related ALF (9,10).

Frequently at the time of hospitalization the viral load is already decreasing while transaminases are still rising. This may reflect the possibility that different factors contribute to the elimination of the virus. Recent experiments indicate that cytokines—namely, interferon (IFN)—are operating through a noncytopathic mechanism to eliminate the HBV genome in hepatocytes, whereas at a later stage T cells infiltrate the liver and destroy hepatocytes (11). Therefore activation of HBV-specific T cells is essential to determine the degree of hepatic injury during ALF.

In the case of HBV/hepatitis D virus (HDV) coinfection, the risk of ALF is increased (12). The exact mechanisms that lead to more pronounced liver failure have not been defined.

**Hepatitis C Virus** The risk of ALF through hepatitis C Virus (HCV) is low (2). In Japan in particular cases of HCV-related ALF have been documented (13). As there are only a few reports in the literature, the pathogenesis of HCV-related ALF is incompletely understood. However, there is evidence that elimination of HCV-specific T cells is associated with chronic HCV infection (14). This indicates that the HCV-specific immune response is involved during acute infection and thus is most likely also the determining factor during ALF.

**Hepatitis E Virus** Acute liver failure owing to hepatitis E Virus (HEV) infection is seldom seen in Western countries. Epidemic outbreaks are known in developing countries including patients with ALF. Pregnant women in particular have a high risk of ALF (up to 20%) (15). The mechanisms operating in these patients have not yet been studied. Therefore there is no clear hypothesis in the literature, and it is only speculative to draw parallels with HAV.

**Rare Cases of Viral Hepatitis** In rare cases, different systemic virus infections can present as ALF owing to a predominant manifestation in the liver. These are the herpes simplex virus, herpes virus type 6, cytomegalovirus, varicella-virus, and Epstein-Barr virus. A few cases have also been described in which an infection with the toga-, paramyxo-, or parainfluenzavirus was documented.

**Non-A/Non-B Hepatitis** ALF cases often have the characteristics of viral hepatitis. However, in these cases none

of the known viruses can be diagnosed, and thus these forms have been classified as non-A/non-B hepatitis (2). Non-A/non-B hepatitis is frequently associated with subacute liver failure that has a lower chance of liver restitution.

**DRUGS/TOXINS/CHEMICALS** Several drugs, chemicals, and toxins can lead to ALF (Table 2), by either direct toxicity or idiosyncratic drug reaction. The most frequent examples are discussed in this review.

**Acetaminophen** Acetaminophen (Paracetamol, Tylenol) is the most frequent drug leading to ALF. In adults, only higher doses (in general more than 10–12 g) are dangerous, and in most cases, acetaminophen was taken in a suicide attempt. Patients who consume alcohol chronically may be more susceptible induced to acetaminophen, cytochrome P450 has been induced in their liver.

The pathogenesis of acetaminophen injury is related to the formation of toxic metabolites through the cytochrome P450 enzymes, especially cytochrome P450 2E1 (16,17). These toxic metabolites are normally conjugated and inactivated through glutathione. However, when glutathione stores are depleted, these toxic metabolites accumulate and result in hepatocyte injury.

A recently published study shows that the measurement of serum acetaminophen–protein adducts can reliably identify acetaminophen toxicity in cases of ALF in which no clinical or historical data are given that would reveal the cause (18).

**Mushroom (*Amanita*) Poisoning** Mushroom poisoning, mainly through the species *Amanita phalloides*, frequently leads to ALF, especially in the Fall. Amanatoxin and phalloidin are the two distinct toxins produced by mushrooms. Phalloidin is not absorbed in the gastrointestinal tract, and the toxic effect of amanatoxin is through inhibition of RNA polymerase II (3,19).

**Halothane** Halothane is the prototype of an idiosyncratic drug reaction that (less frequently) can also be found after anesthesia with other members of the same family. In general, halothane-related ALF is only found after the second exposure to the drug. Halothane hepatitis is a paradigm for immune-mediated adverse drug reactions. The mechanism appears to be related to development of sensitization to both autoantigens (including CYP2D6) and halothane-altered liver cell determinants (20). Specific antibodies are involved in hepatic injury. These antibodies can only be determined in specialized laboratories.

### CARDIOVASCULAR DISORDERS

Cardiovascular diseases can lead to ALF either by ischemia or by impaired blood flow leaving the liver. Examples of ischemic events are hypotension or heart failure. Stasis of blood flow in the liver may occur owing to malignant tumors, venoocclusive disease, or Budd-Chiari syndrome.

**Budd-Chiari Syndrome** Classically, Budd-Chiari syndrome is characterized by a symptomatic occlusion of the hepatic veins and is more frequently found in females (21). Depending on the progression of the disease, Budd-Chiari syndrome may result in ALF when sudden closing of all three main liver veins occurs. Typically, acute Budd-Chiari syndrome presents with ascites, abdominal pain, jaundice, and hepatomegaly (22).

Budd-Chiari syndrome is frequently associated with primary myeloproliferative disorders, a factor V Leiden mutation, anti-cardiolipin antibodies, and protein C and S deficiencies that increase the risk of thrombotic complications (21–23). In general, the course of disease in Budd-Chiari syndrome leads to liver transplantation. Transjugular portosystemic stent shunt (TIPSS) or percutaneous transjugular direct portocaval shunt, in patients with inaccessible hepatic veins, seem to be therapeutic options to decrease the portal pressure gradient, improve synthetic functions, reduce transaminase levels, and control ascites (24,25).

### METABOLIC CAUSES

Different metabolic disorders may present as ALF, for example, Reye's syndrome, which is more common in children; its frequency has declined over the last decades. Also, during pregnancy acute fatty liver of pregnancy (AFLP) or the hemolysis, elevated liver enzymes, and low platelet count (HELLP) syndrome may develop.

**Wilson's Disease** Wilson's disease is an autosomal recessive genetic disorder. The gene is a copper-transporting P-type ATPase involved in copper transport across cell membranes, with over 200 known mutations in the Wilson gene, although its precise location and function is not known (26,27). In general, patients with ALF owing to Wilson's disease present with only moderately elevated aminotransferases. The patients frequently already have liver cirrhosis and therefore do not fall under the real definition of ALF. However, many of the patients were healthy before onset of the disease and therefore are treated like patients with ALF (28).

There is evidence that elevated copper levels are directly toxic for the cell and involve CD95-mediated apoptosis (29). The current hypothesis postulates that excess copper generates free radicals that deplete cellular stores of glutathione and oxidize lipids, enzymes and cytoskeletal proteins.

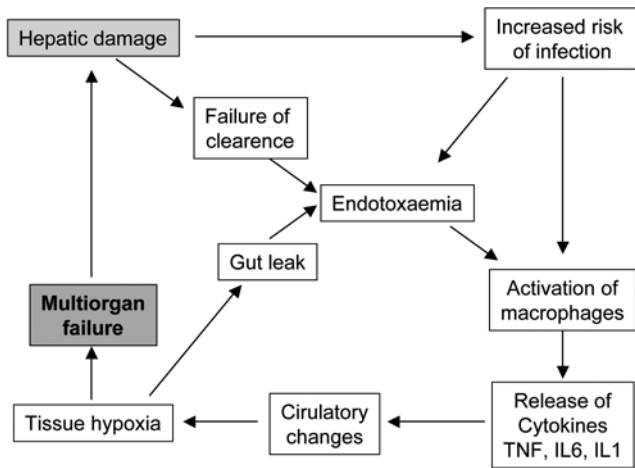
### MECHANISMS OF ORGAN FAILURE

As a consequence of ALF, multiorgan failure develops rapidly (Fig. 1). Different factors contribute to multiorgan failure. Frequent problems that occur during this process are cerebral edema and encephalopathy, an impairment of the immune response with a higher rate of infections, coagulation disorders, and cardiovascular and kidney failure; pulmonary and metabolic complications also develop.

### ENCEPHALOPATHY AND CEREBRAL EDEMA

Hepatic encephalopathy is essential for the diagnosis of ALF, it has four different grades, I to IV. (Table 3). In 75 to 80% of the patients in stage IV, cerebral edema develops independent of the cause of ALF.

The precise pathophysiological mechanisms that lead to hepatic encephalopathy are incompletely understood. However, current studies indicate that the cause is a deficit in neurotransmission rather than a primary deficit in cerebral energy metabolism. Therefore the astrocytes, and the pre- and postsynaptic neurons contribute to the clinical picture (Fig. 2). In contrast, only astrocytes undergo swelling during ALF, and thus determine the degree of cerebral edema (30,31).



**Fig. 1.** Mechanisms that contribute to multiorgan failure during acute liver failure.

**Table 3**  
Stages of Acute Hepatic Encephalopathy

Stage	Mental state
I: prodrome	Mild confusion, slurred speech, slowness of mentation, disordered sleep rhythm, euphoria/depression
II: impending coma	Accentuation of stage I; drowsy but speaking; inappropriate behavior, incontinence
III: stupor	Sleeps most of the time but rousable; incoherent or no speech; marked confusion
IV: coma	Patient may (stage IVA) or may not (stage IVB) respond to painful stimuli

Modified from ref. 44.

Several factors are discussed in the literature that contribute to hepatic encephalopathy, but ammonia (with a consequent dysregulation of the glutamate neurotransmitter system) seems especially relevant for the development of hepatic encephalopathy and cerebral edema.

Arterial ammonia levels at presentation are predictive of outcome in patients with ALF. Patients with encephalopathy grade III and IV show significantly higher serum ammonia levels than patients with lower grade encephalopathy. Possibly patients with advanced cerebral dysfunction can be determined by a serum ammonia cutoff value of 124  $\mu\text{mol/L}$  or more. Ammonia levels can be used for risk stratification (32).

Ammonia has direct effects on cerebral function by direct and indirect mechanisms (Table 4). There is clear evidence that arterial ammonia concentrations directly correlate with cerebral edema and thus herniation (33). Experimental evidence also demonstrates that physiological ammonia concentrations

**Table 4**  
Effects of Ammonia on Brain Function

Electrophysiological effects of the ammonium ion
Effects on the inhibitory postsynaptic potential
Effects on glutamatergic neurotransmission
Effects on brain energy metabolism
Inhibition of $\alpha$ -ketoglutarate dehydrogenase
Effects on astrocyte function
Decreased expression of the glutamate transporter GLT-1
Increased expression of "peripheral-type" benzodiazepine receptors
Alzheimer type II astrocytosis
Effects on the glutamate neurotransmitter system
Direct postsynaptic effects
Impaired neuron-astrocytic trafficking of glutamate
Inhibition of glutamate uptake
Altered glutamate receptors
Effects mediated by formation of glutamine in brain
Cytotoxic brain edema
Increased uptake of aromatic amino acids
Other effects
Stimulation of L-arginine uptake and neuronal nitric oxide synthase (nNOS) expression

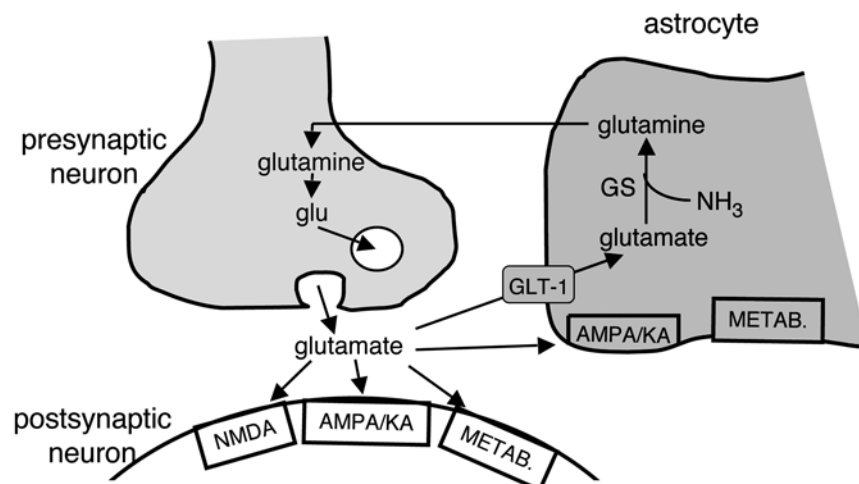
Data from ref. 31.

alone result in astrocyte swelling. Most likely a metabolite of ammonia rather than ammonia itself is the important mediator of astrocyte swelling. Additionally, higher glutamine concentrations are a consequence during this process, and they accelerate cerebral edema (30,31).

Higher ammonia concentrations have a direct effect on the glutamate neurotransmitter system. Glutamate is the major excitatory neurotransmitter in the mammalian brain (Fig. 2). After release at the presynaptic neuron, glutamate binds to glutamate receptors on the postsynaptic neuron (NMDA) or on both the postsynaptic neuron and astrocytes (AMPA/KA). Additionally, glutamate transporters on astrocytes (GLT-1 and GLAST) and neurons (EAAC1) limit the expression of glutamate in the neuronal cleft. After uptake of glutamate in astrocytes via GLT-1, it is transformed into glutamine. Ammonia downregulates GLT-1 expression on astrocytes, and this results in higher and prolonged extracellular glutamate concentrations in patients with ALF. Additionally, there is evidence that the glutamate receptors are differentially expressed during ALF and thus dysregulation of the glutamate system is one of the important determinants for hepatic encephalopathy during ALF (30,31).

Other neurotransmitters that contribute to hepatic encephalopathy are GABA, serotonin, and the opioid system.

A few uncontrolled studies (34–36) show a protective effect of mild hypothermia in ALF and cerebral edema. Hypothermia (32–35°C) can be safely and easily applied. The risk of complications (arrhythmias, myocardial ischemia, infections, coagulopathy) increases with the degree and duration of hypothermia, mainly with body temperatures below 32°C. Hypothermia reduces intracranial pressure and reestablishes disturbed autoregulation of cerebral blood flow. Some studies



**Fig. 2.** The role of glutamate/glutamine in the brain. Shown are the localizations of the glutamate transporter (GLT-1) and glutamate receptor subtypes (NMDA, AMPA/KA, METAB) on astrocytes and neurons involved in glutamatergic neurotransmission. Glu, glutamate. (Modified from ref. 31.)

suggest that hypothermia can reduce the extent of liver injury in ALF (37), in contrast, hypothermia might also lead to impaired liver regeneration. Further research and controlled clinical studies are required to clarify the significance of hypothermia in ALF.

#### CARDIOVASCULAR DYSFUNCTION

Patients with ALF are characterized by hypotension and tachycardia. The basis for this observation is vasodilation in the periphery that results in relative hypovolemia, hypotension, and high output failure. Factors that contribute to this regulation are capillary leakage, low osmotic pressure, and sepsis.

Some patients with ALF may suffer from hypertension. This problem may arise especially in patients with hepatic encephalopathy grade IV and typically occurs when cerebral edema is evolving.

#### INFECTION

Infection and thus sepsis is a major problem in patients with ALF. Patients with a long stay in the ICU have a very high risk in particular, and this may actually be the ultimate reason for death (38). Studies from the King's Collage Hospital group clearly indicated that monitoring by daily cultures (sputum, urine, blood) identifies bacteria in up to 90% and fungal infections in around 30% of these patients (39,40). Frequently the classical signs (fever, leukocytosis, biochemical parameters like C-reactive protein and procalcitonin) in patients with ALF are not directly correlated with infection or are absent. The sites of the body with the most common infections are the lung, urinary tract, and blood (Fig. 3). If antibiotic or antifungal treatment is necessary in these patients, the potential for further liver injury caused by antibiotic drugs should be considered.

Besides the increased risk of patients being managed in an ICU, additional factors contribute to the higher risk of infections in patients with ALF, namely, defects in the immunological defense mechanisms (complement, Kupffer cell function,

polymorphonuclear cell function, cell-mediated immune response). The liver is the main source of complement (e.g., C3 and C5) production. As a consequence of lower complement levels, activity of polymorphonuclear leukocytes and complement-mediated opsonization is reduced. Therefore phagocytosis and killing of polymorphonuclear cells is inhibited in ALF patients. Through the portal circulation bacterial toxins are regularly brought to the liver tissue that are cleared by the resident Kupffer cells of the liver. In ALF there is a correlation between hepatic damage and Kupffer cell dysfunction. Additionally, Kupffer cells are a major source of cytokines, and their dysregulation also contributes to the impaired immune response. Defective lymphocyte function has been attributed to impaired interleukin-2 (IL-2) production in these patients. Thus the defect in immune response can be explained on different levels of the immune system (3,39).

#### PULMONARY COMPLICATIONS

Pulmonary complications are frequent (41). Different mechanisms contribute to this observation. Up to 50% of the patients have infections, especially after intubation and subsequent mechanical ventilation (Fig. 3) (42). The possible consequent capillary leakage can result in an ARDS-like syndrome that is further augmented by the often required infusion of albumin, fresh-frozen plasma, and coagulation factors.

Besides these local mechanisms systemic causes, as a result of liver failure, also lead to intrapulmonary vasodilation and pulmonary edema, which further increase the risk of hypoxic complications (43).

#### RENAL FAILURE

Renal failure with oligo- and anuria is found in 40 to 50% of patients with ALF (44,45). In acetaminophen and *Amanita* poisoning, a direct toxic effect additionally contributes to kidney failure. Therefore, in these patients the rate of kidney failure is increased to 70%.

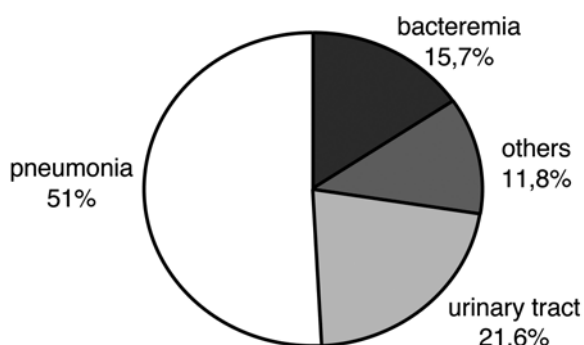


Fig. 3. Sites of infections during acute liver failure. (from ref. 39.)

The association of liver failure and kidney failure is functional and is known as the hepatorenal syndrome. The syndrome is characterized by contraction of the vessels with distinctively reduced renal perfusion. At this stage the kidney impairment is completely reversible. In the further course of the disease, at a more advanced stage, hepatorenal syndrome may progress to tubulus necrosis, which is not reversible (44).

Additional severe complications in patients with hepatorenal syndrome such as long periods of hypotension or sepsis have a fatal effect on kidney function and significantly reduce the prognosis of patients with fulminant hepatic failure.

#### METABOLIC COMPLICATIONS

The liver is essential for several metabolic functions. Two particular problems are frequent in patients with ALF: hypoglycemia and acid–base disturbances.

Different mechanisms lead to hypoglycemia during ALF. The damaged liver loses its capacity to mobilize glycogen stores and to perform gluconeogenesis. Additionally, the liver is the major site of insulin metabolism, and the consequently reduced disintegration of insulin results in elevated insulin serum levels. All three mechanisms contribute to hypoglycemia, and this may also aggravate mental status. In terms of treatment, it might be important to differentiate between hypoglycemia and hepatic encephalopathy as possible causes for disturbed mental status at certain stages.

Both acidosis and alkalosis may be present. Metabolic alkalosis is most frequent, as urea synthesis in the liver is impaired, which results in the accumulation of the two precursor substrates bicarbonate and ammonium. Alkalosis is associated with hypokalemia, which is further aggravated by high sodium reabsorption in patients with ALF.

Acidosis is found in up to 30% of patients with acetaminophen-dependent ALF. In patients with a different etiology acidosis is evident in only 5%. In which lactate acidosis is present because of tissue hypoxia owing to a disturbed microcirculation and the inability of the injured liver tissue to metabolize lactate.

#### COAGULATION DISORDERS

Because of the central role of the liver in coagulation and thrombolysis, severe coagulation disorders are a major problem

in ALF. As a result of reduced coagulation factors and a deficit of inhibitors of fibrinolysis, the hemostasis situation in ALF is complex (46,47).

Factors I, II, V, VII, IX, and X are synthesized in the liver. Therefore prothrombin time is a useful parameter—besides the measurement of single factors—to assess the lack of production of coagulation factors. An additional factor that may contribute to the decrease in blood coagulation factors is disseminated intravascular coagulation (DIC), which may be associated with sepsis during ALF.

Antithrombin III (AT-III) is also synthesized in the liver and is thus reduced. The decrease in AT-III concentration further contributes to coagulation problems.

The number of blood platelets is frequently decreased, and additionally the function and morphology of blood platelets are impaired. Together, these changes result in adhesion abnormalities, leading to decreased aggregation and increased adhesion.

#### DYSREGULATION OF THE CYTOKINE NETWORK IN ACUTE LIVER FAILURE

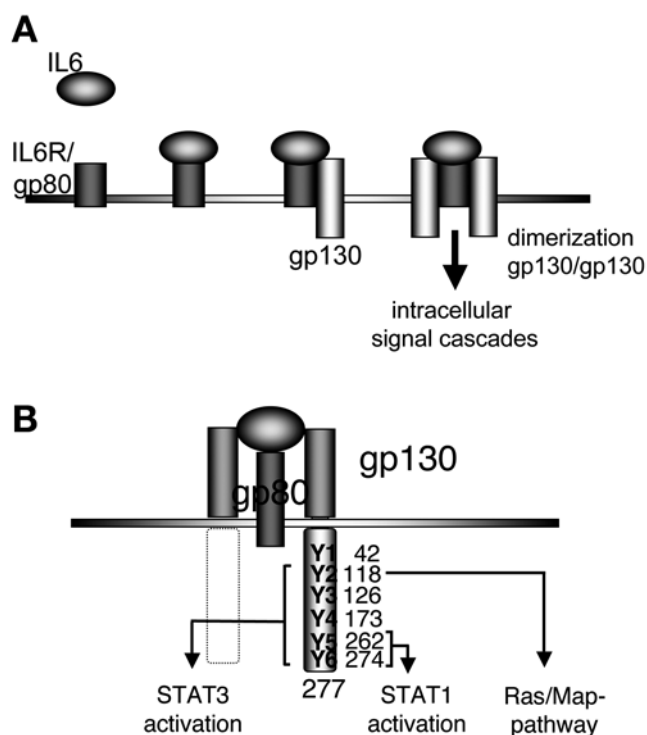
In recent years it has become obvious that there is a dysregulation of cytokine expression during ALF in humans. For example, it has been shown that mediators of the acute phase response—IL-6 and tumor necrosis factor (TNF)—are strongly elevated in the liver and serum of ALF patients. The meaning of this observation becomes more evident through the development of animal models where by the role of each molecule can be more clearly defined. As there is evidence that several cytokines might be involved in the pathogenesis of ALF, all the different aspects can not be covered in this review. We found here on two cytokines, TNF and IL-6 and review recent data in this field.

##### IL-6/GP130-DEPENDENT SIGNALS

IL-6 interacts on the cell surface with the IL-6 receptor (gp80). This complex associates with two gp130 molecules, which results in activation of Janus kinases and in turn in phosphorylation of tyrosines at the intracellular part of gp130. After tyrosine phosphorylation, the ras/map kinase pathways and transcription factors Stat1 and-3 become activated (Fig. 4) (48). In hepatocytes, IL-6 is one of the main inducers of the acute-phase response, and in recent years it has become evident that IL-6 also contributes to the regulation of additional pathophysiological conditions in the liver (49–51).

One of the simplest models for studying the loss of liver tissue is the removal of two-thirds of the liver by surgical resection (52). This model has been applied mainly in rodents (e.g., rat and mouse), and after 1 to 2 wk liver tissue has been restored by hepatocyte proliferation. In recent years it has become obvious that IL-6 and TNF are involved in the restoration of liver mass (53). The ultimate proof of this hypothesis was the observation that liver regeneration was impaired in IL-6 and TNF receptor 1 (TNF-RI) knockout mice after two-thirds hepatectomy. The defect in regeneration in both knockout strains could be restored through IL-6 stimulation (54,55). The current model of how IL-6 and TNF



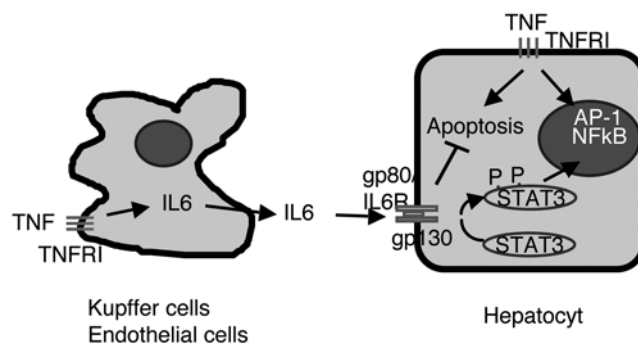


**Fig. 4.** Interleukin-6 (IL-6)/gp130-dependent signaling. **(A)** On the cell membrane IL-6 first interacts with the gp80/IL-6 receptor (IL-6R). This complex interacts with gp130 molecules that dimerize and induce intracellular signaling cascades. **(B)** gp130 dimerization results in the activation of Janus kinases that phosphorylate distinct tyrosines (Y) at the intracellular part of the gp130 receptor. Phosphorylation of the second tyrosine is essential for activation of the Ras/Map pathway. Phosphorylation of the four distal tyrosines results in Stat3 activation; the two most distal tyrosines can also activate Stat1.

may work in concert during liver regeneration after partial hepatectomy is shown in Fig. 5.

The role of IL-6-dependent signals in liver regeneration was further analyzed in more detail using conditional gp130 knockout mice. In these mice the gp130 receptor—the common signal transducer of all IL-6 family members—was deleted in the liver. After hepatectomy, these mice had only an impairment of liver regeneration when the animals were also stimulated with lipopolysaccharide (LPS) after hepatectomy (56). Therefore these experiments demonstrate that IL-6 activates protective pathways in hepatocytes that are important to guarantee liver regeneration but that have no direct impact on the cell cycle progression of hepatocytes.

In humans suffering from ALF, IL-6 serum levels are highly elevated, and in the liver infiltrating cells express tremendous (10-fold higher compared with controls) amounts of IL-6 (49,50,57). In animal models of ALF, IL-6 serum levels are also greatly increased (58), and treatment with an hyper-IL-6 designer molecule reduces liver cell damage in several animal models (59,60). Therefore not only during liver regeneration after partial hepatectomy, but also during ALF it is obvious that IL-6 plays a protective role for hepatocytes; cDNA arrays



**Fig. 5.** Role of interleukin-6 (IL-6) and tumor necrosis factor (TNF) during liver regeneration. Activation of IL-6- and TNF-dependent pathways in nonparenchymal (Kupffer and endothelial cells) and parenchymal (hepatocytes) during liver regeneration after partial hepatectomy is shown.

further demonstrate that IL-6 activates antiapoptotic pathways, e.g., Bcl-x1 in hepatocytes (61,62).

In recent experiments our group generated a hepatocyte-specific knockout mouse for gp130. These animals show normal embryonal development. After IL-6 and also after LPS stimulation, regulation of the acute-phase response is completely blocked. After LPS injection the hepatocyte-specific gp130 knockout mouse shows a phenotype with a strong increase in transaminases in the serum and apoptosis in the liver (49–51).

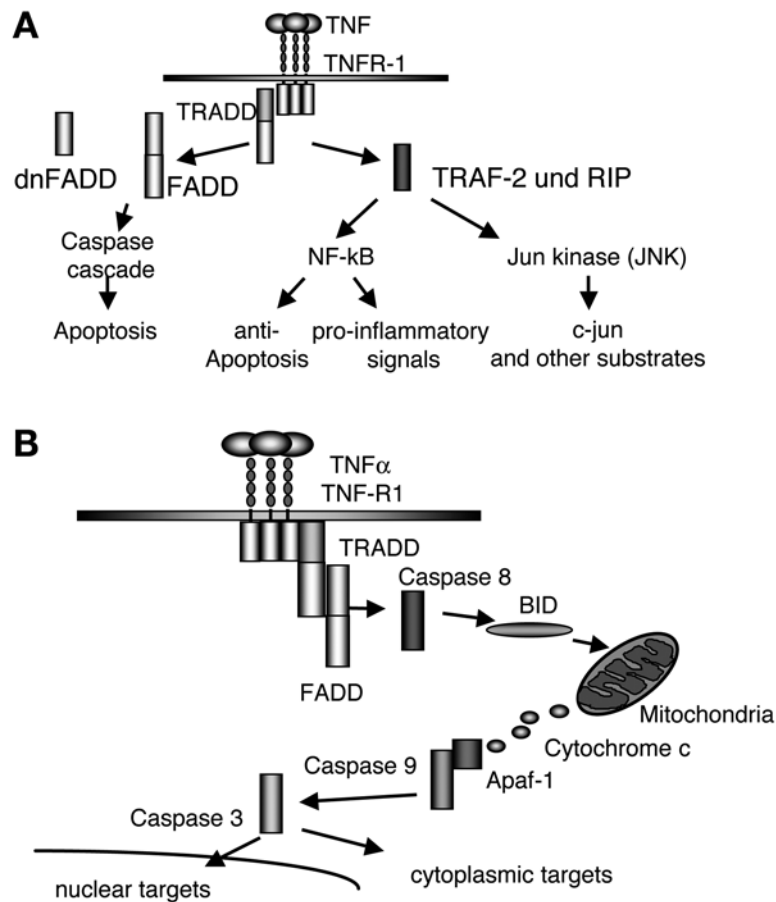
In summary, all the IL-6 data in animal models show that gp130-dependent pathways in hepatocytes activate protective mechanisms, and in humans it is also likely that IL-6 renders hepatocytes more resistant. Therefore it might be promising to modulate IL-6/gp130-dependent pathways in humans during ALF as a potential therapeutic approach.

#### TNF-DEPENDENT PATHWAYS

TNF belongs to a family of several known of Fas and TNF receptor apoptosis-inducing ligands (TRAIL). There is also evidence for an involvement in the pathogenesis of fulminant hepatic failure. At present the role of TNF has been studied in more detail in both human and animal models.

TNF binds to two receptors, TNF-R1 and TNF-R2, on the cell surface. After ligand binding, the intracellular domains of the receptors interact with adapter molecules that activate different pathways (Fig. 6). In the case of TNF-R1, first the molecule TNF-R-associated death domain (TRADD) and then additional molecules bind that activate the caspase cascade either via Fas-associated death domain (FADD) or via TNF-associated factor/ receptor-interacting protein (TRAF/RIP) jun kinase (JNK) and nerve factor- $\kappa$ B (NF- $\kappa$ B) (63).

In practically all the current animal models, TNF seems to be involved in the pathogenesis of ALF. In humans it has also been shown that TNF serum levels correlate with prognosis in ALF patients (57). In animal models, blocking experiments using anti-TNF attenuates liver failure, and therefore it is obvious that TNF plays a central role in the pathogenesis of ALF.



**Fig. 6.** Tumor necrosis factor (TNF)/TNF receptor 1 (TNF-R1)-dependent signaling. (A) The molecules and pathways that are involved in TNF/TNF-R1-dependent signaling are depicted. Activation of the specific intracellular TNF-dependent pathways in the cell has different effects as indicated. (B) The caspase cascade, resulting in apoptosis of the cell is shown in more detail. Activation of caspase 8 via Fas-associated death domain (FADD) triggers a cascade of events including cytochrome c release from mitochondria that results in caspase 3 activation and apoptosis of hepatocytes.

However, further studies indicated that TNF does not have a uniform role in the different models. Depending on the model, the TNF-dependent effect might be related to a different cell in the liver or another intracellular pathway. Three models of acute liver failure and the role of TNF will be discussed.

**Endotoxin/Galactosamine Model** During LPS/galactosamine (GalN)-induced liver injury, TNF induces the transcription of several proinflammatory genes, e.g., chemokines, nitric oxide, and adhesion molecules like intracellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and P-selectin (64–66). These changes in the liver are essential to trigger the extravasation of neutrophils into the liver parenchyma, which results in cytotoxic liver cell damage. During this scenario a stepwise cascade has been described consisting of three events: (1) sequestration of neutrophils in the liver vasculature (2) transendothelial migration, and (3) adherence-dependent cytotoxicity against hepatocytes (67).

Therefore, in the LPS/GalN model, TNF obviously triggers an inflammatory mechanism mediated via NF-κB that results

in liver cell damage. In this model, parenchymal as well as nonparenchymal cells are involved in this process.

**Galactosamine/TNF Model** Administration of GalN and TNF triggers apoptosis of hepatocytes *in vivo* and *in vitro*. The essential role of TNF-R1 in this model has been demonstrated by TNF-R1 knockout mice that are resistant to TNF/GalN treatment (68). GalN will directly inhibit transcription and thus synthesis of antiapoptotic signals. Therefore, in this model the FADD-dependent pathway leading to apoptosis is the essential step in ultimately inducing liver cell damage. In contrast, the NF-κB and JNK pathway does not seem to be involved in the pathogenesis of liver damage, and also nonparenchymal cells play no role. In this model, simple administration of an adenoviral construct expressing a dominant molecule blocking the FADD pathway is protective (57). These data indicate that the caspase cascade activated by TNF might be a relevant target during ALF.

**Concanavalin A Model** Concanavalin A (ConA) is a lectin with high affinity to the hepatic sinus (69). Accumulation of ConA in the hepatic sinus results in activation of liver natural

killer T (NKT) cells, i.e., NK1.1 CD4<sup>+</sup>/CD8<sup>-</sup> T-Cell receptor (TCR) $\alpha\beta^+$ , and NK1.CD4<sup>+</sup>/CD8<sup>-</sup> TCR $\alpha\beta^+$ , which are essential to trigger the early phase of ConA-induced liver injury (70,71). Consecutively CD4-positive and polymorphonuclear cells are attracted to the hepatic sinus and trigger an increase of cytokines like TNF, IL-2, IFN- $\gamma$  IL-6, granulocyte macrophage colony-stimulation factor (GM-CSF) and IL-1 (58). TNF- $\alpha$  and IFN- $\gamma$  have direct implications for the induction of liver cell injury, as anti-TNF- $\alpha$  and anti-IFN- $\gamma$  antibodies protect from ConA-induced liver injury (72,73) and IFN<sup>-/-</sup> and TNF<sup>-/-</sup> mice are resistant to ConA induced liver cell damage.

Until now a stepwise process of liver damage, as shown for the endotoxin/LPS model, could not be defined for the ConA model. Adhesion molecules like ICAM-1 or VCAM-1 seem to play a minor role. Mice pretreated with antibodies against both adhesion molecules or ICAM-1 knockout mice still undergo liver cell injury (74).

The role of TNF-dependent pathways has been further studied in this model using adenoviral vector expression of the inhibitor- $\kappa$ B (I- $\kappa$ B) superrepressor or the dominant negative FADD molecule. Neither constructs has an impact on the degree of ConA-induced liver injury, indicating that NF- $\kappa$ B-dependent targets and the FADD-dependent caspase cascade in hepatocytes are of minor relevance in this model. In contrast, there is a close correlation of TNF-dependent JNK activation with ConA-induced liver injury (49–51). Additionally, first results using an JNK inhibitor indicate that ConA-induced liver injury can be inhibited. Therefore the current data indicate that in the ConA model TNF-dependent JNK activation is essential to trigger liver cell injury.

#### Translation of TNF-Dependent Pathway Results in Animal Models Into Therapeutic Approaches in Humans

The current data in animal models and humans indicate that TNF plays an essential role in the pathogenesis of ALF. However, as demonstrated for the three animals models discussed—depending on the pathogenesis—the intracellular pathways that are activated by TNF could have opposing effects. Therefore, at present it is too early to translate the results into humans, and potential therapeutic approaches cannot be deduced, as it is unclear which of the TNF-dependent pathways are involved in triggering liver failure in individual patients.

#### CONCLUDING REMARKS AND OPEN QUESTIONS

ALF is characterized by sudden onset in patients without evidence of chronic liver disease, by which ALF is differentiated from end-stage chronic liver disease. According to the time between first symptoms and encephalopathy, ALF is divided into three subgroups: hyperacute, acute, and subacute. The prognosis of ALF patients is determined by the metabolic situation resulting from the loss of liver cell mass, the release of mediators and toxic metabolites from injured liver tissue, and the capacity of remaining vital hepatocytes to restore functional liver mass.

Suicidal acetaminophen ingestion is the most frequent cause of drug-induced liver failure worldwide, with approx 500 deaths a year in the United States. Other important

mechanisms are viral hepatitis (e.g., hepatitis B and non-A/non-C hepatitis), and cardiovascular and metabolic disorders.

ALF leads to multiorgan failure, especially cerebral edema and encephalopathy. Owing to the diminished liver function, higher rates of infections and coagulation disorders are observed. Cerebral edema, infections, and renal failure are important clinical complications limiting the survival. For risk stratification in patients with ALF and subsequent hepatic encephalopathy, serum ammonia levels can be used. Advanced cerebral dysfunction is expected at serum ammonia levels of 124  $\mu$ mol/L or higher.

Cardiovascular dysfunction is characterized by peripheral vasodilation that results in relative hypovolemia, hypotension, and high output failure. Capillary leakage and high-volume therapy can lead to an ARDS-like syndrome and cause hypoxic complications.

Prothrombin time is a useful parameter to assess the extent of remaining liver function.

Intensive care therapy is crucial for patients with ALF to manage multiorgan failure, and mild hypothermia to reduce cerebral edema should be considered. Further research and controlled clinical studies are needed to evaluate the importance of hypothermia.

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# **HEPATOTOXICITY OF MEDICATIONS**

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**VI**

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# 28 Immune Mechanisms in Drug-Induced Hepatotoxicity

## *Therapeutic Implications*

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ZHANG-XU LIU AND NEIL KAPLOWITZ

### KEY POINTS

- Drug-induced liver injury (DILI) is a common cause of acute liver failure and also the leading cause of drug withdrawal from the pharmaceutical market. Clinically, most drug-induced liver injuries are unpredictable (idiosyncratic). Only a small fraction of individuals exposed to a drug associated with liver injury will develop hepatotoxicity.
- Many of the examples of idiosyncratic DILI are immune-mediated allergic reactions with the presence of clinical features such as fever, rash, eosinophilia, and other symptoms related to the adaptive immune system.
- In drug-induced immune-mediated hepatic injury, the drug metabolites trigger an immune response directed against the drug-modified liver components (haptentization), resulting in liver injury presenting as acute or chronic hepatitis (similar to autoimmune hepatitis), acute or chronic cholestasis, or mixed disease.
- The effector mechanism for the tissue damage may involve both autoantibodies reacting with liver-specific antigens expressed on the surface of hepatocytes and cell-mediated immunity against hepatocytes. Activation of innate immunity may play an important role in initiation and induction of drug-specific adaptive immune responses.
- The mechanism of hepatotoxicity of acetaminophen (paracetamol) in animal models and humans is well established and could be extrapolated to provide insights into idiosyncratic DILI in humans, particularly the role of the innate immune system and cell-death pathways.
- Progress in understanding the causes of drug-induced idiosyncratic liver toxicity will require identification of specific determinants both in drug-metabolism pathways and in pathways involved in immune-mediated cell injury and protection.

### INTRODUCTION

Drug-induced liver injury (DILI) is a common cause of liver disease. It accounts for approximately one-half of cases of acute liver failure and significant numbers of deaths in the United States and many other countries (1–5). An estimated 1000 or more drugs have been implicated in causing liver disease on more than one occasion (5). Clinically, DILI mimics all forms of liver diseases, with the liver damage varying in severity from mild and transient increases in serum aminotransferases to fulminant hepatic failure. This represents an important diagnostic and therapeutic challenge for physicians. Idiosyncratic drug toxicity refers to toxic reactions occurring in a small subset of patients; it usually cannot be predicted during preclinical or early phases of clinical trials. The occurrence of idiosyncratic drug hepatotoxicity is also a major problem in all phases of clinical drug development and the most frequent cause of postmarketing warnings and withdrawals (1–5). DILI is usually initiated by a toxic drug and its metabolite, followed by either immune-mediated mechanisms and/or intracellular biochemical mechanisms of hepatocytes (2–5). This chapter provides an overview of recent advances in knowledge of the immune mechanisms of drug-induced hepatotoxicity, together with the disease characteristics and possible therapeutic implications associated with drug-induced hepatotoxicity.

### CLASSIFICATION

Adverse hepatic reactions caused by drugs can be either predictable (high incidence) or unpredictable (low incidence) (5). Early onset within a few days, particularly if there has been no previous exposure, is strong evidence for direct toxicity of the parent drug or its metabolite. This pattern of presentation is characteristic of a predictable adverse drug reaction, which is usually dose dependent. Hepatotoxicity caused by acetaminophen, the most commonly used over-the-counter analgesic and antipyretic drug, is a typical example (5,6).

Clinically, most DILI that occurs in humans is unpredictable (idiosyncratic). Only a small fraction of individuals exposed

**Table 1**  
**Clinical Signatures of Drugs Associated With Allergic Drug-Induced Liver Injury**

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**Acute hepatitic reactions**

Allopurinol  
 Dihydralazine<sup>a</sup>  
 Germander and other herbal medicines  
 Haloalkane anesthetics  
 Methyldopa<sup>a</sup>  
 Minocycline<sup>a</sup>  
 Nitrofurantoin<sup>a</sup>  
 Phenytoin  
 Propylthiouracil  
 Diclofenac<sup>b</sup>

**Acute cholestatic and mixed reactions<sup>c</sup>**

Angiotensin-converting enzyme (ACE) inhibitors  
 Amoxicillin-clavulanic acid  
 Chlorpromazine  
 Erythromycin  
 Sulfonamides  
 Sulindac  
 Phenothiazine  
 Tricyclic antidepressants

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<sup>a</sup>Chronic hepatitis also described if drug continued.

<sup>b</sup>Elicits allergic and nonallergic mechanisms.

<sup>c</sup>All cholestatic reactions have some propensity to become chronic (in the absence of drug).

to a drug associated with liver injury will develop hepatotoxicity. Depending on the drug, the incidence of hepatotoxicity occurs from 1/100 to 1/100,000 of exposed individuals (6,7). Unpredictable reactions, manifested as overt or symptomatic disease, can occur with intermediate (1–8 wk) or long (up to 1 yr) periods of latency. A typical example of the former is phenytoin (8), and an example of the latter is isoniazid (9). Long-latency-type reactions usually do not appear to be immune-mediated and probably represent metabolically based idiosyncratic reactions (nonallergic). Intermediate latency is characteristic of allergic hypersensitivity reactions. However, some allergic reactions occur after very long latency, and individual drugs can induce either allergic or nonallergic DILI.

### CLINICAL FEATURES OF ALLERGIC DRUG-INDUCED LIVER INJURY

Idiosyncratic allergic DILI may present as acute hepatitis, acute cholestasis, or mixed disease (2,3,10,11), and the signature for a particular drug is relatively specific (Table 1). Symptoms typically appear within a few weeks after initiation of therapy, but they may appear several weeks after treatment has been discontinued (e.g., antibiotics). In rare cases, the acute hepatitis reaction is not recognized or is not severe, leading to continued use of the drug and possible chronic hepatitis or cirrhosis. Typically, the acute hepatitic reactions improve rapidly and resolve completely within 1 to 2 mo after discontinuation of the drug. The cholestatic reactions, characterized by jaundice, pruritus, high serum alkaline phosphatase levels, and low-grade serum aminotransferase elevations, seem to involve microscopic bile duct injury, and they may involve a prolonged recovery. Occasionally, they may progress to the vanishing duct syndrome

or biliary cirrhosis. Liver histology shows apoptosis, necrosis, and inflammatory infiltrates including mononuclear cells, neutrophils, eosinophils, and lymphocytes or cholestasis and paucity of bile ducts with moderate portal inflammation. The clinical features are low frequency, dose independence, typical immunologic manifestations such as fever, rash, and/or eosinophilia, delayed onset (1 wk to 2 mo after initiation of treatment), rapid recurrence of hepatotoxicity on reexposure to the drug, and the frequent presence of autoantibodies (for example, anti-nuclear antibodies [ANA] and/or smooth muscle antibodies [SMAs]). (2,3,5,10). These events can be viewed as representing immune-mediated hypersensitivity, and most drug-induced autoimmune-like hepatitis belongs to this type of adverse drug reaction. In contrast to self-perpetuating autoimmune liver disease, idiosyncratic allergic DILI depends on continuous exposure to the drug to induce a drug-dependent acute or chronic liver disease and nearly always disappears or becomes quiescent when the drug is removed (3).

### PATHOGENIC MECHANISMS

Although the pathogenesis of drug-induced hepatotoxicity is still largely unknown, particularly for idiosyncratic toxicity, clear evidence indicates the important roles of both drug metabolism and immune-mediated mechanisms.

#### THE ROLE OF DRUG METABOLITES

Administered drugs are largely metabolized by biotransformation reactions in the liver, which account for the organ's susceptibility to metabolism-dependent DILI. For most drugs, metabolism involves two biotransformation phases. Phase I reactions are catalyzed by cytochrome P450 proteins (CYPs), which are the major oxidative catalysts involved in drug



metabolism (3,12). These enzymes catalyze oxidation reactions, resulting in generation of reactive intermediates. Phase II reactions conjugate the metabolic products of the phase I reactions with small endogenous molecules such as glucuronic acid, glutathione, acetate, or sulfate, in order to increase water solubility and elimination from the body (detoxification). Such molecules are catalyzed by various enzymes, i.e., glucuronyl transferases, glutathione *S*-transferases, *N*-acetyltransferases, or sulfotransferases (3,12).

The drug metabolites can be electrophilic chemicals or free radicals which can undergo or promote a variety of chemical reactions, such as the depletion of reduced glutathione (GSH), covalent binding to proteins, lipids, or nucleic acids or induction of lipid peroxidation. All of these have consequent direct effects on organelles such as the mitochondria, endoplasmic reticulum, cytoskeleton, microtubules, or nucleus (2–4,13). They may also indirectly influence cellular organelles through the activation and inhibition of signaling kinases, transcription factors, and gene-expression profiles. The resultant intracellular stress leads to hepatocyte death caused by either cell shrinkage and nuclear disassembly (apoptosis) or swelling and lysis (necrosis) (4,13).

The reactive metabolite in the phase I reaction can produce hepatic injury or be detoxified in the phase II reaction. Defects in detoxification may result in hepatotoxic events. Therefore drug metabolism (toxification and detoxification) determines exposure to toxic metabolites (11,13). This is a prerequisite for both allergic and nonallergic reactions. Genetic polymorphisms of drug metabolism contribute to the risk of allergic reactions (e.g., sulfonamides and dihydralazine) (7,11). In addition, a potential role for drug transport/elimination, the so-called phase III, is emerging.

#### HAPTEN HYPOTHESIS

A hapten-like autoimmune response may be involved in the mechanism for induction of idiosyncratic DILI (10,11,14–16). Small and low-molecular-weight compounds, such as drugs, are usually not immunogenic (hapten) but may become so when bound to a carrier macromolecule such as protein. Thus, if a reactive metabolite covalently binds to a hepatic protein, it may modify the protein. The altered protein would be perceived as non-self by the immune system and could induce an autoimmune response against the normal hepatocellular constituents through crossreaction. The hapten-like autoimmunity may involve the phagocytosis, processing, and presentation of antigen (altered protein) by antigen-presenting cells (APCs) (e.g., Kupffer cells), activation of T-helper cells, induction of hapten-specific cytotoxic T cells, and production of autoantibodies by B cells against target antigens in the liver, e.g., the drug (hapten), or part of the carrier protein, or both. Indeed, the presence of autoantibodies and drug-specific T-cell responses in patients with DILI support the pathogenic role of adaptive immune responses (17–20).

However, the hapten hypothesis cannot explain many aspects of immune-mediated DILI. For example, many drugs that form reactive metabolites, such as acetaminophen, are not associated with hypersensitivity reactions. It is possible that a reactive metabolite may also have to injure or stress hepatocytes,

in addition to modifying a protein, to induce activation of liver innate immunity and inflammation that would help provide sufficient costimulatory signals to break self-tolerance in order to induce an autoimmune response in the liver (11).

#### DANGER HYPOTHESIS

The danger hypothesis proposed by Matzinger (21,22) may help to explain why drugs that frequently form reactive metabolites are not associated with a high frequency of hypersensitivity reactions. Based on this hypothesis, the immune system does not directly differentiate self and non-self, and it only responds to a foreign antigen if the antigen is associated with a danger signal, namely, the “immune system is more concerned with damage than with foreignness, and is called into action by alarm signals from injured tissues, rather than by the recognition of non-self” (21). The driving force of the immune system is the need to detect and protect against danger. Intracellular stress and cell death caused by a reactive metabolite are likely to be danger signals in DILI (11,22). Thus, a reactive metabolite may not only have to modify protein, it may also have to injure or stress cells to induce an immune response. The danger that primes a genetically susceptible immune system might include a background mild hepatic injury or concomitant infection or inflammatory conditions. For example, some drugs like halothane may cause a mild liver injury owing to direct toxicity. This injury may in turn provide a danger signal, which triggers an immune-mediated hepatitis in susceptible individuals (10,11,22). A concomitant viral infection, such as hepatitis C virus (HCV) or human immunodeficiency virus (HIV), may also be a danger signal. Allergic hepatotoxicity is more common in AIDS patients (23).

The altered cytokine milieu of chronic viral disease can also influence susceptibility to nonallergic toxicity and helps to explain the suggested increased susceptibility of patients with HIV or chronic viral hepatitis to isoniazid hepatotoxicity (24,25). This is also supported by recent animal experiments demonstrating that a mild inflammation can enhance the sensitivity to idiosyncratic DILI. The modest inflammatory response induced by nonhepatotoxic doses of bacterial lipopolysaccharide (LPS) has sensitized animals to ranitidine-induced idiosyncratic hepatotoxicity (26). Similar results have been observed with other drugs, e.g., chlorpromazine and trovafloxacin, for which idiosyncratic liver injury in humans is well documented (27,28).

It seems that concomitant inflammation may decrease the threshold of drug toxicity, rendering individuals more susceptible to DILI. Some idiosyncratic DILI may therefore result from sporadic inflammatory episodes during drug therapy.

#### THE P-I CONCEPT

Recently Pichler et al. proposed the *p-i concept* to explain the cellular mechanisms of T-cell mediated drug hypersensitivity (29,30). The *p-i concept* stands for “direct pharmacological interaction of drugs with immune receptors.” According to this hypothesis, certain drugs may directly bind to some of the highly variable antigen-specific T-cell receptors (TCRs) and MHC molecules. The activation and expansion of antigen-specific T cells largely depends on the unique structure of a drug that

**Table 2**  
**Autoantibody Targets in Drug-Induced Liver Disease**

<i>Autoantibody target</i>	<i>Drug</i>
CYP 2C9	Tienilic acid
CYP 1A2	Dihydralazine
CYP 3A	Anticonvulsants (e.g., phenytoin)
CYP 2E1	Halothane
Microsomal epoxide hydrase	Germander

fits into a matching TCR together with costimulatory signaling provided by MHC molecules. It does not involve the function of APCs. In this way, drug-specific T-cells are distinct from classical T-cell activation in the hapten hypothesis. Direct drug-TCR binding may cross-activate peptide-specific memory T cells, leading to bypass of the induction of a primary immune response, which may explain the strong early allergic reactions observed in some patients without previous exposure to certain drugs. This concept may supplement the hapten hypothesis; however it is largely based on studies of drug-induced hypersensitivity reactions in the skin (29–31). It remains to be determined whether this concept applies to DILI. It is also possible that in some cases the liver is an innocent bystander in severe drug-induced systemic allergic reactions, which cause collateral damage to the liver by sequestering inflammatory cells (32).

#### THE ROLE OF ANTI-CYP AUTOANTIBODIES

The drug is first converted into a reactive metabolite by a specific CYP in phase I of biotransformation. The resultant metabolite could covalently bind to the specific CYP and form a neoantigen that could trigger an immune response characterized by the production of autoantibodies against the native and/or the modified CYP (10,11,19). Table 2 lists examples of anti-CYP autoantibodies against the particular CYP isoenzymes that metabolize the parent drug. For example, dihydralazine is transformed by CYP1A2 into reactive metabolites that covalently modify the CYP1A2 protein (17). Serum anti-liver microsome (LM) autoantibodies from patients with dihydralazine hepatitis were found to recognize CYP1A2 (33). Anti-CYP autoantibodies are present in the serum when the disease is diagnosed, and they decline and may disappear after recovery. For halothane hepatitis, an antibody-dependent cell-mediated cytotoxicity (ADCC) has been reported (34), and the antibodies may play a role in the destruction of hepatocytes through the activation of natural killer (NK) cells and the complement system (12). Furthermore, CYPs can be detected on the surface of rat and human hepatocytes by anti-CYP antibodies or autoantibodies (19,35), suggesting a possibility that these cells can be recognized and damaged by the autoantibodies. However, the formation of metabolite adducts and generation of autoantibodies frequently occur in patients who take the drugs but have no evidence of liver injury. For example, many anesthetic-exposed individuals develop antibodies but do not experience hepatitis (36,37). Whether these antibodies actually cause liver injury or are consequences of liver injury caused by other mechanisms remains to be elucidated.

#### IMMUNE-MEDIATED MECHANISMS: WORKING MODEL

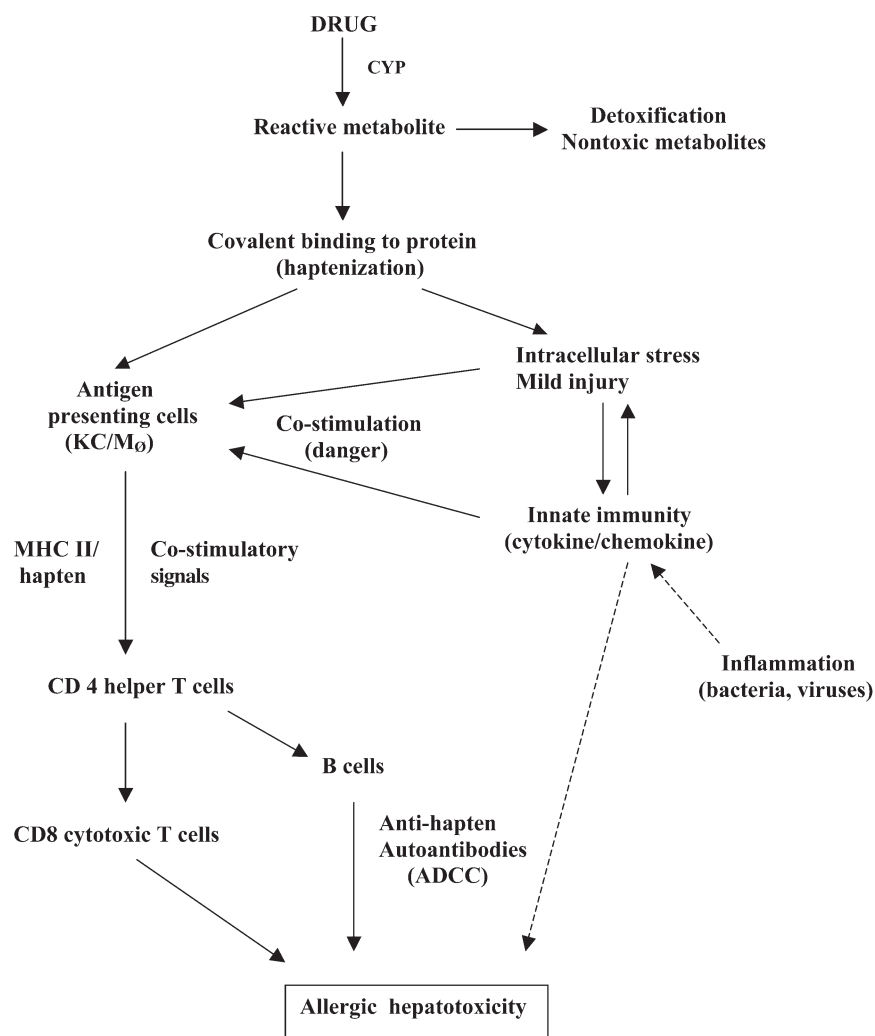
A working model of the immune mechanisms of idiosyncratic DILI is illustrated in Fig. 1. A reactive metabolite of a drug may bind to cellular proteins or macromolecules (haptenization), leading to a direct toxic effect on hepatocytes (intracellular stress or death) and release of the modified proteins (10,11,34,38). APCs in the liver, i.e., Kupffer cells, may capture, process, and then present the hapten peptides on MHC class II molecules to CD4 T-helper cells, resulting in activation of CD4 T cells. Cell stress, cell death, and activation of innate immunity may provide danger signals to promote APC function, thereby stimulating the induction of adaptive immunity. With the help of activated CD4 T cells, drug-specific CD8 cytotoxic T cells and antibody-producing B cells could be generated. As effector cells, CD8 cytotoxic T cells could then recognize and kill hepatocytes that present a hapten peptide or similar antigen in the complex of MHC class I molecules; autoantibodies secreted by plasmacytes (mature B cells) could recognize the hapten peptides expressed on the plasma membrane of hepatocytes and mediate cytotoxic effects through ADCC or the complement system.

#### ACETAMINOPHEN HEPATOTOXICITY AND THE INNATE IMMUNE SYSTEM

There are still many open questions on the working hypothesis just described. A lack of animal models is a big hurdle for the study of immune-mediated DILI. In this regard, extensive animal studies on acetaminophen (APAP) hepatotoxicity in the last decade may provide important insights into the immune mechanisms of DILI, although APAP-induced liver injury is a typical example of dose-dependent, predictable adverse drug reactions.

Overdose of APAP in both humans and animals can lead to acute liver failure characterized by centrilobular hepatic necrosis. APAP hepatotoxicity is currently the most common form of acute liver failure in the United States, accounting for at least 42% of acute liver failure cases and one-third of the deaths in a recent multicenter study (39). Historically, studies on the pathogenesis of APAP hepatotoxicity in both human and animal models have focused on the initial biochemical and metabolic events that occur intracellularly in parenchymal hepatocytes in the early stages of toxicity. However, growing evidence indicates the importance of the innate immune response in determining the progression and severity of APAP hepatotoxicity (40,41).

The toxic response to APAP is initiated by a highly electrophilic intermediate, *N*-acetyl-*p*-benzoquinone-imine (NAPQI), generated by hepatic cytochrome P450, particularly CYP2E1 (42,43). NAPQI is detoxified through conjugation with hepatic GSH after a therapeutic dose; however, after a toxic dose of APAP, the hepatic GSH is depleted, and excessive NAPQI subsequently covalently binds to cellular proteins to form APAP-protein adducts (42,44,45), resulting in oxidative stress reactions, dysfunction of mitochondria, and DNA damage (42,46,47). These initiation events caused by APAP metabolites ultimately lead to direct hepatocyte death (48). However, the threshold for cell death can be modulated by intrahepatocyte



**Fig. 1.** Pathogenesis of immune-mediated idiosyncratic drug-induced liver injury. With costimulatory signals provided by mild injury, activation of innate immunity, or inflammation, liver antigen-presenting cells (Kupffer cells [KC] and macrophages [M $\phi$ ]) present hapten peptides on MHC class II molecules to promote activation of T-helper cells, leading to an adaptive immune response to the antigen. Drug-specific cytotoxic T cells can then recognize and kill hepatocytes that present hapten peptide or a similar antigen in the complex of MHC class I molecules; anti-hapten or autoantibodies secreted by mature B cells could mediate an antibody-dependent cell-mediated (ADCC) or complement-induced hepatotoxicity. The activated innate immune system may also directly contribute to hepatotoxicity. CYP, cytochrome P450.

signal transduction and transcription factors for protective or injurious pathways such as nerve factor (NF)-E2-related factor 2 (Nrf2) and c-Jun-N-terminal kinase (JNK) (11,41). Nrf2 regulates GSH synthetic and detoxification enzymes. Thus *Nrf2*<sup>-</sup> mice are more susceptible to APAP toxicity (49).

Recent evidence has emerged to support the view that these chemical effects of NAPQI may activate intrinsic cell death pathways. Thus, a role of JNK activation, presumably as a consequence of oxidative stress, is supported by marked protection against APAP hepatotoxicity afforded by a chemical JNK inhibitor as well as the silencing of JNK expression with anti-sense oligonucleotide treatment (50). The targets of JNK in this cell death-inducing pathway are currently unknown, but candidates may include Bcl2 family members and mitochondrial proteins (50). Sustained activation of JNK therefore may recruit

death-promoting activities at the level of mitochondria. Since mitochondria are viewed as the key organellar target in APAP toxicity (40), one could speculate that the effects of APAP on mitochondria render this organelle more vulnerable to JNK-mediated effects. The metabolism of APAP in hepatocytes, including GSH depletion, activates protective mechanisms (Nrf2) and injurious mechanisms (JNK). The balance of these events in response to APAP plays an important initial role in determining the threshold for toxicity (11,41).

In addition to upstream factors involved in and responding to APAP metabolism, the downstream factors that lead to modulation of liver innate immunity, e.g., mediators released from Kupffer cells and NK/NKT cells, may affect the individual's risks for developing severe APAP hepatotoxicity (40,41). APAP-induced hepatocyte death may trigger the activation of

resident innate immune cells in the liver, resulting in release of inflammatory mediators and recruitment of inflammatory cells, particularly neutrophils (40,41,51–54). There is growing evidence in the past decade that the inflammatory mediators such as cytokines, chemokines, and reactive oxygen and nitrogen species released by innate immune cells participate in the progression of liver injury (40,41). This notion is further supported by different gene-deficient mice showing altered susceptibility to APAP hepatotoxicity but without effect on GSH depletion and APAP-adduct formation (11,40,41). The important role of the proinflammatory cytokine interferon- $\gamma$  (IFN- $\gamma$ ) is underscored by the decreased susceptibility to APAP in IFN- $\gamma$  null mice (51,54). Interleukin-6 (IL-6) and IL-10 play protective roles in APAP hepatotoxicity. IL-6 null mice are more susceptible to APAP hepatotoxicity owing to a deficiency in the expression of cytoprotective hepatic heat shock proteins (55). Studies in IL-10 knockout mice suggest that IL-10 caused antiinflammatory activity in the liver (56), because the increased susceptibility in these mice correlates with an elevated expression of proinflammatory cytokines in the liver (tumor necrosis factor- $\alpha$  [TNF- $\alpha$ ], IL-1, and IFN- $\gamma$ ), as well as inducible nitric oxide synthase (iNOS).

The precise role of chemokines is controversial. Some studies have shown that they contribute to liver injury by promoting migration of inflammatory cells (51,54,57,58). For example, mice deficient in CXCR2, an important chemokine receptor for neutrophils, have decreased APAP hepatotoxicity associated with reduced neutrophil accumulation in the liver (58). Furthermore, depletion of neutrophils showed a protective effect on APAP hepatotoxicity (58,59). However, others suggest that chemokines or their receptors may in fact protect liver injury through the induction of anti-inflammatory cytokines by infiltrating cells or through their direct hepatoprotective roles and stimulation of hepatocyte proliferation (60–62). Thus mice with targeted gene deletion for CCR2, the receptor for monocyte chemoattractant protein-1 (MCP-1), were more sensitive to the toxic effects of APAP, which was associated with increased expression of TNF- $\alpha$  and IFN- $\gamma$  in the liver (60). Together these studies suggest that the balance between pro- and anti-inflammatory cytokines/chemokines produced in the liver may contribute to susceptibility to APAP hepatotoxicity.

Recent evidence demonstrated that the activation of NK/NKT cells, the major components of resident lymphocytes in the liver, plays a pivotal role in the progression of APAP hepatotoxicity (51). Depletion of these cells by anti-NK1.1 antibody significantly protects mice from APAP-induced liver injury, and the protection is associated with inhibition of mRNA expression for IFN- $\gamma$ , FasL, and chemokines and reduced neutrophil accumulation in the liver (51). One key feature of these cells is the production of IFN- $\gamma$ , a proinflammatory cytokine (51). Downstream of IFN- $\gamma$ , a variety of cytokines and chemokines are expressed in APAP-treated mice, which are presumed to promote inflammatory cell infiltration in the liver (51,54). A recent study suggests that osteopontin (OPN), an important immune mediator produced by NK and NKT cells among other cells, may play a role in APAP-induced liver

injury, presumably by promoting migration of inflammatory cells into the liver (63–65). Thus OPN knockout mice were less susceptible to APAP-induced liver injury than wild-type mice (63). Depletion of NK and NKT cells also inhibited APAP-induced upregulation of hepatic FasL expression at both mRNA and protein levels (51). Furthermore, Fas- and FasL-deficient mice were protected against APAP toxicity (51), and silencing of Fas has also been shown to decrease APAP toxicity (66). Although apoptosis is not a prominent feature in APAP hepatotoxicity (40,67,68), it is not clear whether the Fas system is involved in APAP-induced necrosis (upstream of mitochondria) or is promoting inflammation that leads to amplification of APAP hepatotoxicity (51,69,70).

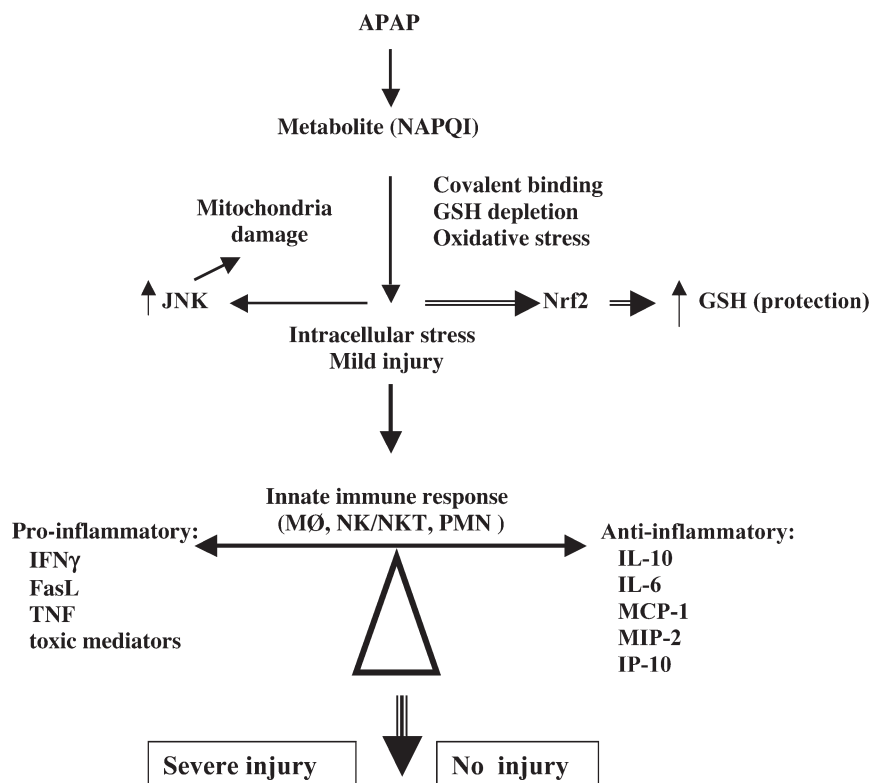
The important lessons from the model of APAP hepatotoxicity are that at the same time as a proinflammatory cascade is activated by the initial intracellular stress of an APAP metabolite, a counterregulatory antiinflammatory or hepatoprotective cascade is simultaneously activated, so that the interplay of pro- and antiinjurious mechanisms modulates the susceptibility to APAP (Fig. 2) (11,41). APAP is the most widely studied model of drug hepatotoxicity, and it is hoped that mechanistic insights from these studies will be relevant to an understanding of the basis for mechanisms of idiosyncratic drug hepatotoxicity.

## EXAMPLES OF IDIOSYNCRATIC DRUG-INDUCED LIVER DISEASES

### DRUG-INDUCED HEPATITIS

The liver diseases induced by halothane, dihydralazine, anticonvulsants, and tienilic acid have been intensively investigated, and they are models of immune-mediated idiosyncratic drug-induced hepatitis.

Halothane is the best studied drug related to immune-mediated DILI. Most patients with halothane-induced hepatitis had multiple exposures to this drug (3). There is also considerable evidence supporting the role of immune-mediated mechanisms in the pathogenesis of the injury because many patients who develop hepatitis have serum autoantibodies that react with specific hepatic proteins such as CYP2E1 (71,72). Halothane is oxidatively metabolized by CYP2E1, with concomitant generation of the reactive intermediate trifluoroacetyl chloride (TFA), which covalently binds to liver proteins and forms TFA adducts (73). After exposure to halothane, TFA adducts can be presented on the plasma membrane of hepatocytes (34,73). This is supported by observations that TFA-modified CYP2E1 has been detected by immunochemical staining in the livers of halothane-treated rats (72). Patients with halothane hepatitis were reported to develop autoantibodies specifically targeting CYP2E1 (71,72,74). However, similar autoantibodies are also detectable in anesthesiologists exposed to halogenated anesthetic gases (37). A recent study further analyzed serum CYP2E1-specific Ig subclass levels and found that persons environmentally exposed to halogenated volatile anesthetics develop CYP2E1-specific IgG1 autoantibodies, which may form immune complexes cleared by classical activation of the complement system (75). In contrast, patients with idiosyncratic drug-induced hepatitis develop CYP2E1-specific IgG4 autoantibodies,



**Fig. 2.** Pathogenesis of experimental acetaminophen (APAP) hepatotoxicity. Following the hepatocellular upstream events, such as covalent binding and glutathione (GSH) depletion, intracellular events influence the threshold for toxicity with c-Jun N-terminal Kinase (JNK) activation promoting toxicity and Nrf2 protecting against toxicity. Upstream events promote intracellular stress, and mild injury activates the downstream innate immune system, which represents a balance of pro- and anti-inflammatory responses, the interplay of which determines progression to severe injury or no injury. APAP, acetaminophen; GSH, glutathione; IFN $\gamma$ , interferon; IL, interleukin; JNK, c-Jun N-term Kinase; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; MØ, macrophage; NAPQI, *N*-acetyl-p-benzoquinoneimine; NK, natural killer (cell); NKT, NKT (cell); Nrf2 nerve factor-E2-related factor 2; PMN, polymorphonuclear neutrophil; TNF, tumor necrosis factor.

which form small, nonprecipitating immune complexes that may escape clearance and cause liver injury (75).

Long-term treatment with the antihypertensive drug dihydralazine has been reported to cause hepatitis (3,10,17). Dihydralazine-induced hepatitis affects women more than men and occurs mainly in the slow acetylators of sulfamethazine. The onset of hepatitis is usually delayed, with a latent period of several months, but chronic hepatitis has been reported after several years of treatment. The hepatitis resolves after discontinuation of the drug, and rechallenge with dihydralazine results in recurrence of the liver injury. Dihydralazine hepatitis is associated with LM autoantibodies that recognize rat liver but not rat kidney microsomes (17,34,76). The molecular target of the anti-LM autoantibodies was identified as CYP1A2 (17), which metabolizes dihydralazine to reactive metabolites in human and rat liver. Anti-LM autoantibodies are also frequently found in autoimmune hepatitis, suggesting that similar autoimmune pathogenetic mechanisms can lead to liver injury in susceptible individuals irrespective of the primary defect.

Anticonvulsant-induced hepatitis has been observed after treatment with drugs such as carbamazepine, phenytoin, and phenobarbital (3,10). The onset of liver injury is usually within

6 wk, and it is usually accompanied by severe rash and eosinophilia. The hepatic damage is mainly cytotoxic, with high serum aminotransferase levels. Histological features include diffuse hepatocellular degeneration, multifocal areas of intense necrosis, multiple acidophilic bodies, and clusters of eosinophils or lymphocytes in the inflammatory infiltrate. A subset of patients with hypersensitivity reactions to the anticonvulsants generates antibodies that recognize the rat cytochrome CYP3A subfamily (77,78). However, these antibodies do not recognize related human CYP3A proteins despite their high degree of structural similarity.

Tienilic acid (TA) is a uricosuric drug that was used in the treatment of hypertension prior to its withdrawal from the U.S. market in 1980 because of its hepatotoxic potential (79). Tienilic acid-induced hepatitis was dose independent and occurred with a delay ranging from 14 to 240 d after starting therapy. After discontinuation of treatment, the liver damage resolved, but rechallenge resulted in recurrence within a shorter period. In some cases, fulminant hepatitis, chronic hepatitis, and/or cirrhosis occurred (79). A specific anti-liver/kidney microsome type 2 antibody (LKM-2) directed against unmodified liver and kidney microsomal protein was detected in the sera of patients

with hepatitis (79,80). Antibodies to LKM-2 were detected only in patients with TA-induced hepatitis, and they appeared to be a specific marker of the disease. TA is mainly metabolized by CYP2C9, which was identified as the molecular target of autoantibodies to LKM-2 (79,81). Autoantibodies recognize CYP2C9 in humans (11,34,79,80) and CYP2C11 in rats (82). CYP2C11 is a major isoform in the adult male rat liver, and it exhibits 85% sequence identity with human CYP2C9 (82). The reactive metabolite-mediated chemical modification of CYP2C9 generates circulating anti-CYP2C9 antibodies in sensitive patients. The appearance of these modified proteins on the hepatocyte surface may lead to formation of antigen-antibody complexes and hepatocyte destruction (11,34,79).

A number of other drugs have been reported to produce autoimmune hepatitis-like syndrome. The serologic findings that resemble type I autoimmune hepatitis in these patients include ANA, SMA, and hyperglobulinemia (3,10). Diclofenac (83), germander and other herbal medicines (84,85) allopurinol (86),  $\alpha$ -methyl dopa (87), nitrofurantoin (88), pemoline (89), and propylthiouracil (90) are within this genre. These drugs can produce acute or chronic hepatitis depending largely on the severity of the initial reaction and whether the drug is continued after onset of the injury.

#### DRUG-INDUCED CHOLESTATIC REACTIONS

A number of drugs are associated with hypersensitivity-type reactions that are either exclusively or predominantly cholestatic in nature (3,10). Among these are chlorpromazine, erythromycin amoxicillin-clavulanic acid, sulindac, angiotensin-converting enzyme (ACE) inhibitors, sulfonyleureas, tricyclics, and sulfonamides (Table 1). The specific immunological targets of these adverse reactions are poorly understood, but presumably they are related to the bile ducts since the predominant histological features are portal inflammation and biliary injury. Importantly, drugs inducing cholestatic hepatitis may cause bile ducts to vanish (vanishing bile duct syndrome) and biliary cirrhosis to develop even after drug withdrawal (3,91). Most drug-induced cholestatic reactions, with the exception of those associated with androgens, estrogens, and cyclosporin A, are accompanied by hypersensitivity manifestations. Drugs such as sulindac and glibenclamide inhibit the bile salt excretory process in hepatocytes, and it is unclear how these mechanisms can induce an immune-mediated cholestatic disease. One theoretical possibility is that toxic metabolites (e.g., reactive glucuronides) undergoing canalicular excretion from hepatocytes react with macromolecules in duct cells or undergo further metabolism within these cells. The finding that hepatitic and cholestatic injury patterns can coexist in some patients (mixed reactions) and that the hepatitic pattern can predominate in some patients emphasizes the variability of the pathogenic mechanisms.

#### SUSCEPTIBILITY AND RISK FACTORS

Genetic polymorphisms in metabolic pathways involved in bioactivation or detoxification of therapeutic drugs may have a strong influence on susceptibility to drug-induced liver injury (7,92). Several genetic polymorphisms of drug-metabolizing

enzymes, particularly CYP, have been identified, some of which cause expression of inactive enzymes or enzymes with altered metabolic activity (7,93). These individual differences in the generation of reactive metabolites may influence the formation of protein adducts and thereby affect susceptibility to drug-induced hepatotoxicity. Polymorphisms of the MHC molecules may be another important factor affecting the susceptibility to drug-induced hepatitis. Halothane-induced hepatitis occurs more frequently in individuals with specific HLA haplotypes (93), and there are other examples of HLA associations with certain drug reactions (94,95). A recent study found that the frequencies of HLA alleles *DRB1\*15* and *DQB1\*06* were significantly increased in patients with the cholestatic/mixed type of liver damage in patients with drug-induced idiosyncratic DILI compared with healthy subjects (95). By contrast, the frequencies of alleles *DRB1\*07* and *DQB1\*02* were significantly decreased (95). This result suggests that the genetic influence associated with HLA class II alleles appears to play a role in cholestatic/mixed hepatotoxicity and may explain why a given drug may cause different patterns of liver damage (95). Other factors, such as age, gender, nutritional status, concomitant viral illnesses, and use of other drugs, may also affect an individual's susceptibility (7).

Recent animal studies, particularly on APAP hepatotoxicity, suggest that host genetic factors regulating the innate immune system, e.g., the expression of cytokines, chemokines, reactive oxygen species, adhesion molecules and Toll-like receptors, may have important influences on individual susceptibility to drug-induced liver injury (11,40,41).

#### DIAGNOSIS

There are generally no specific markers or laboratory tests for the diagnosis of DILI. Therefore, the diagnosis of DILI is mainly one of exclusion with initial suspicion based on circumstantial evidence. It relies largely on the temporal association between the drug and the clinical syndrome, the rate of improvement after discontinuation of the drug (rapid in hepatitis, slow in cholestasis), and the presence of concurrent allergic manifestations, such as rash, eosinophilia, and fever. A positive rechallenge is the strongest clinical evidence of a cause-effect relationship, but it usually should not be performed, since a recurrent injury is usually more severe, especially if the injury is immune mediated. Knowledge of the track record of the drug is also useful, particularly if there are several candidates. Ultimately, it is best to be suspicious of a drug reaction in all cases of hepatitis and to stop any possible agent.

Specific autoantibodies present in serum, for example, anti-CYP antibodies, are potentially important diagnostic markers; however, the diagnostic accuracy of these tests has not been convincing, and they are not widely available (7).

Some investigators have advocated that drug-specific T-cell reactivity to the implicated drug be tested to confirm the diagnosis (18). This approach measures the proliferative response (thymidine incorporation) of peripheral blood lymphocytes to the parent drug. Test sensitivity might be improved by using the metabolites that are covalently bound to protein.

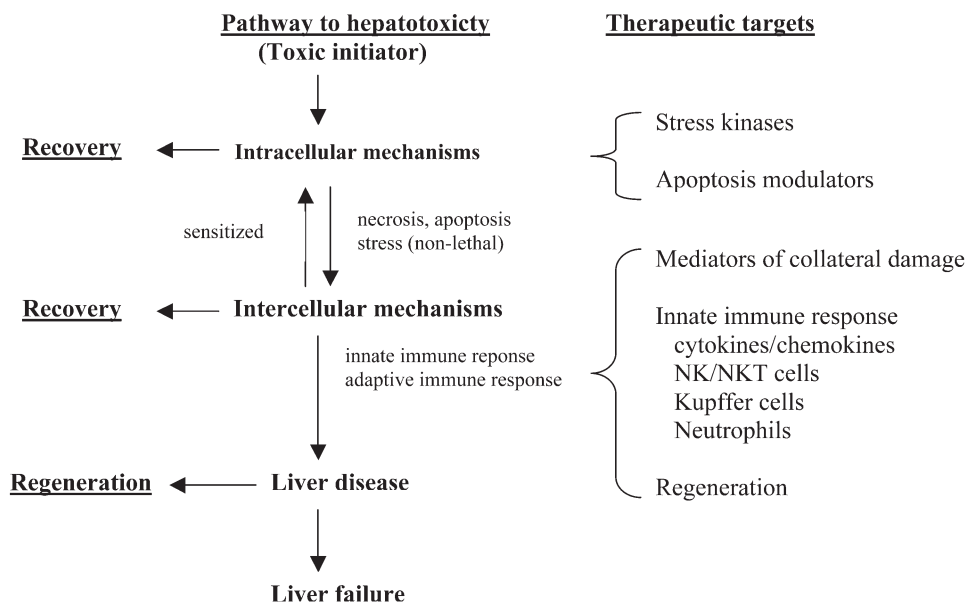


Fig. 3. Overview of pathogenesis and therapeutic targets of drug-induced liver injury.

## THERAPEUTIC IMPLICATIONS

In most cases, there are no specific proven treatments for drug-induced liver disease. The first and most important step is to discontinue the suspected drug and provide standard supportive management. Both patient and physician must be alert to the emergence of the first symptom or sign so that the drug can be stopped in a timely fashion. Specific therapy against drug-induced liver injury is limited to the use of *N*-acetylcysteine in the early phases of acetaminophen toxicity. L-Carnitine is potentially valuable in cases of valproate toxicity.

Remember Hy's law, which states that patients with drug-induced hepatocellular jaundice have 10% mortality or more even if the offending drug is discontinued (3). For those patients, liver transplantation should be considered early because it represents the best chance of survival. A short course of high-dose corticosteroids might be considered for patients with severe acute or chronic drug-induced hepatitis, particularly if associated with severe hypersensitivity syndrome, e.g., dermatological manifestations, or autoimmune features. Steroids may suppress the systemic features associated with hypersensitivity or allergic reactions. Prolonged cholestatic reactions may benefit from empiric treatment with ursodeoxycholic acid (UDCA). Finally, as a preventive strategy, other crossreacting drugs in certain classes, such as phenothiazines, erythromycins, anticonvulsants, halogenated anesthetics, tricyclics, ACE inhibitors, and other, should be avoided.

In the future, better understanding of the immune mechanisms of DILI may provide therapeutic targets at the level of molecular and cellular pathways involved in liver toxicity. The experimental model of APAP-induced hepatotoxicity has revealed important pathophysiological targets for protecting the liver. It is conceivable that these molecular and cellular pathways may also be potential therapeutic targets for idiosyncratic DILI (Fig. 3).

As a preventive strategy, screening analysis of genetic polymorphisms of drug-metabolizing enzymes and genetic profiles of host immune systems might be used to identify at-risk individuals before drug therapy is initiated in order to maximize therapeutic effects while minimizing drug-induced hepatotoxicity. Another promising strategy is the use of metabonomics to predict susceptibility. For example, urinary metabolome is a composite of the phenotypic influence of genetics and environment. Proof of principle has been reported in predicting APAP toxicity in rats (96).

## CONCLUDING REMARKS AND OPEN QUESTIONS

The mechanism of immune-mediated DILI usually involves haptenization of a reactive metabolite coupled with some type of "danger signal" (mild injury, infection, inflammation), which may activate the innate immune system to costimulate an adaptive immune response by inducing an efficient intrahepatic antigen presentation and/or breaking immune tolerogenic environment of the liver (97,98). However, the importance of anti-hapten and specific autoantibodies (e.g., anti-CYP) in pathogenesis and diagnosis is unconvincing. Characterization of the autoantigen-autoantibody repertoire continues to be an attractive and important research field. Recent studies show that drug-specific T cells may play an important role in patients with idiosyncratic drug reactions in the skin (31,99). Drug antigen-specific T cells from these patients are isolated, cloned, and further characterized for their cellular phenotype and functionality (29–31,99). Drug stimulation results in release of cytokines and enhanced cytotoxic activity by drug-specific T cells (29–31). Evidence on the role of T cells in idiosyncratic DILI is lacking. It is important to clarify whether drug-specific T cells exist in both humans and animal models of idiosyncratic DILI and what the characteristics and functions of these cells

are in idiosyncratic DILI. It is also possible that the balance between the different T-cell subsets with different functions, i.e., enhanced helper T cells and impaired regulatory T cells, may be critical in the development of immune-mediated DILI.

Recent evidence clearly demonstrates the important role of the innate immune system in experimental APAP hepatotoxicity (40,41). The balance between the pro- and anti-inflammatory mediators determines the susceptibility and severity of liver injury (11,41). It is conceivable that activation of liver innate immunity and the resultant inflammation may also play an important role in immune-mediated idiosyncratic DILI. A cascade of increasingly efficient intrahepatic antigen presentation might be promoted by inflammation initiated by the innate immune system before the onset of an adaptive immune response to the drug-modified neoantigens. The molecular and cellular mechanisms that lead to activation of NK/NKT cells in experimental APAP hepatotoxicity are not known (51). A recent study suggests that drug metabolites may induce ligand expression on hepatoma cells for activating receptor NKG2D on NK cells, thus rendering these cells susceptible to lysis by NK cells (100). Infiltration of eosinophils is a histological feature of idiosyncratic DILI, and activated NKT cells produce IL-5, a critical cytokine for eosinophil proliferation and activation (101). A recent study indicates that both NKT cell-derived IL-5 and eosinophil infiltration in the liver play critical roles in concanavalin A-induced hepatitis (101). It remains to be determined what causes activation of NK and NKT cells and whether these cells play a role in both initiating innate immune responses and promoting adaptive immunity in immune-mediated idiosyncratic DILI. Clinical studies of the association between the innate immune system and drug hepatotoxicity are needed to determine the applicability of findings in animal models to hepatotoxicity in humans.

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# 29 Acute and Chronic Liver Diseases Induced by Drugs or Xenobiotics

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## KEY POINTS

- Drug-induced liver disease is a major clinical and economical problem. Such drug reactions are associated with high patient morbidity and mortality and are one of the most frequent causes of postmarketing withdrawal.
- A substantial proportion of clinically relevant hepatotoxins acts through idiosyncratic mechanisms, which by definition occur in only a small proportion of individuals exposed to the drug. These idiosyncratic reactions can be further classified as allergic or nonallergic (metabolic).
- Drug-induced hepatotoxicity is generally the result of multiple, discrete processes for both intrinsic and especially idiosyncratic drug toxicity (“multiple determinant hypothesis”).
- Drug metabolism is one of the main contributing determinants to drug-induced liver disease, and the formation of reactive metabolites is frequently involved in mechanisms of liver toxicity.
- Direct and indirect interference with mitochondrial function and integrity is a second, common mechanism in drug-induced liver disease.
- Drug-induced immune-mediated liver disease arises as the result of the formation drug–protein conjugates.
- The intrahepatic immune system is generally associated with inductions of tolerance, and regulatory T cells play a central role in this response.
- The immune response to drug–protein conjugates requires presentation of the immunogen by antigen-presenting cells and additional costimulatory signals. These signals are activated by “danger” signals released as a result of cellular stress or inflammation.
- Inflammation is both a role and a susceptibility factor for drug toxicity and plays a major role in the hepatotoxicity of many drugs.
- The major challenge in this area is to identify the specific and common determinants of severe (idiosyncratic) liver injury that could provide opportunities to design predictive

test strategies identifying toxic properties of drugs and susceptibility factors associated with these reactions.

## INTRODUCTION

The incidence of adverse drug reactions to any given drug may be relatively low, but the total clinical impact of adverse drug reactions is actually substantial because of the number of drugs used and the number of patients treated. It has been estimated that around 7% of patients experience serious adverse drug reactions and that adverse drug reactions are the 4th to 6th leading cause of death (1). Hundreds of drugs and chemicals have been associated with hepatotoxic effects (2–5), and drugs are the most common cause of acute liver failure in the United States and Europe (6,7). In addition, serious drug-induced hepatotoxicity has become one of the most frequent causes of postmarketing withdrawal, labeling changes, and restriction in use of medications (Table 1) (8–10).

Acetaminophen (APAP; paracetamol) poisoning is the leading cause of acute liver failure in the western world (7,8,10), it is marked by prompt onset and is dose dependent. In most other cases of drug-induced liver disease are owing to idiosyncratic hepatotoxicity. Idiosyncratic drug reactions, by definition, occur in a small proportion of patients and are characterized by erratic temporal and dose relationships. They can be broadly classified as allergic and nonallergic reactions. The characteristics of allergic reactions include intermediate latency (days to weeks), eosinophilia, rash, and fever. Antibodies to drug-modified protein and/or nonmodified proteins (autoantibodies) may be detectable in most patients, and hepatotoxicity promptly recurs on rechallenge with the drug. Nonallergic idiosyncratic reactions, conversely, are generally marked by a long latency period (several months) and the consistent absence of markers of hypersensitivity. The response to rechallenge following resolution of liver injury is variable, and hepatotoxicity may not recur following subsequent exposure to the drug.

Idiosyncratic drug reactions are associated with high patient morbidity, and mortality, and they constitute over 18% of acute liver failures in the United States (4,7). These reactions are not detected during preclinical and clinical safety evaluations

**Table 1**  
**Examples of Drugs Associated With Serious Liver Injury**

<b>Drugs withdrawn because of hepatotoxicity</b>
Iproniazid (1959)
Tienilic acid (1979)
Benoxaprofen (1982)
Bromfenac (1998)
Troglitzone (2000)
Nefazodone (2004)
<b>Drugs with significant use limitations owing to hepatotoxicity</b>
Labetalol (1989)
Pemoline (1995)
Felbamate (1997)
Talcapone (1998)
Trovafloxacin (1999)
Bosentan (2001)

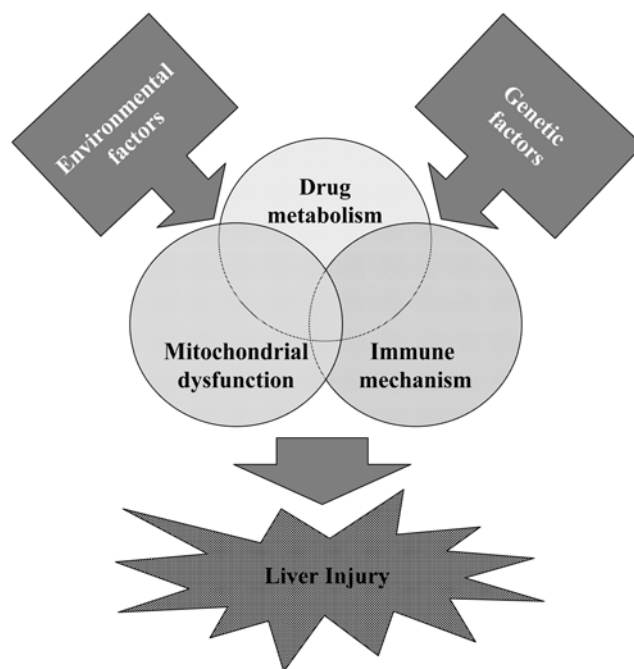
of new drug candidates, and, as a result, are only noticed after the compound has been in clinical use for some time. In addition, idiosyncratic drug reactions are difficult to diagnose and frequently go unrecognized.

The main processes involved in the molecular basis of drug-induced hepatotoxicity are thought to be drug metabolism, immune sensitization, and mitochondrial dysfunction. In addition, there is increasing evidence that environmental factors and especially concurrent inflammation play important contributing roles in the etiology of hepatotoxicity (Fig. 1). For most drugs, metabolic activation to reactive intermediates is necessary step in the generation of idiosyncratic drug reactions (9,11). Some idiosyncratic drug reactions appear to have an immunological etiology (2,3,12–15). They are thought to occur in response to the formation of drug-protein conjugates, which act as immunogens and initiate an immune response against the drug-protein complex or cellular components (3,11,12,14,16–18). The mechanisms of nonallergic idiosyncratic drug reactions are currently poorly understood; a growing body of evidence suggests that direct or secondary mitochondrial dysfunction might be involved (19–21). Concurrent inflammation during drug treatment is also emerging as a determinant of susceptibility to adverse drug effects in the liver (22) and may decrease the threshold for toxicity.

Drug toxicities, especially idiosyncratic drug reactions, are the result of multiple, discrete but necessary processes and depend on pharmacogenetic, immunogenetic, and environmental factors, as discussed in this chapter.

## DRUG METABOLISM

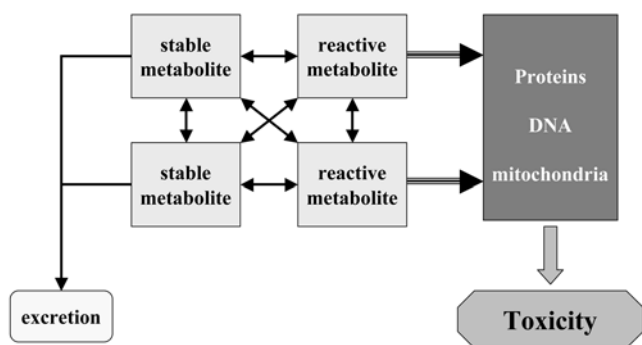
Drug metabolism plays an essential role in the mechanisms involved in drug-induced liver diseases (11,16,18,23–25). Most compounds, including drugs, entering the body are lipophilic and need to undergo biotransformation into more hydrophilic, water-soluble metabolites in order to be cleared efficiently. Drug metabolism will reduce the duration of exposure to the drug and prevent bioaccumulation and therefore can generally be regarded as a protective process.



**Fig. 1.** The “multideterminant hypothesis” for drug toxicity: multiple, discrete processes are required for the development of drug toxicity, and the mechanisms are influenced by genetic and environmental factors (including inflammation).

Biotransformation normally involves an initial activating reaction, converting the parent compound into a slightly more water-soluble metabolite (phase 1 metabolism). The metabolites can then undergo conjugation reactions to generate hydrophilic complexes, which will be readily excreted via the bile and urine (phase 2 metabolism). For most drugs, metabolism involves multiple activation and conjugation reactions and not one single sequential metabolic route. Bioactivation (phase 1 metabolism) may also result in the formation of electrophilic intermediates, which either can be detoxified by cellular protection mechanisms or react with nucleophilic groups on cellular macromolecules. The covalent binding of drug metabolites to cellular components is associated with intrinsic and idiosyncratic toxicity (Fig. 2). Binding to critical proteins in the cell will cause perturbation of the cell homeostasis and can lead to apoptosis or necrosis (15,24,26). Drug-protein adducts can also act as immunogens and trigger an immune response (11,16,18,24,26).

Bioactivation reactions are performed principally by the cytochrome P450 enzymes that are located in the membrane of the endoplasmic reticulum (27). These enzymes are found at the highest level in the liver but are also expressed in other organs like the intestine, kidney, lungs, and skin. There are over 50 known cytochrome P450s in humans, which are categorized into 17 families and 42 subfamilies by their sequence similarities. The members of cytochrome P450 families 1 to 3 are predominantly involved in the metabolism of drugs and



**Fig. 2.** General scheme of drug metabolism in the liver in relation to direct and immune-mediated toxicity.

chemicals, and most compounds are metabolized by one or a few specific forms of cytochrome P450. The expression of cytochrome P450 enzymes is influenced by environmental and genetic factors, and a remarkable interindividual variability in the rate and route of drug metabolism exists. For example, CYP3A4, one of the most important cytochrome P450 isoforms responsible for drug metabolism by humans, has a marked (5–20-fold) interindividual variability as a consequence of both genetic and nongenetic factors (28). Environmental factors, which influence drug metabolism, include general nutrition state and exposure to foreign chemicals including medical products, food additives, environmental chemicals, and life style. Prolonged exposure to chemicals, including drugs can cause an induction of specific cytochrome P450 enzymes. Enzyme induction may increase the formation of reactive intermediates, causing disequilibria between metabolic activation and conjugation rates and therefore predispose to toxicity (11,17,18).

Much of the interindividual variations in drug responses are attributed to genetic differences in drug metabolism. Genetic polymorphisms in drug metabolism arise from the occurrence of variant alleles in the population, which lead to quantitative and qualitative changes in gene expression. Polymorphisms will divide the population into at least two distinct phenotypes, (extensive and poor metabolizers) and have been described for most cytochrome P450 isoenzymes including CYPs 1A1, 1A2, 2A6, 2C9, 2C19, 2D6, 2E1 and 3A4/5 (27,29,30).

Phase 2 biotransformation reactions are normally regarded as detoxification pathways. The enzymes mediating the conjugation reaction, such as UDP-glucuronosyl transferases (UGT), *N*-acetyl transferase, and glutathione-*S*-transferases (GST), are also influenced by genetic and environmental factors. Exposure to drugs and chemicals may cause either inhibition or induction of the enzymes, thereby altering the rate and route of certain metabolic pathways. Several phase 2 enzymes are also polymorphically expressed, and genetic variations have also been linked to predisposition to cancer (29,30).

The role of genetic polymorphisms in the predisposition to drug-induced liver disease has not been firmly established,

but it can be envisaged that variations in metabolic rates and routes can contribute to the susceptibility to drug reactions (16,29,30).

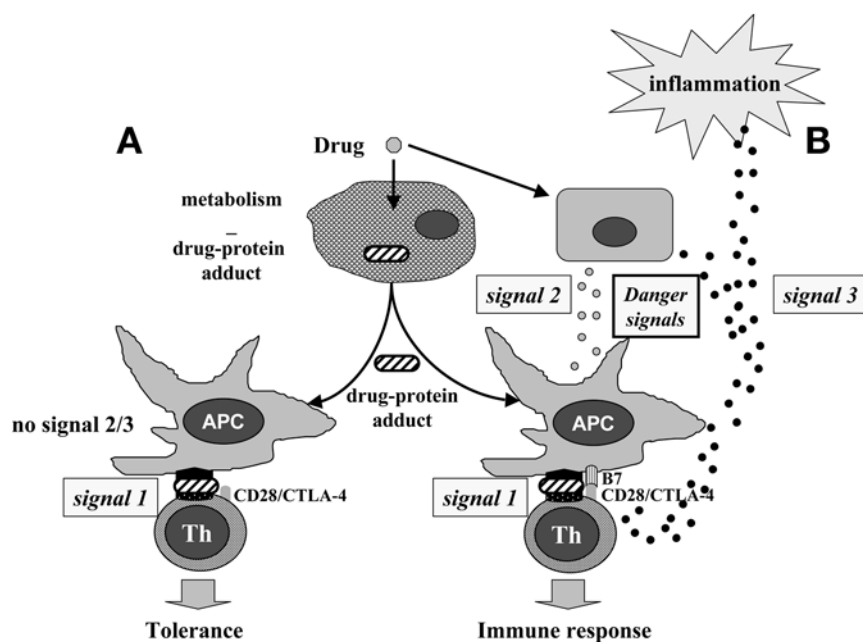
## MITOCHONDRIAL DYSFUNCTION

Mitochondria play a vital role in cell homeostasis; they are involved in fatty acid  $\beta$ -oxidation, tricarboxylic acid cycle, and oxidative phosphorylation, all critical processes involved in cellular energy production. Primary and secondary mitochondrial dysfunction is an important mechanism in drug-induced liver injury (19–21).

Severe impairment of mitochondrial fatty acid  $\beta$ -oxidation causes microvesicular steatosis: the accumulation of tiny lipid vesicles in the cytoplasm. Numerous compounds have been associated with impairment of mitochondrial fatty acid  $\beta$ -oxidation, causing microvesicular steatosis through various mechanisms. Valproic acid and salicylic acid are examples of drugs that sequester intramitochondrial coenzyme A (CoA). In addition, valproic acid also causes depletion of intramitochondrial CoA. Inhibition of  $\beta$ -oxidation enzymes has been demonstrated for numerous drugs such as 2,4-diene- valproyl-CoA (a reactive metabolite of valproic acid), tetracyclines, aryl propionate nonsteroidal anti-inflammatory drugs (e.g., ibuprofen), and tricyclic antidepressants. The antiretroviral dideoxynucleosides, like zidovudine (AZT) and stavudine (d4T), are incorporated in the mitochondrial DNA (mtDNA) by polymerase  $\gamma$  and prevent replication, causing mtDNA depletion, whereas interferon- $\alpha$  (IFN- $\alpha$ ) impairs mtDNA transcription. These metabolic effects disrupt mitochondrial respiration and inhibit of fatty acid  $\beta$ -oxidation (19–21).

Nonalcoholic steatohepatitis (NASH) may progressively develop in patients with chronic steatosis, leading to liver cell death, Mallory bodies, polynuclear cell infiltrates, fibrosis, and cirrhosis. This severe condition can be induced by chronic administration of drugs such as amiodarone, perhexiline, and diethylaminoethoxyhexestrol. These cationic amphiphilic drugs concentrate in mitochondria, causing inhibition of fatty acid  $\beta$ -oxidation and mitochondrial respiration (19–21). Respiration inhibition leads to formation of reactive oxygen species (ROS), causing lipid peroxidation, which in turn can cause cell death. In addition, ROS and lipid peroxidation may enhance the formation and release of cytokines (transforming growth factor- $\beta$  [TGF- $\beta$ ], tumor necrosis factor- $\alpha$  [TNF- $\alpha$ ] and interleukin-8 [IL-8]) which may also contribute to the disease development.

Mitochondria dysfunction may also contribute to cytolytic hepatitis, a severe liver lesion that can cause liver failure. Cytolytic hepatitis is frequently caused by the formation of reactive metabolites but can also be induced by inhibition of fatty acid  $\beta$ -oxidation or mitochondrial respiration (19–21). An addition mechanism is onset of mitochondria permeability transition (MPT) caused by opening of the MPT pores in the mitochondrial inner membrane. Opening of these pores results in mitochondrial depolarization, uncoupling of oxidative phosphorylation, matrix swelling, and outer membrane rupture. The



**Fig. 3.** The “danger hypothesis” in relation to drug-induced hypersensitivity. (A) Antigen presentation in the absence of costimulatory signal (signals 2 and 3) results in tolerance. (B) Antigen presentation in combination with co-stimulatory signals (signals 2 and 3) results in sensitization and immune response. APC, antigen-presenting cell; CTLA-4, cytotoxic T-lymphocyte antigen-4.

consequences of the MPT are the breakdown of mitochondrial membrane potential, inhibition of ATP synthesis, and ultimately necrotic cell death. However, if the insult is more moderate and MPT occurs only in some mitochondria, the unaffected mitochondria will continue to synthesize ATP. Cell death may then occur at the release of cytochrome *c*, which activates caspases promoting apoptosis (an energy-dependent process). MPT can be directly induced by compounds such as lonidamide, atractyloside, and ROS. In addition, extensive reactive metabolite formation may cause DNA damage, overexpression of p53 and bax, glutathione depletion, and increase in cytosolic  $\text{Ca}^{2+}$ . All these events may open the MPT pore, causing breakdown of mitochondrial membrane potential, inhibition of ATP synthesis, and release of cytochrome *c*.

### IMMUNE RESPONSES TO DRUGS

In general, a specific immune response requires two distinct phases: sensitization to an antigen, which involves recognition of a non-self constituent, and the development of memory for subsequent accelerated and amplified responses. Subsequent contact with the antigen results in the development of clinical reactions (12,31,32).

Our current understanding of drug-induced hypersensitivity reactions is based on the hapten hypothesis (11,16,32). For a xenobiotic to be immunogenic, it needs to be large enough to be recognized by the immune system; the molecular weight needs to be at least more than 1000. Most drugs lack intrinsic immunogenicity because of their low molecular weight, but they can act as haptens when bound strongly to larger carrier molecules such as proteins. Most of drugs are not chemically

reactive, to avoid toxicity, but reactive metabolites formed during biotransformation can react with macromolecules to form hapten-carrier complexes that may be immunogenic (11, 12,16,24,26,32).

These adducts need to be processed by local antigen-presenting cells (APCs) (12,24,31,32). In the liver these are Kupffer cells, and they are presented on the cell surface as peptide fragments by major histocompatibility complex (MHC) molecules. Specific T-cell receptors (TCRs) on lymphocytes can interact with the antigen-carrying MHC complexes (signal 1), but additional signals (signal 2 and 3) are required for complete activation of a specific immune response against the peptide fragment presented. In the absence of the costimulatory signals, binding of the MHC molecules to the TCRs will lead to apoptosis of the lymphocyte and tolerance to the antigen (Fig. 3A). A principle component of the second, costimulatory signal (signal 2) consists of the binding of B7 ligands on APCs to the CD28/cytotoxic T-lymphocyte antigen-4 (CTLA-4) receptors on lymphocytes (Fig. 3B). The expression of B7 ligands is tightly regulated, with little expression on nonstimulated APCs, but following activation expression of the B7 ligand is upregulated. It has been postulated that the costimulatory pathway is activated by “danger” signals (pro-inflammatory cytokines such as IL-2, TNF- $\alpha$ , and interferon- $\gamma$  [INF- $\gamma$ ]) released following cellular stress (drug-induced or caused by infection or surgery; Fig. 3B) (31,33,34). In addition, polarizing cytokines released following cellular stress can act directly on T cells, leading to Th1 and/or Th2 immune responses.

Generally, one or more copies of the hapten will be part of the peptide fragment presented on the MHC molecules.

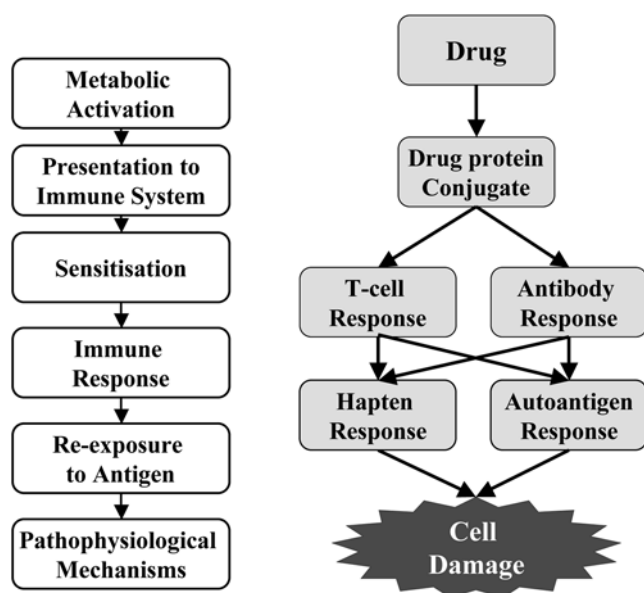


Fig. 4. Mechanism of drug-induced immune-mediated liver injury (hapten hypothesis).

The sensitization of the immune system will produce T and/or B cells with an immunologic memory toward these epitopes. The immune response can be against the drug (metabolite; haptenic epitope), against the carrier molecule (autoantigenic epitope), or against a new epitope formed by the drug-carrier complex (neoantigenic epitope); (Fig. 4). In some cases, the immune response will be predominantly against one epitope, whereas with other drugs the immune response will be against multiple epitopes, as discussed in the selected examples. The nature of the carrier, the nature of the hapten, and the hapten, density can have major influences on both immunogenicity and the immune response (35).

Following sensitization, subsequent contact of the memory cells with the antigen will trigger the production of antibodies by B-lymphocytes and activation of cytotoxic T-lymphocytes (CTLs), macrophages, and natural killer cells. These effector cells and antibodies are involved in the pathophysiological mechanisms leading to cellular and tissue damage (Fig. 4). Several antibody- and cell-mediated immune mechanisms can be subsequently or simultaneously involved in the clinical manifestation of drug hypersensitivity reactions.

The formation of drug-protein adduct is generally regarded as one of the prerequisite steps in drug-induced immune-mediated reactions. Conversely, adduct formation *per se* does not lead to an immune response. For example, high doses of the direct-acting hepatotoxin acetaminophen (APAP) causes the formation of the electrophilic metabolite *N*-acetyl-*p*-benzoquinoneimine (NAPQI), and to his reactive intermediate binds to various cellular macromolecules, which could act as antigens (signal 1). NAPQI also induces extensive hepatic necrosis, which should provide signals 2 and 3 that initiate an immune response according to the danger hypothesis. However, the mechanisms of APAP-induced injury have been extensively investigated

and there are no indications for the involvement of acquired immune effector mechanisms. Conceivably, cell injury may be so extensive that it prevents induction of immune response (8,34).

The formation of drug-protein adducts has also been well established for a number of drugs associated with immune-mediated hepatotoxicity, including halothane, tienilic acid, phenytoin, and carbamazepine. The adducts implicated in the immune responses can be reproducibly generated in animal models. Furthermore, there are indications that adduct formation is not restricted to patients with drug-induced liver injury but occur in all patients exposed to these drugs, with immune-mediated liver disease being rare. Thus drug exposure and antigen formation may occur without immune response.

### IMMUNOREGULATION

There is increasing evidence in the literature to support the possibility that the same mechanisms employed for regulation of humoral and cellular responses to “normal” antigens may also be broadly applicable to the regulation of immune responses to drug-altered antigens (22,36). Regulatory T cells are seen as central players in immune regulation, through the induction and maintenance of tolerance to antigens (37–44). Although several T-cell subpopulations exhibit immune-suppressive activity, two broad categories of regulatory/suppressor cells are currently recognized. These include “naturally arising” T-regulatory cells (T-regs) and inducible T-regs. “Naturally arising” T-reg lymphocytes are a mature and functionally distinct population of CD4<sup>+</sup> T cells produced in the thymus (39,41,45). These cells can be recognized by their constitutive cell surface expression of the CD25 molecule (i.e., the IL-2 receptor  $\alpha$ -chain) and also by their specific expression of *Foxp3*. In contrast to CD25, which is transiently upregulated on all activated CD4<sup>+</sup> and CD8<sup>+</sup> T cells, *Foxp3* expression is a more specific marker for this cell lineage and appears to be essential for their development and function (41). In addition to these “naturally arising” T-regs, naïve T cells in the periphery are induced to become T-regs under certain conditions of antigen encounter. These T cells can be either CD25<sup>+</sup> or CD25<sup>-</sup>. It is thought that low-affinity antigen encounters, for instance with tolerogenic or immature dendritic cells (DCs), or impaired TCR signaling favor the development of these cells (39).

Certain antigen exposure routes, including intranasal or oral administration, also appear to select preferentially for the development of T-regs. Inhibitory Fc $\gamma$ RIIB receptors on DCs were recently shown to have an important role in tolerance induced via these routes of exposure (46). These receptors recognize the Fc portion of IgG molecules and provide inhibitory signaling through immunoreceptor tyrosine-based inhibitory motifs (ITIMs) (47,48). Antigen exposure via the portal vein has also long been recognized for its tolerogenic qualities (49). Another critical tolerogenic factor is the local cytokine environment. The suppressive cytokines TGF- $\beta$  and IL-10 have both been shown to support the development of tolerant and tolerogenic cells (50–52). For instance, TGF- $\beta$  reportedly impairs T-cell activation by raising the T-cell activation threshold through its effects on the Ca<sup>2+</sup> calcineurin pathway (51,52).

Factors involved in the induction of T-regs are also inherently related to T-reg functions in the induction and maintenance of tolerance. T-regs mediate their effects through the production of cytokines such as IL-10 and TGF- $\beta$ , or through cell-cell contact using negative costimulatory molecules such as CTLA-4 (38,50,53–55). This can suppress the development of adaptive humoral and cellular responses (1) by interfering with the development and function of APCs, (2) by reducing the production of positive costimulatory signals, and (3) by increasing negative costimulation. In addition to their suppressive effects on lymphoid cells, cytokines produced by regulatory cells can also inhibit the recruitment, development, activation, and/or effector functions of many other cell types (54–56). These include cells of the innate immune system, which are significant mediators of inflammation and tissue damage (57). IL-10 is also well known for its ability to suppress TNF- $\alpha$  production (55). TNF- $\alpha$  plays a critical role in inflammatory processes and is currently a major therapeutic target (58).

The apparent lack of immunogenicity of numerous drugs and other low-molecular-weight chemicals (LMWCs) may in many cases be owing to immune ignorance. However, it is clear that immune tolerance to LMWCs can also be mediated in an active and “drug-specific” manner. Because of the suppressive nature of these effects, they have often been overlooked in studies primarily focused on hypersensitivity. Recent studies involving nickel, a model hapten, have shown that T-regs can silently maintain immune tolerance to haptens in nonallergic individuals (36,59,60). T-reg activity has also been associated with immune tolerance to other LMWCs including drugs such as penicillamine, procainamide, and gold sodium thiomalate (36,61–63). Although in some instances cell-cell contact is required for the suppressive effects of LMWC-induced T-regs, the regulatory cytokines IL-10 or TGF- $\beta$  are commonly implicated in the suppressive effects of these cells (36,61). Animal studies involving the nonsteroidal anti-inflammatory drug (NSAID) diclofenac also suggest a role for T-regs in mediating immune tolerance to this drug (64,65). Interestingly, studies in humans have shown that increased polymorphisms in the IL-10-encoding gene and the subsequent decrease in IL-10 transcription are associated with diclofenac hepatotoxicity (66). It has even been proposed that the anti-inflammatory potential of drug-induced regulatory cells may contribute to the beneficial effects of some immunogenic NSAIDs (67). However, as immunosuppressive and anti-inflammatory agents are capable of pharmacologically inducing tolerogenic DCs and T-regs by interfering with DC maturation and inhibiting the upregulation of costimulatory molecules, the exact roles of “drug-specific” T-regs in regulating drug tolerance require further clarification (68).

The liver has long been recognized for its particularly tolerogenic properties (69). Recent evidence suggests an important role for T-regs in this phenomenon that is as still, poorly defined (70). Other T-cell populations are present in the liver and may also contribute significantly to liver tolerogenesis. These include the natural killer (NK) and NKT cells, which are present at exceptionally high frequencies in the liver (71). Although NK cells are important in T-cell recruitment, they

may contribute toward the maintenance of tolerance through their cytotoxic activity. NKT cells have a positive role in host defense but have also been shown to have an important protective effect by regulating autoimmunity in nonobese diabetic mice (72,73). The liver is the site of massive apoptosis following T-cell activation in secondary lymphoid tissue and may contribute toward general systemic tolerance by removing activated T cells (71,74).

The liver also possesses several populations of APCs that appear somewhat skewed toward the production of tolerance. For instance, resident hepatic DCs are largely of an immature phenotype, have limited antigen-presenting and costimulatory capacity, and may therefore be inherently more tolerogenic than mature DCs, found in other tissues (75). A study using mouse hepatic DCs has shown that these cells preferentially induce the synthesis of IL-10 by CD4<sup>+</sup> T cells, whereas bone marrow-derived DCs favor the induction of the Th1 cytokine IFN- $\gamma$  (76). Recent data also indicate that intrahepatic APCs may be hyporesponsive to stimulation via certain Toll-like receptor (TLR) ligands such as lipopolysaccharide (LPS) and may instead respond to endotoxin by production of IL-10 (77–79).

Other hepatic APCs include liver sinusoidal endothelial cells (LSECs) and Kupffer cells. Both cell types constitutively express IL-10 and TGF- $\beta$  and upregulate these cytokines following stress. Recent studies have identified tolerogenic roles for these cells in limiting T-cell responses to antigens and LMWCs and indicate that these cells may have additional important regulatory functions in antibody responses to drug-protein adducts, but this has yet to be clearly demonstrated (80–83). Hepatocytes and liver lipocytes have also been shown to produce and respond to regulatory cytokines and may therefore be significant contributors a tolerogenic liver micro-environment (75). A recent study in IL-10 transgenic mice indicates that liver-derived IL-10 can also play an important role in the induction of oral tolerance to antigens (84).

Evidence to date suggests an important role for the liver in regulation of tolerance to antigens and LMWC. However, the regulatory contributions of individual hepatic cell types in controlling immune-mediated adverse drug reactions both within the liver and at the systemic level require further investigation.

## INFLAMMATION AND DRUG-INDUCED LIVER INJURY

In addition to the established susceptibility factors for drug-induced liver injury, concurrent inflammation during drug treatment is also emerging as a determinant of susceptibility to adverse drug effects in the liver. Inflammation is one of the manifestations of the host response to infection or physical injury. Excessive or uncontrolled inflammation results in or is a component of a variety of diseases that include some highly prevalent conditions such as allergy and asthma, various types of arthritis, and a multitude of autoimmune conditions such as multiple sclerosis and systemic lupus erythematosus. Whether the presence of inflammation is owing to a chronic inflammatory condition, an infection, or an injury, the likelihood



of the concurrence of drug treatment with an inflammatory episode in an individual could potentially be quite high.

Inflammation is a multifaceted process involving activation of cells and release of mediators important for host defense but also potentially injurious to tissues if not tightly controlled. The inflammatory cascade comprises multiple cell types such as neutrophils, macrophages, lymphocytes, endothelial cells, parenchymal cells, and the mediators they release such as cytokines and arachidonic acid metabolites. Depending on the nature of the inflammatory stimulus, the cells and the receptors that trigger the inflammatory response following infection or injury may vary. Members of the TLR family play a critical role in detection of microbes and viruses. In particular, TLR4 has been identified as the mammalian endotoxin sensor (85).

The inflammatory response to LPS, also known as endotoxin, can result in organ damage and in certain circumstances multiple organ failure and death. The liver plays an important physiological role in LPS detoxification and clearance of endotoxin derived from the intestine. The hepatic response to large doses of LPS is well characterized in many rodent models of endotoxemia and shock. LPS administration leads to endothelial cell activation and the accumulation of neutrophils and platelets in the liver (80,86). In addition, transcription factors are activated, and pro-inflammatory mediators are synthesized and released. These mediators include cytokines (e.g., TNF- $\alpha$ , IL-1, various chemokines), cyclooxygenase-2 products and other lipid mediators (e.g., prostaglandins), ROS, and toxic proteases (85,87). The role that inflammatory mediators play in drug-induced hepatotoxicity will be discussed in this section using a few examples.

APAP-induced liver injury may not be solely attributable to direct cytotoxic effects. Recent studies point to a role for various cytokines and nitric oxide (NO) in the production of tissue injury following APAP ingestion at high doses. Inflammatory stimuli, such as LPS and inflammatory cytokines, can induce inducible NO synthase (iNOS) in a variety of cells including macrophages and neutrophils. Induction of iNOS leads to high levels of NO, resulting in damage to endothelial, neuronal, and epithelial cells (88). Overproduction of NO in the liver plays an important role in various models of hepatic inflammation and injury (89,90). In the case of APAP hepatotoxicity, NO has been implicated as playing a role. Oral ONO-1714, an iNOS inhibitor, protects against APAP-induced hepatic inflammation/injury, suggesting that NO produced by iNOS plays a key role in the pathogenesis of this drug-induced hepatotoxicity (91). In contrast, low, physiological levels of NO in the liver have the opposite effect, resulting in significant protection of the liver from APAP-induced damage (92). NO appears to produce these beneficial actions through several mechanisms, including the suppression of IFN- $\gamma$ , TNF- $\alpha$ , Fas/Fas ligand and iNOS mRNA accumulation caused by APAP.

Studies suggest that exposure to bacterial endotoxin and the products induced by LPS are major contributing factors in chronic ethanol-induced liver injury. LPS blood concentrations are increased in humans and rats following chronic ethanol consumption (93,94). Excessive alcohol consumption is thought

to lead to bacterial overgrowth in the gut, leading to excessive generation of endotoxin and/or increased intestinal permeability, allowing bacteria access to the portal circulation (95). Exposure of Kupffer cells to LPS in the portal circulation leads to Kupffer cell activation, with the production of many inflammatory mediators implicated in the progression of this disease such as TNF- $\alpha$  and ROS (96).

A large body of evidence supports the involvement of Kupffer cells in the early pathogenesis of alcohol-induced liver injury. For example, Kupffer cell depletion using gadolinium chloride significantly blunted increases in serum transaminase levels, fatty changes, inflammation, and necrosis caused by chronic ethanol (97). In addition, destruction of Kupffer cells blocks formation of ethanol-derived free radicals (oxidants) after chronic enteral ethanol treatment, implicating these cells as potential sources of damaging oxidants (97).

Serum concentrations of TNF- $\alpha$  and several of the TNF- $\alpha$ -inducible cytokines such as IL-1, IL-6, and IL-8 are elevated in patients with alcoholic steatohepatitis and in some cases are associated with greater disease severity and reduced long-term survival (98–102). Animal studies also support the involvement of LPS and the products induced by LPS in the pathogenesis. Antibiotic or probiotic treatment to reduce endotoxemia or treatment with TNF- $\alpha$ -neutralizing antibodies inhibits alcohol-induced liver injury in the rat (103–105). Not only are there increased pro-inflammatory cytokines in alcoholic liver disease, there are also reduced protective anti-inflammatory cytokines such as IL-10. Monocytes from alcoholic cirrhotics produce significantly less LPS-stimulated IL-10 than control monocytes (106). IL-10 knockout mice are markedly more susceptible to ethanol hepatotoxicity and exhibit increased levels of pro-inflammatory cytokines such as TNF- $\alpha$  (107). Collectively, all these studies highlight the importance of inflammation and of the inflammatory cascade triggered by LPS in chronic ethanol-induced liver injury.

The cause of idiosyncratic hepatotoxicity is not known. Conventional wisdom suggests that metabolic mechanisms are involving important, for example, polymorphisms in drug-metabolizing enzymes or the formation of specific immune responses to drug haptens. However, recent research suggests that environmental factors such as concurrent inflammation initiated by bacterial LPS can increase an individual's susceptibility to these reactions. Inflammation induced by LPS enhances the hepatotoxicity of several xenobiotics in rats such as monocrotaline, aflatoxin B1, halothane, cocaine, and chlorpromazine (108–112). These observations spurred attempts to develop animal models of idiosyncratic drug reactions. The case of the histamine-2 receptor antagonist ranitidine provides a good example of such a model. A small percentage of people taking ranitidine develop idiosyncratic liver injury (113). Luyendyk and co-workers observed idiosyncrasy-like liver toxicity in rats exposed to LPS and ranitidine but not in rats treated with either ranitidine or LPS alone (114).

An underlying theme in the role of inflammation and drug-induced liver injury appears to be the omnipotent LPS and the inflammatory cascade it induces. Thus, important risk

factors may be genetic polymorphisms in inflammatory genes, e.g., genes that encode for or control the expression of cytokines, ROS, lipid mediators, adhesion molecules, TLRs, and others. These genes exert control over the magnitude of a pro-inflammatory response. There are many examples of such polymorphisms identified in humans. Schwartz and co-workers showed that polymorphisms in the TLR4 gene exist in humans and control the pulmonary response to inhaled LPS (87). Patients with diclofenac hepatotoxicity and patients susceptible to advanced alcohol-induced liver injury have polymorphisms in the gene encoding the anti-inflammatory cytokine IL-10 (66,115). Polymorphisms in the TNF- $\alpha$  gene have been identified in humans and can affect the magnitude of response to an inflammatory stimulus (116).

Thus there is strong evidence that concurrent inflammation plays a major role in the hepatotoxicity of many drugs. A modest inflammatory response may enhance tissue sensitivity to chemicals, decrease the threshold for toxicity, and render an individual more susceptible to an adverse drug reaction, which otherwise might not occur.

## SELECTED EXAMPLES OF DRUGS ASSOCIATED WITH LIVER INJURY

### ACUTE DOSE RELATED HEPATOTOXICITY

APAP (paracetamol) overdose is the single most common cause of acute hepatotoxicity in both the United States and Europe, with more than one-third of all cases of acute liver injury attributed to APAP (2,6,7,10,117). In nearly all cases, APAP-induced liver injury occurs following either acute (intentionally) or subacute (unintentionally) exposure to super-therapeutic doses of the drug (117).

The contribution of metabolic activation to APAP-induced liver injury is well established. At therapeutic doses, APAP undergoes primarily direct conjugation reactions with glucuronic acid and sulfate. A minor fraction of the drug is metabolically activated by cytochrome P450 to the electrophilic metabolite NAPQI. This reactive intermediate reacts with glutathione (GSH) spontaneously or mediated by glutathione-S-transferases to form a GSH adduct, which is mainly excreted in the bile. When taken in overdose, the principal metabolic pathways (glucuronidation and sulphation) become saturated, and an increased portion of the drug dose is bioactivated by cytochrome P450. The initial result of the increased levels of NAPQI is the depletion of hepatocellular GSH in both the cytosolic and mitochondrial compartments. Once the cellular GSH stores are depleted, NAPQI will react with cellular proteins.

The covalent binding of NAPQI to cellular proteins was originally thought to be the cause of cellular necrosis and organ damage (118–121). Recent studies have indicated that APAP-induced liver injury is not due to general binding to cellular proteins but rather occurs as a result of interaction with specific targets, especially mitochondrial proteins. These initial events lead to disturbances of the cellular Ca<sup>2+</sup> homeostasis, mitochondrial oxidative stress, and peroxynitrite formation (122). This in turn causes induction of the MPT and collapse of the mitochondrial membrane potential (122). This is further

accompanied by the release of endonucleases, which cause DNA fragmentation, and intracellular proteases, which can proteolytically cleave structural proteins. These combined effects result in oncotic necrotic cell death. In addition, recent findings have implicated the innate immune system as an important modulator in the progression and severity of AAP-induced liver injury (*see Inflammation and Drug Induced Liver Injury* and ref. 8).

### NONALLERGIC IDIOSYNCRATIC REACTIONS

Troglitazone (TGZ), a peroxisome proliferator-activated receptor (PPAR)- $\gamma$  agonist, was approved for the treatment of type 2 diabetes in the United States in March 1997. Later that year it was launched in Europe, only to be withdrawn within 6 wk because it was associated with the development of acute liver failure. By November 1997, there were 135 cases of serious hepatotoxicity and six deaths. In the United States, TGZ remained on the market until March 2000, by which time 94 cases of TGZ-induced liver failure had been reported (123). In 2000, TGZ was the second most common cause of drug-induced liver injury with fatal outcome, after APAP, reported to the World Health Organization Collaborating Center for International Drug Monitoring (10). During this period approx 2 million patients were treated with the drug, and the incidence of TGZ-induced acute liver failure has been estimated at between 1/8000 and 1/20,000 patients treated. TGZ-induced liver damage is most commonly associated with characteristic hepatocellular injury, with rare instances of either mixed hepatocellular/cholestatic injury or predominant cholestatic reaction. TGZ is regarded as an idiosyncratic hepatotoxin, but the exact mechanisms of TGZ-induced liver injury remain highly controversial and poorly understood. Various potential mechanisms, largely based on *in vitro* investigations, have been proposed to contribute to TGZ hepatotoxicity (reviewed in ref. 124 and 125).

TGZ undergoes rapid metabolism in the liver by a number of pathways and the predominant metabolite is the TGZ-sulfate (metabolite 1 [M1]) mediated by a specific isoform sulfotransferase (ST1A3). Other metabolic reactions include conjugation to a glucuronide metabolite (M2) and oxidation to a quinone (M3). The latter reaction has been shown to be mediated by two specific isoforms of cytochrome P450, namely, P4503A4 and P4502A8, and it is likely that in humans P4503A4 is mainly responsible for this reaction. M3 can undergo further metabolism to TGZ hydroquinone, probably formed by a NAD(P)H quinone oxidoreductase 1 (NQO1)-mediated reduction, and to TGZ quinone epoxide as a result of further oxidation by cytochrome P450. In general, quinone-metabolites are regarded as reactive metabolites (126,127), which directly or following further metabolism can elicit critical cellular damage through GSH depletion, redox cycling, and binding to cellular proteins. In addition, TGZ can also undergo cytochrome P4503A4 oxidation, leading to ring opening and formation of reactive electrophilic intermediates. TGZ quinone (M3), which has a chemical structure similar to that of vitamin E, is relatively stable and has not been found to be

toxic in at doses up to 320-fold over the therapeutic TGZ dose. The M3-derived reactive metabolites bind covalently with GSH and proteins and could conceivably contribute to TGZ hepatotoxicity.

Direct cytotoxic effects of TGZ have been reported in a number of (mainly) *in vitro* studies using hepatocytes from numerous species. Mitochondrial dysfunction has been proposed as the mechanism for the direct toxicity of TGZ (124). TGZ has been shown to produce rapid decline in MTP followed by ATP depletion, increased plasma membrane permeability, and increased cytosolic  $\text{Ca}^{2+}$ . All these effects may lead to either apoptosis or necrosis depending on the cellular ATP status (*see* Mitochondrial Dysfunction). In addition, some studies have implicated TGZ-sulfate in cellular damage (124). It has been suggested that both TGZ and TGZ-sulfate might contribute indirectly to the development of liver injury by inhibiting bile salt excretion. Accumulation of bile salts in liver cells has been shown to cause mitochondrial dysfunction and apoptosis owing to their intrinsic detergent properties. The predominant pathway in bile acid-induced apoptosis appears to involve the Fas receptor (one of the major death receptors). Bile salts promote Fas aggregation at the cell surface, triggering the caspase cascade and apoptosis. It has been argued that the results of these studies have limited clinical correlation, owing to the interspecies variation and the experimental models used (125).

The contribution of host factors has been investigated in TGZ patients, and several genetic polymorphisms and acquired susceptibilities have been associated with TZG-induced liver injury including CYP3A, CYP1A1, NQO1, GLUT-1, PPAR- $\gamma$ , Bsep, and/or GST (125). The mechanisms by which TGZ induces hepatotoxicity remain unidentified but metabolic idiosyncrasy can be considered the main factor.

#### ALLERGIC IDIOSYNCRATIC REACTIONS

Halothane is a volatile anesthetic agent that has been widely used since its introduction in 1956. The drug has been associated with two distinct types of liver injury (128,129). Mild and transient damage is observed in around 25% of patients, and severe hepatic necrosis occurs in a very small proportion of treated patients (1/3000–1/30,000). The clinical features of the severe form of halothane-induced liver damage suggest an immune-mediated reaction (16,23,130,131). The interval between anesthesia and onset of symptoms is commonly between 6 and 12 dy, and signs of hypersensitivity are frequently observed including fever, rash, eosinophilia, circulating immune complexes, and autoantibodies. Histological examination reveals mainly centrilobular necrosis with an inflammatory infiltrate. Liver damage either resolves over time or can develop into fulminant hepatitis, which can be fatal. Most of patients have been previously exposed to halothane, and reexposure results in a prompt recurrence of the disease with shortening of the latency period and increased severity of symptoms (131,132). Reported susceptibility factors include female sex, middle age, and obesity (131,132).

Sera from most of patients with halothane hepatitis (70%) contain antibodies that react with specific proteins in liver

samples from halothane-treated animals and humans. The antibodies are specific for patients with halothane-induced liver injury and are not observed in normal individuals, patients with liver disease other than halothane hepatitis, or patients who received multiple halothane exposures without developing hepatic damage (133–136). The nature of the halothane-induced antigens has been extensively investigated using immunochemical techniques. The halothane-induced antigens, which are recognized by patient sera, are concentrated in the endoplasmic reticulum (133,137,138).

The formation of these antigens is dependent on cytochrome P450-mediated metabolism. Halothane is metabolized in the liver via two distinct pathways. The reductive metabolic pathway leads to the formation of free radical metabolites that can trigger lipid peroxidation and direct cellular toxicity. The oxidative pathway, which is mediated by cytochrome P4502E1 (CYP2E1), predominates under normal conditions and leads to the formation of the reactive trifluoroacetyl chloride (TFA) intermediate. This metabolite can bind covalently to cellular macromolecules to form drug-protein adducts, which can act as immunogens. An antiserum specific for the TFA group has been used to detect the hepatic drug-protein adducts formed following halothane exposure. The antiserum reacted strongly with several microsomal proteins in liver samples from halothane-treated animals, and the reactive metabolites target a distinct set of proteins in the endoplasmic reticulum (16,130,134,136,137,139–141). The antigens recognized by patients' antibodies correspond to these major TFA modified proteins. The protein targets have been identified, and they are all abundant hepatic proteins resident in the endoplasmic reticulum. They include CYP2E1, the primary enzyme responsible for the metabolic activation of halothane (138,42–144) and several peripheral membrane proteins that are resident in the lumen of the endoplasmic reticulum (16,135,137,145,146). These latter proteins are not associated with the metabolic activation of halothane.

In addition, a proportion of patients express antibodies against a distinct group of halothane-induced antigens that are not detectable by Western blotting analysis (135). These antigens are integral microsomal membrane proteins with epitopes, which appear to be conformational. The formation of halothane-induced liver antigens is not restricted to patients with halothane hepatitis and appears to be expressed in all individuals exposed to halothane (147). However, an immune response to halothane-induced antigens is only observed in the small proportion of patients who develop halothane hepatitis.

Patients with halothane hepatitis develop an immune response that not only is directed against TFA-modified proteins but also expresses autoantibodies to the native unmodified carriers (138,142,146,148,149). These autoantibodies are generally difficult to detect with nonhuman liver samples, but they can be recognized by using human liver samples or purified human liver proteins for analysis.

The pattern of (auto)antigen recognition, based on immunoblotting analysis, varies considerably between patients, with some sera containing antibodies against most antigens, some

recognizing only one or two antigens, and some not reacting with any of the antigens. Some of the antigens can become translocated from the endoplasmic reticulum to the plasma membrane, as has been shown by antibody-dependent cell-mediated cytotoxicity experiments (130,150–153). Furthermore, the major (auto)antigen associated with halothane hepatitis, cytochrome P4502E1, is expressed on the plasma membrane of hepatocytes (138,142,143,154). However, the role of halothane-induced (auto)antibodies in the immunopathology has not been firmly established.

## CONCLUDING REMARKS AND OPEN QUESTIONS

Drug-induced liver disease is a significant problem in clinical practice, public health, and drug development. Toxicity is generally the result of multiple, discrete processes for both intrinsic and especially idiosyncratic drug toxicity (“multiple determinant hypothesis”). Even in the case of the intrinsic hepatotoxin APAP, multiple determinants (biotransformation, mitochondrial dysfunction, and inflammation) contribute to the mechanisms of liver injury and susceptibility of disease development. Idiosyncratic drug reactions occur in only a small number of patients, with no apparent dose relationship, and by definition cannot be predicted by preclinical studies. Routine animal toxicity studies are currently not very accurate in identifying the risk of subsequent problems in clinical drug development and general drug use. Predisposition to drug-induced liver disease is multifactorial and depends on pharmacogenetic, immunogenetic, and environmental factors. This hampers the development of predictive animal models for preclinical safety assessments. The major challenge we face is to identify the factors contributing to the development of organ damage in susceptible individuals.

Understanding the mechanisms of toxicity is essential to the design of new testing strategies. Investigation of the underlying mechanisms of drug-induced liver injury requires the availability of patient material to researchers studying these reactions. Such samples could be used for investigations of the metabolism, immune responses, and molecular/genetic basis for the different responses to hepatotoxins. Investigations of samples from patients who have suffered severe hepatotoxicity reactions to a drug are particularly important because these patients have the specific characteristics associated with severe injury. Technological developments in the wake of the genomic revolution now provide opportunities to detect genetic variations commonly observed in patients and to assist in identifying susceptibility factors.

During preclinical studies, detailed investigations of drug metabolism are required, with an emphasis on the detection of chemical reactive intermediates and the formation of drug-protein conjugates. The use of *in vitro* models expressing specific drug-metabolizing enzymes should be increased for the identification of specific metabolic pathways and the possible involvement of polymorphic routes of metabolism. In addition, screening for GSH depletion, gene induction responses, and mitochondrial dysfunction at an early stage of safety evaluation will provide warning signs for potential hepatotoxic effects.

The further development and validation of more integrated models for the investigation of multifactorial hepatotoxicity should be encouraged.

Detection of idiosyncratic drug reaction depends heavily on postmarketing surveillance, and spontaneous reporting schemes form the cornerstone of postmarketing drug safety surveillance in most countries. One of the main problems with spontaneous reporting is that less than 10% of all serious adverse drug reactions are reported (15,155). As a result, the true incidence of adverse drug reactions is underestimated. Furthermore, the underreporting cause a lack of awareness about the association between a drug and liver injury, which in turn results in under-recognition of the reactions. The regulatory agencies should encourage the reporting of adverse drug reactions regularly. Spontaneous monitoring should be supplemented by the systematic monitoring of cohorts of users of new drugs, using record-linkage to track their subsequent health.

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# TRANSPLANTATION

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**VII**

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# 30 Clinical Use of Immunosuppressive Drugs to Control the Immune Response

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JOHN M. VIERLING

## KEY POINTS

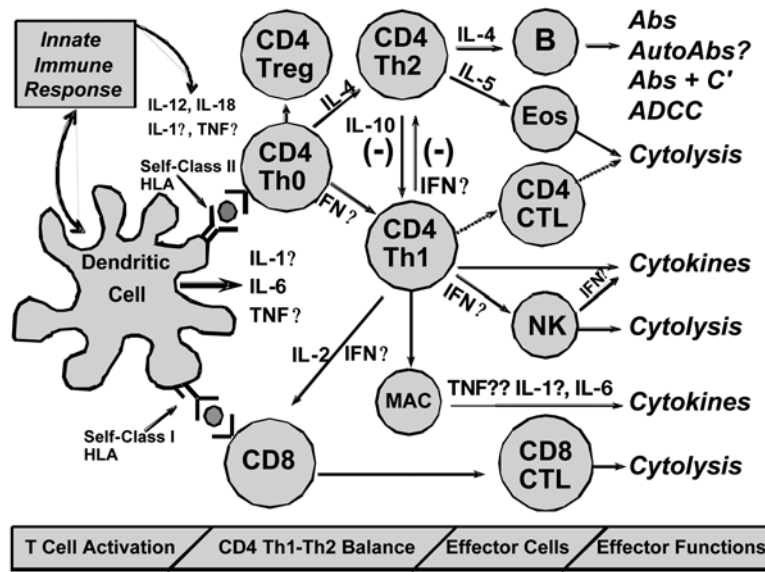
- Both innate and adaptive immunity involve receptor-mediated activation and subsequent sequential events for optimal effector responses and generation of tissue and organ injury.
- Multiple immunosuppressive and anti-inflammatory drugs have been developed that inhibit distinct pathways involved in either innate or adaptive immunity.
- Immunomodulation of innate immunity is currently focused on the development of antagonist or agonist ligands for extracellular and intracellular pattern-recognition receptors and blocking of intracellular signaling pathways.
- Several innate immune antagonist or agonist ligands for Toll-like receptors are now in clinical trials to improve the efficacy of hepatitis B virus vaccines and treat hepatobiliary diseases.
- Immunosuppression and immunomodulation of adaptive immunity can now target T-cell activation, costimulation, clonal proliferation, differentiation and maturation of effector functions, egress from lymph nodes, and transendothelial cell migration into tissues.
- The availability of a variety of immunosuppressive and anti-inflammatory agents provides novel opportunities to suppress concurrently multiple sites involved in the immunopathogenesis of not only allograft rejection but also autoimmune and immune-mediated inflammatory diseases (IMIDs) of the liver and bile ducts.
- Despite concern that established immunological diseases may not be susceptible to immunosuppressive therapies that target activation phases of the adaptive immune response, evidence exists that such drugs may be efficacious in several liver diseases.
- Concurrent immunosuppression of distinct immunopathogenetic processes has reduced the incidence of allograft rejection and the dose-dependent toxicities of individual drugs, indicating the potential applicability of combination therapy in nontransplant hepatobiliary diseases.
- Understanding the mechanisms of action of available and experimental immunosuppressive and anti-inflammatory agents is a prerequisite for the rational design of future clinical trials to prevent hepatic allograft rejection and treat autoimmune and IMIDs of the liver.

## INTRODUCTION

It is increasingly clear that, regardless of etiology, inflammatory and immunological mechanisms are involved in the pathogenesis of virtually all hepatobiliary diseases and hepatic fibrogenesis. Studies of immunopathogenetic mechanisms have identified multiple therapeutic targets in both the innate and adaptive immune responses that portend the future ability to prevent hepatic allograft rejection and control progression of chronic viral hepatitis, alcoholic and nonalcoholic fatty liver disease, drug-induced hepatotoxicity, graft-versus-host disease (GVHD), and autoimmune and immune-mediated inflammatory diseases (IMIDs) of the liver. The current availability of multiple immunosuppressive and anti-inflammatory drugs with distinct, complementary sites of action provides the opportunity and impetus to study their therapeutic potentials in both transplant and nontransplant settings. Concurrent immunosuppression of several specific sites involved in immunopathogenesis may ultimately enhance efficacy, while minimizing the dose-dependent toxicities of individual drugs. Thus, the goals of this chapter are to review the mechanism(s) of action of established and new immunosuppressive and anti-inflammatory agents and to discuss their current and future therapeutic potentials in the prevention of hepatic allograft rejection and nontransplant hepatobiliary diseases. It is important to emphasize, however, that the safety and efficacy of immunosuppressive drugs for autoimmune and immune-mediated inflammatory liver diseases must be studied in appropriately powered, randomized, controlled trials (RCTs) due to the risks of toxicities, malignancies, and potential for teratogenicity or adverse impact on fertility.

## INNATE IMMUNITY

The innate immune response is a rapid, receptor-mediated host response to invariant microbial ligands called pathogen-associated molecular patterns (PAMPs) (1,2). Extracellular



**Fig. 1.** Adaptive immune response and the generation of effector mechanisms of cell and tissue injury. Antigen-specific activation of CD4 and CD8 T cells is modulated by the innate immune response. See text for detailed discussion. MAC, macrophage; NK, natural killer; MHC, major histocompatibility complex; IL, interleukin; IFN- $\gamma$ , interferon- $\gamma$ ; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ , Th, T helper; CTL, cytotoxic T lymphocyte; Abs, antibodies; AutoAbs, autoantibodies; C', complement; ADCC, antibody-dependent cellular cytotoxicity; T-reg, regulatory T Cell.

and intracellular pattern-recognition receptors (PRRs) are activated by PAMP ligands. Extracellular PRRs include Toll-like receptors (TLR1, TLR2, TLR4, TLR5, TLR6, and TLR11), macrophage mannose receptors, and complement (C') receptors; intracellular PRRs include the cytoplasmic nucleotide-binding oligomerization domain (NOD) proteins and TLR7, TLR8, and TLR9 localized to the endosome. Innate immune responses are mediated by phagocytes (neutrophils, monocytes, and macrophages, including Kupffer cells, and dendritic cells), cells containing preformed inflammatory mediators (basophils, mast cells and eosinophils), natural killer (NK) and natural killer T (NKT) cells and activated C' proteins, acute-phase reactants, cytokines, and chemokines. Innate immunity directly influences antigen-specific adaptive immune responses by adjusting activation thresholds of T and B cells and providing the costimulatory signals, cytokines, and chemokines necessary for functional differentiation of T cells and B cells (Fig.1) (3).

## ADAPTIVE IMMUNITY

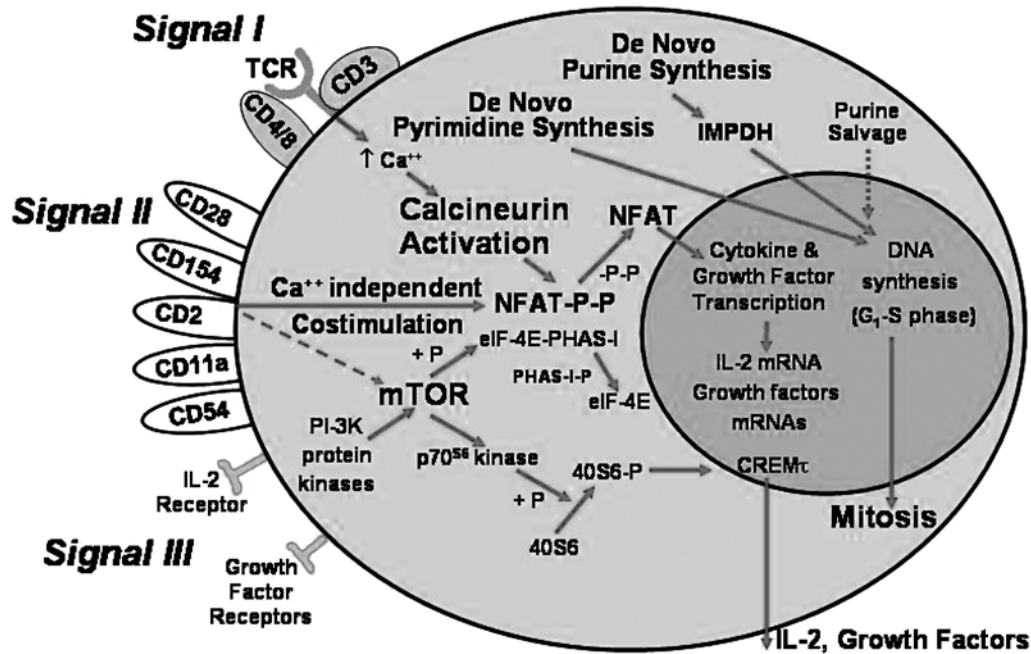
### ACTIVATION OF T AND B CELLS

Adaptive immunity involves antigen-specific responses by its cellular (T cells) and humoral (B cells) limbs (Fig. 1). Development of functional T cells requires three distinct signals (Fig. 2). Activation signal 1 results from binding of T-cell receptors (TCRs) with processed peptide antigens presented by professional antigen-presenting cells (APCs) in the antigen-binding grooves of MHC class II (CD4-restricted) and I (CD8-restricted) molecules. Costimulatory signal 2 is transduced by the binding of several different receptors on T cells with costimulatory ligands expressed by APCs. Signal III is required for T-cell clonal proliferation and maturation and is mediated

by receptors for mitogenic cytokines and growth factors. Activated CD4 T cells differentiate into distinct, functional subsets: T-helper 1 (Th1), T-helper 2 (Th2), and T-regulatory (T-reg) cells. CD4 Th1 and Th2 cells secrete unique assortments of cytokines that inhibit each other's proliferation and cytokine secretion. This cross-inhibition establishes the ultimate balance between Th1 and Th2 subsets within tissues. A predominance of Th1 cells results in more intense tissue immunopathology. Th1 predominance is favored in an environment of innate immune reactions generating proinflammatory interferon- $\gamma$  (IFN- $\gamma$ ), interleukin-6 (IL-6), and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ). CD4 Th1 and Th2 cells promote the differentiation and maturation of CD8 cytotoxic T lymphocytes, chemoattraction and activation of monocytes and eosinophils, and immunoglobulin (Ig) secretion by B cells. This coordinated response leads to multiple, complementary effector mechanisms involved in tissue and organ injury (Fig. 1).

### TRANSENDOTHELIAL LEUKOCYTE TRAFFICKING AND COSTIMULATION

Both innate and adaptive immunity require transendothelial leukocyte migration (4). Dendritic APCs migrate from tissues to regional lymph nodes to present processed peptide antigens to naïve T and B cells (Fig. 3). Activated T cells then egress from the lymph node into the circulation. Transendothelial migration of activated T cells and other leukocytes produces and maintains inflammatory infiltrates within tissues. Tissue injury or cellular stress results in the innate immune production of chemokines that are taken up and displayed by endothelial cells (4). Activated leukocytes roll along the endothelial cell surface under the control of leukocyte selectin adhesion molecules. Firm adhesion occurs when specific chemokine receptors



**Fig. 2.** Functional activation of T cells requires three interdependent signals and proliferation for clonal expansion and differentiation. The interdependent signals are: (1) T-cell receptor (TCR) engagement of the MHC-antigen complex on APCs during appropriate adhesion between the cells, conferring antigen specificity; (2) non-antigen-specific costimulatory signals, generated by the interaction between other T cell receptors and ligands expressed by APCs; and (3) signals for mitogenesis and functional maturation provided by IL-2 and multiple growth factors. Proliferation requires *de novo* synthesis of both purines and pyrimidines. Lymphocytes lack robust pathways for purine salvage and are particularly susceptible to inhibitors of purine synthesis. IL, interleukin; NFAT, nuclear factor of activated T cells; mTOR, mammalian target of rapamycin; IMPDH, inosine-5'-monophosphatase dehydrogenase; CREM, cyclic adhesive monophosphate response element modulator.

and integrin adhesion molecules on activated leukocytes bind to endothelial chemokines and adhesion molecules. Diapedesis of leukocytes through endothelial cell tight junctions and basement membranes allows the extravasated leukocytes to migrate toward the source of chemokines recognized by their chemokine receptors. The costimulatory T cell costimulatory receptor CD154 (also termed CD40 ligand, [CD40L]) induces endothelial cell expression of adhesion molecules E-selectin, intracellular cell adhesive molecule-1 (ICAM-1 [CD54]), and vascular cell adhesion molecule-1 (VCAM-1).

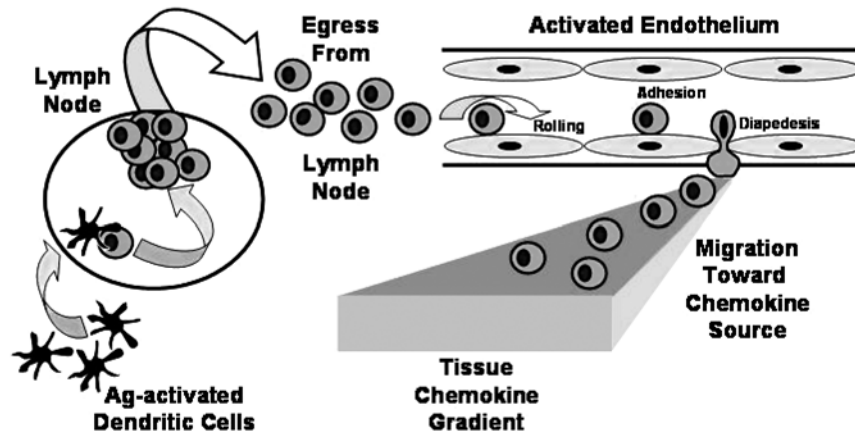
## INHIBITION AND MODULATION OF INNATE IMMUNITY

Advancing knowledge of the structures, ligands, and signaling pathways of TLRs and NODs has resulted in the rapid development of novel therapeutics (1,2). Currently efforts are directed toward the: (1) development of small-molecule analogs to act as antagonists or agonists for TLRs or NODs, (2) inhibition or modulation of PRR intracellular signaling pathways, and (3) creation of more potent adjuvants for preventative and therapeutic vaccines. The applicability of these emerging therapeutic agents in multiple diseases, including acute and chronic hepatobiliary diseases, remains tentative but very promising (Table 1). These new therapeutics may have important impacts on the prevention and/or treatment of allograft ischemia/reperfusion injury (5), hepatitis B (6–9), hepatitis C (10–14), acute liver

failure (15,16), autoimmune liver diseases, (17,18), alcoholic hepatitis (19), nonalcoholic fatty liver diseases (20), and hepatobiliary cancers. (21). Several of these agents will likely become available for clinical use in the near future.

## INHIBITION OF ADAPTIVE IMMUNITY

The sequential events required to generate and maintain adaptive immune responses afford multiple sites for therapeutic inhibition (Fig. 4). These targets include T-cell activation (signal 1), costimulation (signal 2), clonal proliferation (signal 3), differentiation and maturation of effector mechanisms, trans-endothelial migration, and accumulation within tissues expressing specific antigens. As shown in Fig. 4, multiple drugs and therapeutic antibodies are available that immunosuppress and/or immunomodulate adaptive immune responses, and several agents act at more than one site. The availability of multiple therapeutic agents now permits the use of combination therapies to achieve optimal effects, while minimizing dose-dependent toxicities (22). Immunosuppressive drugs administered at the time of organ or tissue transplantation to prevent allogeneic immune-mediated rejection are also efficacious in the treatment of chronic immune-mediated hepatic diseases (*see below*). Evidence of such efficacy clearly indicates that the immunopathogenetic mechanisms of many chronic hepatic diseases involve an obligatory cycle of perpetual T-cell activation and tissue infiltration that can be therapeutically



**Fig. 3.** Transendothelial migration of activated T cells. After uptake of antigen in organ tissues, dendritic cells migrate to regional lymph nodes, where they present processed peptide antigens to CD4 and CD T cells. Activated T cells proliferate and egress from the lymph node to the circulation. They circulate until they encounter cytokine/chemokine-activated endothelial cells expressing concentrated chemokines in luminal, cytoplasmic projections and adhesion molecules. Rolling T cells bearing specific adhesion molecules, and chemokine receptors become static and undergo transendothelial migration into the tissues (diapedesis). T cells then migrate toward the source of the chemokine gradients. Ag, antigen.

**Table 1**  
Toll-Like Receptors as Therapeutic Targets and Potential Applications as Immunosuppressive and Immunomodulatory Agents in Acute and Chronic Liver Diseases

<i>Toll-like receptor</i>	<i>Natural ligand(s)</i>	<i>Synthetic analogs</i>	<i>Therapeutic applicability</i>
<b>TLR1</b>	<i>Borrelia burgdorferi</i> , <i>Neisseria</i> , mycobacterial lipoproteins	Triacyl lipopeptides	Attenuate bacterial (Gram <sup>+</sup> ) and fungal infections/sepsis
<b>TLR2</b>	Proteoglycan Lipotechoic acid Atypical lipopolysaccharide Mycobacterial lipoproteins Lipoproteins Glycolipids Lipoarabinomannan HSV Yeast zymosan HSP70	Di- and triacyl lipopeptides	Attenuate bacterial (Gram <sup>+</sup> ) and fungal infections/sepsis <i>Overcome HCV inhibition of dendritic cell responses to TLR2?</i>
<b>TLR3</b>	Double-stranded RNA	Poly I:C	Viral infections Treatment of ovarian cancer <i>Potential to enhance clearance of HBV or HCV infections?</i> <i>Potential to prevent glomerulonephritis by inhibiting renal mesangial cell responses to immune complexes in viral hepatitis?</i>
<b>TLR4</b>	Lipopolysaccharide Mycobacteria RSV Fibrinogen peptides HSP60 Taxol	MPL adjuvant  RC529 E5564 (Eritoran)	Treatment of allergy Improved vaccines <i>Improved HBV vaccine</i> Prevent endotoxemia by blocking TNF- $\alpha$ secretion. <i>Potential role in treatment of acute liver failure. PBC, PSC, alcoholic and non-alcoholic steatohepatitis?</i>
		CRX-526	Prevent LPS activation of genes (autoimmune diseases, IMIDs, atherosclerosis)

(Continued)

Table 1 (Continued)

<i>Toll-like receptor</i>	<i>Natural ligand(s)</i>	<i>Synthetic analogs</i>	<i>Therapeutic applicability</i>
<b>TLR5</b>	Bacterial flagellin	Discontinuous 13 amino acid peptide	Attenuation of bacterial infections
<b>TLR6</b>	Mycoplasma lipopeptides Lipotechoic acid Fungal zymosan	Diacyl lipopeptides	Undefined
<b>TLR7</b>	Single-stranded RNA (mouse)	Imiquimod Resiquimod  Loxoribine Bropirimine Isatoribine	Induction of IFN- $\alpha$ and other cytokines that enhance cutaneous immune responses to genital warts and basal cell carcinoma <i>May increase host endogenous IFN-<math>\alpha</math> production during HBV and HCV infections (see isatoribine)</i> Anticancer effects Anticancer effects <i>Potential in cholangiocarcinoma and hepatocellular carcinoma?</i> <i>Dose-dependent reduction of HCV RNA</i>
<b>TLR8</b>	Single-stranded RNA (human)	Imiquimod	Induction of IFN- $\alpha$ and other cytokines <i>Potential to increase endogenous IFN-<math>\alpha</math> production during HBV and HCV infections?</i>
<b>TLR9</b>	Bacterial DNA  Viral DNA Other DNA with low content of nonmethylated CpG sequences	CpG oligodeoxy-nucleotides  HBV-ISS-1018 (HepIisav) CPG10101 (Actilon)	Improved vaccines Treatment of melanoma Treatment of non-Hodgkin's lymphoma <i>Improved vaccine for HBV</i> <i>Treatment of refractory HCV infection in combination with pegylated IFN and ribavirin</i>

Abbreviations: TLR, toll-like receptor; HSV, herpes simplex virus; HSP, heat shock protein; LPS, lipopolysaccharide; RSV, respiratory syncytial virus; IMID, immune-mediated inflammatory disease; HBV, hepatitis B Virus; HCV, hepatitis C Virus; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; PBC, primary biliary cirrhosis; PSC, primary sclerosing cholangitis; IFN- $\alpha$ , interferon- $\alpha$ .

disrupted. In addition, it is now clear that hepatic fibrogenesis leading to cirrhosis is dependent on cytokines and growth factors resulting from hepatic inflammation and adaptive immune reactions (23). Thus, it is increasingly important for the hepatologist to understand the available and investigational immunosuppressive and anti-inflammatory agents that will be used increasingly to treat nontransplant viral hepatitis, acute liver failure, hepatotoxicity, alcoholic hepatitis, nonalcoholic fatty liver diseases, and autoimmune and IMIDs of the liver.

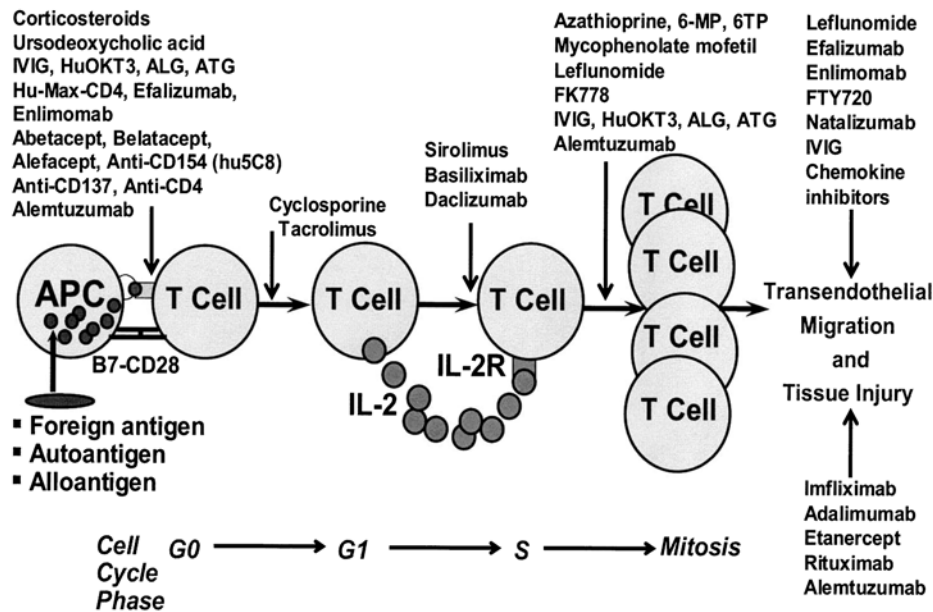
### INHIBITION OF T-CELL ACTIVATION

**Corticosteroids** Corticosteroids have immunosuppressive and antiinflammatory properties at the level of T-cell activation by APCs (24). By reducing CD4 T-cell secretion of IL-2, they inhibit T-cell activation and clonal proliferation. They also inhibit secretion of the proinflammatory cytokines, IL-1, IL-6, and TNF- $\alpha$  by APCs, reducing the efficiency of antigen presentation and the proinflammatory milieu (Fig. 1).

*Prednisone* is rapidly converted to *prednisolone* in the liver, despite the presence of necroinflammatory disease. Both have shown efficacy in the treatment of autoimmune hepatitis (AIH),

(25), primary biliary cirrhosis (PBC) (26), primary sclerosing cholangitis (PSC) associated with autoimmune pancreatitis (27), and severe alcoholic hepatitis (28) and in the prevention of allograft rejection when combined with calcineurin inhibitors (29). Concern that prednisolone might increase production of hepatotoxic deoxycholic acid led to use of a combination of prednisolone and ursodeoxycholic acid (UDCA) in one RCT in PBC (30). Significant dose-related adverse events associated with chronic use of corticosteroids include osteopenia, hypertension, cataracts, glaucoma, glucose intolerance, acne, weight gain, hirsutism, insomnia, and dyspepsia. Concern about osteopenia in PBC has dampened enthusiasm for chronic corticosteroid therapy.

*Budesonide* is a potent corticosteroid with reduced potential for systemic toxicity owing to its high first-pass hepatic clearance and the weak glucocorticoid effects of its metabolites (31). However, portal systemic shunting and the unpredictability of hepatic metabolic function may affect adverse events and efficacy. For example, significantly higher peak concentrations and areas under the plasma concentration curves were observed in cirrhotic vs precirrhotic patients with PBC (32).



**Fig. 4.** Sites of action of immunosuppressive and immunomodulatory drugs and antibodies. Multiple agents can inhibit the activation, clonal proliferation, differentiation, and transendothelial cell migration of T cells. See text for a detailed description of each agent. ALG, anti-lymphocyte globulin; APC, antigen-presenting cell; ATG, antithymocyte globulin; IL, interleukin; IVIG, intravenous immunoglobulin.

Budesonide has been studied in RCTs and open-label trials in AIH (33) and PBC (34). The results have been encouraging enough to warrant larger RCTs.

**Ursodeoxycholic Acid** Among its multiple mechanisms of action, UDCA is weakly immunosuppressive, immunomodulatory, and antiapoptotic (35). However, the therapeutic value of its immunological effects, compared with the cytoprotective effects of increasing UDCA concentration in hepatic bile and decreasing concentrations of toxic bile acids, remains unclear. UDCA is licensed for treatment of PBC (36) but has also been studied in AIH (37), PSC (38), nonalcoholic fatty liver disease (39), and hepatic GVHD (40). Controversial results of efficacy in studies of PSC patients receiving doses of 20 to 30 mg/kg/d (reviewed in ref. 38) are being assessed in a multicenter trial of high-dose therapy in the United States.

Adverse events associated with UDCA are infrequent, but they include abdominal pain, dyspepsia, nausea, vomiting, diarrhea, dizziness, symptoms of upper respiratory illness, and alopecia. Long-term use also caused weight gain in PBC (41). Rare, serious adverse events of leukopenia and anaphylaxis have been reported.

**Calcineurin Inhibitors** Both cyclosporine and tacrolimus inhibit signal 1 of T-cell activation (Figs. 1 and 2). This signal 1 is mediated by  $Ca^{2+}$ -dependent activation of calcineurin, which dephosphorylates transcription factors, such as nuclear factor of activated T cells (NFAT), required for transcription of essential mitogenic cytokines and growth factors, including IL-2, IL-3, IL-4, TNF- $\alpha$ , IFN- $\gamma$ , and granulocyte-macrophage colony-stimulating factor (GM-CSF) (42). As a result, both block cell cycle progression from phase G0 to G1 (Fig. 4).

**Cyclosporine** Cyclosporine inhibits calcineurin phosphatase by binding to cytoplasmic cyclophilin receptors. These cytokines and growth factors are required for activation and proliferation of functional T-cell clones (Figs. 1 and 2) (43). In vitro, cyclosporine also induces expression of transforming growth factor- $\beta$  (TGF- $\beta$ ), a suppressor of T-cell functions. The absorption, hepatic metabolism, and pharmacokinetics of cyclosporine are preserved in chronic liver diseases and are unaffected by prolonged use (44). Adverse events are dose-dependent and include hypertension, nephrotoxicity, neurotoxicity, hypercholesterolemia, and (more rarely) lymphoproliferative disorders.

Cyclosporine is primarily used to prevent hepatic allograft rejection, but it is used less frequently than tacrolimus (45). Cyclosporine-induced production of TGF- $\beta$  by cholangiocytes may contribute to the frequency of ductopenia by inhibiting mitogenesis and inducing senescence protein p21, which is observed in ductopenic rejection (46).

Cyclosporine has been efficacious in uncontrolled and RCTs in AIH (47,48) and PBC (49). In addition, some beneficial effects of long-term therapy in PBC were noted, including improved bone metabolism (50) and reduced hypercholesterolemia (51). However, hypertension and nephrotoxicity constrain the use of cyclosporine in these diseases. Randomized, concentration-controlled trials could help define the optimal, effective serum levels of cyclosporine, as has been done for tacrolimus to minimize its toxicity in multiple sclerosis (52).

**Tacrolimus** Tacrolimus (also termed FK-506) inhibits calcineurin, but its mechanism of action involves formation of complexes with cytoplasmic FK-binding protein-12 (FKBP12)

within T cells (53). These bind to calcineurin-calmodulin complexes, preventing calcineurin phosphatase dephosphorylation of the same transcription factors inhibited by cyclosporine. Tacrolimus also promotes secretion of the pluripotent cytokine TGF- $\beta$ , which is capable of acting as a T-cell immunosuppressant and growth factor. In contrast to cyclosporine, tacrolimus does not increase cholangiocyte expression of senescence protein p21, possibly explaining its superiority in prevention of ductopenic rejection. In addition to inhibiting IL-2 secretion, tacrolimus also inhibits expression of the IL-2 and IL-7 receptors responsible for signal 3 of T-cell activation. Most of tacrolimus's adverse events are identical to those of cyclosporine. However, tacrolimus is more frequently associated with diabetes mellitus, pancreatitis, gastrointestinal symptoms, bone marrow suppression, and allergic reactions.

Tacrolimus is the most frequently used immunosuppressive drug to prevent hepatic allograft rejection (45). No RCTs of tacrolimus have been reported in AIH, but it has been successfully used to treat steroid-refractory disease (47,48,54). The absence of RCTs of tacrolimus in PBC (34) is unfortunate, since biliary inflammation in PBC results in ductopenia, (55), and tacrolimus more effectively prevents ductopenic rejection than cyclosporine (45).

#### IMMUNOGLOBULIN AND MONOCLONAL ANTIBODIES

**Intravenous Immunoglobulin** Intravenous immunoglobulin (IVIG) contains IgG antibodies from pooled human serum with a multitude of antigen specificities, including natural autoantibodies and anti-idiotypes. IVIG has concurrent immunosuppressive, immunomodulatory, and anti-inflammatory effects on dendritic cells, B cells, T cells, antibodies, cell proliferation, apoptosis, production of TGF- $\beta$  and IL-4, fibrogenesis, and transendothelial migration of leukocytes into tissues (56–58). Anti-idiotypic antibodies in IVIG inhibit antigen-specific TCRs, preventing T-cell activation. IVIG also inhibits effector mechanisms (Fig. 1) mediated by autoantibodies, immune complexes, C' activation, and proinflammatory cytokines. Adverse events are usually well tolerated and include headache, fever, chills, anemia, back pain, temporary hypotension, nausea, perspiration, and venous thromboses (59). IVIG has not been used to treat acute liver failure, alcoholic hepatitis, or autoimmune or alloimmune liver diseases; however, its mechanisms of action provide the rationale for pilot feasibility studies.

#### HUMANIZED MONOCLONAL ANTIBODIES

Several monoclonal antibodies (MAbs) have been developed to inhibit signal 1 of T-cell activation. Further clinical studies are needed to determine their safety and efficacy.

**HuMax-CD4** Two humanized anti-CD4 MAbs (HuMax-CD4 and TRX1) have reduced immunogenicity, and alteration of their Fc domains prevents depletion of CD4 T cells. By disrupting activation, HuMax-CD4 produced dose-dependent decreases in memory CD4 T cells in psoriasis vulgaris (60), while TRX1 prevented humoral responses in baboons, and repeated doses produced tolerance (61). These results provided

proof of principle that inhibition of memory CD4 T cells can ameliorate chronic disease.

**Efalizumab** Signal 1 of T-cell activation can also be prevented by disrupting adhesion between ICAM-1 (CD54) on APCs and lymphocyte function antigen-1 (LFA-1; heterodimer of CD11a and  $[\alpha L]$ -CD18  $[\beta 2]$  integrin) expressed on T cells. Efalizumab, an injectable humanized MAb against the CD11a monomer of T-cell LFA-1 (62), was effective in prolonged therapy of psoriasis (63), oral lichen planus (64), dermatomyositis (65), and cutaneous systemic lupus erythematosus (SLE) (66). Mild adverse events occurring in 1 to 2% included headache, chills, fever, nausea, and myalgia after the first two injections and arthralgia, asthenia, and edema later (67). The relative contributions of inhibition of signal 1 in memory T cells and transendothelial T-cell migration into tissues are unclear, but it is likely that both mechanisms are involved.

**Enlimomab** Enlimomab is a MAb reactive against ICAM-1. It was ineffective in preventing transendothelial trafficking of activated leukocytes in patients with burns or stroke (68,69). It is unclear whether it blocks T-cell signal 1.

None of these humanized MAbs have been studied in orthotopic liver transplant (OLT) or in acute or chronic liver diseases. HuMax-CD4 and efalizumab are attractive candidates for the treatment of refractory allograft rejection, AIH, and overlap syndromes.

#### INHIBITION OF T-CELL COSTIMULATION

Signal 2 provides the costimulatory signaling required for functional activation and clonal expansion of antigen-specific T cells. Multiple receptors transduce costimulatory signals (Fig. 2), including CD28, CD154 (also termed CD40-ligand [CD40L]), CD11a (portion of the LFA-1 heterodimer), CD2, CD137, and CD152. Therapeutic agents have been developed to inhibit each of these costimulatory receptors. Although such inhibition is most appealing for prevention of alloimmune activation mediating allograft rejection or GVHD, the repetitive process of T-cell activation required for clonal proliferation of memory T cells mediating chronic diseases suggests that inhibition of signal 2 might be beneficial in the treatment of established hepatic diseases. For example, in autoimmune and IMIDs, it could reduce the activation and maturation of antigen-specific memory T cells responsible for immunopathology. Inhibition of signal 2 at the time of organ or hematopoietic stem cell transplantation could theoretically induce polyclonal T-cell anergic tolerance.

#### FUSION PROTEIN INHIBITORS

**CTLA4-Ig Inhibitors** Both CD28 and cytotoxic T-lymphocyte antigen-4 (CTLA-4, also termed CD152) are T cell receptor for the B7.1 (CD80) and B7.2 (CD86) ligands expressed on APCs. In contrast to the activating costimulatory signal transduced by CD28, CTLA-4 signaling is immunosuppressive (70). A CD28-Ig fusion protein has been developed to block CD80/CD86, but it has not been used clinically (71).

**Abatacept** Abatacept, a fusion product of human CTLA-4 and the modified Fc portion of human IgG1, is approved by



the Food and Drug Administration (FDA) to block CD80/CD86 by binding competitively to T-cell CD28 in patients with refractory rheumatoid arthritis. (72). Adverse events, like headache, nasopharyngitis, dizziness, and cough, were similar in frequency to those seen with placebo. Serious adverse reactions, including cardiorespiratory symptoms and anaphylaxis, were rarely observed during infusions.

**Belatacept** Belatacept (also termed LEA29Y) is a derivative of abatacept, which differs from abatacept by two amino acid substitutions that increase avidity of binding to CD80/CD86 on APCs (73). In contrast to abatacept, which is ineffective in prevention of allograft rejection in nonhuman primate models, belatacept prevented rejection in nonhuman primates and was as effective as cyclosporine in the immunosuppression of patients undergoing renal transplantation (74). Adverse events were comparable for patients treated with either belatacept or cyclosporine.

The therapeutic potential of either abatacept or belatacept in prevention of hepatic allograft rejection or treatment of acute or chronic liver diseases is unknown but promising. CTLA-4-Ig costimulatory blockade has been effective in animal models of liver transplantation (75) and suppressed injury in a model of fulminant hepatitis (76). In addition, adenoviral delivery of the CTLA-4-Ig gene to the liver effectively prevented infiltration of leukocytes and apoptosis of hepatobiliary cells (77). The effectiveness of these agents in rheumatoid arthritis suggests potential benefit in the treatment of severe autoimmune and IMIDs of the liver.

**Alefacept** Alefacept is a humanized CD2 fusion protein exhibiting two mechanisms of action: (1) inhibition of costimulatory signaling by T-cell CD2 receptors engaging with CD48 (expressed on all APCs and T cells) (78) and (2) increased apoptosis of activated CD4 and CD8 memory subsets (79). Alefacept has produced long-term remissions in psoriasis without evidence of immunogenicity, infectious complications, or malignancy (80). Preliminary studies indicate its effectiveness in lichen planus (81) and cutaneous GVHD (82). Adverse events were minor, including headache, nasopharyngitis, influenza-like symptoms, upper respiratory tract infection, pruritus, arthralgia, fatigue, nausea, and increased aminotransferase levels (83). Alefacept has not been studied in acute or chronic liver diseases, and the mechanisms and potential consequences of abnormalities of liver tests are unknown.

### MONOCLONAL ANTIBODY INHIBITORS

Three MABs have been developed to antagonize costimulatory signal 2. Their greatest therapeutic potential will most likely be prevention of alloimmune responses in OLT and hematopoietic stem cell transplantation.

**hu5C8** The humanized anti-CD154 (CD40L) MAB hu5C8 inhibits costimulatory signaling mediated by CD40 on APCs (84). In animal models, hu5C8 significantly delayed rejection of MHC-mismatched primate allografts (84) and inhibited autoimmunity (85). Although promising in clinical trials in SLE and idiopathic thrombocytopenia, thrombotic complications have raised concerns about this agent (85).

**Anti-CD137** The costimulatory signal transduced by CD137 (4-1BB) increases Bcl-x(L) and c-FLIP(short), preventing apoptosis of antigen-activated T cells (86) and promotes signaling required for CD8 CTLs (87). In experimental autoimmune uveitis, anti-CD137 MAB induced apoptosis of newly activated T cells (88) but had no effect on activated memory T cells. Of concern is evidence that anti-CD137 may also prevent activation of CD4 T-reg cells responsible for antigen-specific tolerance (89). It has not been studied in liver diseases or in the prevention of hepatic allograft rejection.

**Efalizumab** As noted above, efalizumab not only inhibits the costimulatory effects of the T-cell LFA-1 subunit CD11a but also blocks its functions in cellular adhesion and trans-endothelial migration (62). Its safety and efficacy in clinical trials of several human IMIDs make it an attractive candidate for trials in chronic inflammatory hepatobiliary diseases.

### INHIBITION OF CLONAL T-CELL PROLIFERATION AND DIFFERENTIATION

Signal 3 of T-cell activation results in clonal expansion and maturation of T cells. The mitogenic cytokine IL-2 stimulates clonal proliferation by binding to IL-2 receptors (IL-2R [CD25]). In addition, growth factors induce receptor-mediated signaling required for differentiation of T-cell effector functions. Two approaches have been used to inhibit signal 3: (1) blocking of IL-2Rs with MABs during the initiation of T-cell activation and (2) prevention of signaling by inhibiting the mammalian target of rapamycin (mTOR).

**Sirolimus** Sirolimus (also termed rapamycin) forms complexes with cytosolic FKBP12 that inhibit mTOR signaling (90). This inhibition results in reduced translation of proteins and disruption of cell cycle transition, which prevent clonal T-cell proliferation. Everolimus (also termed RAD) is a derivative of sirolimus with lesser affinity for FKBP12 (91). Both sirolimus and everolimus prevent G1 to S cell cycle transition in T cells by inhibiting Ca<sup>2+</sup>-independent activation of T cells by IL-2, IL-4, and IL-6 (Figs. 2 and 4) and costimulatory effects of B7.1(CD80)/B7.2(CD86)-CD28 binding (signal 2) (92). Neither is nephrotoxic, which provides a distinct advantage over cyclosporine or tacrolimus for chronic use. However, sirolimus inhibition of wound healing led to an FDA Black Box Warning, proscribing its use as primary immunosuppression in OLT owing to an increased risk of hepatic arterial and venous thrombosis. In contrast, sirolimus has been safe and effective in renal transplantation (93). Currently, sirolimus is used to immunosuppress OLT patients with calcineurin-inhibitor induced azotemia after healing of wounds and vascular anastomoses (94). Investigationally, it has been used to treat AIH before (48) and after OLT for AIH (95). Other studies have evaluated sirolimus in the prevention of GVHD (96), cancer (97), and hepatic fibrosis (98). Common adverse events include headache, hypertension, edema, hyperlipidemia, asthenia, gastrointestinal symptoms, and arthralgia. Serious adverse events include infections, lymphoma, other malignancies, leukopenia, and thrombocytopenia.

Since the effects of sirolimus on IL-2 signaling complement the effects of cyclosporine and tacrolimus inhibition of IL-2 secretion, combination therapy with sirolimus and a calcineurin inhibitor could theoretically prevent all three signals of T-cell activation. The successful use of calcineurin inhibitors in AIH (47,48) and PBC (34) indicates that sirolimus alone or combined with low doses of cyclosporine or tacrolimus should be studied in RCTs. The mechanisms of action of sirolimus also suggest therapeutic potential in PSC (99).

**Monoclonal Anti-IL-2R (CD25) Antibodies** Daclizumab and basiliximab are therapeutic MAbs that bind to the Tac/CD25  $\alpha$ -subunit of the IL-2 receptor (IL-2R, [CD25]). Both are approved for use as induction therapy to prevent renal allograft rejection (100,101). The combination of blockade of IL-2R-mediated mitogenesis and inhibition of IL-2 synthesis with cyclosporine or tacrolimus effectively inhibits both signals 2 and 3 of T-cell activation.

Daclizumab is a humanized MAb, whereas basiliximab is a mouse chimeric MAb with increased risks of anaphylaxis when readministered to patients who have developed anti-mouse IgE (102). Both MAbs effectively suppressed alloimmune T-cell responses at the time of OLT (103,104). Basiliximab has also been used in severe ulcerative colitis (105) and to prevent GVHD (106). Nontransplant uses of daclizumab include prevention of ischemic-reperfusion injury (107), treatment of ulcerative colitis (108), GVHD (in combination with infliximab) (109), uveitis (110), and refractory autoimmune diseases such as thrombocytopenia purpura (111) and multiple sclerosis (112). The observed benefits of signal 3 disruption in established autoimmune and IMIDs suggest that daclizumab or basiliximab should be studied in autoimmune and IMIDs of the liver.

### INHIBITION OF T-CELL PROLIFERATION

Antiproliferative agents, including azathioprine, 6-mercaptopurine, 6-thioguanine (antiproliferative inhibitors of purine metabolism), cyclophosphamide, and methotrexate, have been used to reduce inflammatory infiltration and effector functions of lymphocytes in hepatic autoimmune and IMIDs (47,48). Azathioprine continues to be used because of its proven efficacy in AIH (48). In addition, small numbers of patients with AIH have been treated with either 6-mercaptopurine or 6-thioguanine to reduce the proliferation of effector T and B cells (113). Because toxicities may result from variation in the activity of thiopurine methyltransferase (TPMT), the enzyme responsible for metabolism of azathioprine, 6-mercaptopurine and 6-thioguanine, testing should be performed before commencing treatment (114). Azathioprine has been abandoned as immunosuppression for OLT recipients and proved to be ineffective in RCTs of treatment in PBC (115) and PSC (116). Cyclophosphamide toxicities precluded long-term use in hepatic diseases. A large RCT of methotrexate in PBC was negative (117). However, recent development of drugs with greater antiproliferative specificity for T- and B- effector cells affords new opportunities to selectively inhibit proliferation of T and B cells without indiscriminately inhibiting other rapidly dividing cells.

### PURINE SYNTHESIS INHIBITION

**Mycophenolate mofetil** Mycophenolate mofetil is a prodrug of mycophenolic acid, which is a non-competitive, reversible inhibitor of the rate-limiting enzyme for *de novo* purine synthesis, inosine monophosphate dehydrogenase (Fig. 2) (118). Mycophenolic acid preferentially inhibits T- and B-cell proliferation because they require *de novo* purine synthesis to compensate for deficient purine salvage pathways. Mycophenolic acid has multiple immunosuppressive effects, which make it difficult to assess the mechanisms involved in clinical efficacy. Its immunosuppressive effects include: (1) decreased expression of IL-2R, HLA-DR, transferrin receptors, and chemokines involved in signals 1 and 3 and leukocyte migration; (2) decreased glycosylation of adhesion molecules required for signal 1 and cytotoxic effector functions; and (3) decreased T-cell-independent B-cell secretion of Ig. Mycophenolic acid, however, does not reduce secretion of proinflammatory IL-1 $\beta$ , IL-6, or TNF- $\alpha$  by APCs, or T cell secretion of IL-2, IL-4, and IL-13, or neutrophil superoxide or chemotaxis.

In OLT, mycophenolic mofetil has been successfully used alone (119) or in combination with calcineurin inhibitors to preserve renal function and prevent rejection (120). Although anecdotally successful in refractory (47,48) and *de novo* AIH post OLT (121), mycophenolate was unsuccessful in PBC (122) or PSC (123). Adverse events led to discontinuation of mycophenolate in 24% of treated patients with PBC (122). Common, dose-dependent adverse events include abdominal pain, nausea, vomiting, diarrhea, and abdominal pain. Rare, serious adverse events include neutropenia, thrombocytopenia, infections, lymphoma, other malignancies, and gastrointestinal ulceration or perforation. A RCT comparing the combination of corticosteroids with azathioprine vs mycophenolate has been proposed.

### PYRIMIDINE SYNTHESIS INHIBITION

Development of more lymphocyte-specific antiproliferative agents is now focused on pyrimidine synthesis inhibition. Two promising drugs being studied are leflunomide and FK778, which not only inhibit pyrimidine synthesis but also exhibit additional immunosuppressive properties and antiviral activity against cytomegalovirus (CMV) (124).

**Leflunomide** The active metabolite of leflunomide (A771726) inhibits dihydro-orotate dehydrogenase, the rate-limiting enzyme for pyrimidine synthesis required for cell proliferation (125). In addition, A771726 also inhibits trans-endothelial migration of peripheral blood mononuclear cells, potentially reducing inflammatory infiltration of tissues and organs (125). Leflunomide has been successful in RCTs in rheumatoid arthritis and psoriasis (126). Leflunomide has also been used in transplantation (127) and is a candidate for the treatment of autoimmune diseases (128). Leflunomide prevented experimental collagen- and adjuvant-induced arthritis, myasthenia gravis, systemic lupus erythematosus, experimental autoimmune encephalomyelitis (129,130), and hepatic injury by suppressing intrahepatic T-cell functions and inhibiting proinflammatory cytokine production by Kupffer cells (131). Leflunomide has

not been studied in hepatic autoimmune or IMIDs, but its mechanisms of action, tolerability, and effective use in combination therapies make it an attractive candidate for study.

Adverse events associated with leflunomide alone or in combination with methotrexate were either less than or comparable to those with methotrexate alone (132). However, serious adverse events have been reported, including vasculitis (133), hypertriglyceridemia (134), interstitial lung disease (135), and hepatitis associated with a specific CYP2C9 allele (136).

**FK778** FK778, a leflunomide derivative, also inhibits dihydro-orotate dehydrogenase (137), prevents maturation of human dendritic cells (138), reduces upregulation of adhesion molecules in experimental transplantation (137), attenuates interactions between lymphocytes and endothelial cells (139), and exhibits antiviral activity against CMV (124). In animal models, FK778 effectively prevented: (1) obliterative airway disease in combination with sirolimus or tacrolimus (140), (2) acute cellular rejection (124), and (3) arteriosclerosis in chronic rejection (137,141). Further clinical trials in transplantation are on hold, and its potential use in other diseases is unidentified.

#### DEPLETION AND IMMUNOMODULATION OF T CELLS

Antisera, including antilymphocyte globulin (ALG) and antithymocyte globulin (ATG), contain polyspecific antibodies that react with T-cell surface antigens. These antisera have been used successfully to prevent hepatic allograft rejection (142–144) and GVHD (145). The murine MAb OKT3 also has been extensively used to deplete effector T cells in patients with steroid-refractory rejection, but host immune responses to mouse proteins remain problematic (146). Humanized MAbs are being developed and tested to permit more effective depletion and immunomodulation of activated T cells.

##### Humanized Chimeric Monoclonal Antibodies

**Recombinant Humanized OKT3** Humanized OKT3 MAbs are designed to eliminate immunogenicity and prevent lethal syndromes of cytokine-release and flash pulmonary edema observed with murine OKT3 (147). Humanized OKT3 MAbs deplete CD3<sup>+</sup> T cells from both blood and inflamed tissues and generate T-regs (148,149). The single-chain variable fragment (scFv) IgM agent known as scOKT3- $\gamma\delta$  IgM VAEVD is a chimeric MAb containing light and heavy variable binding domains of OKT3 and CH3 and CH4 domains of human IgM variants bound by a human IgG3 hinge region (150). Binding to CD3 does not activate T cells but, instead, inhibits their functions without causing cytokine release. Hu291, another humanized anti-CD3 MAb, induces more apoptosis of human T cells than OKT3 (151). Humanized hOKT3- $\gamma$ 1(Ala-Ala) was also antiproliferative for T cells (148).

Humanized OKT3 MAbs are promising not only for the treatment of transplant patients with refractory rejection but also for use in other hepatic T-cell-mediated autoimmune or IMIDs. The prospect that humanized OKT3 MAbs could deplete effector T-cells, modulate production of proinflammatory cytokines, and generate T-regs provides a rationale for

aggressive, temporary treatment of established diseases (152). Following reduction of disease activity, more conventional immunosuppression could be used to prevent reactivation of memory T cells, reaccumulation of an effective mass of effector cells, and transendothelial migration into the liver.

**Alemtuzumab** Alemtuzumab (Campath 1H) is a humanized MAb specific for CD52, which is expressed on T and B cells, malignant lymphoid cells, but not progenitor cells (153). It has been successfully used to prevent GVHD (154) and hepatic allograft rejection (155). Unfortunately, posttreatment lymphopenia predisposes to infection and reactivation of hepatitis B (153,156). Adverse events include chills, fevers, nausea, vomiting, hypotension, urticaria, fatigue, dyspnea, pruritus, and depression. Cardiac events, pancytopenia, seizures, pancreatitis, and liver failure have also been reported as serious adverse events. These toxicities make it unlikely that alemtuzumab will be used in pilot studies of hepatic diseases.

#### INHIBITION OF TRANSENDOTHELIAL MIGRATION OF LEUKOCYTES INTO TISSUES

To mediate effector functions, activated leukocytes must migrate transendothelially into tissues (Fig. 3). Therapeutic regulation of leukocyte transendothelial migration mechanisms involving chemokines, chemokine receptors, adhesion molecules, and integrins could prevent inflammatory tissue injury and fibrogenesis in acute or chronic liver diseases.

**Leflunomide** Inhibition of leukocyte transendothelial migration (125) is one of the effects of leflunomide. Others include antiproliferation (157) and modulation of cytokine production, effector cell functions, and metalloproteinase production (130,158,159).

**Efalizumab and Enlimomab** Transendothelial migration requires the arrest of activated leukocytes on the endothelial cell surface, which is caused by the strong adhesion between leukocyte LFA-1 (CD11a/CD18) and endothelial cell ICAM-1 (CD54). This obligatory adhesion can be prevented by competitive binding of efalizumab to the CD11a moiety of LFA-1. The lack of effect of enlimomab (anti-ICAM-1) in prevention of transendothelial leukocyte trafficking in patients with burns (68) makes it unattractive for further studies.

**FTY720** FTY720 is an oral immunosuppressive agent that causes sequestration of lymphocytes in lymphoid tissues and the thymus, preventing lymphocyte circulation and transendothelial migration into tissues (160,161). The metabolite of FTY720 is a potent agonist of sphingosine-1-phosphate (S1P), which causes long-lasting downregulation of lymphocytic and dendritic S1P, preventing the migration of such cells (160,162). Cumulative consequences of FTY720 include modulation of monocytic dendritic cell functions, lymphocyte apoptosis, inhibition of transendothelial migration of activated T cells, prevention of activation of tissue-infiltrating lymphocytes, and inhibition of germinal centers resulting in suppression of humoral immunity (162–166). In contrast, clonal T-cell activation, proliferation, or differentiation of effector functions remain unaltered (161). FTY720 has been successfully tested with cyclosporine in renal transplantation, opening the way for

further human trials (167). It has also been used to prevent experimental AIH (168), ischemic-reperfusion injury (169), colitis (170), autoimmune encephalomyelitis (171), neoplastic angiogenesis (172), and rejection of cardiac allografts (173). Adverse events included bradycardia and lymphopenia (167, 174). FTY720 has not been used in autoimmune or IMIDs of the liver or other organs. It is a particularly attractive candidate for RCTs in AIH, PBC, and PSC.

**Natalizumab** Natalizumab is a humanized MAb that reacts with  $\alpha 4\beta 1$  integrin to prevent transendothelial leukocyte migration mediated by leukocyte  $\alpha 4\beta 1$  integrin (also termed very late antigen-4 [VLA-4]) binding to VCAM-1 on activated endothelial cells. Although natalizumab was efficacious in early therapeutic trials of relapsing multiple sclerosis (175) and Crohn's disease (176), clinical trials were stopped when several treated patients developed progressive multifocal leukoencephalopathy (177). The unanticipated toxicity of natalizumab serves as a warning for future trials of potent inhibitors of transendothelial migration.

**Inhibition of Chemokines and Chemokine Receptors** Chemokines, which are expressed by activated endothelial cells in response to their local production in tissues (Fig. 3) (4), promote transendothelial migration of activated leukocytes bearing specific chemokine receptors (178). Chemokine gradients within tissues chemoattract leukocytes and induce effector functions. Thus, chemokines produced by activated leukocytes and epithelial cells (55) within tissues and organs control the ultimate composition, quantity, and functions of inflammatory cells migrating to the site. A recent report indicated the potential of developing broad-spectrum chemokine inhibitors capable of blocking leukocyte migration mediated by a variety of chemokine receptors (179). Conversely, multiple inhibitors of chemokine receptors have been identified, including antagonists of CCR1, CCR3, CCR5, CXCR1, CXCR2, CXCR3, CXCR4, and CXCR6 (180–184). Preliminary studies indicate the potential therapeutic value of chemokine receptor inhibition in hepatic inflammatory diseases.

N-terminal modifications of CC chemokine ligand 5 (CCL5, also termed Regulated on Activation Normal T cell Expressed [RANTES]) produced analogs that prevented binding of chemokine receptors CCR1, CCR3 and CCR5 with multiple chemokines (CCL5/RANTES, CCL3/macrophage inhibitory protein-1 $\alpha$  (MIP-1 $\alpha$ ), CCL4/MIP-1 $\beta$ , CCL8/monocyte chemoattractant protein-2 (MCP-2), CCL7/MCP-3, CCL13/MCP-4, CCL11/eotaxin, CCL24/eotaxin-2, CCL26/eotaxin-3, and CCL15/lkn-1) (185). These inhibitory analogs include the methionyl CCL5/RANTES variant (Met-RANTES) (180), the rationally designed aminooxypentaine (AOP)-RANTES (181), and its potent derivative, PSC-RANTES (186). Met-RANTES antagonizes all three CCR receptors, whereas AOP-RANTES antagonizes CCR1 and CCR3. AOP-RANTES is an initial agonist for CCR5 that ultimately inhibits its receptor functions by sequestering it intracellularly. These inhibitors have been effective in experimental models of tissue and organ inflammation. (180,187). They have not been studied in RCTs of human diseases but may be harbingers of agents that can selectively

prevent hepatic inflammation and cytokine-mediated fibrogenesis regardless of disease etiology.

Th1 cells mediating immunopathology predominantly express the CXC chemokine receptor CXCR3 (182). The CXCR3 ligands CXCL9 (Mig), CXCL10 (IP-10), and CXCL11 (ITAC) are generated by leukocytes and epithelial cells in sites of inflammation (55). Inhibition of CXC3 with MAb prevented both inflammatory infiltration and bronchiolitis obliterans in an animal model of lung transplantation (188). The small-molecule CXCR3 antagonist NBI-74330 is a candidate for pilot studies in autoimmune and IMIDs of the liver (182). CXCR6 was recently found to play a key role in the recruitment of CD8 T cells to the inflamed liver in a model of GVHD, suggesting a new target for inhibition in inflammatory liver diseases (183).

### INHIBITION OF EFFECTOR MECHANISMS MEDIATED BY CYTOKINES AND IMMUNOGLOBULINS

**CD4 Th2 Cytokines** The immunopathology of AIH and PBC is characterized by infiltrates predominantly containing CD4 Th1 cells and CD8 CTLs. Theoretically, CD4 Th2 cytokines such as IL-10 and IL-4 could reduce hepatic secretion of the Th1 cytokines IL-2 and IFN- $\gamma$ , promoting a new regulatory balance between hepatic CD4 Th1 and Th2 cells (Fig. 1). Although recombinant human IL-10 therapy has been studied in inflammatory bowel disease (189), rheumatoid arthritis (190), the prevention of pancreatitis after endoscopic retrograde cholangiopancreatography and (191) chronic hepatitis C (192). Concern about Guillian-Barre syndrome terminated plans for studies in AIH. Immature human dendritic cells transfected with recombinant adenovirus containing the human IL-10 gene selectively activated CD4 Th2 cells producing IL-10 (193). These results raise the prospect of a gene therapy to increase expression of IL-10 for organ-specific effects, which has been demonstrated in experimental cardiac transplantation (194).

**Inhibition of TNF- $\alpha$**  Three FDA-approved agents are available to inhibit the injurious effects of TNF- $\alpha$ . To date, TNF- $\alpha$  antagonists have been used in hepatology to treat hepatic GVHD (195) and sarcoidosis (196) and as adjunctive therapy for chronic hepatitis C (197).

**Infliximab and Adalimumab** Infliximab and adalimumab are MAbs that antagonize TNF- $\alpha$  effects in vivo and cause apoptosis of human monocytes in vitro (198). Infliximab is a chimeric MAb that is a safe and effective treatment for Crohn's disease (199), rheumatoid arthritis (200), and sarcoidosis (196). It was also successfully used to treat psoriasis vulgaris with arthritis in a cirrhotic patient (201). The most common adverse event is an infusion site reaction, but a minority of patients develop allergic reactions or hepatotoxicity (202). Adalimumab is a human recombinant IgG1 mAb (203), which is also a safe and effective treatment for rheumatoid arthritis (204) and Crohn's disease in patients with inadequate responses or allergies to infliximab (205). Long-term monitoring is ongoing to ascertain the risks of neoplasia, infection, or immune disorders.

**Etanercept** Etanercept is a recombinant dimeric fusion protein combining the ligand-binding domain of the p75 TNF- $\alpha$

receptor and an Fc fragment of human IgG1 (206). Soluble etanercept binds TNF- $\alpha$ , preventing its deleterious effects. It is a safe and efficacious antagonist of TNF- $\alpha$  in rheumatoid arthritis (207) and psoriasis (208). An RCT showed that etanercept improved the response of patients with chronic hepatitis C to IFN and ribavirin (197). Etanercept also prevented TNF- $\alpha$  induction of tissue inhibitor of metalloproteinases-1 in rats treated with CCl<sub>4</sub> (209). It is currently being investigated as a therapy in alcoholic hepatitis.

### DEPLETION AND IMMUNOMODULATION OF ACTIVATED B CELLS

The role of Ig-mediated mechanisms in the immunopathogenesis of acute and chronic liver diseases is poorly defined. Pathogenetic autoantibodies have been proposed in AIH (25). In PBC, identification of a shared PDC-E2 antigenic motif for antimitochondrial antibodies and the TCRs of intrahepatic CD4 and CD8 T cells (210) suggested a pathogenetic link between cellular and humoral immunity. Despite these rationales, therapeutic modulation of Ig has not been reported in acute or chronic liver diseases.

#### Monoclonal Antibodies against B Cells

**Rituximab** Rituximab is a mouse chimeric antihuman MAb specific for CD20. It eliminates Ig-secreting CD20<sup>+</sup> B cells but does not ablate plasma cells (211). Treatment has been successful in non-Hodgkin's B-cell lymphomas (212), CD20<sup>+</sup> lymphoproliferative disorders (213), prevention of rejection in ABO-incompatible liver transplantation (214), and several autoimmune diseases (211,215). Treatment of patients with hepatitis C and B-cell lymphoma resulted in increased HCV replication (216). Fatal hepatotoxicity after bone marrow transplantation also has been reported (217). Pilot feasibility studies have been proposed for selected patients with refractory AIH or PBC.

**Alemtuzumab** As noted above, alemtuzumab eliminates B cells expressing CD52, resulting in significant lymphopenia (153). The documented risks of serious infections to ablation of all mature T and B cells makes it an unattractive candidate compared with rituximab.

### ANTI-INFLAMMATORY PROPERTIES OF STATINS

It is now clear that statins exert potent antiinflammatory and antioxidant effects (218). In addition to lowering elevated cholesterol in patients with PBC, simvastatin also significantly reduced alkaline phosphatase,  $\gamma$ -glutamyl transferase, and the concentration of IgM (219). Atorvastatin significantly reduced secretion of hepatic acute-phase reactant proteins in patients with hypercholesterolemia (220) and was beneficial in combination with UDCA in the treatment of nonalcoholic steatohepatitis (221). Studies in cultured human hepatocytes have shown that statins inhibit transcription of C-reactive peptide, even in the presence of proinflammatory cytokines (222). Available data support a role for statins in the prevention of the pathogenetic effects of subclinical inflammation of white adipose tissue in the development of nonalcoholic fatty liver diseases (223). Whether chronic statin inhibition of hepatic

gene expression induced by proinflammatory cytokines is also beneficial remains speculative.

### CONCLUDING REMARKS AND OPEN QUESTIONS

Although the pathogenesis of multiple hepatic diseases involves inflammatory and immunological mechanisms, we still lack understanding of the precise pathogenesis of virtually all hepatobiliary diseases. Studies in animal models and patients must be conducted to clarify pathogenetic mechanisms, if we are to take full therapeutic advantage of the availability of immunosuppressive and immunomodulatory agents with the potential to alter liver disease progression selectively. The spectrum of available therapies allows for the rational selection of combination therapies to interrupt multiple sites involved in immunopathogenesis, while minimizing dose-dependent toxicities of individual drugs. The drugs and agents discussed in this chapter have the capacity to prevent or ameliorate hepatic allograft rejection, the inflammation and fibrosis accompanying chronic viral hepatitis, alcoholic and nonalcoholic fatty liver disease, drug-induced hepatotoxicities, GVHD, and autoimmune and IMIDs of the liver. Our challenge is to select the most promising therapies and to evaluate their safety and efficacy in animal models, pilot feasibility trials, and, ultimately, well-designed, appropriately powered RCTs.

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# 31 Hepatic Complications of Hematopoietic Cell Transplantation

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## KEY POINTS

- Liver disease before transplantation can have an impact on the type of transplant chosen and on the frequency of hepatic complications after transplant, for example, sinusoidal obstruction syndrome and fulminant hepatitis B.
- During periods of severe immune suppression after transplant, prophylaxis must be given to prevent hepatic infection caused by HSV, VZV, HBV (for patients at risk), and fungi; fulminant hepatic failure caused by adenovirus is the only common hepatic infection among patients receiving proper prophylaxis.
- The differential diagnosis of liver disease after transplant is guided by the type of conditioning therapy, the choice of hematopoietic stem cell donor, and the period when the liver disorder occurs.
- Transvenous liver biopsies with measurement of the wedged hepatic venous pressure gradient provides a safe and accurate way to diagnose sinusoidal obstruction syndrome (previously called venoocclusive disease) when the clinical diagnosis is in doubt.
- Elevation of serum ALT of more than 1000 U/L is often not caused by infection, but rather by zone 3 hepatocyte necrosis in sinusoidal obstruction syndrome, by hepatitis as a manifestation of graft-versus-host disease (GVHD), by drug-liver injury, or by ischemia caused by septic shock.
- Cholestatic liver diseases are very common after transplant, caused by circulating cytokines (interleukin-6, in septic patients), acute GVHD, drug-liver injury, and (rarely) biliary obstruction. Prophylaxis with ursodiol lowers the frequency of jaundice and improves survival.
- The histologic diagnosis of liver GVHD requires biopsies that contain sufficient numbers of evaluable portal spaces; narrow-gauge needles or forceps that distort portal spaces should be avoided. The characteristic findings are bile duct damage or destruction. The degree of cholestasis and inflammation are highly dependent on the duration of liver GVHD, the presence of concomitant gut GVHD,

immunosuppressive drugs, use of ursodiol, and proximity to donor lymphocyte infusion.

- There is no clear demarcation between acute and chronic liver GVHD nor is there a validated histologic grading system for liver GVHD. Evidence-based observations indicate that the time to recovery of jaundice is proportionate to the severity of bile duct destruction.
- In the current milieu, GVHD rarely if ever causes cirrhosis but may lead to severe cholestatic liver disease caused by ductopenia. With proper management using immunosuppressive drugs, some patients with apparent destruction of all interlobular bile ducts will recover, but the risk of infection is very high during this time.
- Cirrhosis caused by chronic hepatitis C infection has emerged as an important late consequence of hematopoietic cell transplantation, affecting a third of HCV-infected transplant survivors after 25 yr.

## INTRODUCTION

In no other medical situation is a patient at risk for so many liver diseases as during a hematopoietic cell transplant (HCT). The preparation for transplant includes either liver-toxic myeloablative therapy or intense immunosuppression that allows host microchimerism with allogeneic donor hematopoietic cells. As a result, patients are profoundly immunosuppressed until engraftment of infused hematopoietic stem cells; full recovery of immune function is often delayed for a year or longer; and infection with viruses, fungi, and bacteria is common. Recipients of allogeneic stem cells are also at risk for graft-versus-host disease (GVHD) involving the liver, and some patients who have received autologous or syngeneic stem cells may also develop bile duct injury resembling GVHD. Thus, a patient undergoing transplant is at risk for toxic, infectious, and immunologic liver injury. Jaundice after transplant is an ominous prognostic sign, with total serum bilirubin in the 4 to 7 mg/dL range conferring 50% mortality and bilirubin values more than 10 mg/dL conferring more than 70% mortality at d 200 posttransplant (1). Fortunately, the incidence of serious liver injury following transplant has fallen dramatically over the last decade, for several reasons. The most hepatotoxic myeloablative regimens have been abandoned, prophylaxis has

eliminated most hepatic infections (2,3), and GVHD is less frequent. To reduce the morbidity of liver disease after transplant, emphasis should be placed on the recognition of risk factors for liver problems before the transplant process starts, implementation of measures to prevent liver damage, and early recognition of liver disorders that have specific treatments.

## LIVER PROBLEMS BEFORE HEMATOPOIETIC CELL TRANSPLANT

### VIRAL HEPATITIS IN ALLOGENEIC HCT DONORS

Infected donors may transmit hepatitis viruses (3). When equally suitable HLA-matched donors are available, the donor who is not infected should be chosen. Hepatitis B surface antigen (HBsAg)<sup>+</sup> and Hepatitis C virus (HCV) RNA<sup>+</sup> donors can be treated with antiviral therapy to reduce the risk of transmission, but HBV may persist in donor peripheral blood stem cells despite clearance from serum (4,5). Thus, the goal of antiviral therapy of infected donors should be serum and buffy coat cells that are negative for virus by polymerase chain reaction (PCR) before stem cell harvest. Anti-HB core (c)-positive but HBV DNA-negative donors can be used. A donor who is naturally anti-HBs-positive is preferred for an HBV-infected recipient, as adoptive transfer of immunity can effect clearance of virus (6).

### CHRONIC LIVER DISEASE IN CANDIDATES FOR HCT

The risk of fatal hepatic sinusoidal injury (sinusoidal obstruction syndrome [SOS], formerly known as venoocclusive disease of the liver) after some myeloablative regimens is increased 10-fold among patients with inflammatory liver diseases such as chronic hepatitis C, steatohepatitis, sinusoidal fibrosis related to extramedullary hematopoiesis, and amyloidosis (Fig. 1A–C) (7). The risk of fatal SOS is also increased if patients have received recent treatment with gemtuzumab ozogamicin (8) or with other drugs that may cause liver injury, for example, subacute hepatic necrosis caused by imatinib (Fig. 1D) (9,10). Cirrhosis poses a prohibitive risk for developing SOS following most myeloablative regimens and increases the risk from hepatic decompensation after nonmyeloablative regimens (11). In transplant candidates who have risk factors for fatal SOS, modification of the conditioning regimen to exclude the more liver-toxic agents may increase the chance of survival. There is a 35% risk of post-HCT reactivation of HBV in patients with isolated anti-HBc antibodies, usually during treatment for acute GVHD (3). Severe hepatitis B after transplant has been seen in anti-HBc<sup>+</sup>/anti-HBs<sup>+</sup> patients and in a patient with occult hepatitis B (12). In the absence of antiviral prophylaxis, fatal fulminant hepatitis develops in approx 15% of hepatitis B-infected HCT recipients, sometimes after reactivation of occult HBV (3,12). Lamivudine prophylaxis has virtually eliminated HBV-related liver failure after transplant (13).

### GALLBLADDER AND BILE DUCT STONES

Patients with asymptomatic gallstones do not require operative intervention, but cholecystectomy should be considered before transplant if symptomatic cholelithiasis or

choledocholithiasis are found. The risk of cholangitis and uncontrolled sepsis is high and the therapeutic options limited if gallstones cause obstruction during a time when a patient lacks neutrophils and platelets post-transplant.

### IRON OVERLOAD

Hepatic iron levels may be very high in diseases such as thalassemia, aplastic anemia, and chronic leukemia or lymphoma. In patients with extreme iron overload, effective pre-HCT chelation therapy improves post-HCT survival (14). In most patients, quantitation of tissue iron stores and its mobilization can be deferred until after transplant.

### FUNGAL LIVER INFECTIONS

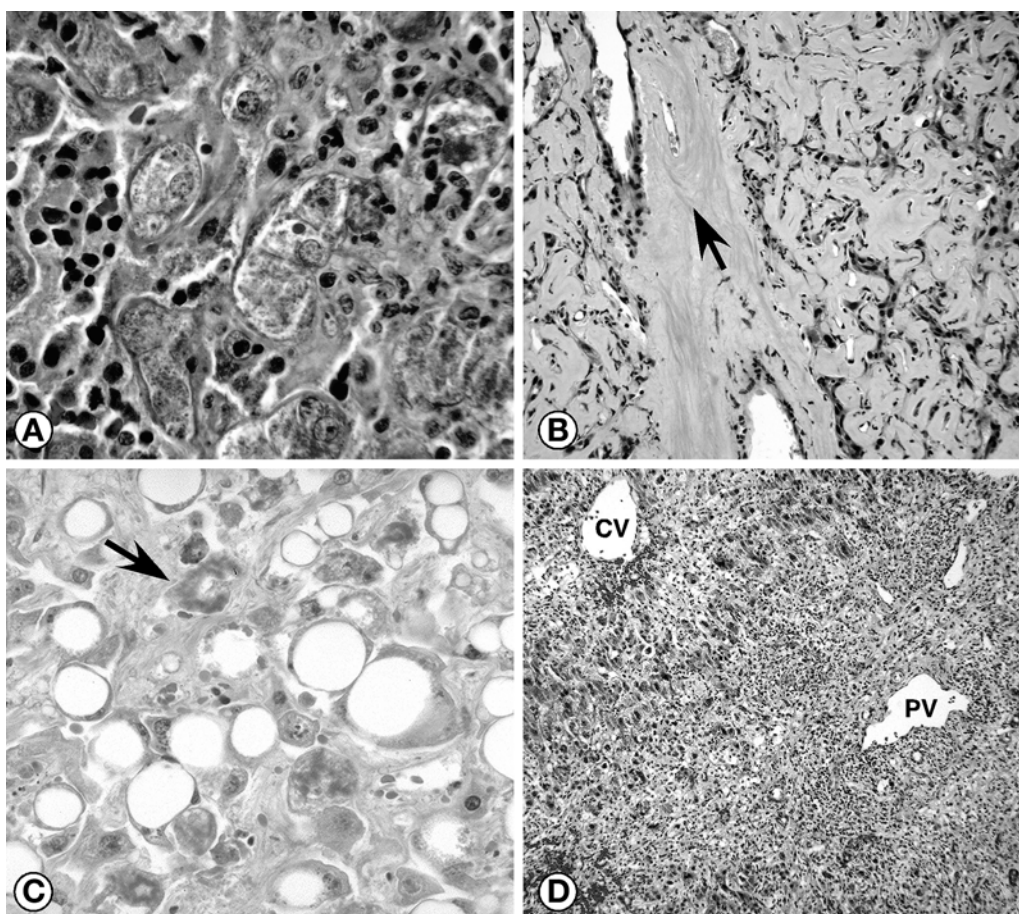
Hepatic fungal infection is best identified by liver pain, positive serum tests for fungal antigens or DNA, magnetic resonance imaging, and, if necessary, histology (15). Laparoscopic biopsies of focal liver lesions provide the opportunity to distinguish lesions containing identifiable fungal organisms from fibrosis and granulation tissue surrounding sterile necrotic debris. Liposomal amphotericin, voriconazole, or caspifungin should be given until engraftment is established (16). Prophylaxis with antifungal drugs will prevent almost all candidal infections after transplant, but azole drugs inhibit hepatic cytochrome P450 enzymes, affecting metabolism of many drugs, including cyclophosphamide (17).

## LIVER PROBLEMS IN THE FIRST 200 D AFTER TRANSPLANT

### HEPATIC DRUG TOXICITY

Except for sinusoidal liver toxicity caused by high-dose myeloablative therapy, which causes a distinct clinical syndrome, proving that a drug is causing hepatic injury in this milieu is difficult. Polypharmacy is the rule after transplant, with most patients receiving drugs for infection prophylaxis (usually acyclovir, fluconazole, and trimethoprim-sulfamethoxazole), GVHD prophylaxis (usually tacrolimus or cyclosporine plus methotrexate or mycophenolate mofetil), antiemetics, antihypertensives, and ursodiol. When specific infections or GVHD are diagnosed, a wide range of other medicines is used. The diagnosis of hepatic drug injury should be considered when the histologic features in the liver biopsy are not typical for liver GVHD or liver tests have worsened despite adequate treatment for GVHD. For example, a pseudo-ground-glass hepatocyte change has been recently described in immunosuppressed patients on numerous medications; the ground-glass hepatocytes contain periodic acid-schiff (PAS)-distase variable accumulations of abnormal glycogen (18).

**Sinusoidal Obstruction Syndrome** Toxins contained in myeloablative conditioning regimens may damage hepatic sinusoids, leading to hepatomegaly, fluid retention, weight gain, and elevated serum bilirubin in the first 20 to 30 d after transplant. This form of injury is termed SOS (19). The term “venoocclusive disease” is a misnomer, as the primary pathology is in the sinusoids (19), and venules are patent in up to 25% of fatal cases (Fig. 2) (20). Individual variability in cyclophosphamide (CY) metabolism, total body irradiation



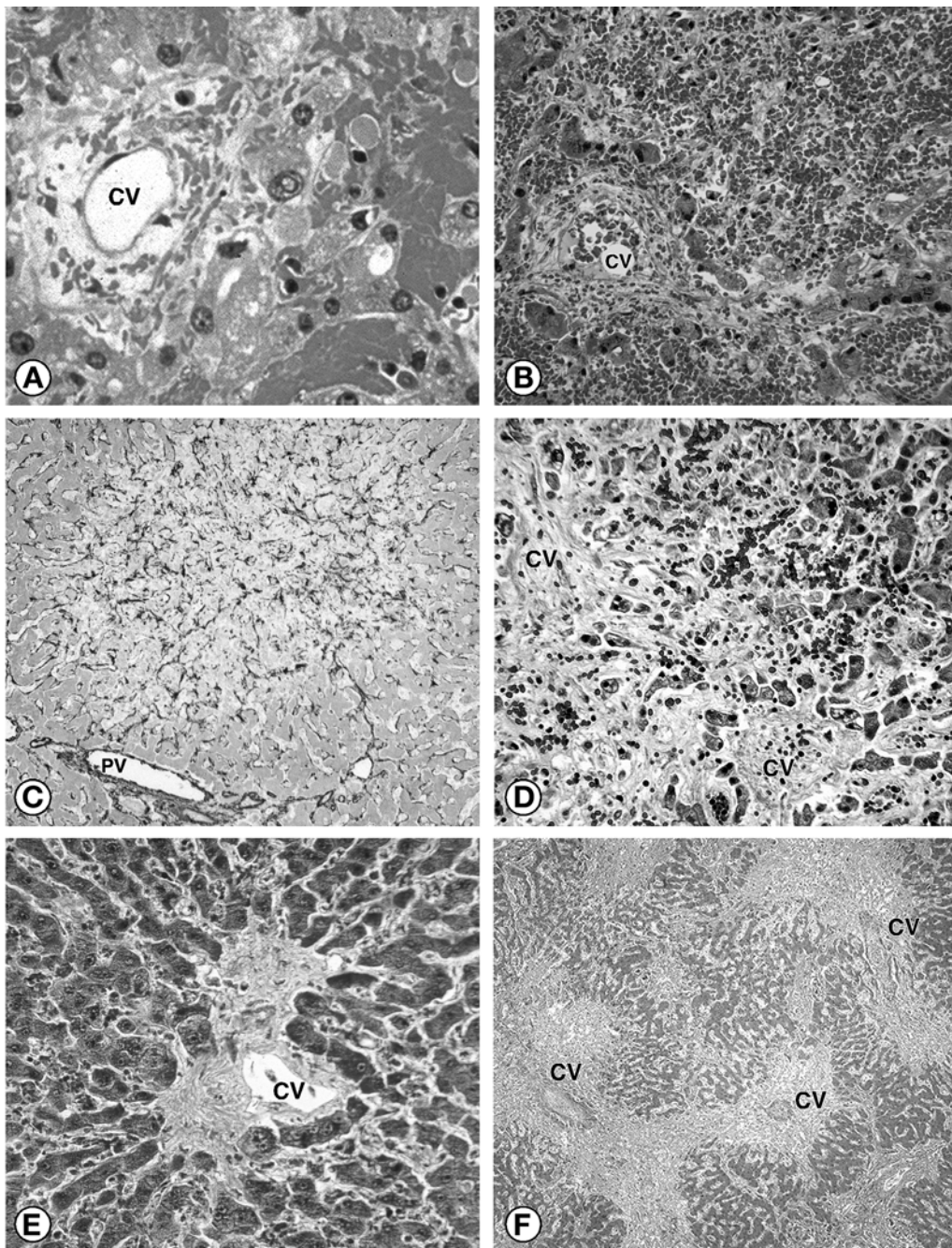
**Fig. 1.** Histology of some uncommon hepatic disorders that increase the risk of fatal outcome following myeloablative hematopoietic cell transplant. Not pictured here is the histology of chronic viral hepatitis and alcoholic liver disease, which are also risk factors. (A) Extramedullary hematopoiesis with extensive matrix deposition in sinusoids, from a patient with agnogenic myeloid metaplasia and myelofibrosis. (Masson trichrome). (B) Amyloidosis with extensive amyloid deposition within a hepatic artery (arrow) and in sinusoids, compressing remaining periportal hepatocytes. (H&E). (C) Steatohepatitis with macrosteatosis, multiple hepatocytes containing hyaline and pericellular fibrosis. (H&E). (D) Subacute hepatic necrosis with extensive portal inflammation and periportal collapse caused by imatinib. CV, central vein; PV, portal vein. (Masson trichrome).

(TBI) dose, use of gemtuzumab ozogamicin, and preexisting liver inflammation and fibrosis are risk factors (7,21,22). The frequency of SOS varies in proportion to these factors; the case fatality rate, however, is relatively constant from center to center, at 15 to 20%. At our center, the overall incidence of SOS among patients with hematological malignancy conditioned with CY 120 mg/kg plus TBI 12 to 13.2 Gy is 38% (7% severe), and among patients with myelodysplastic syndrome conditioned with targeted busulfan (BU) plus CY 120 mg/kg, it is 12% (2% severe). A myeloablative regimen of fludarabine and targeted BU does not appear to cause similar sinusoidal damage (23,24). There is no sinusoidal liver toxicity from a nonmyeloablative regimen of fludarabine plus low-dose TBI (11).

A clinical diagnosis of sinusoidal injury may suffice if typical signs develop before day +20 post-transplant, but Doppler ultrasound, measurement of the wedged hepatic venous pressure gradient, and liver histology may be needed

in difficult cases (25). Initial histologic changes of SOS are dilation of sinusoids, extravasation of red cells through the space of Disse, necrosis of perivenular hepatocytes, and widening of the subendothelial zone in central veins (Fig. 2) (19). The later stages are characterized by extensive collagenization of sinusoids with variable degrees of obstruction of venular lumens by collagenized walls (Fig. 2). A less severe form of liver toxicity from conditioning therapy may result in only focal sinusoidal damage or phlebrosclerosis, an eccentric perivenular fibrosis without luminal narrowing (Fig. 2E).

Staining of liver biopsies with trichrome is the most useful and accurate way to confirm the diagnosis of SOS (Fig. 2B, D, and E). Within a few weeks of toxin exposure, immunofluorescent staining demonstrates accumulation of fibrin adjacent to the adventitia of veins and in the subendothelial zone through which sinusoidal pores must penetrate to reach the lumen of the vein, thus leading to sinusoidal obstruction (26). Immunostains for the sinusoidal endothelial cell markers



**Fig. 2.** Histology of sinusoidal obstruction syndrome (SOS) following high-dose myeloablative therapy. CV, central vein; PV, portal vein. **(A)** Central vein and zone 3 hepatocytes in an early phase of SOS, with disruption of sinusoidal anatomy, red blood cells extending through the space of Disse, hepatocyte necrosis, and subendothelial edema in a patent central vein. (H&E). **(B)** Central vein and zone 3 of the liver acinus, with widespread hepatocyte necrosis and dropout, disruption of sinusoids, extravasation of red blood cells throughout zone 3, and subendothelial fibrosis. (Masson trichrome). **(C)**  $\alpha$ -Actin-positive stellate cells within zones 2 and 3 that contain areas of extensive hepatocyte necrosis; periportal hepatocytes are intact. ( $\alpha$ -Smooth muscle actin immunohistology). **(D)** A later phase of SOS, showing extensive collagenization of sinusoids adjacent to two central veins, with hepatocyte dropout and extinction of hepatocyte cords in between the veins. (Masson trichrome). **(E)** Central vein and zone 3 hepatocytes later after transplant, illustrating eccentric phleboscclerosis and collagen deposition in sinusoids. (Masson trichrome). **(F)** Lower power view of confluent fibrosis in and around adjacent central veins, with central to central bridges forming a picture of “reverse” cirrhosis 2 mo after transplant. (Masson trichrome).

CD31 (PecaM), Ulex, and FVIII/vWF demonstrate a loss of staining for sinusoidal endothelial cells in zone 3 as well as cyokeratin staining of hepatocytes (26). At the interface between viable zone 2 and nonviable zone 3 hepatocytes, large numbers of CD8-positive macrophages accumulate. Within a few weeks from the toxin exposure, immunostaining for smooth muscle actin, a marker of hepatic stellate cell activation and proliferation, demonstrates a continuous staining in the damaged zone 3 sinusoids (Fig. 2C). In severe SOS—if patients survive beyond d 50 post transplant—a pattern of reverse cirrhosis may develop with extensive linkage between obliterated central venules by fibrous bridges, collapse, and acinar extinction (Fig. 2F).

The severity of SOS has been classified as mild (clinically obvious, requires no treatment, and resolves completely), moderate (signs and symptoms require treatment such as diuretics or pain medications, but resolve completely), or severe (requires treatment but does not resolve before death or d 100). A range of clinical and laboratory findings corresponds to these operational definitions of disease severity (2). A statistical model has been developed that predicts the outcome of SOS after CY-based regimens, derived from rates of increase of both bilirubin and weight in the weeks following transplant (2). A poor prognosis correlates with the rate of bilirubin elevation and weight gain, higher serum ALT values, higher portal pressure, development of portal vein thrombosis, and multiorgan failure. Treatment of severe SOS is unsatisfactory; the best current results (45% response) are with intravenous defibrotide (25 mg/kg/d), a porcine oligonucleotide that has effects on microvascular endothelial cells (27).

Whenever possible, patients with severe SOS should be enrolled in clinical trials. Prevention of sinusoidal injury is likely to be more effective than treatment. If a CY/TBI regimen must be used for a patient at high risk for fatal SOS, modifications should be considered for both CY and TBI dosing, with the understanding that clinical trials to prove efficacy and safety have not been done. The total dose of CY should be in the 75 to 100 mg/kg range, and TBI doses should not exceed 12 Gy (28). If a BU/CY regimen must be used for a patient at high risk for fatal SOS, liver toxicity appears to be less frequent if CY is given before targeted BU (this requires intravenous BU, as oral BU is poorly tolerated after CY infusions) (29) or if dosing of CY is delayed for 1 to 2 d after completion of BU (30). It is not clear whether intravenous BU offers any advantage over oral BU with regard to liver toxicity from a BU/CY regimen when both are dosed to the same steady-state concentration. Alternatively, substituting fludarabine for CY may reduce liver toxicity (23,24). There may be value in prophylaxis of SOS with repletion of intracellular glutathione (GSH), or inhibition of matrix metalloproteinase enzymes, or infusion of defibrotide. Large-scale clinical trials of these modalities have not been reported. Prospective studies have shown no benefit from use of heparin, ursodiol, or antithrombin III for prevention of fatal SOS (reviewed in ref. 19). Although a recent meta-analysis suggests that prophylactic ursodiol results in a lower frequency of SOS (30a), it is likely that what is being prevented by ursodiol is cholestatic jaundice, not sinusoidal injury.

In the 30-d period following transplant, the most common cause of serum ALT elevation over 1500 U/L is hepatic necrosis caused by SOS, with peak values at  $d 23 \pm 9$  post transplant, a result of ischemia in zone 3 of the liver acinus, related to poor perfusion. Extreme elevations of ALT portend a poor prognosis. Septic shock with prolonged hypotension may present similarly.

**Calcineurin Inhibitors** Cyclosporine and tacrolimus inhibit canalicular bile transport and contribute to mild jaundice, particularly when blood levels are above the therapeutic range (31); the effect is solely on bilirubin levels, as ALT and alkaline phosphatase levels remain normal (32).

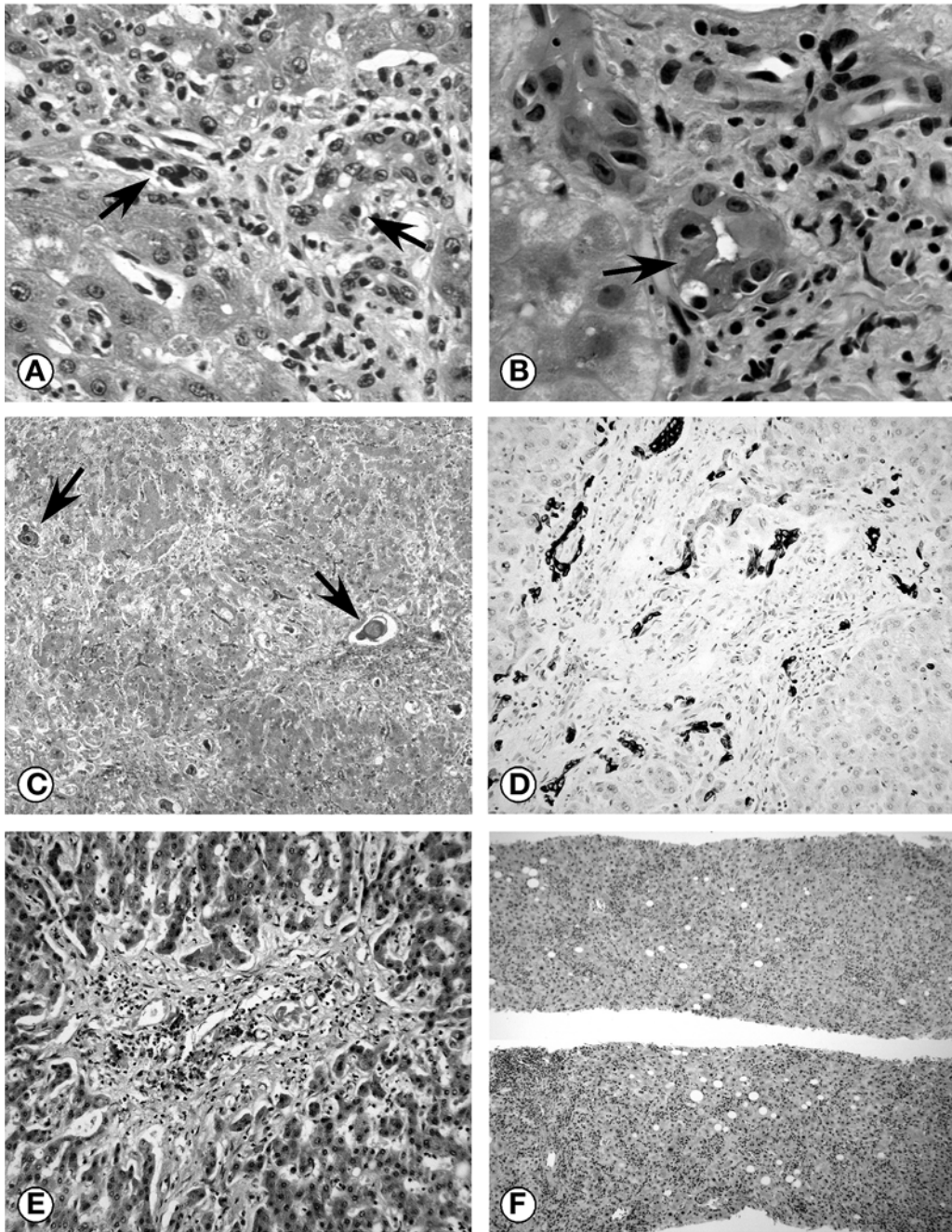
**Antimicrobial Drugs** Of drugs commonly used in the transplant setting, trimethoprim-sulfamethoxazole, itraconazole, voriconazole, and fluconazole are the most commonly associated with elevations of serum ALT and alkaline phosphatase.

**Gemtuzumab Ozogamicin** When gemtuzumab ozogamicin (a conjugate of the toxin calicheamicin with anti-CD33) is given for relapsed acute myeloid leukemia following transplant, liver damage may result, probably because of CD33<sup>+</sup> cells resident in sinusoids (Kupffer cells, myeloid leukemia cells, and possibly stellate cells). Clinical manifestations are hepatomegaly, ascites, and jaundice, similar to SOS following high-dose myeloablative therapy (33). Liver histology reveals activated stellate cells and intense deposition of collagen in sinusoids with varying degrees of venular obstruction.

### GRAFT-VERSUS-HOST DISEASE

Acute GVHD is the most common cause of severe cholestatic injury, as alloreactive T cells recognize foreign major and minor histocompatibility antigens as well as adhesion molecules expressed on biliary epithelial cells. Hepatic GVHD usually follows cutaneous and/or intestinal GVHD and is heralded by a gradual rise in serum bilirubin, alkaline phosphatase, and aminotransferase enzymes (2). In allograft recipients on minimal immunosuppression or after donor lymphocyte infusion, GVHD may present as an acute hepatitis (34,35). A cholestatic condition identical to GVHD occurs rarely in autologous HCT recipients (36). Characteristic liver biopsy findings in GVHD include lymphocytic infiltration of small bile ducts, nuclear pleomorphism, and epithelial cell dropout (Fig. 3) (37).

The histological diagnosis of liver GVHD is based on the global assessment of dysmorphic or destroyed interlobular bile ducts infiltrated by lymphocytes along with cholestasis and inflammatory changes (37–39). The histological interpretation is affected by the quality, size, and timing of the sample. The diagnosis may be obscured if the biopsy is obtained with thin core needles, partially crushed by transvenous forceps biopsy, or of short length with few portal spaces. Liver biopsies obtained shortly after the onset of liver abnormalities may not have developed bile duct damage (37). The inflammation in liver GVHD is variable; it may be scant with the use of multiple immunosuppressive agents or exuberant after donor lymphocyte infusion or when immunosuppressive agents are tapered. Characteristic bile duct changes include an irregularity and redundancy of the bile duct outline, nuclear pleomorphism,



**Fig. 3.** Histology of graft-versus-host disease (GVHD) involving the liver. (A) Portal area showing small bile ducts (arrows) with a distorted appearance, lymphocyte infiltration, and epithelial dropout. (H&E). (B) High-power view of adjacent small bile ducts, showing dysmorphic features, cytoplasmic eosinophilia, apoptosis (arrow), atypical nuclei, and lymphocytic infiltration. (H&E). (C) Low-power view of liver lobules from a patient with severe multisystem acute GVHD, showing expanded, fibrotic portal spaces and periportal bile thrombi (arrows). (Masson trichrome). (D) Immunohistochemical stain for cytokeratin 19 in a patient with long-standing liver GVHD, illustrating ductular reaction at the periphery of a portal space along with staining of putative progenitor cells in the lobule but without an identifiable interlobular bile duct. (E) High-power view of a portal space showing absence of recognizable bile duct epithelium in a patient with long-standing refractory chronic GVHD. (H&E). (F) Low-power views of diffuse lobular inflammation, from a patient with a hepatic onset of GVHD following discontinuation of immunosuppressive drug therapy. (H&E).



atypia, hyperchromatism, and uneven nuclear spacing with broad segments of nuclear dropout (Fig. 3A,B). The characteristic damaged interlobular bile duct has a withered appearance, with cytoplasmic eosinophilia and syncytia formation with only a few atypical nuclei (Fig. 3B). Apoptosis of bile duct epithelium is an uncommon histologic finding despite the immune-mediated bile duct damage. In addition to the interlobular bile ducts, the peribiliary glands in the hilar connective tissue are also targets (40).

Patients with concomitant gut GVHD may develop a picture that resembles cholangitis lenta with extensive ductular reaction, and a proliferation of ductules at the margin of portal spaces, along with periportal bile thrombi and increased inflammation (Fig. 3D). This change is thought to be secondary to chronic exposure to tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) from the denuded GVHD-damaged gut showering endotoxin into portal venous blood (41). The proliferated ductules appear to be a target of GVHD because cytological changes are similar to those seen in interlobular bile ducts. The ductular reaction is also a reparative effort in response to damage to interlobular bile ducts, resulting in default activation of hepatic progenitor cells (evinced by cytokeratin 19 staining of cells along the margin of the portal space as well as a small number of cells in the parenchyma, Fig. 3D). The resulting ductular reaction also promotes portal fibrosis (42).

Although GVHD usually presents with jaundice and a cholestatic picture, histologically there is often a component of hepatocyte necrosis, and under some circumstances the presentation is that of an acute hepatitis with serum ALT levels in the 400 to 2000 U/L range (Fig. 3F) (34,35,43). The most common circumstances for a hepatitic presentation of GVHD are following donor lymphocyte infusions in the absence of immunosuppressive drugs (usually done because of relapse of leukemia, to achieve a graft-versus-leukemia effect) and following a taper or discontinuation of immunosuppressive drugs, usually after d 100. Liver biopsy is necessary to make a diagnosis, and treatment is with prednisone and tacrolimus. If untreated, hepatitic GVHD may rapidly progress to ductopenia (Fig. 3E) and deep jaundice (34). The striking amount of inflammation and hepatocyte acidophilic body formation reflects the involvement of the fas/fas ligand system induced by cytokines, with hepatocytes being innocent bystanders (44). Other changes included perivenular inflammation with hepatocyte dropout and numerous pigment-laden macrophages. Despite the similarity of GVHD to chronic liver allograft rejection, endothelialitis is uncommon. The hepatitic onset of GVHD may resemble an autoimmune hepatitis with a prominent plasmacytic inflammatory component that obscures the bile duct damage. This possibility should be considered since rare cases of autoimmune hepatitis have been documented after allogeneic transplantation (45).

The chief difficulty in the differential diagnosis of GVHD is distinguishing immune-mediated bile duct damage from the background reactive or destructive changes seen in other inflammatory liver diseases such as viral hepatitis B and C and

drug toxicity. In a coded histological study, the bile duct changes of HCV were different from those with liver allograft rejection (46), which are similar to those of GVHD (47,48). Nonetheless, some cases of long-standing liver GVHD with portal fibrosis and ductular reaction could not be readily distinguished from HCV (37). Patients with refractory liver GVHD maintained on chronic immunosuppression develop chronic cholestasis with ductopenia and stellate fibrosis with some bridging (Fig. 3E) (49). The earlier anecdotal reports of cirrhosis from GVHD are tainted by a high frequency of HCV infection or inadequate immunosuppression.

The role of liver biopsy in determining the prognosis of patients with liver GVHD is unsettled. Although the prognosis in patients with persistent jaundice after transplant is very poor (1,50), patients can recover from widespread bile duct injury and loss of bile ducts after both liver allografts (51) and allogeneic hematopoietic cell transplant (34). There are no data that indicate whether additional immunostaining using cytokeratin 19 staining of bile ducts, p21<sup>WAF1</sup> (an antibody for senescent cells [52]), or Ki-67 (an antibody for cell proliferation) can clarify the prognosis. It is also unclear whether serial liver biopsies can provide greater insight into prognosis or the need for additional immunosuppressive therapies than examining serum bilirubin, ALT, and alkaline phosphatase values.

#### OTHER CHOLESTATIC LIVER DISORDERS

Cholestasis is the most common mechanism for jaundice following HCT, but dissecting out the exact cause is difficult, as there may be overlapping causes. Prophylaxis with ursodiol, started several weeks before transplant and continued to d 80 post HCT, has been shown to reduce the frequency of jaundice and ALT elevations and to reduce mortality, compared with placebo (53).

**Cholangitis Lenta** Sepsis-associated cholestasis is an important contributor to hyperbilirubinemia after HCT, mediated by endotoxins, interleukin-6 (IL-6) and TNF- $\alpha$  (54). In the past, what was assumed to be GVHD involving the liver in patients with a rapidly progressing skin rash and protein-losing enteropathy was probably a consequence of translocation of endotoxin and bacteria into the portal circulation, causing IL-6 release and cholestasis, as histology of the liver in these patients often fails to show typical bile duct injury (37).

**Extrahepatic Obstruction and Cholecystitis** Gallbladder sludge composed of calcium bilirubinate is found at autopsy in 100% of HCT patients (55). Biliary passage of sludge may cause epigastric pain, nausea, and abnormal serum liver enzymes. Endoscopic papillotomy is rarely indicated. Biliary sludge may be a cause of acute "acalculous" cholecystitis, acute pancreatitis, and bacterial cholangitis (56,57). Diagnosis of cholecystitis is difficult because of the high frequency of gallbladder wall thickening and sludge on ultrasound following HCT, but pericholecystic fluid, gallbladder wall necrosis, or localized tenderness suggest cholecystitis. A radionuclide bile excretion study, with morphine infusion to enhance gallbladder filling, can be useful; nonvisualization of the gallbladder

suggests cholecystitis (58). Persistent biliary obstruction is a rare event, caused by a variety of disorders (e.g., lymphoblastic infiltration of the common bile duct and gallbladder in Epstein-Barr virus [EBV] lymphoproliferative disease; CMV-related biliary disease; dissecting duodenal hematoma complicating endoscopic biopsy; inspissated biliary sludge; and leukemic relapse (chloroma) in the head of pancreas) (59).

## LIVER INFECTIONS

**Viral Infections** Acute hepatitis caused by herpes simplex virus (HSV), varicella zoster virus (VZV), adenovirus, and HBV virus are now very uncommon but can be fatal after HCT (Fig. 4) (3,60,61). Hepatic infections caused by CMV and HCV are seldom severe (7). The mechanisms of hepatocyte necrosis varies with the virus: HSV, VZV, and adenovirus often develop during severe immune suppression, whereas HBV and HCV replicate during immune suppression but seldom cause hepatitis until there has been recovery of immunity (3). With prophylactic acyclovir, acute hepatitis caused by HSV and VZV is now rare, but after acyclovir has been discontinued, VZV hepatitis can present with abdominal bloating, pain, and elevations of serum ALT (62). HHV-6 and HHV-8 reactivation have been associated with the development of fever, rash and hepatitis in HCT recipients. An important histologic point of distinction between SOS and infections from herpesviruses or adenovirus is the random distribution of the necrotic foci with infection, whereas in SOS the necrosis is always in zone 3. The PAS stain is very useful for demonstrating necrotic foci because dead hepatocytes contain no glycogen (Fig. 4E).

When there is uncertainty about the cause of rising serum ALT, DNA blood tests for herpesviruses, adenovirus, and HBV, transvenous measurement of the wedged hepatic venous pressure gradient, and liver biopsy are indicated (Fig. 4). If acyclovir is not being given, it should be started empirically, particularly if the patient presents with the abdominal bloating and elevated serum ALT typical of VZV infection (62). If the patient has concomitant pulmonary, renal, bladder, or intestinal symptoms, adenovirus should be suspected; the most effective treatments are cidofovir and donor leukocyte infusions (61,63–65). Fulminant hepatitis B may develop during immune reconstitution in patients at risk, including those who activate occult HBV, but it can be prevented with prophylactic lamivudine or adefovir (3,13). HCV infections are seldom severe; asymptomatic elevation of ALT is commonly seen from d +60 to +120, frequently coinciding with the tapering of immunosuppressive drugs (7). Therapy directed at chronic HCV infection should be deferred for at least a year post-transplant (see Liver Problem in Long Term Transplant Survivors below). EBV lymphoproliferative disease was commonly seen in allogeneic HCT recipients at d +70 to 100, with the highest incidence in recipients of HLA-mismatched T-cell-depleted grafts and after potent anti-T-cell therapies, manifest in the liver by abnormal serum alkaline phosphatase and massive hepatosplenomegaly (Fig. 4F) (66). This disease is now infrequent because of EBV-DNA surveillance and preemptive treatment (67).

**Fungi and Molds** Prophylaxis prevents almost all candidal infections in the liver; fungi in the liver after transplant are likely to be molds or resistant *Candida* species (68). The signs are fever, tender hepatomegaly, and increased serum alkaline phosphatase levels, but the sensitivity of imaging tests for disseminated military fungal lesions is less than 30% (69). Assays for fungal elements are useful in diagnosis—serum galactomannan assay for mold infection (70,71), and  $\beta$ -D-glucan assay for *Candida* infection (72). Identification of fungal elements in liver tissue that is obtained by guided liver biopsy may be subject to a large sampling error, as only some of the lesions detected by imaging methods contain an active infection with identifiable organisms (Fig. 4A).

**Bacterial Infections** Reactivation of latent mycobacterial infection, including bacillus Calmette-Guérin (BCG), within the liver may occur with prolonged immunosuppressive therapy (73). Bacterial liver abscesses are rare after transplant, probably owing to prompt antibiotics for neutropenic fever. Disseminated clostridial infection and gallbladder infection with gas-producing organisms may lead to air in the liver and biliary system (74).

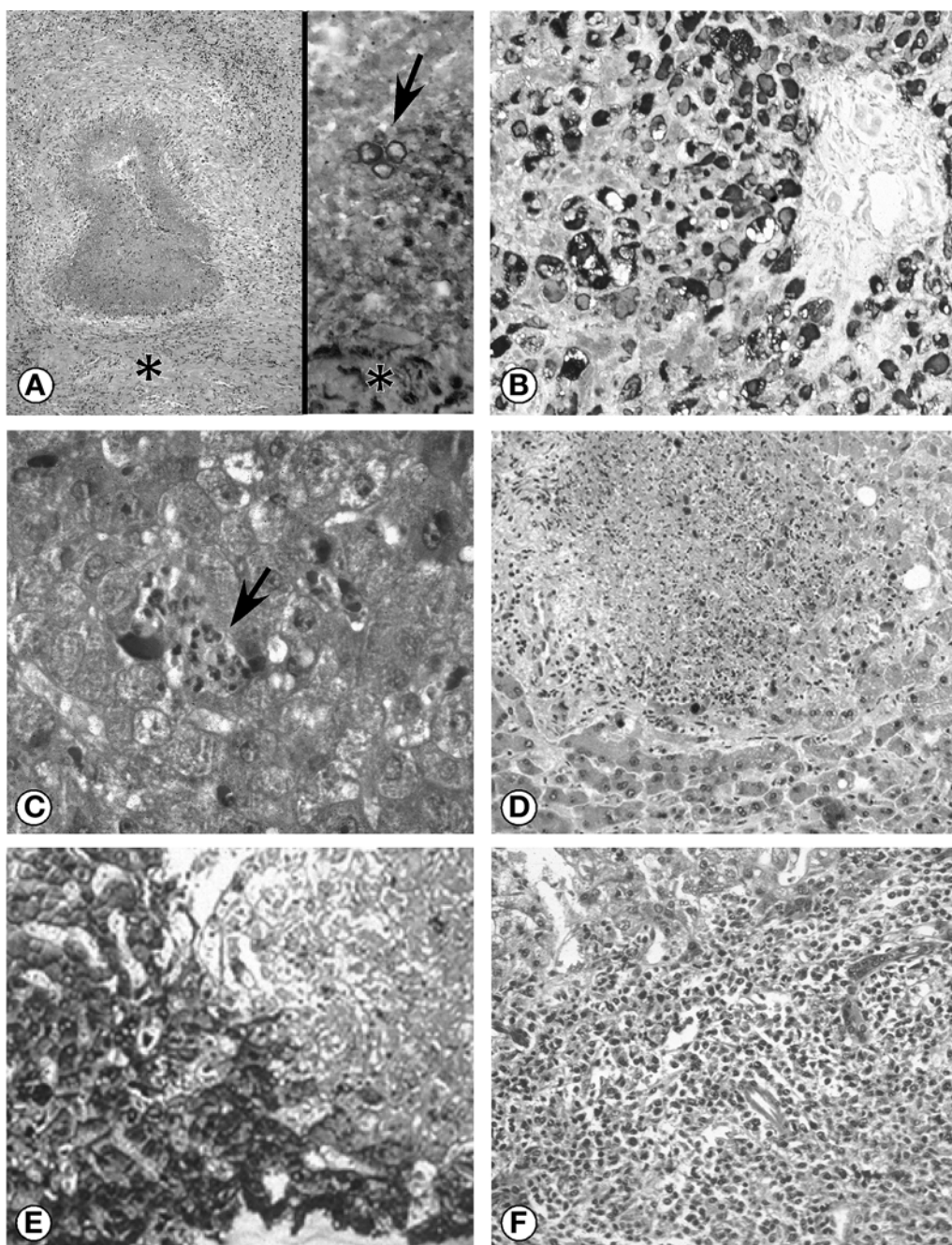
## IDIOPATHIC HYPERAMMONEMIA

A syndrome of hyperammonemia and coma occurs rarely after transplant (75). The presentation is with progressive lethargy, confusion, weakness, incoordination, vomiting, and hyperventilation. The diagnosis is confirmed when the plasma ammonia exceeds 200  $\mu$ mol/L and there is no evidence of liver failure. The outcome is usually fatal.

## LIVER PROBLEMS IN LONG-TERM TRANSPLANT SURVIVORS

### CHRONIC GVHD

Cholestasis is present in 80% of patients with extensive chronic GVHD; however, bile duct damage in a transplant survivor is not considered to be diagnostic of chronic GVHD, but rather a manifestation of protracted acute GVHD (76). The spectrum of liver disease in patients with protracted liver GVHD ranges from elevations of serum ALT and alkaline phosphatase to jaundice. By the time jaundice develops, liver biopsy shows extensive damage to small bile ducts (Fig. 3). Thus, development of jaundice in a patient with biopsy-proven liver GVHD is an indication for more aggressive immunosuppressive therapy, as a completely ductopenic stage of GVHD may not be reversible in some patients. In patients receiving no, or tapering, doses of immunosuppression, liver GVHD may also present with abrupt elevations of aminotransferase levels to over 2000 U/L (34). Infusion of donor lymphocytes in a patient whose immunosuppressive drug therapy has been discontinued may result in a similar acute hepatitis (35). Liver biopsy and PCR of serum are essential to exclude acute viral hepatitis caused by a herpesvirus (HSV or VZV) or a hepatitis virus (Fig. 4) and to make a definitive diagnosis of hepatic GVHD (77). The outcome of acute hepatitis as a manifestation of GVHD in the liver is dependent on prompt recognition and treatment with calcineurin inhibitor and prednisone; delayed treatment may result in death (34). Immunosuppressive drug



**Fig. 4.** Infections in the liver following hematopoietic cell transplant. (A) Fungal liver abscesses, from laparoscopic biopsy, demonstrating the variability of findings in different samples. Left, a sterile healing abscess with a necrotic center devoid of fungal elements, surrounded by inflammatory cells and a pseudocapsule (asterisk). (H&E). Right, an acute abscess with a small focus of red-staining fungal elements (arrow) in a field of degenerative neutrophils, surrounded by a pseudocapsule. (PAS). (B) Immunohistochemistry for hepatitis B core antigen, in a patient with fulminant hepatitis B after transplant, showing extensive periportal hepatocyte cytoplasmic and some nuclear staining. (C) Focal microabscess (arrow) in the liver lobule, caused by cytomegalovirus (CMV), in which lymphocytes and neutrophils are seen adjacent to enlarged, brick-red cells containing CMV. (H&E). (D) Confluent hepatocyte necrosis caused by adenovirus infection; in the rim of hepatocytes surrounding the necrotic area are darker “smudged nuclei” typical of adenovirus. (H&E) Confirmation of adenovirus would come from immunohistochemistry or viral culture. (E) Confluent hepatocyte necrosis (upper right) caused by varicella zoster virus (VZV) infection, with absence of PAS staining of necrotic cells; confirmation of VZV would come from immunohistochemistry or viral culture. (F) Diffuse infiltration by plasmacytoid cells and immunoblasts with displacement of portal structures, caused by Epstein-Barr virus lymphoproliferative disease. (H&E).

treatment of chronic GVHD is successful in 50 to 80% of patients with extensive multiorgan disease. The addition of ursodeoxycholic acid (15 mg/kg/d) may result in biochemical improvement in those with liver involvement (78). In some patients who are long-term survivors of allogeneic transplant, ductopenia caused by GVHD does not appear to be reversible, resulting in persistent deep jaundice. Liver transplantation may be the only option for treatment (79).

### CHRONIC VIRAL HEPATITIS

HCV infection in HCT survivors almost always results in chronic hepatitis (7,80). In the first 10 yr of HCV infection after HCT, there is little liver-related morbidity. However, cirrhosis of the liver related to chronic HCV infection is rising in frequency among patients who were transplanted more than 20 yr ago (45,80). Patients with chronic HCV should be offered therapy with combination pegylated interferon- $\alpha$  (IFN- $\alpha$ ) plus ribavirin (81). Pegylated IFNs, with their longer half-lives, should be administered with caution, as some HCT patients experience rapid falls in platelet and granulocyte counts. Interferon- $\alpha$  may also activate chronic GVHD, but the risk of this complication in patients with only a remote history of chronic GVHD is small.

### IRON OVERLOAD

Iron overload is particularly severe in thalassemic patients who have undergone HCT (82). Iron overload is caused by a combination of multiple red cell transfusions and dyserythropoiesis, leading to increased iron transport by the intestine. After HCT, iron accumulation stops, and body iron stores fall slowly over time (83). The consequences of extreme iron overload in HCT survivors are primarily those of cardiac, pituitary, and pancreatic endocrine dysfunction. Iron overload may also be a cause of persistent elevations of serum ALT after HCT (84,85). Patients with liver iron content more than 15,000  $\mu\text{g/g}$  dry weight should be treated aggressively with both phlebotomy and chelation; when liver iron content is 7000 to 15,000  $\mu\text{g/g}$  dry weight, phlebotomy is indicated; when liver iron content is under 7000  $\mu\text{g/g}$  dry weight, treatment is indicated only if there is evidence of liver disease (86). Mobilization of iron from heavily overloaded patients improves cardiac function, normalizes serum ALT levels, and results in improved liver histology (84–87).

### OTHER CAUSES OF LIVER INJURY

Drug–liver injury may be related to antihypertensive drugs, lipid-lowering agents, hypoglycemic agents, nonsteroidal anti-inflammatory drugs, antidepressants, antibiotics, and herbal preparations. A particular risk of nonsterile herbal remedies in immunosuppressed individuals is the potential for fungal contamination of herbal preparations, leading to translocation of fungal spores into the portal circulation and liver abscesses (88). Compared with the general population, patients who survive over 10 yr post HCT have an eight fold risk of developing a new solid malignancy; the risk of hepatocellular carcinoma is particularly elevated (89). There is a higher than expected incidence of gallstones and stone-related biliary problems after HCT than in an age-matched population, probably related to

formation of biliary sludge (calcium bilirubinate) as nucleating factors immediately after transplant (55). Chronic cyclosporine or tacrolimus dosing may also lead to gallstones, biliary symptoms, and pancreatitis (90).

### CIRRHOSIS

Cirrhosis has emerged as an important late complication of transplantation as a result of a high frequency of hepatitis C in patients transplanted before the mid-1990s. The rate of progression of chronic hepatitis C to cirrhosis appears to be accelerated after transplant, with 25 to 35% of such patients developing cirrhosis within 25 yr (45,80). Transplant survivors whose immune reconstitution is complete and who do not evince chronic GVHD should be strongly considered for antiviral therapy (81). Liver transplantation should be considered in any HCT survivor with incipient liver decompensation; in some cases, the original allogeneic cell donor can be a partial liver donor (79,91).

### CONCLUDING REMARKS AND OPEN QUESTIONS

Over the last decade, hepatobiliary complications of HCT have become better understood and less common, coincident with avoidance of the most liver-toxic conditioning regimens, more accurate HLA matching of donor–recipient pairs, and infection control. At the same time, the process of HCT has been evolving, with new conditioning regimens, a larger repertoire of immunosuppressive drugs, expanded sources of hematopoietic stem cells, application of HCT to older patients, and inclusion of a wider range of diseases as indications for transplant, including autoimmune disorders such as systemic sclerosis, Crohn's disease, and PBC. Even with the current state of knowledge, the hepatology consultant can effect improved outcomes by careful screening of patients before transplant, by recommendation of less liver-toxic conditioning regimens for those at risk for fatal SOS, by institution of prophylactic antiviral therapy for patients at risk of fulminant hepatitis B, and by routine use of prophylactic ursodiol to lessen the impact of cholestatic liver diseases.

The pathologist plays a critical role in the evaluation of patients with liver dysfunction, as histology can distinguish among a number of serious liver diseases with similar clinical presentations (for example, SOS vs GVHD vs viral infection). However, information from the liver biopsy is reliable only if the sample is adequate to address the clinical questions. The utility of liver biopsies to monitor therapeutic response of treatments for GVHD or to provide prognostic information has not been validated. To do so will require standardization of timing of biopsies in relation to immunosuppressive therapy, the use of uniform histologic criteria, and linkage to a large multiinstitutional data base (77). The use of immunohistochemistry for cytokeratin and cellular markers of senescence may aid in assessing the extent of bile duct injury and estimating the time until recovery. However, ductopenia caused by GVHD remains a therapeutic enigma—not dissimilar to the problem of ductopenia related to liver transplant or after drug exposure.

The major unresolved questions in this field are likely to be answered by genomic research, specifically studies that examine polymorphisms responsible for aberrant drug metabolism, toxic liver injury, acute and chronic GVHD, and infection. The use of biological agents is likely to expand—for example, molecules that interfere with the actions of IL-6 may prevent cholestasis in patients with sepsis. The ultimate challenges, however, lie in achieving a deeper understanding of the balance among tolerance to allogeneic donor cells, GVHD, and graft-versus-tumor effects.

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# 32 Acute and Chronic Rejection of the Liver Allograft

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JAMES NEUBERGER

## KEY POINTS

- There are three patterns of liver allograft rejection: hyperacute, acute, and chronic.
- Hyperacute, cellular rejection is rare and is seen primarily in the context of ABO incompatibility.
- Acute (or cellular) rejection occurs in up to 40% of recipients and is most commonly found in the first 3 mo of transplantation.
- Acute cellular rejection is characterized by the triad of bile duct damage, endothelialitis, and portal inflammation.
- Acute cellular rejection is diagnosed histologically.
- Most cases of early cellular rejection respond to a single treatment of high-dose corticosteroids.
- There is no evidence that single episodes of early rejection adversely impact on the survival of the graft, and they may, possibly through the induction of tolerance, lead to improved graft survival.
- Chronic ductopenic rejection, also known as vanishing bile duct syndrome, usually occurs during the first year.
- Ductopenic rejection is characterized by absence of bile ducts and the appearance of foam cells.
- Some instances of ductopenic rejection may resolve (if more than 50% of portal tracts contain bile ducts); use of sirolimus may be helpful in preventing progression.
- Risk factors for chronic rejection include transplant for chronic rejection or for autoimmune diseases and low levels of immunosuppression.

## INTRODUCTION

For the great majority of liver allograft recipients, immunosuppression is required life-long to prevent rejection. Spontaneous tolerance does develop in a small number of recipients. Although major advances have occurred in the numbers and modes of action of immunosuppressive agents available for clinical use, the modes of action of these agents is relatively wide and nonspecific: thus, the need to prevent rejection must be

balanced against the risks of immunosuppression, which may be general (such as the increased risk of infection and some malignancies) or drug specific, such as the renal failure associated with the calcineurin inhibitors. Despite advances in care, death associated with rejection remains a concern (Fig. 1).

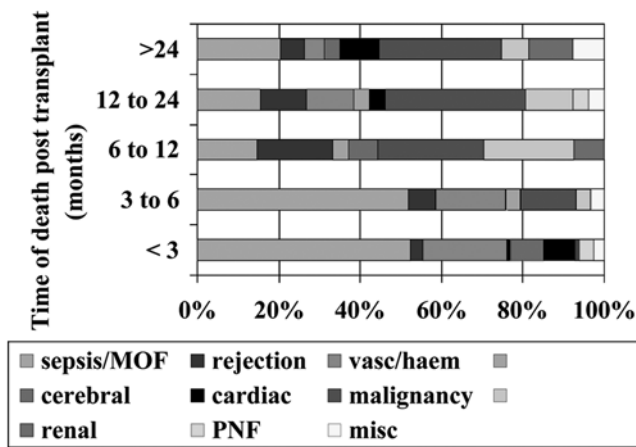
There are many strategies to reduce the risk and impact of rejection, but none has been shown to be superior. Liver rejection is most commonly seen in the first few postoperative months but can occur at any time. Thus, rejection of the liver allograft remains a potential problem, and the clinician needs to be aware of the possibility of rejection.

Liver transplantation differs from transplantation of other solid organs in several important aspects:

- Role of HLA: liver transplantation is done in the absence of matching for HLA and cross-matching; there is little evidence that HLA matching has a significant effect on outcome (1,2). Large retrospective studies have shown that there is a higher rate of graft failure in those who have a zero HLA match (1,2). That this is a true effect of HLA is suggested by the fact that the graft loss occurs early. It has been suggested that HLA matching may play a role in those transplanted for autoimmune hepatitis (AIH) (1). Although recurrence of AIH is well described, the role of HLA matching remains controversial. However, as with renal transplantation, ABO identity or compatibility is observed. When ABO-incompatible grafts are used, there is a greatly increased risk of hyperacute rejection (*see* next section below), although good results have been reported in very young children (3).
- Acute rejection in the liver allograft is not harmful to the graft. Indeed, current data suggest that when liver grafts undergo rejection they actually have a greater survival than nonrejected grafts. Furthermore, grafts with severe rejection have a longer survival than those with mild rejection (4,5). The reasons for this paradox are not fully established but may relate to the theory proposed by Calne of Window for Immunological Engagement (WOFIE) that early immune engagement may lead to the development of tolerance (6).

There are classically three patterns of liver allograft rejection:





**Fig. 1.** Main causes of death post liver transplantation (results from the Liver Unit, Birmingham). MOF, multiple organ failure; PNF, primary graft non-function.

1. Hyperacute rejection.
2. Acute or cellular rejection.
3. Chronic or ductopenic rejection.

## HYPERACUTE REJECTION

Hyperacute liver allograft rejection presents in the early postoperative period as a fulminant hepatic failure. Although it may develop in the first few days after transplantation, later presentations up to the end of the second week may occur. This cause of graft loss was seen more commonly in the early days of transplantation, when the procedure was done without avoiding ABO-incompatible matches. However, much has been learned from animal studies and from xenotransplantation. With the increasing use of living donors, there has been a resurgence of ABO-incompatible transplants, and much interest has focused on the prevention of hyperacute rejection. A number of approaches have been adopted to overcome the effects of complement activation and damage to the vascular endothelium, such as plasmapheresis, intravenous infusion of immunoglobulin (IVIG) or anti-CD20 monoclonal antibody (rituximab), or more invasive approaches like splenectomy and/or hepatic arterial perfusion with prostaglandin E<sup>1</sup> (7). These approaches may be helpful, but there is an associated morbidity and, because about 20% of recipients of ABO-incompatible livers develop such problems, a controlled study is really needed to demonstrate a benefit.

### DIAGNOSIS

**Clinical** The clinical picture of hyperacute rejection is the onset of the signs and symptoms of acute liver failure, usually seen within hours of implantation of the graft.

**Serological** The serum transaminases become rapidly elevated, often reaching levels of 100 times the upper limit of normal. The clotting becomes profoundly deranged, and lactic acidosis and hypoglycemia are seen. A thrombocytopenia is also seen.

**Histological** The histological features of hyperacute rejection are usually identified in the removed liver (either

post mortem or after re-graft) since the coagulopathy usually precludes liver biopsy. The histological features are sinusoidal infiltrates of neutrophils, fibrin, and erythrocytes, progressing to hemorrhagic infarction. There is focal IgM, fibrin, and C1q and C4d deposition (8).

**Radiological** Imaging of the liver is needed to exclude hepatic artery thrombosis.

### RISK FACTORS

As outlined above, preformed antibodies and transplantation across the ABO barriers are the most common risk factors.

### TREATMENT

Supportive treatment and early liver replacement is the only therapeutic option.

**Differential Diagnosis** The main differential diagnoses are primary non-function and hepatic artery thrombosis.

## ACUTE CELLULAR REJECTION

With currently used immunosuppressive protocols, acute cellular rejection is seen most commonly in the first 10 d following liver transplantation (Fig. 2A). The diagnosis is made primarily on the basis of the histology.

### DIAGNOSIS

**Clinical** The literature describes the association of rejection with generalized feelings of poor health, malaise, and poor appetite. Fever is often present and may be associated with rigors. Graft enlargement and tenderness may be detectable. Where there is external biliary drainage, the bile will appear pale.

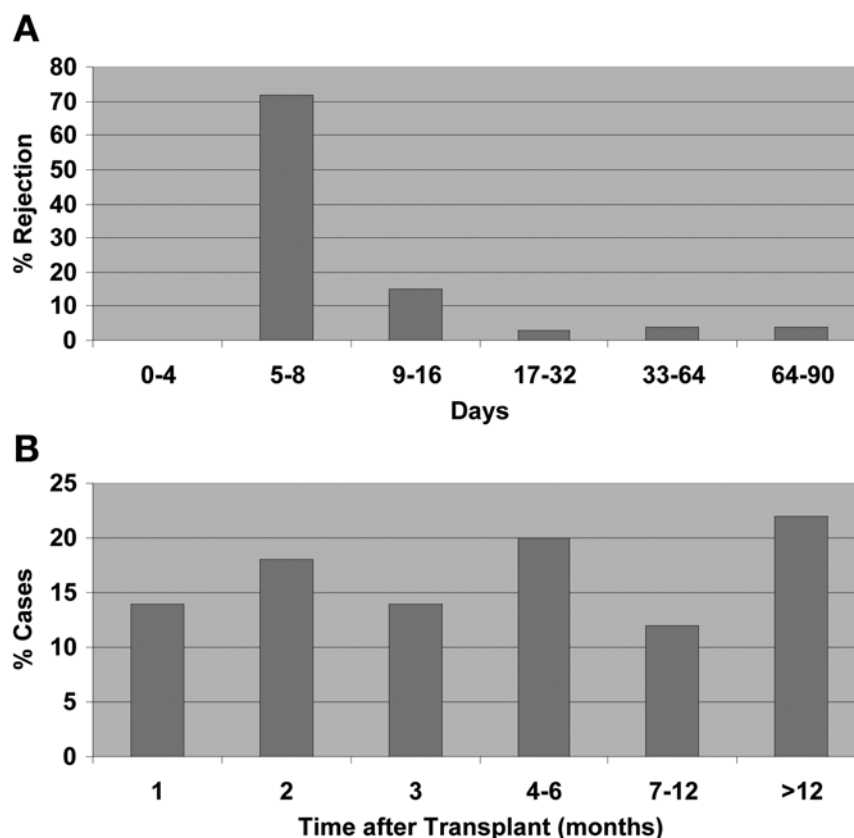
**Serological** The liver test changes in acute rejection are non-specific, and the abnormalities focus more on the serum bilirubin rather than the transaminases. Analysis of serial liver tests shows that there is no reliable test for rejection and that there is little correlation between any blood analyte and the histological features of acute rejection (Table 1) (9). A reduction in the rate of fall or rise in serum bilirubin is probably the best guide to the presence of rejection.

Eosinophilia has been reported to be associated with acute cellular rejection, but this is controversial. The use of peripheral eosinophilia to diagnose or exclude rejection is affected by the use of corticosteroids (10).

Autoantibodies are seen in some patients with acute rejection (11); indeed, those with antibodies against biliary epithelial cells have a greater risk of acute rejection (12); reasons for this observation are not clear.

A number of studies have evaluated noninvasive markers of acute rejection (13), but to date none has had adequate sensitivity or specificity to make or refute the diagnosis.

**Radiological** Measurement of portal blood flow velocity and splenic pulsatility can be assessed using Doppler ultrasonography. In acute rejection, the portal venous blood flow is reduced, the wave form is dampened, and the splenic pulsatility index is slightly increased. These observations can be used noninvasively to detect rejection with a reasonable specificity and sensitivity (14); however, this has not been widely adopted in practice.



**Fig. 2.** Time of diagnosis of (A) acute cellular rejection in the first 3 mo after transplantation and (B) chronic rejection. (Date is taken from the first biopsy showing irreversible rejection.) (Data from the Birmingham Liver Transplant Unit.)

**Table 1**  
**Correlation of Liver Tests With Severity of Rejection<sup>a</sup>**

Test <sup>b</sup>	Nil	Mild	Moderate	Severe
Bilirubin ( $\mu\text{mol/L}$ )	81 (16–466)	109 (17–490)	129 (24–425)	205 (43–699)
AP (IU/L)	277 (109–1123)	336 (73–1213)	521 (198–1444)	476 (218–1877)
AST (IU/L)	27 (12–490)	36 (10–872)	49 (14–1060)	57 (17–440)

<sup>a</sup>Values are shown as medians (with range in parentheses).

<sup>b</sup>Normal ranges: bilirubin  $<15 \mu\text{mol/L}$ ; AP (alkaline phosphatase) 80–320 IU/L, AST (aspartate aminotransferase)  $>35 \text{ IU/L}$ .

**Histological** The diagnosis of acute, cellular rejection is based on the triad of portal inflammation, bile duct damage, and endothelialitis (15) (Table 2) (Fig. 3). The inflammatory response consists of eosinophils, monocytes, and lymphocytes (both CD8 and CD4 in the portal tracts and mainly CD8 around the bile ducts). The portal inflammation consists of lymphocytes, macrophages, eosinophils, and monocytes (Fig. 3). The vascular endothelialitis affects primarily the venules in the portal tract, but the central veins may also be affected. The activated lymphocytes and monocytes invade the vascular endothelium, and this may result in lifting of the membrane.

Other histological features that may be seen include a lobular portal tract infiltrate of lymphocytes, cell necrosis and apoptosis in the lobular areas, and an arteritis of the small arteries.

The severity of rejection can be graded according to the extent of the triad of features (Table 2). The Banff criteria are most widely adopted for this (15). It is not clear whether the severity of the rejection episode reflects the response to increased immunosuppression or the eventual outcome. Although some have found a significant correlation (16,17), our own data (18) show no effect of either the total score or the various subscores on either the response to steroids or the graft outcome. Indeed, we did find that a low score for endothelialitis was associated with a worse outcome.

Most centers use needle biopsy material to confirm the diagnosis of rejection. Fine-needle aspiration has been used and is of similar specificity; however, little information is given about other factors that may affect the graft, such as ischemic/reperfusion injury, structural changes, and some infections, so this approach is not widely used.

#### RISK FACTORS

A number of risk factors for acute, cellular rejection have been identified (Table 3) (19,20). The type of immunosuppression plays a significant role: those grafted with tacrolimus-based immunosuppression have a lower probability of rejection

**Table 2**  
**The Banff Criteria for Acute Liver Allograft Rejection**

Category	Criteria	Score
Portal inflammation	Lymphocytic inflammation involving a minority of triads	1
	Expansion of most or all triads with a mixed infiltrate containing occasional blasts and eosinophils	2
	Expansion of most triads by a mixed infiltrate containing numerous blasts and eosinophils with spillover into periportal parenchyma	3
Bile duct inflammation/damage	Minority of bile ducts infiltrated by inflammatory cells, mild reactive change	1
	Most bile ducts infiltrated by inflammatory cells, more than occasional degenerative changes (pleomorphism, disorder polarity, vacuolation)	2
	As above, but most ducts showing degenerative changes or focal luminal disruption	3
Venous endothelial inflammation	Subendothelial lymphocytic infiltrate of some hepatic/portal venules	1
	Subendothelial infiltration of most or all portal and/or hepatic venules	2
	As above plus moderate to severe perivenular inflammation extending adjacent parenchyma and associated with perivenular hepatocyte necrosis	3

Data from ref. 15.

than those receiving cyclosporine, although comparisons of tacrolimus with cyclosporin using C2 monitoring (adjusting the dose according to the levels 2 h after dosing) do not show such an effect (21). The indication for transplant is another significant factor (Table 4), with those grafted for autoimmune diseases having a greater risk and those grafted for hepatitis B Virus (HBV)-related disease and alcoholic liver disease having less risk. Thus immunosuppression could be tailored to the individual, but this is rarely done in practice.

Cytomegalovirus (CMV) infection may also be implicated; Slifkin and colleagues (22) found that prophylaxis with ganciclovir for 3 mo was associated with a decreased risk of developing rejection (HR 0.78).

Several studies have looked at gene polymorphisms and the risk of liver allograft rejection: polymorphisms within interleukin-6 (IL-6), transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1), RANTES-28, monocyte chemoattractant protein (MCP-1-2518), and CCR5-59029 are not associated with the risk of rejection, whereas IL-10 polymorphism at 1082 and cytotoxic T lymphocyte antigen-4 (CTLA-4) at the 49 A/G single nucleotide polymorphism (SNP) (but not at 318) may be associated with a lower risk of acute cellular rejection (23–25).

### TREATMENT

The need for treatment will depend on the clinical, serological, and histological situation. For those with histologically mild rejection and without progressive deterioration as seen by liver tests, no additional immunosuppression may be needed. With histologically mild rejection, an increase in dose of the calcineurin inhibitor may be sufficient to control the rejection episode. When the rejection is moderate or severe, then additional immunosuppression is required. In our own experience, only 30% of those with histologically mild rejection required treatment with high-dose steroids, compared with 97% of those with severe rejection.

Most centers use a bolus of high-dose corticosteroids for 3 d; there is scant evidence on which to base selection of the dose, duration, or type of steroid used. Centers typically use prednisolone 200 mg/d for 3 d or intravenous methyl prednisolone 0.5 to 1 g daily for 3 d.

Most cases (up to 80%) of rejection will resolve using this approach. When the rejection is recurrent or fails to respond, several options are advocated: a further steroid bolus, use of in antilymphocyte monoclonal or polyclonal antibody (such as OLT3 or ATG) or antibodies to the IL-2 receptor, or a switch in immunosuppression regime (26).

More aggressive approaches have been to use plasmapheresis and radiotherapy of the graft (27).

### IMPLICATIONS FOR GRAFT FUNCTION

As indicated above, most instances of early acute cellular rejection respond well to increased immunosuppression. Of those that fail to respond fully or when recurrent episodes occur, there is a strong likelihood of progression to chronic ductopenic rejection. In contrast, acute rejection occurring late after transplant is associated with a worse outcome and a significant risk to progress to ductopenic rejection and graft loss (28). In our experience, those treated for early acute rejection had a response rate of 75%, and less than 5% proceeded to ductopenic rejection, whereas only 51% of those treated for late acute rejection responded to increased immunosuppression and 27% developed ductopenic rejection.

### DIFFERENTIAL DIAGNOSIS

The main difficulty in making the diagnosis of acute cellular rejection occurs in patients grafted for hepatitis C Virus (HCV) infection. Here, recurrence (or, more accurately, graft infection) will occur in almost all, and thus the pathologist's difficulty is to distinguish HCV re-infection from HCV reinfection and rejection. Since increased immunosuppression encourages viral replication and is associated with earlier graft damage from the virus, this distinction is important. In HCV infection alone, portal tract inflammation, bile duct damage, and interface hepatitis may all be present. The presence of eosinophils and neutrophils in the portal tract infiltrate and significant bile duct damage are more suggestive of rejection. The presence of C4d has recently been more associated with rejection than HCV infection (29). Other viral infections and (rarely) drug toxicity may also give a picture resembling acute cellular rejection.

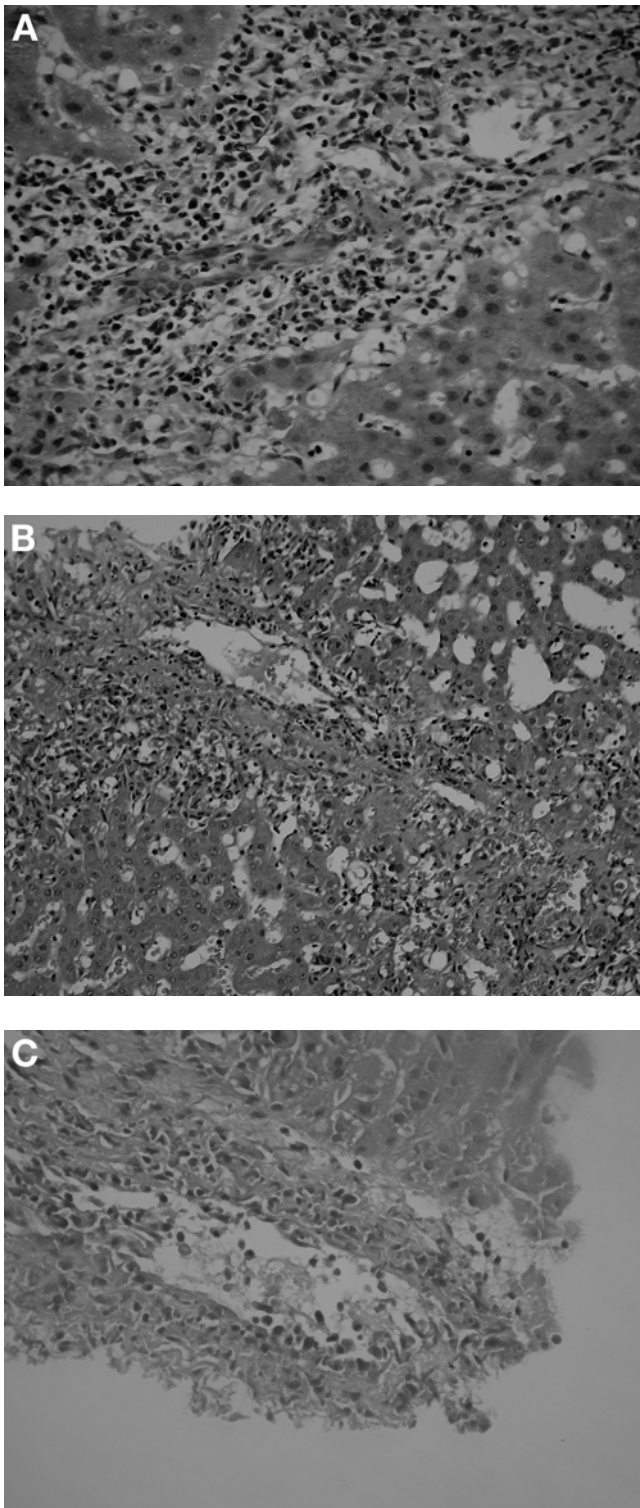


Fig. 3. Histological features of acute rejection.

### CHRONIC DUCTOPENIC REJECTION

Chronic liver allograft rejection has been also termed late allograft rejection, or vanishing bile duct syndrome. The term “chronic” may be misleading, as it implies long-standing

Table 3  
Risk Factors for Acute Rejection

Type and degree of immunosuppression
Indication for transplant
Recipient age
Donor age
Serum creatinine
Some polymorphisms ( <i>see text</i> )

Data from ref. 19–25.

Table 4  
Percentage of Patients with Severe Acute Rejection on Routine Day 7 Biopsy According to Indication for Transplant

Diagnosis	%
Hepatitis C	69
PBC	63
Autoimmune Hepatitis	61
FHF, non-A/non-B	55
Primary sclerosing cholangitis	50
Hepatitis B	46
Cryptogenic cirrhosis	45
Alcoholic liver disease	42
FHF, paracetamol	37

Abbreviations: FHF, fulminant hepatic failure; PBC, primary biliary cirrhosis.

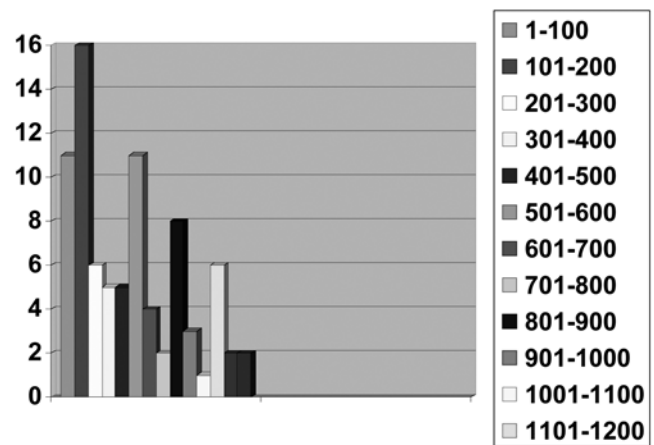


Fig. 4. Incidence of chronic rejection by 100 transplants. (Data from Queen Elizabeth Hospital, Birmingham.)

status, which is not necessarily the case. Nevertheless, the term has become established.

### INCIDENCE

The incidence of chronic rejection has fallen from the level of 20% seen in the early decades of liver transplantation to the current levels of about 3 to 5% (Fig. 4). Reasons for this decline are not clearly established but are probably to be related to improved immunosuppression. Although chronic rejection can develop at any stage during the post-transplant course, it is most commonly seen in the second half of the first year (Fig. 2B).

**Table 5**  
**Features of Early and Late Chronic liver Allograft Rejection**

Structure	Chronic rejection	
	Early	Late
Bile ducts <60 μm	Degenerative changes in most ducts; ducts only partially lined by BECs	Loss in >50% portal tracts
Terminal hepatic venules and zone 3 hepatocytes	Intimal/luminal inflammation; lytic zone 3 necrosis and inflammation	Focal obliteration Severe fibrosis
Portal tract arterioles	Occasional loss, involving <25% portal tracts	Loss involving >25% portal tracts
Large perihilar hepatic artery branches	Focal foam cell deposition	Luminal narrowing by subintimal foam cells
Large perihilar bile ducts	Inflammation damage; focal foam cell deposition	Mural fibrosis
Other		Marked cholestasis Sinusoidal foam cell accumulation

Abbreviations: BECs, biliary epithelial cells. From ref. 32.

## DIAGNOSIS AND NATURAL HISTORY

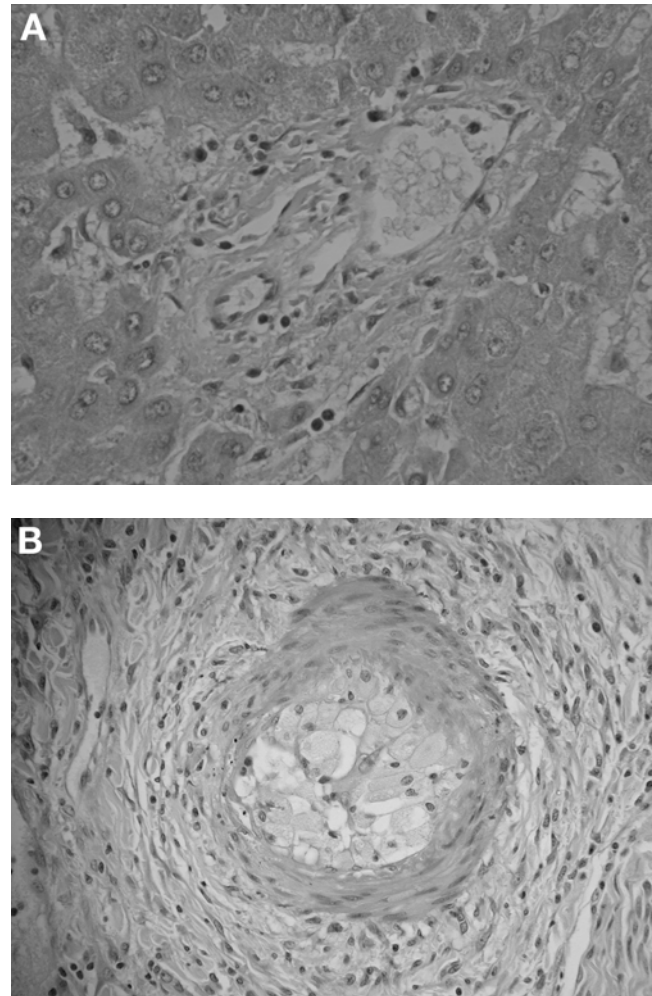
**Clinical** There are four presentations of chronic rejection:

1. Progressive cholestasis. The patient is well, but the liver tests show progression of the cholestatic pattern, with a gradual rise in serum alkaline phosphatase and other markers (such as  $\gamma$ -glutamyl transferase). As the condition progresses, serum bilirubin rises, and the patient develops cholestatic symptoms such as pruritus.
2. Following recurrent, late, or nonresponsive acute cellular rejection. Unlike early acute rejection (within 6 mo of transplantation), late acute rejection is more likely to progress to chronic rejection. Multiple episodes of acute cellular rejection or episodes of acute cellular rejection that fail to respond to higher doses of immunosuppression often herald the progression to chronic rejection.
3. Late chronic rejection. The well recipient starts to develop cholestatic liver tests. Whether this represents the consequences of inadequate immunosuppression or other factors is unclear.
4. Resolving Chronic Rejection. Not all cases of chronic rejection progress to graft failure. Some studies have shown some patients with histological features of chronic rejection, but less than 50% portal tracts having bile ducts can recover with increased immunosuppression and conversion to tacrolimus-based immunosuppression (30).

**Serological** The serology of chronic rejection is one of progressive cholestatic liver tests with late rises in serum bilirubin and fall in synthetic function. There are no serological markers that differentiate chronic rejection from other causes of progressive cholestasis. As in acute rejection, autoantibodies are observed (31).

**Radiological** The liver may be small or normal. Arteriography may show pruning of the smaller hepatic arteries.

**Histological** The diagnosis of chronic rejection is made on liver histology (Table 5) (Fig. 5) (32). In the early stages of the syndrome, the histological hall marks are a vanishing bile



**Fig. 5.** Histological features of chronic rejection.

**Table 6**  
**Risk Factors for the Development of Chronic Rejection**

Immune associated
Transplantation for chronic rejection
Transplantation for autoimmune disease (PBC, PSC, AIH)
Severity and number of episodes of acute rejection
Late (>6 mo) acute rejection
Immunosuppression (lack of azathioprine)
Donor/recipient associated
Donor recipient sex mismatch
Non-Caucasian recipient
Donor age >40 yr
Controversial
HLA matching
CMV infection
Treatment with interferon

Abbreviations: PBC, Primary biliary cirrhosis; PSC, primary sclerosing cholangitis; AIH, autoimmune hepatitis; CMV, cytomegalovirus.  
Data from ref. 28 and 32–36.

duct syndrome in the absence of degenerative changes in the bile ducts with eosinophilic transformation of the cytoplasm, increased nuclear/cytoplasmic ratio, and ducts only partly lined by biliary epithelial cells. Significant inflammation is not a major feature. As the condition progresses, the degree of bile duct loss increases, histological evidence of cholestasis becomes apparent, and sinusoidal foam cells accumulate and may occlude the large perihilar hepatic artery branches. These may not be apparent in needle biopsies.

Following a cell-mediated response against the alloantigens of the graft, CD4- and CD8-activated cells activate the macrophages, leading to their proliferation and the release of chemotactic factors. In particular, platelet-derived growth factor, released from platelets, macrophages, and damaged endothelium stimulate smooth muscle cell proliferation, and release of TGF- $\beta$  leads to their proliferation to myofibroblasts, which secrete extracellular matrix resulting in subintimal fibrosis.

### RISK FACTORS

The risk factors that have been associated with chronic rejection are shown in Table 6. These factors (28,32–36), some of which are immune associated, are not universally described in all series. The most important factor is the indication for transplant: chronic rejection being the greatest risk factor. It should also be remembered that demonstration of a statistically significant association does not prove a causal association.

The role of CMV in the development of chronic rejection remains controversial. On the one hand, studies such as those of Evans et al. (35) do indicate an association and suggest that the virus can infect the biliary epithelial cell and so trigger an immune attack; other studies have failed to show this effect and suggest that the development of chronic rejection may be more associated with the iatrogenic reduction of immunosuppression associated with the treatment of a viral infection. Likewise, the evidence suggesting a role for HLA mismatching is uncertain. A role for minor antigens (such as the HY antigen

or those associated with a mismatch between donor and recipient with respect to ethnicity) in increasing the risk for chronic antigens is likely, but not all studies show the same effect. Part of the explanation for the various and sometimes conflicting conclusions is that most studies are relatively small and use different definitions for chronic rejection.

### TREATMENT

If diagnosed early in the course of disease, there may be a significant response. In those on cyclosporin, conversion to tacrolimus has been of some benefit. Others have adopted strategies of switching to more potent agents, such as sirolimus and mycophenolate, but these studies are small and there are no large prospective studies in this field to guide the clinician.

It has been suggested that sirolimus, which has an effective action against smooth muscle cell proliferation, may be of benefit in either the prevention or the treatment of ductopenic rejection, but there are little clinical data, as yet, to support this hypothesis (37,38).

For those who reach end-stage disease, retransplantation is indicated, although the same process may affect the new graft. There is no clear evidence on which to base management to prevent recurrent disease.

### DIFFERENTIAL DIAGNOSIS

In the early stages, the differential diagnosis is with other causes of vanishing bile duct syndrome, including:

- Drug toxicity.
- Sepsis.
- Viral infection (especially CMV).
- Recurrent disease (especially primary biliary cirrhosis and primary sclerosing cholangitis).
- Hepatic artery thrombosis.
- Biliary obstruction.

### OTHER FORMS OF REJECTION

If liver allograft rejection is defined as an immune-mediated process reacting with donor antigens that results in graft damage, then it could be argued that the *de novo* autoimmune hepatitis seen following liver transplantation (discussed in Chapter 20) is a form of rejection since some data show that the immune response is directed against a donor antigen not seen in the host. Similarly, it has been recognized that after liver transplantation many patients have chronic hepatitis, which cannot be readily explained by processes such as recurrent disease. Whether this is a form of inadequately treated rejection remains uncertain at this time.

### CONCLUDING REMARKS AND OPEN QUESTIONS

Liver allograft rejection remains an important cause of graft damage, but the advent of newer agents has resulted in a lower incidence of both acute, cellular rejection and chronic, ductopenic rejection. The diagnosis of rejection may be suggested clinically or serologically but must be confirmed histologically. The characteristic histology of rejection is now well described.

The major questions for the future are:

1. What should be the optimal approach to immunosuppression be? Most studies have focused on eliminating acute rejection, but some studies suggest that early, acute rejection may encourage tolerance and lead to greater graft survival. The advent and introduction into clinical practice of an increasing array of drugs and other agents offer potentially exciting opportunities to develop a logical approach to the management of immunosuppression.
2. How does one best induce tolerance? Long-term graft failure relates primarily to recurrent disease (which may be affected by immunosuppression) and the consequences of immunosuppression. Achieving tolerance remains a goal for the future; the translation of tolerogenic regimes, effective in smaller animals, has not yet been applied successfully to humans.

## ACKNOWLEDGEMENTS

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# 33 Immunological Tolerance in Allo- and Xenografts

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AFTAB A. ANSARI AND KOVIT PATTANAPANYASAT

## KEY POINTS

- **Allorecognition and its role in transplant rejection.** Whereas T-cell receptors (TCR) expressed on CD4<sup>+</sup> T cells predominantly recognize specific antigenic peptides in association with MHC class II molecules on antigen-presenting cells (APCs), TCRs on CD8<sup>+</sup> T cells predominantly recognize antigenic peptides in association with MHC class I molecules on APCs. In allogeneic combinations, the TCR of the responding cells (recipient T cells) recognizes not only the foreign MHC of the donor tissue cells but also self (recipient) peptides that are processed and presented by donor MHC molecules; thus these self-peptides assume different orientations and conformations to be perceived as “foreign.” Thus, it is the composite response of the many clones of T cells that leads to a robust T-cell activation response. Not only major histocompatibility complex (MHC) molecules but in some cases a composite of minor histocompatibility molecules can serve to induce robust alloimmune responses and lead to transplant rejection. Most MHC typing is now being performed based on sequencing data of MHC molecules and has clearly advanced our understanding of MHC polymorphisms. A composite between MHC typing results and cold ischemia is now being utilized for identifying donor/ recipient combinations awaiting transplantation.
- **Cell lineages involved in the rejection process.** Most acute rejection is a result of preformed donor-specific antibodies against recipient antigens (humoral rejection), and chronic rejection occurs a result of cell-mediated recognition of recipient antigens by donor T cells by either the direct or the indirect pathway. It is now recognized that in addition to the role of antibody and CD4<sup>+</sup> and CD8<sup>+</sup> T cells, the role of NK cells should not be underestimated and this precise role is new a subject of intense study.
- **An update on immunosuppressive drugs.** Although the use of conventional immunosuppressive drugs continues,

a number of clinical trials involving a combination of chemotherapeutic drugs with depleting or nondepleting (blocking) antibodies are in progress. In addition, the use of inhibitors of select intracellular signaling molecules is being studied intensely, owing to their higher specificity for the effector T cells mediating graft rejection and lower potential for side effects including malignancies.

- **Tolerance induction and maintenance.** Although the concepts of central and peripheral tolerance have been recognized for some time, the precise mechanisms of tolerance induction and maintenance continue to be the “holy grail” in the field of immunology. The issue of non reactivity is complex and ranges from ignorance to active suppression involving multiple pathways that include energy, apoptosis, and specific vs nonspecific suppression.
- **Unique mechanisms of immune tolerance in liver transplantation.** The liver has the unique intrinsic ability to induce transplant tolerance across MHC barriers in the absence of immunosuppression. This inherent tolerogenic potential is supported by its large mass, unique circulation, and diverse cellular composition, but most importantly by its capacity to function as a hematopoietic organ.
- **Xenotransplantation.** With increased knowledge of the processes of immune activation and regulation and with new techniques for the development of genetically defined minipigs for organ donors, the routine practice of cross-species organ transplantation may be in sight. Advances have already been made in the prevention of hyperacute and to some extent acute rejection, but the problem of chronic rejection remains a formidable challenge. In addition, the risk of transmission of new infectious agents from xenospecies to humans provides yet another cautionary note.
- **Summary and future directions.** Clearly some of the important issues of this decade involve the ethical issues concerning embryonic stem cell transplants and the use of stem cell transplants for the therapy of a myriad of diseases such as ischemic cardiac disease. Most of these strategies are in their infancy, but they appear to have enormous promise, and their clinical application has attracted a number of new industrial interests. At a more conventional level, the recent advances toward achieving prolonged survival and accommodation of transplanted organs are based

on our growing understanding of the immunologic basis of antigenic recognition, of how immune regulatory networks are governed by contact-dependent and contact-independent interactions between lymphoid and nonlymphoid cells, of the phenotypic and functional diversities that are expressed by different cellular lineages and maturational stages, and of new molecular, chemotherapeutic, and genetic techniques for manipulation and improving organ transplant survival.

## INTRODUCTION

Advances in surgical techniques and ancillary care, in parallel with advances in clinical immunosuppression, are the reasons for the enormous success that has been achieved to date in solid organ transplantation. Such success has also provided a considerable boost in the advancement of our knowledge of immunological tolerance in allogeneic and (relatively more recently) xenogeneic transplantation. Thus, success in human organ transplantation has provided the incentive and foundation for unraveling some of the mysteries of organ transplant acceptance vs failure and has been the foundation for the science of transplantation immunology, which includes to a large extent studies of the fundamental mechanisms of immunological tolerance and the understanding of the concepts involved in self- vs nonself-discrimination.

Such tremendous progress has been made in this field that certain surgical procedures such as kidney, heart, liver, and lung transplantation are now considered standard clinical procedures for end-stage diseases for of these organs. The relative success in such human organ transplantation has also propelled plastic surgeons to initiate composite tissue allograft (CTA) transplantation, with more than 50 transplants being performed worldwide including hands, abdominal wall, vascularized bone, nonvascularized peripheral nerve, vascularized tendons, nonvascularized trachea, larynx, isolated muscle, tongue, ears, and cephalocervical skin flaps (summarized in ref. 1), each utilizing varying protocols of immunosuppression developed during the process of optimizing solid organ transplantation. Although clinical progress in organ transplantation continues to be made, with the discovery of more refined methods to prevent rejection and achieve transplant tolerance, the basic underlying cellular and molecular mechanisms of the induction and maintenance of tolerance have yet to be defined.

The purpose of the present review is to summarize our current knowledge of allograft and xenograft tolerance, with an emphasis on studies performed in both experimental models and human clinical liver transplantation. In 2004, 12,972 renal transplants were performed compared with 2096 heart and 6644 liver transplants in the United States; the number of patients on the waiting list for transplants was 61,778 for the kidney, 3249 for the heart, and 17,563 (2) for the liver, emphasizing the growing problem of donor organ shortage and the nearly 3000 deaths that occurred in patients waiting for organ donors. These striking and yearly increasing numbers have prompted the studies of alternate sources for organ donors and gave birth to the science of xenograft transplantation. In

addition, although the 1-yr survival rate of solid organ allografts has markedly increased in the past three decades, there has been relatively little change in the long-term survival rate of transplants that function at 1-yr post-transplant. This poor long-term survival of organ allografts requires long-term use of immunosuppressive drugs, which logically leads to increased compromise of the immune system, with increased cardiovascular disease, malignancies, and opportunistic infections.

These facts underscore our need to define better the conditions that can lead to transplant tolerance, which would decrease and/or hopefully one day eliminate the requirement for the long-term use of immunosuppressive drugs. To understand the progress that has been made so far on the cellular and molecular basis of allo- and xenotolerance, there is a need first to understand the cellular and molecular basis of allorecognition and transplant rejection.

## ALLORECOGNITION AND ITS ROLE IN TRANSPLANT REJECTION

The initial discovery by Bain, Vas, and Lowenstein (3,4) more than 40 yr ago that lymphoid cells from two genetically disparate subjects of the same species underwent proliferation *in vitro* led to an explosive growth of knowledge in the field of transplantation biology. What was puzzling about this initial observation at that time was that the frequency of T cells that proliferated when cocultured with allogeneic lymphoid cells appeared to be significantly higher than the frequency of T cells that proliferated *in vitro* when cultured with an antigen to which the donor of the T cells was immunized. Thus, the frequency of proliferating cells ranged from 0.1 to 10% in the case of mixed lymphocyte reactions, which was difficult to reconcile with the view that these cells were responding to a specific antigen. It is now recognized that this interaction is secondary to the recognition via clonally rearranged heterodimeric T-cell receptors (TCRs; receptors with specificity for antigens) on responding cells following recognition of molecules on the stimulating cells (termed alloantigens), which in large part are molecules encoded by the major histocompatibility complex (MHC).

The genes that code for the MHC molecules are among the most polymorphic known, with hundreds of alleles for each of the five major loci that have so far been documented, which include three MHC class I loci termed HLA-A, HLA-B, and HLA-C and two MHC class II loci termed HLA-DR and HLA-DQ. We inherit one set of these HLA-A, HLA-B, HLA-C, HLA-DR, and HLA-DQ alleles from each parent, giving rise to the diversity characteristic for each population. The normal physiological function of each of the molecules encoded for by the MHC is to present foreign antigens in the form of peptides to their cognate peptide-specific clones of T cells. The stimulator cells in a mixed lymphocyte reaction (MLR) are the same as an antigen-presenting cells (APCs) during immune responses against foreign antigen. The APCs basically process the foreign antigens into small peptides. In general, if the foreign antigen is extracellular to the APCs, the antigen is phagocytosed, processed into small peptides, and associates

with MHC class II molecules, which then traffic to the cell surface and are expressed as peptide-bearing MHC class II molecules. In the case of intracellular antigens such as intracellular viruses, for example, the viral proteins are also processed into small peptides, but these follow a different intracellular pathway and become associated with MHC class I molecules, which then traffic to the cell surface of the APCs and are expressed as peptide-bearing MHC class I molecules.

The TCRs have specificity for such peptide-bearing MHC class I and II molecules. As a general rule, the TCRs of CD4<sup>+</sup> T cells have specificity for peptide-bearing MHC class II molecules, and the TCRs of CD8<sup>+</sup> T cells have specificity for MHC class I molecules. During an MLR, TCRs on clones of responder CD4<sup>+</sup> T cells and clones of CD8<sup>+</sup> T cells thus recognize their cognate peptide-bearing MHC class II and I molecules, respectively, on stimulator cells, and it is this composite response of all the clones of CD4<sup>+</sup> and CD8<sup>+</sup> T cells that accounts for the high frequency of reactive cells in an MLR.

It should also be kept in mind that in addition to such major MHC gene-encoded molecules, there are multitudes of minor MHC molecules that may be minor individually but in a composite form could be cumulatively just as strong an allogeneic barrier as a disparity at one of the major MHC molecules. This has become apparent in results of MHC-identical bone marrow transplant patients such as in HLA-identical siblings in whom severe and life-threatening graft-versus-host disease (GVHD) has been reported (5). The discovery of the MHC molecules and their extensive polymorphisms led to the science of histocompatibility testing or MHC typing under the establishment of tissue typing laboratories worldwide under the overall hypothesis that matching of donors and recipients would benefit organ allograft survival. Identification of the MHC polymorphisms was performed utilizing pools of alloantisera collected primarily from women following pregnancy during which they would develop antibodies against their non-shared of the husbands MHC antigens.

The laboratory protocols for performing such typings continue to evolve and become more sophisticated and automated, but the interpretation of data derived from such typing results continues to present a formidable challenge and a daunting task. Clearly, with the advent of molecular typing, these earlier problems have to a large extent been resolved. Even in cases in which MHC matching between donor and recipient was often being performed at the expense of problems associated with prolonged cold ischemia times, it was recognized early on that survival of the transplanted organ was highly dependent on the use of immunosuppressive drugs. It now appears that more attention is being paid to cold ischemia times than MHC typing results, although to a large extent some sort of compromise has been reached in arriving at the decision for the selection of donors and recipients for solid organ allografts.

### CELL LINEAGES INVOLVED IN THE REJECTION PROCESS

After several decades of conflicting data on a predominant role for either CD4<sup>+</sup> or CD8<sup>+</sup> T cells, it is now clearly accepted

that either of these two cell lineages can mediate organ allograft rejection, with CD8<sup>+</sup> T cells most likely directing their cytotoxic effect by recognizing peptide-bearing MHC class I molecules on the target cells and releasing enzymes such as perforin and granzyme B, and CD4<sup>+</sup> T cells mediating their cytotoxic effect via Fas/Fas-L interactions. CD4<sup>+</sup> T cells can also recognize alloantigens via either the direct or the indirect pathway. Thus, recipient CD4<sup>+</sup> T cells can recognize peptide bearing MHC class II molecules on donor origin dendritic cells (DCs) directly (direct pathway), or the recipient APCs can pick up donor origin MHC molecules, process them, and present the allopeptides in association with recipient MHC class II molecules to recipient CD4<sup>+</sup> T cells (indirect allorecognition). In either case, following alloactivation, the CD4<sup>+</sup> and CD8<sup>+</sup> recipient T cells can release significant amounts of cytokines and chemokines that lead to the attraction of cells of the innate immune system, which can further contribute to the inflammatory graft rejection process. In this latter regard, considerable interest now exists in the role of natural killer (NK) cells in organ allograft rejection (6). Thus, for a considerable period, the role of NK cells in transplantation was primarily thought to be in the setting of bone marrow transplantation but several lines of data now appear to support a role for NK cells in organ allograft rejection. These findings have also led to a search for immunosuppressive drugs that target this cell lineage, as discussed just below in an update on immunosuppressive drugs. It is also important to note that a role exists for regulatory T cells (T-regs) in the prevention of graft rejection and, in particular the maintenance of tolerance, which is also discussed below in tolerance induction and maintenance. Finally, It is important to recognize the major role that antidonor MHC and non-MHC-specific antibodies can play, particularly in acute graft rejection. Recipients are carefully screened for the presence of such donor-specific antibodies, and appropriate decisions are made prior to transplantation to minimize the potential for acute rejection. However, the role of such antibodies during the chronic rejection period continues to be a subject of debate.

### AN UPDATE ON IMMUNOSUPPRESSIVE DRUGS

Basically, drugs that have been successfully utilized as immunosuppressive agents are targeted at inhibiting the activation of T cells either directly or indirectly. A wide range of immunosuppressive drugs have been identified, including inhibitors of DNA synthesis (inhibitors of purine synthesis), inhibitors of either cytokine synthesis or function, inhibitors of antigen processing and presentation, inhibitors of the expression of cell adhesion molecules, and finally more recently, inhibitors of select intracellular pathways of T-cell signaling. Although these drugs have been highly effective in the prevention of acute rejection, their role in preventing the occurrence of chronic rejection has been questioned.

Since chronic rejection remains one of the major obstacles of organ transplant rejection and long-term use of immunosuppressive drugs is associated with increased risk of malignancies, cardiovascular disease, and opportunistic infections, it is clear that alternative strategies need to be sought. Thus, there continues

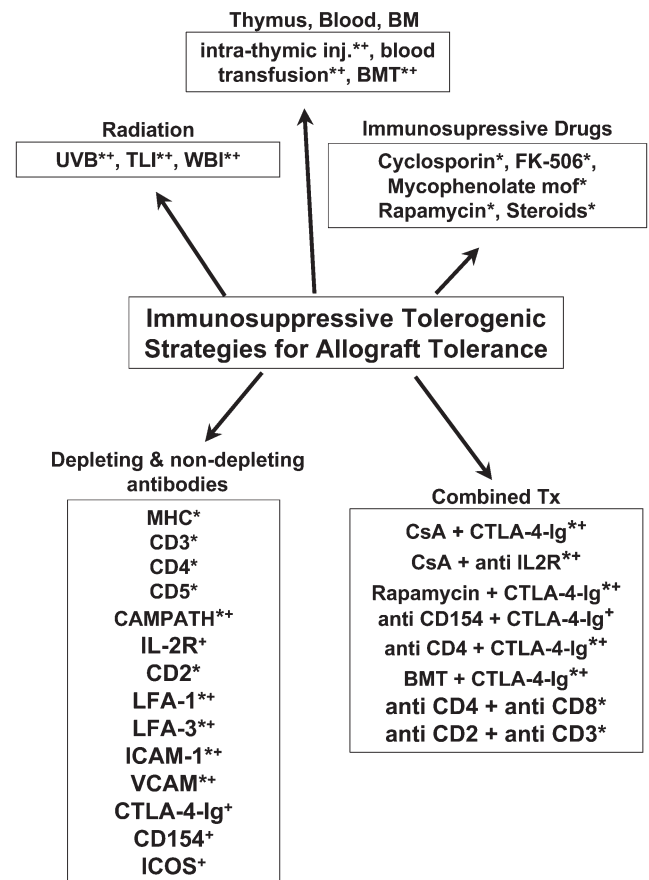
to be considerable interest in identifying novel methods to inhibit chronic rejection. The primary target for kidney and heart transplantation appears to be the vascular bed, whereas for the lung it is the bronchial tree. In the case of renal and heart allografts, chronic insults to endothelial cells results in endothelial cell activation, local smooth muscle proliferation, and fibrosis with the deposition of extracellular matrix proteins, which in concert lead to vascular occlusion.

There has also been an intense search for biological reagents that have the potential to prevent chronic rejection and promote tolerance. These have included antibodies that block costimulatory pathways such as the CD28/B7 and the CD40/CD40L pathways, those that block and/or alter the trafficking patterns of effector cells, and those that block chemokines. One school of thought has proposed a combination of reagents that can be administered at different stages of organ transplantation for minimizing the side effects inherent in each agent and maximizing the induction of true allogeneic tolerance. Most of these, however, are under experimental evaluation.

The general paradigm for the evaluation of these drugs and biological reagents has been to ascertain their efficacy first in small rodent models, then in larger animal models such as dogs and pigs, and then in nonhuman primates before their use in humans. It is also generally accepted that there appears to be a hierarchy in the strength of the allogeneic immune response based on the organ or tissue being studied. Thus, it appears that skin allografts are the most sensitive predictors of allograft rejection and the hardest in which to prevent rejection, followed by the kidneys and islets and then by the heart and liver, the liver being the easiest in which to induce tolerance. There is limited (if any) evidence for chronic liver rejection, and this has been ascribed to the unusual function of the liver in the induction and maintenance of allotolerance. The search for immunosuppressive drugs that inhibit select intracellular signaling pathways is currently gaining considerable interest because they are more selective and not global inhibitors of cell activation, which should limit the long-term side effects. The strategies that have and continued to be utilized for immunosuppression for the prevention of transplant rejection are summarized in Fig. 1.

## TOLERANCE INDUCTION AND MAINTENANCE

Current research in solid organ transplantation is focused on understanding how "self-tolerance" develops naturally in order to develop improved protocols for inducing and maintaining tolerance to transplanted organ allografts. According to the data that have accumulated so far, basically two broad mechanisms have been implicated in the induction and maintenance of tolerance. These include methods and pathways that mediate central tolerance and those that mediate peripheral tolerance. Thus, although for a long time the concept was that all self-reactive T cells eliminated in the thymus (central tolerance), it gradually became clear that central tolerance (thymic deletion of self-reactive T cells) could not account alone for normal physiological T-cell self-tolerance. It was found that a large



**Fig. 1.** Strategies of immunosuppression for the achievement of allograft survival. There are basically five different strategies, which have had variable levels of success in the prolongation of allograft survival. These include (1) use of radiation (ultraviolet B irradiation [UVB] whole-body, introduction [WB]; total lymphoid irradiation, [TLI]); (2) use of thymus (intrathymic injection of donor cells), donor blood transfusion, or donor bone marrow transplant (BMT) to achieve donor-specific tolerance; (3) use of a variety of chemotherapeutic agents; (4) use of a variety of cell lineage-depleting or-blocking key molecules involved in immune interactions by antibodies; and (5) use of a combination of chemotherapeutic agents and depleting/nondeleting antibodies. Those that work by blocking and/or eliminating signal 1 of the immune response are denoted by an asterisk, and those that block and/or eliminate signal 2 are denoted with a plus sign. BM, bone marrow; CsA cyclosporin A; CTLA-4, cytotoxic T-lymphocyte antigen- 4; ICAM, intracellular cell adhesion molecule; IL, interleukin; LFA, lymphocyte function antigen; VCAM, vascular cell adhesion molecules.

number of peripheral tissue antigens never gain access to the thymus and also that other mechanisms besides deletion of self-reactive T cells exist providing evidence for the existence of tolerance mechanisms that are now called *peripheral*. These are briefly discussed below.

## CONCEPTS OF CENTRAL TOLERANCE

The immune system has developed the ability to discriminate self from nonself at the cellular level by deleting bone marrow-derived immature autoreactive T cells in the thymus before they can enter the peripheral circulation. In this process of negative

selection, which provides the basis for central tolerance to self-antigens, immature T cells migrate through cortical and medullary regions of the thymus in search of antigen-specific activation signals necessary to sustain their maturation. Those that fail to encounter cognate antigen do not receive life-supporting growth or activation signals and suffer an apoptotic “death by neglect.” Autoreactive T cells, which express strong affinities for self-antigen presented in the form of individual peptides in association with MHC molecules expressed by thymic epithelial cells, receive a positive lethal apoptosis-inducing signal and are deleted. More than 95% of all T cells entering the thymus succumb to negative selection (7). Immature T cells are only able to survive this decimating process of selection by effecting a type of cognate engagement with self-antigen that is weak enough to avoid triggering positive apoptotic signals yet strong enough to initiate reinforcing maturation signals.

This process of positive selection thus ensures that mature thymic emigrants possess the ability to engage properly in cognate interactions with foreign antigens in the context of self-MHC in the periphery. The deletion mechanisms that define the process of central tolerance are efficient and durable and have therefore been emulated in research and clinical protocols for inducing tolerance to organ transplants (*see* Role for Microchimerism in Liver Transplantation below). A role for the AIRE gene has also been identified in the selection process (8,9), but the precise mechanisms that involve this gene product continue to be a subject of debate. However, the processes involved in positive/negative selection are not perfect (since many of the self-antigens that comprise tissues in the periphery gain limited if any access to the thymus tissue and thus never participate in the negative selection process), and thus nature has also provided backup systems for inducing and maintaining tolerance in mature T cells to self-antigens expressed in the periphery. Gaining insights into these mechanisms has required a more sophisticated understanding of antigen-specific T-cell activation, but such insights have proved even more useful in the development of effective clinical strategies for successful organ transplantation.

### ANTIGEN-SPECIFIC ACTIVATION OF MATURE T CELLS

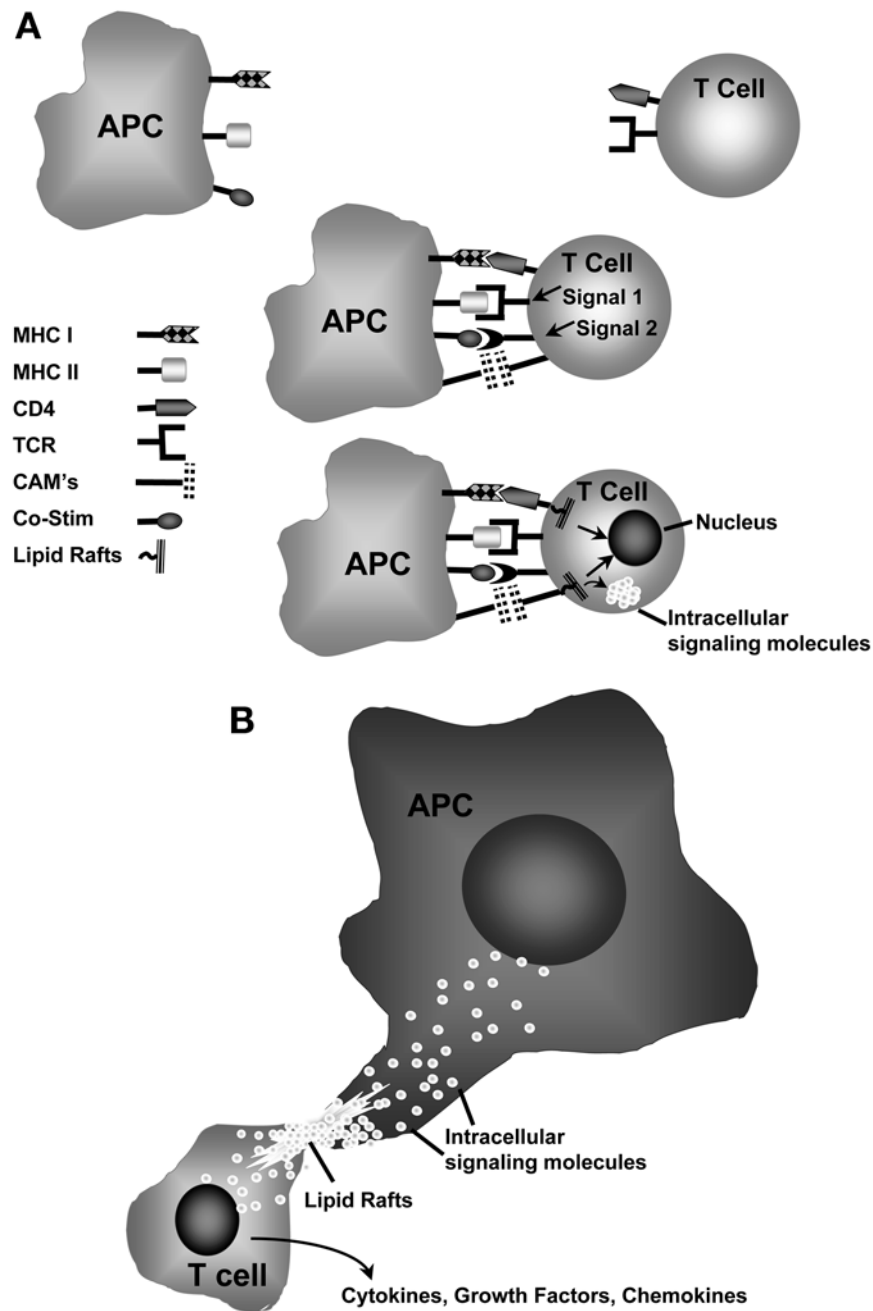
The process of antigen-specific T-cell activation requires at least two signals, as originally proposed by Bretcher and Cohn (10,11) and later revised by Gill and Lafferty (12). These two signals are a result of contact-dependent events between a T cell and an autologous APC. Through their clonally rearranged receptors (TCRs), T cells recognize short linear sequences of antigenic peptides presented in the context of class I or class II MHC antigens expressed by the APCs. The peptides are processed from whole protein antigens by one of two independent intracellular antigen-processing pathways in the APCs (13,14). Cognate interactions between the TCR and the peptide-MHC complex on the APCs results in calcium-dependent signals transduced by transmembrane proteins associated with the CD3 complex on the T cell (15). The TCR-peptide-MHC complex is stabilized by interactions between

CD4 and CD8 accessory molecules with MHC class II or MHC class I, respectively.

These antigen-specific events, referred to as signal 1, initiate the formation of what has been recently termed the “immunological synapse” (IS) (16). The IS represents the intimate contact-dependent association between the T cell and the APC where antigen-specific signals and responses are exchanged and propagated. As the development of the IS matures, critical sets of complimentary costimulatory molecules (CSMs) and cell adhesion molecules (CAMs) on the T cell and APC aggregate and associate around the TCR-MHC complex. The result is attenuation of T-cell migration, enhancement of affinity between other sets of CAMs, (e.g., leukocyte function antigen-1 [LFA-1]/intracellular cell adhesion molecule-1 [ICAM-1] and CD-2/LFA-3) that helps to stabilize T cell-APC interaction and to form a close protected “zone of contact” between the T cell and APC allowing signaling by lower affinity antigen-specific interactions, and generation of potent costimulatory signals (signal 2) for full T-cell activation (Fig. 2A). The formation of the IS, which is also influenced by other signals within the microenvironment (9), can last for hours and serves to ensure the integrity of the antigen-specific (signal 1) and costimulatory (signal 2) signals required for full T-cell activation.

Events that corrupt or compromise this cognate relationship between the mature T cell and its APC influence the quality and strength of the T-cell activation response, which is mediated by disruption of the intracellular signaling pathways, potentially leading to antigen-specific unresponsiveness. What has been difficult to understand is how such interaction between the TCR on the T cell and the cognate peptide-bearing MHC molecule on the APC (in addition to the multitude of other signals that the T cell receives) leads to integration of these signals and the generation of an immune response. It is becoming increasingly clear that during such T cell/APC interaction, lymphocyte surface proteins cluster into microdomains, which consist primarily of membrane lipids and are hence termed *lipid rafts*. Lipid rafts are thus patches of the lymphocyte cell membrane that is rich in cholesterol and sphingomyelin, and it is these lipid rafts that serve as a platform to which intracellular signaling molecules localize. Thus these lipid rafts serve to orchestrate protein trafficking and the regulation of intracellular signaling (Fig. 2B).

Full and productive T-cell activation following interaction with peptide bearing APCs is also strongly influenced by the nature and developmental state of the APC. For cognate T cell-APC encounters in the nonlymphoid peripheral environment, the integrity of the IS may be more greatly influenced by the nature of the APC. APCs can be classified as professional, semiprofessional, and nonprofessional based on their relative abilities to process and present antigen and to functionally deliver activating costimulatory signals to T cells (17). The DC is the prototypic professional APC. DCs get their name from their morphology, which is characterized by finger-like (dendritic) projections. In mature forms, these finger-like projections richly express potent costimulatory molecules such as CD80 and CD86 and MHC-bearing processed antigenic peptides



**Fig. 2.** Interaction between T cells and antigen presenting cells (APC) for the induction of an immune response. T-cells express a heterodimeric molecule called the T cell receptor (TCR), which has specificity for a specific peptide-bearing MHC molecule expressed by APCs. The dialog at the cell surface between the T cell and its cognate peptide-bearing MHC-expressing APCs is converted into biologic activity in a series of sequential steps. The first step following interaction between the TCR and the peptide-bearing MHC molecule (signal 1) is followed by the up-regulation of B7 on the APC and its interaction with CD28 on the T-cell, leading to the generation of signal 2. Such interaction is further facilitated by increasing the stability of the interaction between the two cell lineages by the binding of CD4 with MHC class II and the binding of cell adhesion molecules (CAMs) with their respective ligands to form the immunological synapse. **(B)** The intracellular cytoplasmic tails of these series of molecules become activated and contain signature motifs that attract a series of kinases, and the interaction among these kinases is facilitated by the formation of lipid rafts that serve as scaffolds to promote efficient transmission of signals to the nucleus for the activation and transcription of appropriate promoters for proteins involved in cell activation such as cytokines, chemokines, and growth factors.

and thus are capable of delivering the full set of antigen-specific and costimulatory signals necessary for activation of naïve and mature T cells (18). Mature DCs are present predominantly in the T- and B-cell germinal centers of primary and secondary lymphoid organs, e.g., the thymus, spleen, and regional lymph nodes. In the periphery, DCs exist in an immature state and are more specialized in acquiring and processing exogenous antigen and less competent in antigen presentation for T-cell activation (19,20).

Therefore, T cells trafficking through peripheral tissues are more likely to experience antigen presented “nonprofessionally” either by immature DCs or on parenchymal tissues, which may have limited abilities to process and present antigen and/or which may lack functional expression of appropriate costimulatory molecules. In this setting, the activation signals (antigen-specific, nonspecific, or both) encountered by T cells will be altered or incomplete and will result in T-cell tolerance. Therefore, multiple mechanisms, both deletion- and nondeletion-based, are governed by contact-dependent relationships between APCs and mature T cells and can be proposed as likely for the induction and maintenance of self-tolerance in the periphery.

### CONCEPTS OF PERIPHERAL TOLERANCE

As just noted central tolerance is achieved by a process of deletion involving both active and passive apoptotic mechanisms resulting from contact-dependent cognate interactions between peptide-bearing MHC molecules expressed by mature APCs and immature T cells. On the other hand, peripheral tolerance involves a more complex set of both deletion and nondeletion processes that are defined by unique interactions between mature T cells and APCs of variable levels of maturity and immune potential as well as by conditions in the surrounding microenvironment. Ultimately, the pathways to peripheral tolerance develop from five events related to T-cell activation: ignorance, apoptosis, immune deviation, anergy, or generation of regulatory T cells (T-regs). Each of these events is discussed briefly below.

### IMMUNOLOGIC IGNORANCE

Although the purging mechanisms of central tolerance are efficient, they are by no means perfect, and thus some autoreactive T cells escape into the periphery. Furthermore, T cells undergoing the selection process within the thymic tissue are never exposed to certain self-antigens, owing to their unique temporal and spatial expression in the peripheral environment. The peripheral environment, defined as tissues outside the lymphatic and circulatory systems, is comprised of cells possessing a wide spectrum of antigen-processing and presentation capabilities that are skewed toward a functional antigen-processing cell function termed nonprofessional. During inflammation, cytokines, e.g., interferon- $\gamma$  (IFN- $\gamma$ ), are released and can induce the expression of MHC antigens on neighboring tissues in the microenvironment. However, since most of these tissues are comprised of nonprofessional APCs, they have a limited ability to process and present self-antigens. Therefore, autoreactive memory T cells (CD45R0<sup>+</sup>), which infiltrate in response to inflammatory signals and

encounter MHC-bearing somatic tissues, may fail to engage in antigen-specific interactions owing to the inability of the nonprofessional APCs to process and present self-peptides with requisite specificities. This type of *nondeletion*-based peripheral tolerance occurs because autoreactive T cells experience *immunological ignorance* owing to the complete absence of TCR signaling (signal 1) (21–23). Such antigenic encounters have no lasting effects on the T cells, which maintain their ability to respond normally to self-antigens presented by professional APCs.

### APOPTOSIS

Interactions between APCs and cognate T-cells resulting in T cell activation obviously do occur in peripheral somatic tissues, which, as previously stated, exhibit a wide range of antigen-processing and presentation abilities. Two apoptotic deletion mechanisms (similar to those employed in central tolerance for the removal of self-reactive immature T cells) occur in the periphery with infiltrating mature T cells. Passive apoptotic death (PD) can result from the lack of or from ineffective costimulation (signal 2) in the presence of effective TCR-mediated antigen-specific stimulation (signal 1). This may occur when somatic nonprofessional APCs effectively process and present MHC-associated antigenic peptides but fail to express functional costimulatory molecules. The absence of vital costimulation leads to the down regulation of survival genes e.g., *Bcl-2* and *Bcl-xL*, leaving the partially activated T cells susceptible to apoptotic death (24–25).

A model has been proposed by Lechler et al. to explain how activated autoreactive T cells may be generated during inflammation (26). Tissue DCs recruited to zones of inflammation at sites of viral or bacterial infection take up and process both foreign antigens and self-antigens that result in T-cell activation expressed or shed by infected tissues. These DCs then migrate to the spleen or regional lymph nodes and differentiate into mature APCs, where they engage in cognate interactions with foreign antigen and self-antigen-specific T cells. As the activated T cells emigrate out of the secondary lymphoid organs and migrate into peripheral tissues toward zones of inflammation, foreign antigen-specific effector T cells are reinforced by cognate interactions with professional APCs, e.g., infiltrating macrophages, while autoreactive T cells are deleted after encountering self-antigen on nonprofessional APCs.

An alternate pathway of activation-induced cell death (AICD) also exists for the deletion of activated T cells (27). Immune activation induces increased levels of expression of FAS (a member of the tumor necrosis factor [TNF] receptor family) on T cells. FAS ligand (FAS-L) is induced on many lineages of somatic tissues in response to proinflammatory cytokines, e.g., IFN- $\gamma$  (28). Furthermore, FAS-L is constitutively expressed in immunoprivileged sites, e.g., the eye and testis (29–31). FAS-FAS-L interactions induce strong apoptotic signals in activated T cells leading to their death (32,33). Thus, the induced and constitutive expression of FAS-L on tissues in the periphery can attenuate proinflammatory responses by inducing AICD in T cells.

## IMMUNE DEVIATION

During prolonged immune activation, effector T-helper cells assume fixed functional phenotypes, which have been utilized to classify T cells based on the patterns of cytokines they express: TH1 cells express interleukin-2 (IL-2) and IFN- $\gamma$  and are associated with strong inflammatory cell-mediated responses; TH2 cells express IL-4, IL-5, and IL-6 and are associated with eosinophil-mast cell and humoral responses; TH3 or T-reg (*see below*) express IL-10 and transforming growth factor- $\beta$  (TGF- $\beta$ ) and are associated with immunosuppression; and TH0 are less polarized and exhibit more random patterns of cytokine expression (34). In the context of transplantation, both TH2 and TH3 responses are commonly grouped together and are referred to simply as TH2. Both TH1 and TH2 responses reciprocally inhibit the development of the other (35).

The combination of cell contact-dependent and -independent signaling events that occurs during T-cell activation strongly influences the type of effector cell response that evolves. Professional APCs, e.g., DCs, express cytokines e.g., IL-12, during cognate interactions with T cells, which drives TH1 effector responses (36). TH1 T cells interacting with DCs also activate or “commission” DCs to induce TH1 responses with other naïve mature T cells during subsequent cognate interactions, thus propagating the TH1 response (37). Likewise, T-cell activation that develops under the influence of TH2 cytokines, e.g., TGF- $\beta$ , promotes the propagation of suppressive and regulatory T-cell responses (38–40).

In certain microenvironments in the periphery, e.g., the gut and the liver, T-reg responses tend to predominate, a phenomenon termed immune deviation (41,42). The TH2 environment in the gut and liver helps to moderate aggressive immune activation in these peripheral tissues, which come in continuous daily contact with an abundance of otherwise nonpathogenic foreign commensal microorganisms and exogenous antigens. Select strategies to induce tolerance to organ allografts by inducing oral tolerance to transplantation antigens are based on our understanding of how such immune deviation in the gut develops and how to harness such methods for the induction of transplant tolerance (described below in Role of Costimulation and Donor-Specific Tolerance).

The external influence of TH2 cytokines on the downregulation of immune activation is an example of a contact-independent mechanism of peripheral tolerance. TH2 effector responses may also ensue from TCR engagement with altered peptide ligands (APLs) (43,44). The inability of nonprofessional APCs to process antigen properly may sometimes result in peptides that are able to associate with MHC but have a slightly altered sequence than those expressed by MHC molecules on the surface of professional APCs. Cognate interactions of T cells with nonprofessional APCs presenting APLs can result in immune deviation, or even anergy (*see next section*), even in the absence of external TH2 cytokines. Thus, the induction of immune deviation during immune activation in the periphery involves both contact-dependent and contact independent mechanisms, resulting in the propagation of TH2 responses and an overall tolerogenic effect.

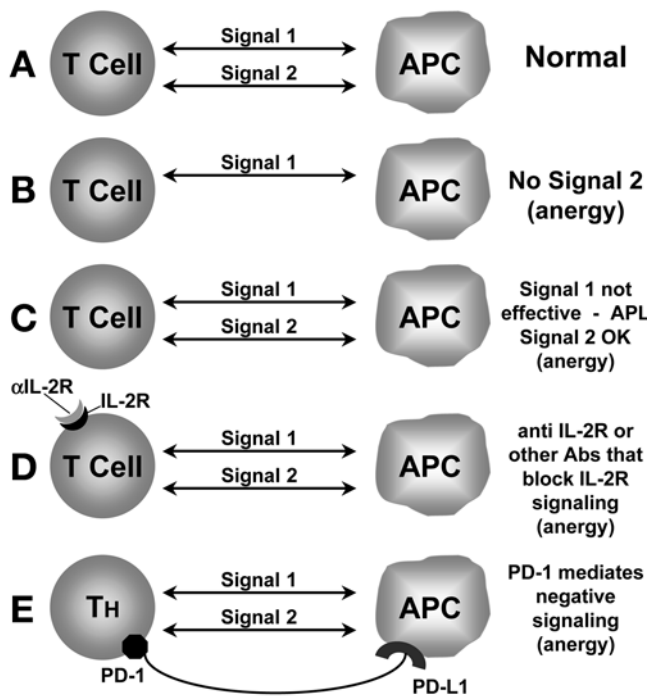
## ANERGY

T-cell anergy is a form of antigen-specific unresponsiveness that differs from apoptosis because it does not cause T-cell death and also differs from immunological ignorance in that once anergy is induced, subsequent cognate interactions with the antigen presented by professional APCs do not cause immune activation. It has been proposed that anergy was merely an intermediate refractory state assumed by a T cell undergoing a slow process of apoptosis. Although this may be true for B cells, there is mounting evidence that this is not the case for T cells, since it has been shown that T-cell anergy can be reversed by exposure to high doses of IL-2 (45). Generation of T-cell anergic responses have been demonstrated both *in vivo* and *in vitro* by number of pathways, which all evolve from inductive encounters between T cells and APCs, including (1) TCR-mediated signaling in the absence of effective costimulation, (2) inhibition of signaling through IL-2 receptor (IL-2R), (3) TCR engagement of APLs, and (4) cognate interactions between T cells and T cell APCs (Fig. 3).

Anergy is not a passively induced state (unlike ignorance) and is difficult to induce in naïve T cells *in vitro* (46). Experimentally, it has been shown that the induction of anergy may arise from perturbed TCR-and/or costimulatory molecule-mediated signaling. Presentation of APLs by nonprofessional APCs effects antigen-specific signaling (signal 1) by decreasing the time of TCR engagement with peptide-MHC complexes (47). Lord et al. have proposed that TCR engagement results in sets of both rapid and delayed signals (48). Rapid signaling is proposed to be associated with induction of anergy, whereas full activation occurs upon sustained TCR-mediated signaling associated with the development of the mature IS. TCR engagement with APLs favors faster off-rates and thus the development of anergy. Jenkins et al. showed that the anergy induction pathway is calcium dependent by demonstrating that T-cell clones became hyporesponsive after treatment with ionomycin in the absence of other stimulation (49). Supporting evidence shows that the immunosuppressive drug cyclosporin A (CsA), which blocks the TCR-induced calcineurin signaling pathway, also blocks the experimental induction of T-cell anergy (50). This is a significant finding because it highlights how in clinical transplantation the use of calcineurin inhibitors to prevent organ transplant rejection may conflict with the goal of inducing durable transplant tolerance.

Evidence that lack of critical costimulation causes extended T-cell unresponsiveness comes from experiments in which T-cell clones were rendered anergic after stimulation by immobilized CD3 in the absence of signaling through a potent CSM, e.g., CD28. During T-cell activation, effective costimulation causes increased expression of the IL-2 receptor (IL-2R) and subsequent proliferative responses by activated T cells. In an elegant study, Powell et al. demonstrated that T-cell anergy can be induced when T-cell cell cycle progression was inhibited by rapamycin even in the presence of effective costimulation (51). In these experiments, T-cell clones were rendered anergic when stimulated with immobilized CD3 (signal 1) and CD28 (signal 2) in the presence of rapamycin, which inhibits IL-2R-mediated





**Fig. 3.** Pathways that lead to T-cell anergy. (A) Normally, interaction between the TCR with its cognate peptide-bearing MHC molecule leads to the generation of signal 1, the interaction between costimulatory molecules such as CD28 and CD40 with their ligands B7 and CD40L leads to the generation of signal 2, and an immune response develops. However, many alternate interactions develop that give rise to anergy. (B) In the absence of signal 2, the T cells become anergic. (C) In some cases such as when the MHC molecule that is involved in antigen presentation contains a peptide that is slightly different from the parent peptide, it leads to ineffective generation of signal 1, which can also lead to the development of anergy in the T cell. (D) Antibodies that block interleukin-2 receptor (IL-2R) signaling prevent T-cell proliferative responses, leading to the anergy of the T cell. (E) T cells can express the molecule termed programmed cell death1 (PD-1), which, when ligated by PD-ligand1 (PD-L1) on an APC, leads to the generation of a negative signal and T-cell anergy. Abs, antibodies; APL, altered peptide ligand.

signaling and cell cycle progression at G1, but not in the presence of hydroxyurea, which inhibits IL-2R-mediated cell cycle progression in the S phase. These data suggest that anergy is induced by intracellular events requiring active calcium-dependent signaling from the TCR in the presence of cell cycle arrest at G1. Thus, these results have important implications for designing effective immunosuppressive antirejection therapies. Unlike calcineurin inhibitors, e.g., CsA and FK506, rapamycin can provide a cover of immunosuppression without preventing induction of prolonged transplantation tolerance.

### REGULATORY T CELLS

Although much remains to be discovered about the generation and action of T-reg, they are emerging as perhaps one of the most important factors in the induction and maintenance of peripheral tolerance. Apparently T-regs are generated in a variety of ways during the induction of peripheral tolerance. Most of the other pathways involve costimulatory blockade,

microchimerism, or interactions with immature DCs (52–55). T-reg, on the other hand, are phenotypically CD4<sup>+</sup>/CD25<sup>hi</sup>/FoxP3<sup>+</sup>/GITR<sup>+</sup>. However, there has been considerable debate as to the preciseness of this phenotype with functional activity; certain schools maintain that there are currently no unique markers to distinguish them from anergic T cells (26,56). More recently, studies have described T-regs as CD4<sup>+</sup>/CD127<sup>lo</sup> cells that are either CD25<sup>+</sup> or CD25<sup>-</sup>, making the point that FoxP3 is perhaps a transient marker of cell activation, and thus activated and memory T cells can also express FoxP3 but that CD127 distinguishes T-regs from activated T cells (57).

An important study by Lombardi et al. demonstrated that defined clonal populations of anergic human T cells were able to suppress proliferation of T-cell clones with specificity for the same antigen (56). The addition of neutralizing antibodies specific for IL-4 or IL-10 failed to inhibit suppression. However, when the experiments were conducted under conditions in which the anergic cells were separated from the APCs and T cells, antigen-specific suppression of T-cell proliferative responses were not observed, indicating that suppression was mediated and the APCs. Furthermore, these anergic T cells were able to suppress proliferative responses of T cells specific for third-party antigens as long as they were presented by the same APC.

These results, which have been confirmed by others (58), characterize a T-regulatory cell phenotype as one that (1) does not proliferate in response to cognate antigen, (2) mediates antigen-specific T-cell suppression not through cytokine-induced immune deviation but in a manner that requires contact with the APC, and (3) is capable of suppressing the responses of T cells with dissimilar antigenic specificities as long as both antigens are presented by the same APC, a characteristic termed “linked suppression.”

In an early model designed to reconcile these observations, Waldmann and Cobbold proposed that these anergic antigen specific suppressor cells behave as “passive civil servants” (59). In this model, T cells with different clonal specificities are assembled in cognate interactions with MHC-peptide complexes arrayed on the finger-like projections of a common DC and participate in a communal immune activation that is driven by T-cell-APC interactions as well as shared interactions between activated T cells. Intercalation of anergic T cells (passive civil servants) dilutes the interactive T-cell effect on immune activation, resulting in suppression owing to the decreased availability of cytokines that act in a paracrine mode. This model also accounts for “linked suppression” as well as another observed characteristic of T-reg known as infectious tolerance or epitope spreading. Such cognate interactions generate an expanding population of anergic T-cell clones, which in turn provide fresh legions of “passive civil servants.”

However, recent evidence is unfolding that supports a more active role for T-reg in inducing and maintaining peripheral tolerance by directly influencing the APC (60–62). It has been shown that antigen-experienced TH cells have the ability to activate or “license” APCs. Thus, after such cognate interactions with a TH cell, activated APCs exhibit a more mature phenotype with an enhanced ability to activate antigen-specific CD8 T cells

without being in direct contact with the licensing TH cell. In this way, TH cells can expand their immunopotentiating influence to a wider field of CD8<sup>+</sup> T cells and thereby drive inflammatory cellular immune responses. Likewise, antigen-experienced T-reg have demonstrated the ability to inhibit APC-mediated immune activation by APC "prohibition". This model shares in common the same characteristics of linked suppression and infectious tolerance as the "passive civil service" model. More recent evidence documents the finding that CD25<sup>+</sup>CD4<sup>+</sup> T-reg synthesize IL-10 in response to antigen activation, leading DCs to revert to an immature and less immunogenic phenotype by downregulating expression of MHC and CSM (63,64). Indeed, there are perhaps multiple pathways of T-reg-mediated induction of peripheral tolerance that are dictated by the context of the microenvironment in which such interactions occur. A role for PD-1/PD-L1 has also been identified (65) and discussed in Role of other costimulatory molecules and Tolerance below.

### ROLE OF COSTIMULATION AND DONOR-SPECIFIC TOLERANCE

There is growing evidence that the combination of immunosuppression with procedures for blocking T-cell signaling mediated through a growing list of select costimulatory molecules significantly reduces acute rejection and promotes allograft acceptance through the establishment of transplant tolerance (66). Costimulatory blockade prevents T-cell activation by blocking signal 2 and to a lesser degree facilitates destabilization of IS formation, thereby weakening or altering signal 1. There are many targets for CSM blockade. Two of the most studied pathways that have perhaps the greatest impact on induction of transplantation tolerance are the CD28-B7 and CD40-CD40L costimulatory pathways.

#### B7-CD28 BLOCKADE

CD28 is constitutively expressed on T cells and has two known ligands, B7-1 (CD80) and B7-2 (CD86), which are expressed on professional APCs. B7-CD28 interactions induce Ca<sup>2+</sup>-independent signaling that synergizes with Ca<sup>2+</sup>-dependent TCR-mediated signaling to promote IL-2 production and T-cell proliferation. The expression of a second (T-cell) receptor for B7, cytotoxic T-leukocyte antigen 4 (CTLA-4), is induced on activated T cells (67). CTLA-4 has a much higher affinity for B7 molecules than CD28. Furthermore, signaling through CTLA-4 in certain settings does not promote but instead inhibits T-cell activation (68). This occurs through several pathways. CTLA-4 stimulation can directly inhibit synthesis of cyclin proteins involved in cell cycle progression and T-cell proliferation (69). CTLA-4 stimulation also leads to a decrease in the synthesis of select set of signal transcription factors: nuclear factor of activated T cell (NFAT) and activator protein-1 (AP-1) which are involved in CD28 costimulation-mediated IL-2 gene transcription (70). Furthermore, CTLA-4 signals cause increased cytoplasmic levels of inhibitor  $\kappa$ B- $\alpha$  (I $\kappa$ B- $\alpha$ ), resulting in inhibition of the nerve factor- $\kappa$ B (NF- $\kappa$ B) pathway of gene expression for other T-cell cytokines, e.g., IL-2, IL-3, IL-4, and IL-10. Finally, the higher affinity of CTLA-4 for

CD80/86 affords a competitive advantage to its overall inhibitory effect on T-cell activation.

A number of laboratories have examined the effects of costimulatory blockade on T-cell alloactivation by testing antibodies and reagents that block either CD28 expressed on T cells or CD80/86 expressed by APCs. Originally it was reasoned that blocking of CD28 was the preferred strategy since not only would activating costimulatory signals be attenuated but also CD80/86 on professional APCs would still be available to activate the T-cell CTLA-4-mediated inhibitory pathway. However, results from studies with CD28 knockout mice have shown that B7 may operate through other costimulatory pathways (e.g., the inducible costimulatory receptor [ICOS]) to mediate T-cell activation and allograft rejection (71–73). On the other hand, blocking B7-mediated signaling in combination with the administration of immunosuppressive drugs has proved quite effective in achieving enhanced suppression and organ allograft survival (74,75).

Support for this view comes from the finding that CTLA-4-Ig, a reagent that has been developed for use in clinical studies, has been shown to block effectively B7-mediated co-stimulation (76). This soluble hybrid fusion protein consists of the extracellular region of the CTLA-4 molecule which binds to the B7 molecule and the Fc portion of human IgG. The latter enhances stability and increases the functional half-life of the CTLA-4 molecule in vivo. In studies using nonhuman primates, Pearson et al. have shown that CTLA-4-Ig works synergistically with a regimen of CsA and steroids to increase the mean survival time (MST) of kidney allografts (MST 150 d vs 22 d for CsA + steroids alone). In more recent studies using an experimental high-affinity form of CTLA-4-Ig (BMS224818), Pearson's group has extended their findings to demonstrate synergistic immunosuppression with other immunosuppressive drugs, e.g., MMF, and with anti-IL-2R-blocking antibodies (77). One explanation for these observed synergistic effects on enhanced immunosuppression and prolonged graft survival is that the administration of both CSM blockade and immunosuppressive drugs is able to block activation of both naïve and mature alloreactive T cells. In addition to TCR-MHC stimulation pathways, other costimulation pathways that synergistically interact with B7-CD28 signaling are now being identified as targets for signal blockade immunosuppressive therapies (78).

#### CD40-CD40L BLOCKADE

As immature precursor T cells leave the bone marrow on their journey into maturity and self (antigen) awareness, they are first subjected to rigorous and unambiguous selection criteria by a selection process within the thymus during the development of central tolerance. Mature thymic emigrants then receive a postthymic education in self-discrimination after more subtle and sophisticated encounters with nonprofessional or semiprofessional APCs in the periphery. Thus, it is important to bear in mind that in the context of transplanted organ allografts, the recipient's maturely developed immune systems must be prepared to receive and respond to tolerogenic signals

delivered by nonprofessional APCs of both host and donor origin in peripheral microenvironments.

CD40 is an important immunoregulatory CSM that is expressed by a range of both professional and nonprofessional APCs, e.g., DCs, macrophages, B cells, T cells, vascular endothelial cells, epithelial cells, smooth muscle cells, fibroblasts, and others (79). Its counterreceptor CD40 ligand (CD40L or CD154) is primarily expressed by blood cells, including DCs, T cells, platelets, and mast cells (80,81). Signaling through CD40 alone or in combination with other costimulatory signaling results in a variety of both activating and tolerizing immunoregulatory responses by DCs, macrophages, T cells, and endothelial cells (79,82). T-helper cell expressed CD154 triggers costimulation through CD40 expressed on DCs causing an upregulation of the expression of the CSMs, CAMS (LFA-3, CD134), proinflammatory cytokines (IL-12 and TNF- $\alpha$ ), and MHC I/II, thus enhancing DC/APC function. Also, as discussed above, these activating events license DCs to activate antigen-specific CD8<sup>+</sup> in the absence of CD4 TH<sup>+</sup> cells. CD40 stimulation has similar effects on macrophages, resulting in increased synthesis of IL-1, IL-12, TNF- $\alpha$ , nitric oxide (NO), CSMs and APC function. B cells upregulate CD80/86 expression, FAS-L, and isotype class switching, epithelial cell increase expression of epithelial cells specific CAMs (VCAM-1, CD62E, and CD54) and synthesis of cytokines (IL-8 and IL-6) after CD40 costimulation. CD40 works synergistically with CD28 cosignaling and TCR-mediated signaling to augment T-cell activation and proliferation.

However, in the absence of costimulation through CD28, CD40 signaling exerts a partial tolerogenic effect, leading to limited T-cell activation and ultimately T-cell apoptosis of some activated T cells (83). Thus, stimulation through the CD40-CD40L pathway serves mainly to support or enhance other costimulatory signals that work in conjunction with TCR-mediated signals for full T-cell activation. Accordingly, protocols that combine CSM blockade of both the CD40 and CD28 signaling pathways in combination with regimens of appropriate immunosuppressive chemotherapy have proved quite effective in immunosuppression and T-cell alloantigen hyporesponsiveness (84,85).

More recently, studies have demonstrated that blocking CD40-CD40L with antibodies that target CD40L induces peripheral tolerance by inducing cell cycle-dependent apoptosis in alloreactive T cells (86). Immunosuppressive drugs that inhibit the calcineurin pathway, e.g., CsA, not only block TCR-mediated signals but also block CD40L expression as well and the tolerogenic effects of anti-CD40L blocking antibody. However, when IL-2R blocking antibodies or rapamycin is substituted for calcineurin pathway inhibitors, the tolerogenic effects of the anti-CD40L blocking antibody on T-cell alloactivation are restored (87). These observations support the idea that anti-CD40L not only blocks stimulation through the CD40-CD40L pathway, but also exerts a direct negative signaling effect through CD40L that cooperates with calcineurin-dependent signals mediated through the TCR. Thus, how the goals of immunosuppression (to achieve graft

survival) and tolerance induction (to achieve graft acceptance) can be realized by combining therapies with different modalities, e.g., immunosuppressive drugs and CSM blockade, should be carefully considered in the context of how they effectively interact to achieve alloantigen-specific hyporesponsiveness.

## ROLE OF OTHER COSTIMULATORY MOLECULES AND TOLERANCE

In addition to CD28/B7, CTLA-4/B7, and CD40/CD40L a number of additional costimulatory molecules and their ligands have been described including a series of five new B7 family members such as the ICOS ligand, programmed cell death ligand-1 (PD-L1, B7-H1), PD-L2 (B7-DC), B7-H3, and B7-H4 and the CD28 family members ICOS, PD-1 and BTLA (88). The B7 family members are expressed on professional APCs as well as cells of nonlymphoid origin (nonprofessional APCs) providing a series of redundant pathways involved in T-cell activation and regulation. The CD28 family members are expressed predominantly by T cells following induction and appear to play a role in the regulation of T-cell activation and tolerance induction. Each of these pathways is currently being studied in detail including the genetic polymorphisms inherent in each of these molecules and their respective promoters. Although the roles of these new members of the CD28 and B7 families have not been studied in detail with regard to transplantation tolerance, a brief summary of the nature of these molecules is provided below because of the importance of such molecules in the induction and/or maintenance of tolerance.

Thus PD-1 was isolated using subtractive hybridization with expression was enhanced by apoptotic stimuli induced in a thymic T-cell line. The extracellular portion of the PD-1 molecule has an IgV line domain, and the intracellular portion contains both an immunoreceptor tyrosine-based inhibitory and a switch motif (ITIM), (ITSM). Although the expression of CTLA-4 is restricted to T cells, the expression of PD-1 is broader, being expressed by B cells, and is myeloid cells and is thus reasoned to play a broader role than CTLA-4 in the regulation of immune responses.

There are two known ligands for PD-1, termed PD-L1 and PD-L2, which were in fact identified by searching databases for molecules that had similarity with B7 molecules. Both the PD-L molecules are transmembrane proteins with IgV- and IgC-like domains in the extracellular regions. Although PD-L1 is constitutively expressed by T cells, B cells, macrophages, DCs, and nonlymphoid cells such as endothelial cells, pancreatic cells, glial cells, and muscle cells, PD-L2 expression appears on more limited cell lineages such as macrophages and DCs. The level of PD-L1 expression is upregulated following cell activation. Since PD-L1 has a wider tissue distribution, it is reasoned that this molecule and the PD-1/PD-L1 pathways may play a wider role in the regulation of immune responses which has important implications for its role in solid organ transplants. Ligation of PD-L1 and -L2 has been shown to inhibit lymphocyte activation and induce peripheral tolerance; it does so by directly inhibiting the effector function of T cells to maintain tolerance (reviewed in ref. 89).

More recently, the role of PD-1 and PD-L1 and PD-L2 has gained further prominence: these molecules play a role in regulating immune responses and a potential role in tolerance. Thus, mice that were chronically infected with lymphocytic choriomeningitis virus (LCMV) appeared to express a high frequency of functionally impaired (exhausted) LCMV-specific CD8<sup>+</sup> T cells that did not clear viral infection. These LCMV-specific CD8<sup>+</sup> T cells expressed high mean densities of PD-1. Administration of PD-L1 to such mice led to a marked decrease in viremia, which was reasoned to be secondary to the blocking of PD-1 interaction with PD-L1 (65). Of interest, the administration of CTLA-4 had no effect in this model, suggesting that PD-1-PD-L1 interaction identifies a unique pathway of regulating effector T cells.

### **ROLE OF THE LIVER IN TRANSPLANTATION TOLERANCE**

Among all the solid organ transplants, the liver has experienced greater success in terms of graft survival than all the other transplanted vascularized organs. Successful transplants across complete MHC barriers in the absence of immunosuppression in murine and in some pig animal models presents strong evidence for the intrinsic tolerogenicity of liver allografts. Furthermore, it has been demonstrated that organ allografts that are not usually tolerated enjoy long-term survival when transplanted in combination with liver allografts from the same donor. The enhanced ability of the liver to induce peripheral tolerance is owing to a variety of factors including its large mass and unique cellular composition, which includes liver sinusoidal endothelial cells that possess a constitutive ability to activate and induce immunosuppressive TH2 responses in naïve CD4<sup>+</sup> and even CD8<sup>+</sup> T cells (by antigen cross-presentation). Liver allografts are also a large source of soluble MHC class I antigens which are able to bind to alloreactive CD8<sup>+</sup> T cells and induce activation and apoptosis in the absence of costimulation. The unique circulation to the liver from the hepatic portal vein plays a major role in oral tolerance to dietary antigens. However, perhaps the most significant influence on its inherent tolerogenicity is the liver's ability to function as a hematopoietic organ.

### **ROLE OF MICROCHIMERISM IN LIVER TRANSPLANTATION**

Evidence that the liver can support hematopoiesis and serve as a source of multiple leukocyte and myeloid lineage cells has been well documented (90). Thus, during transplantation, donor passenger stem cells including precursor DCs can migrate out of the liver allograft and seed/integrate into host lymphoid and nonlymphoid tissues. This phenomenon, known as microchimerism, occurs to a greater or lesser degree during transplantation of all vascularized organ allografts. However, the potential for microchimerism is significantly greater with liver transplantation owing to the larger organ mass, heavier passenger leukocyte load, and the liver's intrinsic hematopoietic capacity. There is growing evidence that microchimerism is not an epiphenomenon but plays a significant role in the

induction and maintenance of long-term (if not permanent) tolerance to donor antigen. Peripheral tolerance will be maintained as long as donor alloantigen is available, and the liver's ability to function as a renewable source of donor stem cells enhances its tolerogenic characteristics. Donor microchimeric cells have been detected within host tissues of liver allograft recipients up to 30 yr post transplant (91,92). Mechanisms of microchimerism-induced tolerance may involve both direct and indirect pathway interactions between donor and recipient APCs. Donor DCs migrating into host spleen or lymph nodes directly engage alloreactive T cells and promote AICD, thus perpetuating transplant tolerance by deleting alloreactive T-cell clones. Indirect pathway mechanisms can also be involved owing to the persistence of donor antigen, which can drive clonal expansion of donor-reactive T cells that are ultimately eliminated upon encountering alloantigen presented indirectly and nonprofessionally in the periphery.

### **ROLE OF DENDRITIC CELLS IN LIVER TRANSPLANTATION**

The hematopoietic potential of the liver is supported by the ability of hepatic stromal cells to provide nurturing growth factors and cytokines, e.g., TGF- $\beta$ , IL-10, and granulocyte macrophage colony stimulating factor (GM-CSF), which are necessary for precursor stem cell development. Their presence helps to produce a continuous supply of immature precursor DCs (pDCs), B cells, and macrophages, all of which have tolerogenic capabilities owing to nonprofessional presentation of alloantigen. Of all the phenotypes of the various precursor leukocytes in the liver, pDCs are the most important in maintaining donor-specific peripheral tolerance (93,94). In their immature state, DCs lack expression of potent costimulatory molecules, express low levels of MHC, and induce anergy in allospecific T cells in vitro. Also, pDCs migrate quickly from the liver and home to secondary lymphoid organs, where they engage with allo-reactive T-cell-inducing tolerance. Cytokines play a role not only in their development but also in the persistence of immature DCs. TGF- $\beta$ , which is present in the liver and expressed by many types of somatic cells including hepatocytes, inhibits the maturation of pDCs while allowing their expansion in the presence of GM-CSF (95). Furthermore, immunoregulatory cytokines, e.g., IL-10, which are produced by hepatocytes, downregulate expression of CSMs and MHC on mature immunogenic DCs, transforming them into immature tolerogenic phenotypes (96). The liver also serves as a biological reclamation site for many apoptotic cells (97). Phagocytosis of necrotic cells causes activation and enhanced APC function in DCs. However, exposure to apoptotic bodies has the opposite effect, relegating DCs to nonprofessional APC status (98). Thus hepatic DCs, interacting with a variety of cells and signals of both donor and recipient origin, play a significant role in inducing and maintaining peripheral hyporesponsiveness to donor antigens.

### **A HISTORICAL REFERENCE**

The unusual immunological role the liver plays in the induction and maintenance of self-tolerance brings to mind

a historical fact that has often been forgotten. The Egyptians, when preparing the body for mummification, always removed most of the internal organs except the heart and discarded the rest except for the liver, intestines, lungs, and stomach, each of which was preserved in separate “Canopic jars”; each jar had unique lid that represented the four sons of Horus (Imsety, Qebensenuf, Hapy, and Duamutef). The jar containing the liver was the only one that had a human head as a lid; the intestines, lungs, and stomach had the heads of a falcon, baboon, and jackal, respectively. The symbolism of a human head provided uniquely to the liver suggests that the ancient Egyptians placed this organ in special and high regard.

## XENOTRANSPLANTATION

Recent advances in achieving transplant tolerance and the limited availability of organs allografts has fueled explorations of the prospects of cross-species organ transplantation, or xenotransplantation. In the consideration of appropriate organ xenograft donors for human recipients, much attention has focused on the pig animal model, for several reasons. Anatomically, the size and physiology of pig organs are compatible with those of humans. Also, pigs are not susceptible to many human viruses, e.g., hepatitis C virus (HCV) and cytomegalovirus (CMV), and therefore the risk of human infection is minimized or eliminated, especially during regimens of immunosuppression to prevent graft rejection. Furthermore, pig donors can be maintained under controlled and sterile conditions, thereby providing availability upon demand. This would make possible the ability to schedule organ transplantation so that protocols providing for more effective tolerance induction in the host could be accommodated. Pigs are now being genetically manipulated and even cloned, thus providing for the availability of genetically engineered organs that could exhibit improved antirejection properties. Also, there are fewer ethical challenges for human transplantation using xenografts from pigs than from nonhuman primates. However, one important unknown risk is the probability of acquiring human infection with unidentified passenger porcine retroviruses.

## HYPERACUTE REJECTION

There are also more general challenges for achieving accommodation of vascularized organ xenografts. The first hurdle to xenograft survival is overcoming hyperacute rejection. Hyperacute rejection is a type of vascular rejection mediated by naturally occurring human antibodies, generated in response to common intestinal bacteria, which crossreact with the polysaccharide antigen Gal $\alpha$ 1, Gal ( $\alpha$ GAL) that is normally expressed on the surface of vascular endothelial cells (VECs), which line the blood vessels of lower mammals but not human or nonhuman primates (99–101). More than 80% of xeno-antibodies involved in hyperacute rejection have specificities for the  $\alpha$ GAL epitope. Vascular damage in hyperacute rejection results from complement activation subsequent to xeno-antibody binding to VEC xeno-antigens. The resulting damage to the intimal VEC layers of the blood vessels exposes

basement membrane extracellular matrix proteins, which results in platelet activation, activation of the coagulation cascade, thrombosis, and ultimately graft rejection. The hyperacute rejection process is aggravated by the fact that certain regulatory proteins for complement activation expressed by porcine VECs are not physiologically compatible with human complement or coagulation proteins and thus fail to inhibit vascular injury.

Strategies to prevent hyperacute rejection do not at present include induction of immunologic tolerance since the rejection is mediated by preformed naturally occurring xeno-antibodies. Plasmapheresis to remove xeno-antibodies before xenotransplant effectively prevents hyperacute rejection. Other experimental approaches have been tested using xenografts from  $\alpha$ GAL knockout mice in an attempt to eliminate the xeno-antigen instead of the xeno-antibody (102,103). However, these have met with variable success because they do not prevent rejection mediated by other xeno-antigens expressed on murine VECs. Other genetic approaches that appear more promising use organs from pigs genetically engineered to express the transgene for decay-accelerating factor on VECs which attenuates complement activation and thus inhibits progression of thrombosis and vascular rejection (104,105).

## ACUTE REJECTION

After hyperacute rejection, acute vascular rejection becomes the most serious threat to xenograft survival. As in hyperacute rejection, acute rejection is mediated by xeno-antibodies to VEC-expressed xeno-antigens. The xeno-antibodies that mediate acute rejection of the xenograft are T-cell-dependent antibodies that appear within 20 d post-transplant. Therefore, immunosuppressive chemotherapies are effective in attenuating acute xenograft rejection. T-cell recognition of xeno-antigens occurs through both the direct and indirect pathways (106). MLR responses with human and porcine leukocytes are comparable (for both MHC class I and II) to allo-MLR. Methods for inducing tolerance to xeno-antigens have mainly been studied in concordant animal models (i.e., cross-species transplants not involving humans). Tolerance-inducing strategies that have been tested include mixed chimerism, T- and B-cell depletion, and CSM blockade.

Since humoral responses play such a significant role in both hyperacute and acute rejection, methods for depletion of xeno-specific B cells have been attempted but have achieved mixed results in concordant models (107,108). Other strategies to prevent acute vascular rejection have been aimed at preventing secondary humoral responses to xeno-antigen by combining mixed chimerism and CSM blockade. Recently Buhler et al. (108) demonstrated that induced baboon humoral responses directed toward  $\alpha$ GAL and non- $\alpha$ GAL determinants on pig VECs could be selectively blocked after inducing microchimerism using high-dose porcine hematopoietic cell transplantation combined with CD40 ligand blockade. More research is still needed. However, although hyperacute and acute rejection may present daunting challenges, they may not be insurmountable barriers to successful xenotransplantation.

## CONCLUDING REMARKS AND FUTURE DIRECTIONS

Since the initial discovery that dizygotic bovine calves, which shared a common blood circulation *in utero*, remained immune to each other's red blood cells into adulthood by Owen in 1947 (109) and the discoveries of Billingham, Brent, and Medawar (110) that the twins would also accept skin grafts from each other but would reject third-party grafts, the science of organ transplant was born. Based on these observations, the latter authors (correctly) proposed that neonatal exposure to alloantigen resulted in life-long tolerance, and they subsequently confirmed their hypothesis in a series of experiments with both mice and chickens (110).

These discoveries revealed an important insight into nature's technique for instructing the immune system to abstain from deliberately responding to particular (self) antigens, a process now called central tolerance. In the half century that has followed, scientists have built on the knowledge and our growing understanding of these basic principles to develop newer more, effective strategies for inducing immunosuppression and peripheral tolerance leading to graft survival and accommodation. Based on the principles of central tolerance and thymic education, effective techniques for the selective deletion of alloantigen-specific T-cell clones, e.g., mixed chimerism, intrathymic antigen injection, and transplantation of donor thymic tissues, have been developed. Investigations of the basic mechanisms of antigen recognition and T-cell activation have revealed the important interrelationships between antigen-dependent TCR-mediated signaling pathways and antigen-independent costimulatory pathways. These studies have helped to define the different signaling pathways that govern T-cell activation and that determine the quality of T-cell effector responses, e.g., ignorance, anergy, or immunoregulation. This knowledge has helped to define better approaches for selecting immunosuppressive methods and blocking of costimulatory pathways as forms of therapies, either alone or in combination, to achieve immunosuppression, tolerance, or both.

The principles of antigen recognition and immune activation also explain how immune responses are regulated in the periphery by T-cell interaction with the host of facultative and professional APCs. They also contribute to our understanding of how the results of such encounters give rise to T-reg cells, which propagate their immunosuppressive influence through immune deviation or licensing and prohibition of DCs. In addition, the unique aspects of liver transplantation have introduced the new concept of microchimerism and have brought a deeper understanding of the nature and significance of DC regulatory functions in establishing and maintaining peripheral tolerance. These insights have inspired more sophisticated approaches to the greater challenges of xenotransplantation.

### FUTURE DIRECTIONS

Clearly among the most important issues to emerge in the field of transplantation is the issue concerning the science of stem cell transplants and embryonic stem cell research. This issue alone has gained national and international prominence

owing to the inherent ethics problems associated with the use of embryonic stem cells and political issues such as potential involvement of discriminatory practices (111). Thus, the curtailment of funding research for the isolation of new lines of embryonic stem cells is viewed by some as restricting the potential benefit of therapies that may potentially evolve only to the ethnic group of individuals who share the genetic background with the currently existing bank of embryonic stem cells. Stem cell therapy has already been initiated for cardiac disease, certain neurological disease, and vascularized bone grafts, to name a few. In addition, there are a plethora of experimental protocols are in progress for the potential use of "engineered" stem cells for the cure of a number of genetically inheritable diseases. Clearly, it seems like the "genie" is out of the bottle when it comes to this entire issue of embryonic stem cell research and stem cell therapeutic strategies.

On a more scientific level, current and future research is evolving in directions that incorporate the current ideas and principles of transplantation immunobiology with newer techniques in genetics, molecular biology, and tissue engineering. Long considered the most important cellular component in initiating positive antigen-specific proinflammatory T-cell responses, DCs are now becoming recognized as equally effective in mediating antigen-specific T-cell hyporesponsiveness and tolerance. Therefore, research efforts are being focused on ways to enhance and apply DC immunomodulatory characteristics to organ transplantation. The basic strategy involves isolating and modifying donor DCs, and then introducing them into the host before or at the time of organ transplant. The goal is to modify donor DCs so that their encounters with recipient T cells results in alloantigen-specific T-cell tolerance. DC modification involves transfer of genes (usually with adenovirus vectors) that govern DC expression of immunomodulatory proteins or DC maturation. Some of these procedures that have shown promise involve modifying DCs to express viral IL-10, CTLA-4-Ig, TGF- $\beta$ , and FAS-L.

Similar approaches have been also been used in manipulating donor organ tissues, especially in xenotransplantation, in which the greatest risk to graft accommodation is immune-mediated vascular injury. Many genes involved in the expression of adhesion molecules and cytokines associated with endothelial activation are commonly regulated through the NF- $\kappa$ B signal transduction pathway and play a role in immune-mediated events during hyperacute rejection and chronic vascular rejection. Adenovirus transfer into donor endothelial cells of a gene (A20) that inhibits apoptosis and the N-F $\kappa$ B pathway, has been shown to make vascular endothelial cells resistant to activation *in vitro* and thus may provide a therapeutic option for preventing or minimizing the risk of vascular rejection. Other genetic approaches have been to create minipigs transgenic for human decay accelerating factor (hDAF). hDAF is an important regulator of complement activity. In concordant pig-to-baboon transplantation models, renal xenografts from hDAF transgenic pigs exhibit increased graft survival (112).

Cellular transplantation to repair or reinforce diseased or defective organs is also being developed as an alternative to

organ replacement. Hepatocyte transfer is an example of this new practice (113). The major technical and biological limitations to successful cellular transplantation may be overcome by the ability to culture cells *in vitro* and manipulate them genetically prior to transplantation. Some approaches that are the focus of current research are genetically manipulating hepatocytes to enhance their function and proliferative capacity, to improve their ability to respond to somatic factors and integrate appropriately into tissues of the host organ, and to avoid rejection.

Our growing understanding of the underlying mechanisms that govern regulation of alloimmune responses has led to significant progress in achieving longer survival of organ allografts and has opened the door to new prospects for xenotransplantation. As the demand for effective and long-lasting solutions to organ replacement persists, the new frontiers in bioengineering and cellular transplantation may open new directions and alternatives to transplantation of vascularized organs from human or nonhuman donors.

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# 34 Autoimmune Diseases in Transplanted Livers

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## KEY POINTS

- Autoimmune hepatitis (AIH) can recur, the prevalence being reported to be as high as 42%.
- Recurrence of primary biliary cirrhosis (PBC) has been debated for a long time, but it is now generally accepted, and it occurs in up to one-third of patients.
- A cholestatic pattern of liver biochemical abnormalities is not specific for recurrence of PBC since cholestasis can arise from multiple causes after transplantation.
- The presence of antimitochondrial antibodies (AMAs) does not mean recurrence since AMAs persist in most patients following transplantation, usually with a small and transient fall in their titer.
- Ursodeoxycholic acid can be used with some benefit for posttransplant PBC patients, although its treatment effects on long-term survival are not known.
- Primary sclerosing cholangitis also recurs following transplantation, although this is particularly difficult to prove.
- Autoimmunity and autoimmune liver disease may arise postliver transplant in adults and children transplanted even for non-autoimmune liver diseases.
- *De novo* AIH should be included in the differential diagnosis of unexplained graft dysfunction.
- Awareness of *de novo* AIH in the posttransplant liver is important since this entity responds well to prednisolone and azathioprine, the standard treatment for AIH, but not for antirejection treatment.
- The use of the term “autoimmune” to define hepatitis affecting an allogenic organ is controversial; thus alternative terms such as “posttransplant immune hepatitis” or “graft dysfunction mimicking autoimmune hepatitis” have been proposed.
- The mechanisms underlying recurrence or occurrence of autoimmune diseases in posttransplant livers are not yet defined, but genetic predisposition, molecular mimicry

with microorganisms, and exposure to new alloantigens may play a role.

## INTRODUCTION

Liver transplantation (LT) is a standard therapeutic approach for the treatment of end-stage acute and chronic liver disease of various etiologies including autoimmune hepatitis (AIH), primary biliary cirrhosis (PBC), and primary sclerosing cholangitis (PSC). Results of LT for these autoimmune liver diseases are good, with a patient survival of 80 to 85% at 5 yr after LT. Transplant recipients, however, experience various complications, such as acute and chronic rejection, recurrence of disease, and chronic hepatitis. Recurrence of disease on the graft may be influenced by the genetic background of the recipient as well as other factors such as the degree of immunosuppression. Interestingly, autoimmunity and autoimmune disease can occur *de novo* following LT even for non-autoimmune liver diseases. However, the mechanisms that lead to autoimmunity or recurrence of autoimmune liver diseases after LT have not yet been defined. This chapter focuses on recurrence and occurrence of autoimmune diseases in transplanted livers.

## RECURRENCE OF AUTOIMMUNE LIVER DISEASES

### AUTOIMMUNE HEPATITIS

Usually patients with AIH respond well to immunosuppressive therapy, but a few AIH patients who are refractory or intolerant to corticosteroids and/or azathioprine therapy develop end-stage liver disease requiring LT. A few but significant number of cases occur with fulminant hepatitis, and they also require LT. Recurrence of AIH after LT is supported by most studies (1–11). Histological evidence of recurrence may precede clinical and biochemical evidence of recurrence (5,11). Recurrence has been noted in both adults and children. It may appear as early as 35 d after surgery, and the incidence of this complication increases with the post-transplant interval.

**Incidence** The incidence of recurrent AIH has been reported to be as high as 42% (range, 20–42%) (7–10). Recurrent AIH usually occurs several years after the immunosuppressive

**Table 1**  
**Frequency of Rejection in Patients**  
**With and Without Recurrence<sup>a</sup>**

Feature	Recurrent	No
	AIH (n = 7)	recurrent AIH (n = 34)
Acute rejection	3 (43)	21 (62)
Steroid-resistant rejection	2 (29)	8 (24)
Chronic rejection	0 (0)	4 (12)
Duration to recurrence (yr)	4.6 ± 1	NA
Follow-up after recurrence (yr)	4.9 ± 0.9	NA
Follow-up after liver transplant (yr)	9.5 ± 1.6	6.3 ± 0.7

Abbreviations: AIH, autoimmune hepatitis; NA, not applicable.

<sup>a</sup>Numbers in parentheses are percentages. Data from ref. 10.

agent is reduced, and the average time to recurrence was reported to be 4.6 yr after transplantation (9).

**Diagnosis** Diagnosis is based on the criteria for AIH, i.e., sustained abnormal serum aminotransferases without a markedly elevated alkaline phosphatase or  $\gamma$ -glutamyl transferase. Liver histology, elevated serum immunoglobulin G of more than 1.5 times the upper limit of normal and circulating auto-antibodies such as antinuclear antibodies (ANAs), smooth muscle antibody (SMA), or anti-liver-kidney microsome (LKM) antibody titer of more than 80 without other possible causes are important as well as a response to prednisolone and azathioprine (7–10).

**Risk** Despite the intensive immunosuppression following LT, patients are at risk for recurrent AIH. Possession of the HLA-DR3 allele appears to confer predisposition to disease recurrence, as it does to the original AIH (2,10). In a report of 43 patients, for example, recurrence occurred in 11 (26%), 9 of whom were positive for HLA-DR3 in whom all the grafts were from donors negative for HLA-DR3 (2). HLA-DR4-positive recipients are also at risk for recurrence regardless of donor HLA status (10). However, this has not been universally confirmed (6). High-grade inflammation in native liver at LT is a strong predictor of recurrent AIH (7). Discontinuation of corticosteroid therapy after LT may increase the risk for recurrent disease. Recurrence may be related to the immunosuppressive regimen used after transplantation (11).

**Prognosis** The consequences of recurrent AIH are not severe, and it does not result in graft failure, progression to cirrhosis, hepatic death, or need for retransplantation. In the study of 41 patients who underwent transplantation for type 1 AIH, acute (43% vs 62%;  $P = 0.4$ ) and steroid-resistant rejection (29% vs 24%;  $P = 0.9$ ) occurred with similar frequencies in patients with and without recurrence. Chronic rejection did not occur in the patients with recurrent disease, but it was recognized in four patients without recurrence (0% vs 12%;  $P = 0.9$ ) (Table 1) (10). However, AIH recurrence can lead to graft failure and to the need for retransplantation.

### PRIMARY BILIARY CIRRHOSIS

Although recurrence of PBC after LT has been debated for a long time, it is now generally accepted that the disease recurs

in the allograft in LT from cadaver (13–23). In recipients of living-donor-related liver transplants, Hashimoto et al. (19) studied the recurrence of PBC, and observed it in three (50%) of six recipients. However, in another study from Japan, no recurrence of PBC was confirmed in 50 case series of living LT (24). Because most donors for living liver transplantation are blood relatives with close HLA matches, the recurrence of PBC in transplant recipients might be different from cadaver-donor transplantation.

Diagnosing recurrent PBC in the transplanted liver is more difficult than diagnosing it in the native liver. After LT, elevated immunoglobulin M and antimitochondrial antibodies (AMAs) often persist, and extrahepatic disorders associated with PBC may recur or appear *de novo*, indicating that the underlying immune defect remains uncorrected after removal of the diseased liver (25). Liver biopsy specimens showing small bile duct damages are seen in both rejection and recurrent PBC.

Van de Water J et al. (26) supported the concept of recurrence of PBC by an immunohistochemical study. They showed intense apical staining in the liver biopsy specimens of 74% of patients transplanted for PBC but in none of the patients transplanted for other conditions using a murine monoclonal antibody (C355.1) specific for the E2 subunit of the pyruvate dehydrogenase complex and apical biliary epithelial antigens. Reactivity to C355.1 was associated with evidence of recurrent PBC on biopsy and biochemical evidence of cholestasis. However, another study failed to show epithelial expression of E2 in a liver transplanted for PBC (27). In liver biopsy specimens from patients after transplantation, the pattern of E2 staining was similar to that of normal control liver, suggesting that E2 overexpression on bile duct cells may not be important in the perpetuation of the bile duct damage in PBC (27). The expression of E2 in the allograft may be modified by immunosuppression, or else PBC does not recur in the allograft.

**Incidence** Recurrent PBC, as diagnosed by standard histologic characteristics, has been shown to occur in 17 to 30% (15–23). In one of the largest series, from Pittsburgh, histologic features of PBC were found in 7.9% patients at 5 yr after liver transplantation and in 21.6% at 10 yr (17). The study from Birmingham, England reported that recurrence was observed in 18% at 5 yrs and 30% at 10 yrs (18).

**Diagnosis** The diagnosis of recurrent PBC must be based on histologic (rather than serologic or biochemical) findings, which show the characteristic portal tract lesions with mononuclear inflammatory infiltrate, formation of lymphoid aggregates, epithelioid granulomas, and bile duct damage (21). The presence of granuloma, which is not a picture of rejection, is important for its diagnosis. However, the interpretation of liver histology may sometimes be difficult because the rejection process is also centered on the bile ducts (Table 2) (20). The recommended criteria to make the diagnosis of PBC are shown in Table 3 (14).

A cholestatic pattern of liver biochemical abnormalities is neither sensitive nor specific for recurrence since cholestasis can arise from multiple causes in the transplant setting, and not all patients with well-documented histologic recurrence

**Table 2**  
**Factors Confounding the Diagnosis**  
**of Recurrent Autoimmune Disease**

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Immune-mediated graft damage (rejection)
Ischemia
Drug toxicity
Preservation damage
Infection: viral, bacterial, or protozoal
Graft-versus-host disease
Altered immune environment: immunosuppressive therapy
ABO host/graft incompatibility, nonautologous target cells

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Data from ref. 20.

have cholestasis. Similarly, the presence of AMAs does not mean that recurrence is present or will develop. AMAs persist in most patients following transplantation, usually with a small and transient fall in their titer.

**Risk** Some authors have reported that histologic disease recurrence appears in patients followed up for at least 3 yrs after LT, whereas others have noted recurrence within the first year (13–18). Immunosuppressive regimens have been implicated in the timing of recurrence, with patients on tacrolimus experiencing earlier recurrence than those on cyclosporine (15,23). Recurrence of the disease on the graft may be influenced by different factors such as the genetic background of the recipient and the degree of immunosuppression. Rapid weaning of antirejection drugs has been reported to favor recurrence (22). The studies identified independent predictors for recurrence, including older recipient age, longer cold ischemia time, treatment with tacrolimus (compared with cyclosporine), and younger donor age although the magnitude of risk associated with these variables (and their validity in other centers) remains to be determined (17). Neither acute rejection episodes nor OKT3 use before diagnosis of recurrence was significant. Donor and recipient gender and HLA were not identified as risk factors.

The effects of immunosuppression may modify or delay disease expression within the graft (23). On multivariate analysis, the only risk factor identified with recurrence was the type of calcineurin inhibitor used. The odds ratio for recurrence on tacrolimus was 2.73 (95% confidence interval [CI]: 1.84–4.10) compared with cyclosporine. For those receiving cyclosporine, the median time to recurrence was 123 mo, and for those on tacrolimus it was 62 mo ( $p < 0.001$ ). Reasons for this difference between the two calcineurin inhibitors are not clear.

**Prognosis** Patients with recurrent PBC demonstrated prolonged survival. When PBC recurs, intermediate-term patient and graft survivals are excellent, but the long-term outcome remains unknown.

**Treatment** Ursodeoxycholic acid (UDCA) appears to improve biochemical tests of recurrent PBC (28), but its effect on the natural history of recurrent PBC has not been determined. Other immunosuppressive agents have been studied with regard to their antirecurrence properties; however, no standard therapy has been established for this group of patients (29).

**Table 3**  
**Histologic Diagnosis of Recurrent PBC**

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Transplant for PBC <i>and</i>
Persistence of AMA <i>and</i>
Liver histology: the characteristic portal tract lesions include the following:
Mononuclear inflammatory infiltrate
Formation of lymphoid aggregates
Epithelioid granulomas
Bile duct damage
Definite recurrent PBC: three of the four portal tract lesions need to be present
Probable recurrence: two are present

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Abbreviation: AMA, anti-mitochondrial antibody; PBC, primary biliary cirrhosis. Data from ref. 14.

### PRIMARY SCLEROSING CHOLANGITIS

PSC is a chronic cholestatic liver disease of unknown etiology that is progressive in most symptomatic patients, advancing toward cirrhosis and liver failure. LT is the only therapeutic option for patients with end-stage PSC. The results of transplantation for PSC are excellent, with 1-yr survival rates of 90 to 97% and 5-yr survival rates of 80 to 85%. Recurrence of PSC after liver transplantation is common.

**Incidence** PSC recurrence is commonly seen after liver transplantation and is known to recur in 15 to 30% of liver transplant recipients. By using strict criteria based on characteristic cholangiographic and histologic findings, Graziadei et al. (30,31) found recurrence in 20% in 120 patients with PSC who underwent orthotopic LT. In a series of a single-center experience with 17 orthotopic LTs, recurrent PSC occurred in approx 12% of cases but did not significantly affect patient survival (32).

**Diagnosis** It is particularly difficult to prove recurrent PSC in the absence of a gold standard for diagnosis and well-established diagnostic criteria. The diagnosis of PSC may be based on the radiographic documentation of biliary tree lesions (33), which, however, can also arise as a consequence of the LT surgery or post-LT complications. Moreover, radiologic and histologic features indistinguishable from those of PSC may result from ischemic biliary complications (34,35). The study from the Mayo Clinic used strict cholangiographic and histological criteria in a large cohort of patients with PSC in whom other causes of biliary strictures were excluded (30,31).

**Risk** More immunosuppression seems to be detrimental to the outcome of patients with sclerosing cholangitis: use of OKT3 is associated with a greater incidence of recurrence (36). Length of corticosteroid use does not affect timing or risk of recurrence, and it has been proved that early corticosteroid withdrawal after liver transplantation is beneficial. A multivariate analysis by Vera et al. (37) showed that being male (relative risk: 1.2; 95% CI: 0.73–2.15) and an intact colon before transplantation (relative risk: 8.7; 95% CI: 1.19–64.48) were associated with recurrence.

**Prognosis** Recurrence appears to have little effect on patient survival, as survival of patients with recurrent PSC

is similar to that of those without evidence of recurrence (37,38). Similarly to PBC, immunosuppression may modify or delay the disease expression within the graft.

#### APPEARANCE OF AUTOANTIBODIES AND LIVER DAMAGE

Appearance of autoantibodies after LT is reportedly common in patients transplanted for non-autoimmune liver diseases (39,40). ANAs are most common, followed by anti-SMAs and LKM-1. Interestingly, the fluorescence pattern for LKM-1 is at times atypical; sera positive for atypical LKM do not react with the microsomal liver fraction, which contains cytochrome P450 2D6, a molecular target of classical LKM-1, but they do react with an as yet unidentified cytosolic antigen (40,41).

The appearance of autoantibodies was shown to be associated with various conditions, including chronic hepatitis, severe graft dysfunction, chronic rejection, and loss of graft (39–44). Interestingly, the presence of ANA or LKM-1 is associated with biochemical and histological evidence of graft dysfunction, suggesting that these autoantibodies are directly involved in liver damage.

The development of autoantibodies associated with late graft dysfunction indicates two clinicohistological entities: *de novo* AIH and early chronic rejection. Portal/periportal inflammatory infiltrate with interface/lobular hepatitis is suggestive of *de novo* AIH. Pericentral hepatocyte confluent dropout with a variable degree of central vein endothelitis, but not with ductopenia (loss of >50% of interlobular bile ducts), is diagnosed as early chronic rejection. In a recent report by Riva et al. (43), 60 (24.3%) of 247 children developed autoantibodies after LT. Graft dysfunction was demonstrated in 22 (37%); 9 patients had *de novo* AIH and 13 early chronic rejection. Five of nine in the *de novo* AIH group were on cyclosporine, and four of nine were on tacrolimus. In the early chronic rejection group, 11 children were treated with cyclosporine A and 2 with tacrolimus. All *de novo* AIH patients had normal liver function tests on corticosteroids and azathioprine. Five patients with early chronic rejection recovered by increasing calcineurin inhibitor dosage, but in 8 of 13, including 7 switched from cyclosporine to tacrolimus, azathioprine and steroids were added to obtain remission of the disease. *De novo* AIH improves after standard treatment for AIH.

Early chronic rejection has a good response to increased doses of calcineurin inhibitors, although ductopenic chronic rejection may occur. Riva et al. (43) concluded that the early differential diagnosis of these conditions and appropriate treatment seem to allow good overall results, reflected by a graft survival of more than 90%.

#### DE NOVO AUTOIMMUNE HEPATITIS

Occurrence of *de novo* AIH after LT for non-autoimmune liver diseases was first reported by Kerkar et al. (40) and has since been confirmed by several other groups in both adults and children (44–48). In contrast to recurrence of disease in patients transplanted for AIH, this newly recognized condition affects patients transplanted for disorders usually

of a non-autoimmune nature other than AIH. They first reported *de novo* AIH as a particular type of unexplained graft dysfunction associated with autoimmune features in children who underwent LT. In their study, 7 (4%) of 180 liver transplant recipients developed an unexplained but characteristic form of graft dysfunction over a 5-yr period of observation at a median post-LT period of 24 mo. The hepatitis was very responsive to corticosteroids. They defined the hepatitis as “*de novo* autoimmune hepatitis” and concluded that autoimmune hepatitis may appear in liver transplant patients while they are on antirejection immunosuppression.

*De novo* AIH may arise from alloimmunologic injury, marked by clinically obvious episodes of acute rejection. Since the target antigen in grafted liver is allogenic, the use of the term “autoimmune” to define hepatitis affecting an allogenic organ has been controversial, and alternative terms such as “posttransplant immune hepatitis,” “graft dysfunction mimicking autoimmune hepatitis,” or “post-liver transplantation *de novo* hepatitis” have been suggested (47–49). However, the concept of recurrence of AIH after LT has been accepted, since the antigenic targets for liver-specific autoimmunity are species specific and are shared by both recipient and donor livers. Therefore, the term “*de novo* autoimmune hepatitis,” with its clinical and therapeutic implications, remains the best until the pathogenesis of the condition is clarified (11). However, whether *de novo* AIH in transplanted livers represents a distinct entity or a form of atypical rejection in individuals at risk of developing autoimmune phenomena is unclear at present.

One can assume that other forms of autoimmune liver disease can also arise *de novo* after LT. A case of post-liver transplant *de novo* overlap syndrome of AIH-PBC, a novel “autoimmune-type” response, has recently been reported (49). However, cases of *de novo* PBC or PSC have not been reported. Since PBC develops insidiously without symptoms over several years, it is difficult to diagnose this condition if the serum biliary enzyme level does not increase (50). It is also hard to diagnose *de novo* PSC since PSC itself is difficult to diagnose before the change in biliary trees appears on cholangiography.

**Incidence** *De novo* AIH occurred in 7 (4%) of 180 recipients in the series of Kerkar et al. (40) and in 9 (3.6%) of 247 recipients in the series of Riva et al. (43).

**Diagnosis** The features of *de novo* AIH greatly resemble classic AIH, showing high immunoglobulin G, serum autoantibodies, and histologic features of plasma cell infiltration, interface hepatitis, bridging fibrosis, and collapse.

**Treatment** *De novo* AIH can be successfully treated with prednisolone and azathioprine, the conventional treatment for classic AIH (40,41). In the series of Kerkar et al. (40), the index case did not respond to antirejection treatment such as infusion of high-dose steroids and an increased dose of calcineurin inhibitor but only to corticosteroid. All patients had serum concentrations of cyclosporin A or tacrolimus within therapeutic antirejection levels at the time of diagnosis of *de novo* AIH.

**Prognosis** This entity responds well to immunosuppressive treatment, avoiding graft rejection and additional transplantation and thus improving long-term survival (51). However, the series by Miyagawa-Hayashino et al. (52) highlights the more severe histologic outcome of *de novo* AIH despite immunosuppressive treatment with longer follow-up.

### POSSIBLE MECHANISMS LEADING TO AUTOIMMUNITY AFTER LIVER TRANSPLANT

The recurrence of autoimmune diseases in transplanted livers is readily understandable. The recipient had an autoimmune liver disease whose immune system has been sensitized to species-specific antigens presented in the recipient liver. After transplantation of the graft from the donor, the immune system is restimulated and activated by either the recipient's antigen-presenting cells, which repopulate in the grafted liver, or by the donor's antigen-presenting cells, which share histocompatibility antigens with the recipient and present target alloantigens to the recipient's immune system. A pool of memory cells subsequently reexpands, and an immune response such as autoantibody production and immune-mediated liver injury occurs.

The pathogenesis that leads to *de novo* autoimmunity and posttransplant autoimmune liver diseases is not yet defined although the occurrence of autoimmune disease in patients with non-autoimmune liver disease is not infrequent. A variety of potential mechanisms have been postulated. One explanation is that it may arise by recognition of alloantigens, i.e., polymorphic antigens that differ between individuals and nonpolymorphic antigens shared by individuals of the same species, which are transferred with the graft. Inui et al. (42) examined autoantibodies serially in a patient with *de novo* AIH and in patients without *de novo* AIH after liver transplantation. Anticytokeratin 8/18 antibodies were detected in the patient's sera after the onset of *de novo* AIH, whereas other patients without *de novo* AIH were seronegative throughout the follow-up period, even with acute cellular rejection or other cause of liver dysfunction. They concluded that the changes in cytokeratin 8/18 in hepatocytes might be one of the sources of pathogenic autoantigens that cause *de novo* AIH after LT.

Aguilera et al. (53) have recently reported on some interesting cases: an antibody directed to glutathione-S-transferase T1 (CSTT1) was present in patients who developed *de novo* immune-mediated hepatitis. GSTT1 is a drug-metabolizing enzyme abundantly expressed in liver and kidney cells; it is encoded by a single gene that is absent in 20% of the Caucasian population. They confirmed that only under one of the four possible genetic combinations (null recipient/positive donor) is an alloimmune response triggered with production of anti-GSTT1 antibodies and concluded that this genetic mismatch can be considered to be a risk factor for *de novo* AIH (54).

Auxiliary LT is an ideal model for investigating whether *de novo* AIH after LT is a form of rejection. If the recipient's immune system reacts against self, both auxiliary and native liver should be similarly affected, since the antigenic targets

are shared by both organs, whereas rejection would affect only the transplanted liver. Miyagawa-Hayashino et al. (55) described a patient with cirrhosis caused by biliary atresia who developed graft dysfunction after auxiliary liver transplant from an almost HLA identical living donor. The patient's graft dysfunction was associated with positive ANAs and elevated IgG, which appeared after immunosuppression was suspended because of a septic episode. A biopsy of the auxiliary liver showed histological features compatible with those of AIH. Tsuji et al. (56) also reported the occurrence of *de novo* AIH in the allograft after auxiliary partial orthotopic LT.

Viral infections, which are frequent post LT, may raise autoimmunity in the graft by the mechanism of molecular mimicry, whereby T cells exposed to viruses sharing similar amino acid sequences with autoantigens are activated to crossreact to the autoantigens, leading to autoimmunity (57). Salcedo et al. (41) reported the observation that supports this hypothesis; all their patients developed *de novo* AIH in relation to infection with cytomegalovirus, Epstein-Barr virus, or parvovirus. Viral infections may also induce autoimmunity through other mechanisms, including polyclonal stimulation, enhancement and induction of membrane expression of MHC class I and II antigens, and interference with the immunoregulatory cells and/or with the idiotype-antiidiotype network (58).

Posttransplant patients treated with immunosuppressants may be predisposed to developing autoimmunity through the influence of cyclosporin A or tacrolimus. Cyclosporin A-induced autoimmunity, also called autoimmune syngeneic graft-virus-host disease, is a thymus-dependent, T-cell-mediated rodent animal model of disease and is considered to be an experimental model for human scleroderma (59–61). This interesting observation was obtained from experimental animals treated during the neonatal period or rendered immunocompromised by irradiation; the use of calcineurin inhibitors predisposes to autoimmunity and autoimmune disease, possibly by interfering with the maturation of T lymphocytes or with the function of regulatory T cells (61,62), with consequent emergence and activation of autoreactive T-cell clones. The relative presence of autoregulatory T cells with a CD45RO low T-helper cell phenotype may be a critical determinant in susceptibility to cyclosporin A-induced autoimmunity.

### CONCLUDING REMARKS AND OPEN QUESTIONS

Autoimmune hepatitis can occur *de novo* following LT, but whether *de novo* AIH represents a distinct entity or merely a form of atypical rejection is not clear at present. Recurrence of autoimmune liver diseases does not appear to be infrequent following LT. The rate of recurrence varies, however, depending on the diagnostic criteria used. Establishment of distinct diagnostic criteria is needed to solve this problem. Occurrence or recurrence of the diseases may be influenced by different factors including the genetic background of recipients and environmental factors. However, the mechanisms that lead to autoimmunity or recurrence of the diseases are not yet defined.

The pathogenesis of *de novo* autoimmunity and posttransplant autoimmune diseases in transplanted livers awaits clarification.

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# 35 Immunopathogenesis and Outcomes of Recurrent Hepatitis C

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JAMES R. BURTON, JR., LUCY GOLDEN-MASON, AND HUGO R. ROSEN

## KEY POINTS

- In the Western world, hepatitis C virus (HCV)-related end-stage liver disease is the leading indication for liver transplantation.
- HCV recurrence is nearly universal, with a significant proportion of patients (20–30%) developing allograft cirrhosis by the fifth year post-transplant associated with a high rate of decompensation and mortality.
- Many published studies investigating the treatment of recurrent disease with interferon with or without ribavirin (no longer the standard of care) have been small, single-center, uncontrolled trials with significant variability in patient selection, type and timing of antiviral therapy administered, and study end points evaluated.
- The human liver transplantation (LT) model provides a unique opportunity and a theoretical framework to examine HCV pathogenesis for many reasons, the most important of which is the accelerated natural history of HCV after LT, which allows the identification of distinct clinical outcomes (mild vs severe) in a relatively short period.
- HCV is most competent in replicating within the allograft. Viral kinetics during and after LT are generally characterized by a sharp decrease in HCV RNA levels during the anhepatic phase and immediately after graft reperfusion, reaching pretransplantation levels by approx 4 d followed by a marked increase (approx 20-fold) by the first postoperative month.
- Factors impacting on the cellular immune response (use of antilymphocyte preparations such as OKT3 and IL-2R antibodies and CMV infection) appear to shape the clinical outcome following LT.
- The vigor and timing of the CD4<sup>+</sup> T-cell response correlate with the histological and clinical outcome of HCV recurrence, with patients who develop mild recurrence demonstrating statistically significant higher responses at 1, 2, and 3 mo post-transplant than patients who develop severe recurrence.
- Strategies to provide adoptive transfer of HCV-specific T cells may represent a promising new approach to immunotherapy in these patients and may diminish the rate of graft loss from recurrent disease.
- The in vitro specificity of these donor HLA allele-restricted cytotoxic T lymphocytes (CTLs) suggests that these CTL clones could be exploited for adoptive immunotherapy in liver transplant patients who develop severe recurrence of HCV infection within their allografts by specifically targeting infected donor organ tissues without triggering generalized alloimmunity against recipient tissues.
- The role that NK cells play in determining the course of HCV infection in patients who have undergone LT for HCV-related cirrhosis is relatively unexplored. Control of HCV by recipient NK cells at the early stage of reinfection appears to be important in determining subsequent outcome.

## INTRODUCTION

In the Western world, hepatitis C virus (HCV)-related end-stage liver disease is the leading indication for liver transplantation (LT). Based on the presence of HCV RNA in the serum, recurrence is immediate and universal, with nearly all patients developing some evidence of histological recurrence. (Acute hepatitis occurs in 60–80% at a median of 4–6 mo post transplantation and chronic hepatitis in 80–100% by 1–4 yr [1–3]). Reports indicate that 20 to 40% of recipients with recurrent HCV disease progress to allograft cirrhosis within 5 yr compared with less than 5% of nontransplant chronic HCV patients; thus, the natural history of recurrent HCV is accelerated compared with the nontransplant setting (4,5). Once cirrhosis develops in the transplant setting, two-thirds will develop decompensation (ascites, variceal hemorrhage, encephalopathy) within 3 yr (5,6). The development of decompensation is associated with a very poor outcome, with only about 10% surviving for 3 yr. Despite this accelerated course, approximately a third of patients demonstrate only minimal fibrosis after 5 yr of follow-up (7). The use of protocol liver biopsies is justified in patients transplanted for HCV based on the increasing probability of severe histological findings (stage 3 or 4) (8,9). It remains unclear whether all HCV liver transplant

**Table 1**  
**Factors Associated With Severe Recurrence and Patient and Graft Survival After Liver Transplantation For HCV**

<i>Viral</i>	<i>Recipient</i>	<i>Donor</i>
Genotype 1b (14,15)	Age (23)	Age (14)
Pre-LT viral load (16,17)	Lack of CD <sup>+</sup> response (24)	Warm ischemia time (27)
Early post-LT viral load (18)	Non-Caucasian (17,23)	Cold ischemia time?
<i>Post-LT</i>	Female gender (13)	HLA-matching?
Rapid steroid taper (14)	CMV infection (25,26)	
Treatment of rejection with steroids (19,20) and antilymphocyte preps (21,22)	Diabetes (23)	HCV(+)?
	Previous HCV treatment?	

Abbreviations: LDLT; live donor liver transplantation.

recipients develop progressive fibrosis and whether the rate of fibrosis is constant or changes over time, e.g., is greater after the first 5 yr.

The presence of rapidly progressive cholestatic HCV is observed in approx 5% of patients transplanted for HCV, typically developing 1 to 3 mo post-LT and resulting in graft failure in 3 to 6 mo (9). Patients typically have very high serum HCV RNA levels with serum bilirubin levels of more than 6 g/dL and alkaline phosphatase levels greater than five times the upper limit of normal. The pathogenesis of this syndrome remains undefined, but preferential Th2 cytokine production by intrahepatic lymphocytes has been implicated (10,11). Optimal treatment remains uncertain but is focused on reducing very high HCV RNA levels by lowering immunosuppression and indefinite use of interferon-based therapy (12).

Although many studies have shown that short-term patient and graft survival is similar for patients undergoing LT for HCV compared with other indications, these studies were likely underpowered to detect small differences. Analysis of the United Network for Organ Sharing (UNOS) database revealed significantly diminished survival at 5 yr after primary LT in HCV-positive patients (56.7% vs 65.6% for HCV-negative transplant recipients;  $p < 0.05$ ) (13). A number of factors have been suggested to affect both severity of HCV recurrence and patient and graft survival. Table 1 outlines these viral, recipient, donor, and post-transplant factors.

Given the accelerated natural history of HCV recurrence, several approaches have been proposed to prevent or slow the progression to HCV-related graft failure. Current treatment strategies fall into three general categories: (1) pretransplant antiviral therapy, (2) preemptive therapy (prophylaxis) started in the early post-transplant period before the development of clinically apparent acute hepatitis, and (3) post-transplant therapy at the time of diagnosis of acute hepatitis or for established and/or severe chronic hepatitis. Unfortunately, many published studies investigating the treatment of recurrent disease with interferon with or without ribavirin (no longer the standard of care) have been small, single-center, uncontrolled trials with significant variability in patient selection and type and timing of the antiviral therapy administered and the study end points evaluated (i.e., histological response, end of treatment

response, sustained virological response [SVR]). Rates of SVR (HCV RNA negative 24 wk after completing antiviral therapy) are far less than those achieved in immunocompetent HCV-infected patients; however, in patients achieving SVR, long-term absence of HCV RNA in the liver and marked histological improvement (inflammatory scores much more so than fibrosis scores) have been described (28). Many transplant centers follow protocols to initiate treatment when clinically significant evidence of recurrent HCV develops. For patients who either fail or do not tolerate antiviral therapy, the only option for those developing allograft failure from recurrent HCV is LT, which is associated with a number of complex issues (29–31).

### HUMAN LIVER TRANSPLANT AS A RESEARCH MODEL TO STUDY HCV PATHOGENESIS

The human liver transplantation model provides a unique opportunity and theoretical framework to examine HCV pathogenesis for a number of reasons (Table 2) (32). First, because the whole organ is removed at the time of transplantation, it is possible to characterize intrahepatic lymphocytes directly ex vivo. Accordingly, we have recently shown that dominant T-cell clones established in vitro using anti-CD3 are poorly representative of dominant clones present within intrahepatic populations examined directly ex vivo from HCV-infected cirrhotic livers at the time of transplant (33). Additionally, following transplantation, patients are followed closely at regular intervals and frequently undergo liver biopsies to evaluate abnormal liver function tests. Because of the telescoped natural history of HCV in this setting, it is possible to define distinct phenotypic outcomes (mild vs severe recurrence) in a relatively short period.

#### VIRAL KINETICS

A direct pathogenic role of HCV replication has been difficult to establish, in part because of the lack of robust tissue culture systems and animal models that allow modulation of immune response (35). Nonetheless, data are emerging in support of the direct causative roles of HCV proteins in apoptosis by virtue of oxidative stress and cell cycle disturbance (36). It has been known for over a decade that liver transplantation leads to a marked increase (approx 20-fold) by 1 mo in circulating viral titers (37,38). However, until recently,

**Table 2**  
**Advantages of Human Liver Transplantation**  
**as a Research Model to Study Hepatitis Virus**

1. Opportunity to characterize directly ex vivo intrahepatic lymphocytes without the need of nonspecific expansion techniques (needed for lymphocytes derived from liver biopsies).
2. Acute infection of the allograft invariably occurs and provides the opportunity to study innate and adaptive immune responses triggered in the early stages of infection.
3. Opportunity to track patients at regular intervals for blood and tissue sampling (e.g., protocol liver biopsies or biopsies performed to rule out rejection.)
4. Accelerated natural history post-transplant makes it possible to define disease outcomes within a relatively short period of follow-up.
5. In contrast to the immunocompetent setting which is associated with stable viral replication that does not vary to significant degree over months to years (34), liver transplantation leads to a marked (approx 20-fold) increase in circulating viral titers. Because viral replication is associated with accumulation of mutations, quasispecies can be characterized longitudinally following liver transplantation.
6. Opportunity to determine the effects of different immunosuppressive drugs and the impact of antiviral therapy given at different time points (preemptively or after histological recurrence has been documented) on the natural history of HCV.

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the early viral kinetics remained undefined. It is important to recognize that viral load measurement indicates the rates of viral production and clearance only when the homeostatic balance of the host is perturbed, e.g., with antiviral therapy or with LT.

An elegant analysis from the Barcelona group (39) demonstrated that a sharp decrease in HCV viral load occurs during the anhepatic phase and immediately after graft reperfusion, most likely related to a lack of virion production and hepatic viral clearance via massive entrance of HCV into the hepatocytes or uptake by the liver reticuloendothelial system. The HCV viral load decay after graft reperfusion follows first-order elimination kinetics, with a mean half-life of approx 3.44 h. In one patient with a prolonged anhepatic phase (20 h), the elimination half-life of HCV was significantly longer than in the other patients in the Barcelona series. Three different kinetic patterns were noted during the first week after LT, with most of the patients demonstrating a rapid increase in HCV viral load (Fig. 1). Interestingly, HCV RNA increased rapidly in patients receiving corticosteroids as part of their immunosuppressive therapy, whereas it continued to decrease in most patients who did not receive corticosteroids. Ultimately, viral kinetics data may facilitate early identification of different subsets of patients, i.e., mild vs severe recurrence, and the design of new antiviral and/or immunosuppressive strategies following transplantation.

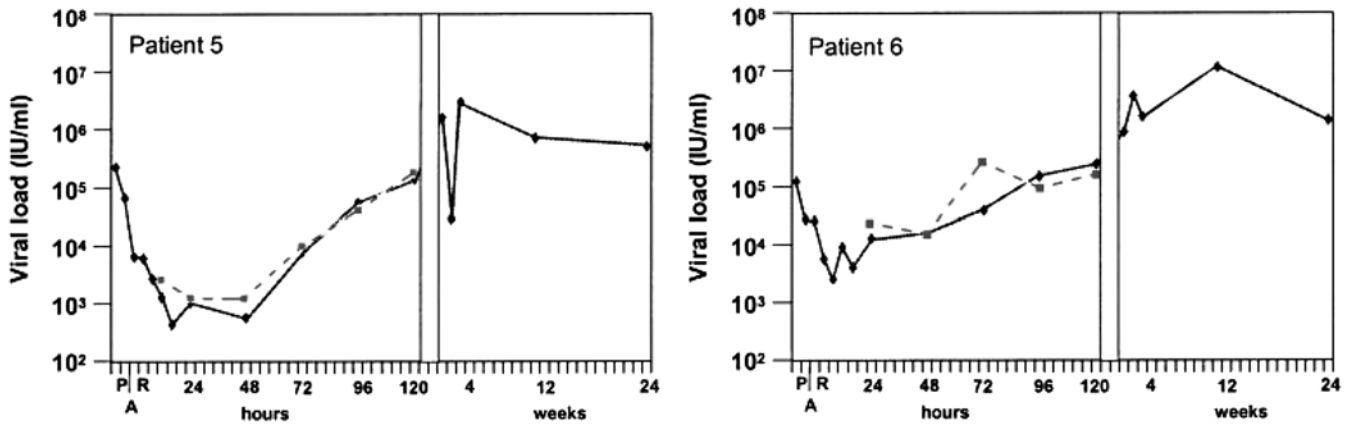
#### **VIRAL QUASISPECIES**

The rapid increase noted in HCV viral loads after transplantation proves the high capacity of HCV to adapt to a new environment. In theory, the viral species present in an individual

are not all equally able to infect and replicate within the allograft, in part because of differences in cell tropism, and selection may occur during the acute phase of infection of the new liver. A variant strain could constitute a minority of the circulating viral population before transplantation but could be more adept at infecting hepatic allograft cells or even extrahepatic sites that sustain viral replication, therefore having a survival advantage. With a rapidly replicating virus like HCV, even small fitness differences may lead to substantial differences in overall replication and survival ability (40). In particular, viral escape from a dominant T-cell response early after LT could play a central role in viral persistence by enhancing viral survival when it is most susceptible to immune selection (i.e., during massive reinfection of the allograft) (41). Transplantation of an HCV-infected liver into an HCV-positive recipient represents a model of superinfection (42), and Vargas and colleagues (43) demonstrated that superinfection of the liver recipient by the donor's strain was associated with significantly milder disease than when the recipient strain became (or remained) dominant. In addition, genotype 1 or 1b consistently predominated over non-1 or non-1b genotypes in recipients of infected grafts, suggesting replicative differences among viral strains.

Despite the coexistence of virus-specific immune responses, HCV is able to persist for a virtually indefinite period in a tug-of-war with the host as a complex of heterogeneous and dynamic quasispecies (44). Most HCV quasispecies analyses in LT patients have focused on HVR1, located at the N-terminus of the E2/NS1 region, and the results have been conflicting. Gretch et al (45) showed that successful propagation of pre-transplant major quasispecies was associated with a more severe form of HCV disease recurrence, a finding that was subsequently confirmed by Doughty and colleagues (46). In contrast, Pessoa et al. (47) found that, in the subset of patients with fibrosing cholestatic hepatitis, divergence of quasispecies was enhanced, resulting in emergence of many new variants. However, differences in quasispecies are not in themselves definitive evidence for the existence of immune selection.

The assumption that RNA viruses are in mutation-selection equilibrium has recently been called into question, i.e., the state of flux in mutants previously ascribed to immune pressure may depend more on the relative fitness of viral subpopulations (48). In this model, lower viral loads in patients with epitopic sequence variation may simply reflect compromised replicative activity of the variant. These considerations are particularly relevant in the LT setting, in which HCV may have a direct viral cytopathic effect and for which no protective role for virus-specific antibody responses has ever been established. Indeed, a recent analysis from our center found that the mean antibody reactivity to E2 was virtually identical in patients with mild ( $n = 52$ ) vs severe ( $n = 12$ ) recurrence (49). Intriguingly, we and others (50) have reported higher levels of antibody reactivity specific to other HCV regions (core, NS4, NS5) in patients with severe compared with mild recurrence. Although HVR1 is the putative target of specific neutralizing antibody, no LT study to date has determined whether there is a temporal relation with viral evolution and emergence of a specific

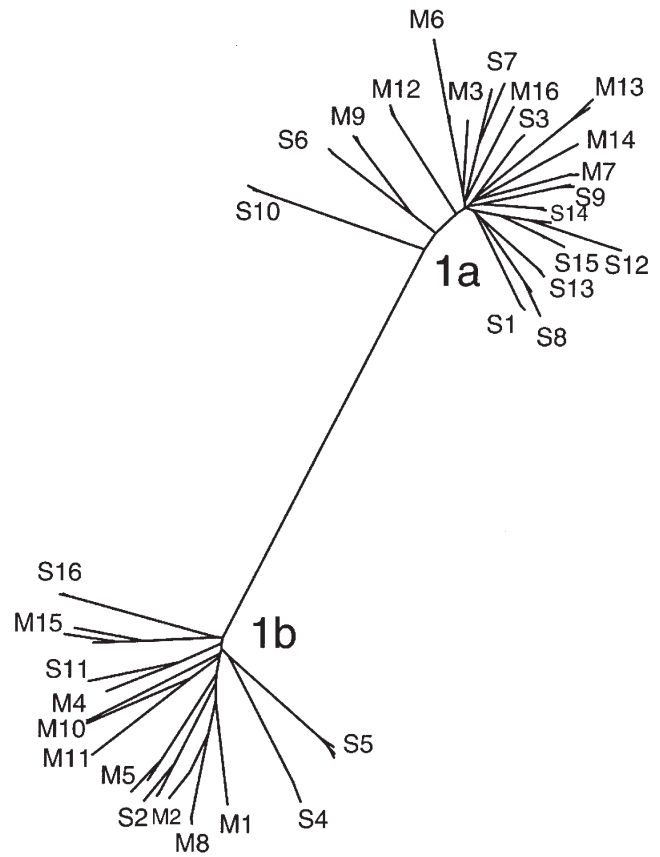


**Fig. 1.** HCV kinetics during and after liver transplantation. Two representative patients demonstrating the most common pattern of rapid increase in viral load, reaching pretransplantation levels by 4 d. A, anhepatic phase; P, pretransplant; R, reperfusion phase. (Reprinted from ref. 39, with permission.)

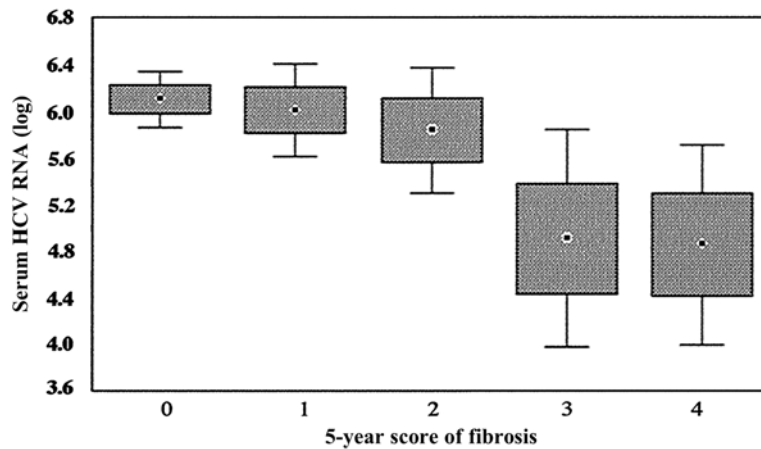
immune response to corroborate the hypothesis of mutation-selection equilibrium.

We characterized viral genetic diversity within regions encoding putative T-cell epitopes, comparing the nucleotide and predicted amino acid protein sequences of the HCV genome obtained from serum samples of 32 HCV-genotype 1-infected LT patients with well-characterized outcomes, i.e., mild vs severe recurrence (51). Because the sum of immunogenic peptides generated in HCV infection likely influences the breadth and strength of the T-cell response, we examined approx 1.8 kb of amplicon products (420 bp in core and 1380 bp in NS3) and the viral peptide sequence of nine different epitopic regions previously shown to elicit strong CD4<sup>+</sup> and CD8<sup>+</sup> T-cell responses. However, we failed to find any association between the presence of mutations within core or NS3 regions and outcome of HCV disease following LT (Fig. 2). Specifically, the changes in the amino acid composition of the quasispecies (nonsynonymous substitutions, or  $d_N$ ) occurred at an extremely low rate, irrespective of disease severity. The lack of evolution of viral protein sequences over time after transplantation suggests that immunosuppression effectively eliminates selective pressure by cytotoxic T lymphocytes (CTLs).

In accord with these findings, Gigou and colleagues (52) observed weak correlations between the  $d_N$  matrix, reflecting the rate of amino acid substitutions in the core protein sequence, and phenotypic matrices in 53 genotype 1 patients assessed 5 yr post-LT. Instead, they found that the nucleotide sequence of the core region, rather than its primary amino acid sequence, correlated with outcome post-transplant. In other words, the shorter the genetic distance between two genotype 1 strains, the more similar the degree of allograft fibrosis and the level of HCV viremia in the corresponding patients. These data support the concept that different HCV configurations may have different degrees of intrinsic pathogenicity. Moreover, they found that viral replication at 5 yr post-LT inversely correlated with the degree of allograft fibrosis, postulating that



**Fig. 2.** Neighbor joining phylogenetic trees constructed from NS3 gene sequences (nt 3882–5202) of all study patients (severe [S1–S16] and mild [M1–M16] recurrence). Distinct clusters of viral sequences corresponding to each individual patient were found. (Reprinted from ref. 51, with permission.)



**Fig. 3.** Serum HCV RNA levels (Taqman PCR) and fibrosis score at the time of 5-yr routine biopsy after liver transplantation. Error bars indicate standard deviations. (Reprinted from ref. 52, with permission.)

a host response that induces fibrosis may paradoxically reduce viral replication (Fig. 3).

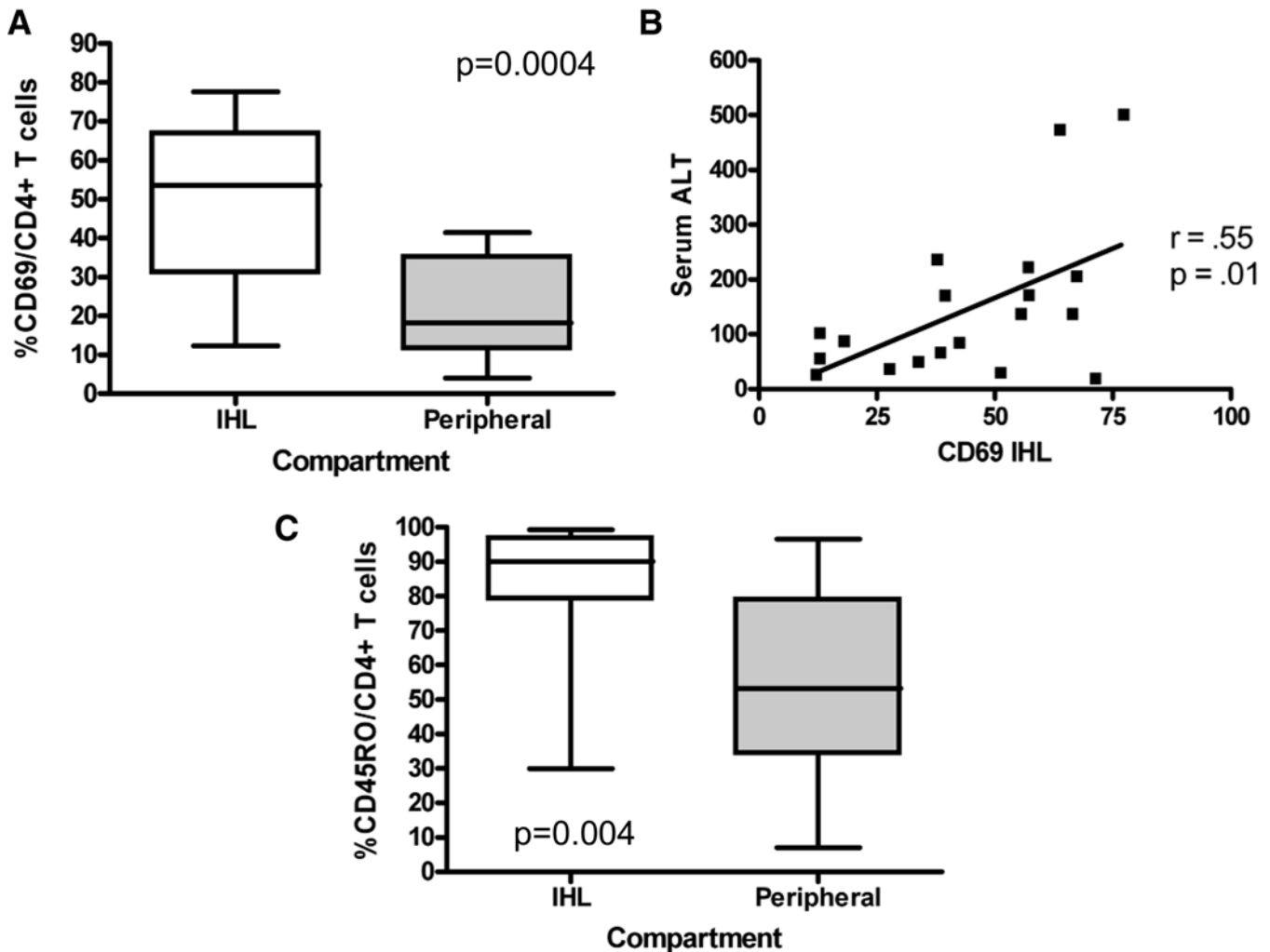
### THE ROLE OF THE IMMUNE RESPONSE IN DETERMINING SEVERITY OF HCV RECURRENCE FOLLOWING LIVER TRANSPLANTATION

#### CD4<sup>+</sup>T-CELL RESPONSES

Several lines of indirect evidence support a role for the cellular immune response in shaping outcome following transplantation. A case control study from the University of California at Los Angeles showed that patients receiving monoclonal the anti-CD3 antibody OKT3, which opsonizes, depletes, and induces cell death in T cells, developed earlier and more severe recurrence compared with a contemporary cohort matched for key variables (21). Five of 19 (26.3%) patients who received OKT3 ultimately developed allograft cirrhosis vs 2 of 33 (6%) of patients with steroid-responsive rejection (not receiving OKT3) ( $p < 0.03$ ). A subsequent analysis demonstrated that HCV-positive patients receiving OKT3 experienced a 10-fold increase in graft loss (53), and this finding has now been confirmed by a number of groups. A recent study from the University of Florida (54) showed that HCV-positive liver transplant recipients who received daclizumab (an interleukin-2 receptor [IL-2R] antibody that blunts T-cell activation) were more prone to develop earlier onset of allograft hepatitis and greater histologic activity compared with a well-matched HCV control population, with 45% of the former group developing advanced disease within 1 yr. Coinfection with cytomegalovirus (CMV), which has been shown to induce cell-mediated immune defects and consequently a higher risk of opportunistic infections following transplantation, has been correlated with a higher risk of development of HCV-related allograft cirrhosis (25). A number of centers, including the Mayo Clinic and University of Pittsburgh, subsequently have confirmed the association between CMV and HCV allograft cirrhosis.

Characterization of the functional features of intrahepatic T cells is essential to understanding the mechanisms involved in recurrent HCV infection. Our group has characterized these intrahepatic T cells using paired liver-derived and circulating CD4<sup>+</sup>T cells, expanded simultaneously and stained with the monoclonal antibodies anti-CD3, CD4, CD69, CD28, and CD56 (authors group, unpublished data). An increased proportion of CD4<sup>+</sup>T cells within the liver allograft expressed the activation marker CD69 compared with the peripheral blood (mean  $48.15 \pm 4.9$  (SEM) vs  $21.7 \pm 2.9$ ,  $p = 0.0004$ , Kruskal-Wallis test), confirming that the cells isolated from allograft specimens actually derive from intrahepatic cellular infiltrates and do not represent a contamination of circulating cells present in the biopsy tissue (Fig. 4A) (55). We found a direct correlation with expression of intrahepatic CD69 and serum ALT (Fig. 4B). We noted a loss of the co-stimulatory receptor CD28 by T cells, evidence for “chronic” T-cell activation; accordingly, we found fewer CD28-positive T cells in the liver than in the peripheral blood ( $6.07 \pm 2.8$  vs  $13.38 \pm 4.6$ ;  $p = 0.06$ ). Moreover, consistent with a memory phenotype, CD4<sup>+</sup>T cells in the intrahepatic compartment expressed significantly higher levels of CD45RO compared with their peripheral blood counterparts (mean 83.3% vs 53.5% in intrahepatic vs peripheral compartment;  $p = 0.004$ , Kruskal-Wallis test) (Fig. 4C). As expected, CD4<sup>+</sup>T cells expressing natural killer (NK) cell markers on the cell surface were nearly absent (mean less than 3%). Thus, these findings indicate for the first time that following LT, there is preferential compartmentalization of activated memory CD4<sup>+</sup>T cells within the liver allograft.

A failure to mount an efficient immune response to HCV antigens, either because of selective defects in the host immune system or because of viral interference with the normal function of the immune cells, could account for why most HCV-infected transplant recipients develop allograft hepatitis. A study from our program demonstrated that approx 40% of patients with minimal or self-limited recurrent HCV demonstrated proliferative

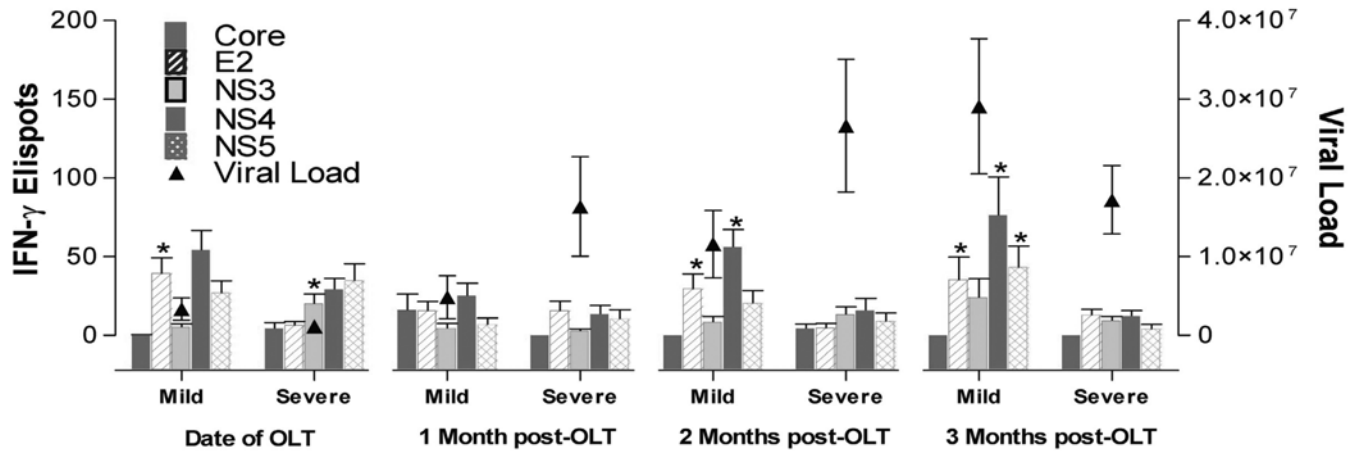


**Fig. 4.** Enrichment for activated, memory CD4<sup>+</sup> T cells within the liver allograft. (A) An increased proportion of CD4<sup>+</sup> T cells within the liver allograft expressed the activation marker CD69 compared with the peripheral blood (mean, 48.15 + 4.9 [SEM] vs 21.7 + 2.9, respectively;  $p = 0.0004$ , Kruskal-Wallis test). (B) A direct correlation with expression of intrahepatic CD69 and serum ALT. (C) CD4<sup>+</sup> T cells in the intrahepatic compartment expressed significantly higher levels of CD45RO compared with their peripheral blood counterparts (mean, 83.3% vs 53.5% in intrahepatic vs peripheral compartment, respectively;  $p = 0.004$ , Kruskal-Wallis test).

responses to HCV antigens, whereas none of the patients with severe recurrence did so (24). In a more recent analysis (56), we prospectively tracked T-lymphocyte responses in three groups of HCV-seropositive patients who underwent LT: patients who received preemptive antiviral therapy (or placebo) starting within the first month after transplantation, patients who received antiviral therapy for severe histological recurrence more than 3 mo after transplantation, and patients with long-term follow-up who have demonstrated minimal evidence of histological recurrence and have not required antiviral therapy. Figure 5 demonstrates that the vigor and timing of the CD4<sup>+</sup> T-cell responses correlated with the histological/clinical outcome of HCV recurrence, with patients who develop mild recurrence demonstrating statistically significantly higher responses at 1, 2, and 3 mo post-OLT than patients who develop severe recurrence. Despite immunosuppression, on the average,

those patients with mild recurrence demonstrated stronger responses compared with chronically infected nontransplant patients ( $n = 50$ ). Of interest, there were no appreciable differences between the severity groups on the day of LT, and whereas HCV-specific CD4<sup>+</sup> T-cell responses decreased further following initiation of immunosuppression in the patients who subsequently developed severe recurrence, the responses directed against E2, NS3, NS4, and NS5 steadily increased. Our data demonstrate that patients with advanced HCV-related liver disease may retain the ability to respond to a broad array of HCV proteins/peptides following LT.

Based on these findings, we have designed a targeted therapy trial in which patients demonstrating vigorous CD4<sup>+</sup> T-cell ELISPOT responses are not treated, whereas those lacking responses in the first few months are randomized to therapy vs expectant observation. Strategies to provide adoptive transfer of



**Fig. 5.** Combined interferon- $\gamma$  (IFN- $\gamma$ ) ELISPOT results in response (SEM) to five recombinant HCV antigens demonstrate differences between patients who subsequently developed mild recurrence ( $n = 7$ ) vs those who developed severe recurrence ( $n = 8$ ).  $*p < 0.05$  between mild and severe groups for specific antigen by two-tailed  $t$ -test. There was no significant difference between viral loads in severity groups at the different time points. (Reprinted from ref. 55, with permission.) OLT, orthotopic liver transplant.

HCV-specific T cells may represent a promising new approach to immunotherapy in these patients and diminish the rate of graft loss from recurrent disease.

#### CD8<sup>+</sup> T CELLS: SELF AND NONSELF RESTRICTED RESPONSES

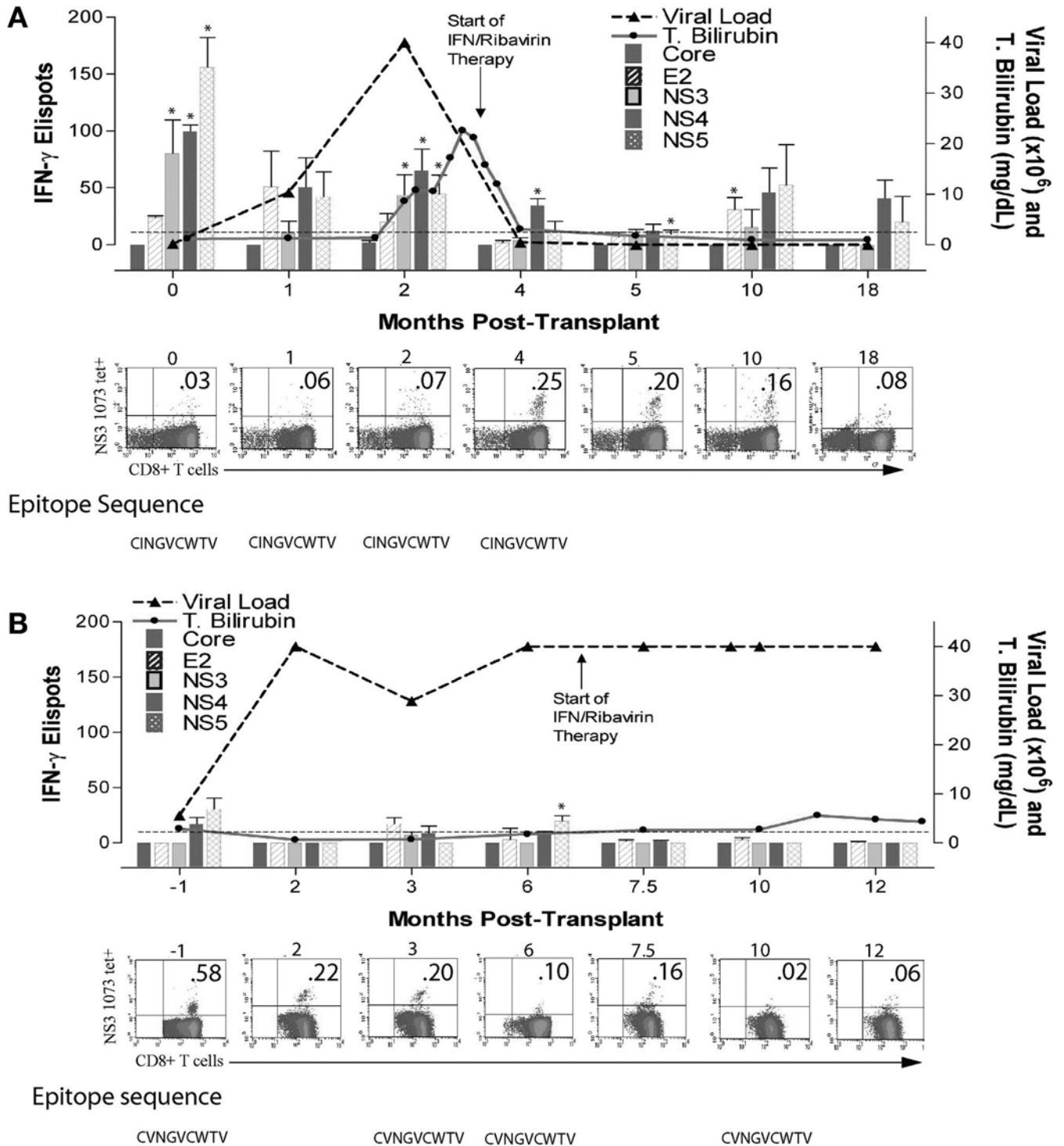
Although incompletely understood, the immune recognition of the HCV-infected allograft may be essential in the containment of infection. CD8<sup>+</sup> T cells are the primary effector lymphocytes for provision of protective immunity against intracellular pathogen infection of parenchymal cells and are effective because of their ability to recognize infected cells as the combination of pathogen-derived peptides in the peptide-binding grooves of MHC class I molecules on the surface of infected cells. Novel CD8<sup>+</sup> T-cell responses were demonstrable in patients who cleared serum HCV RNA with interferon and ribavirin, in agreement with another recent study (57). Figure 6A and B show patients who developed cholestatic HCV recurrence within 6 mo post-LT, prompting antiviral treatment; moreover, both patients were HLA A2-positive recipients of HLA A2-positive liver donors, facilitating comparison of CD8<sup>+</sup> T-cell responses. To determine the origin of the HCV-specific clonotype, we examined liver-infiltrating tetramer-positive cells from the recipient explant (liver removed at time of LT), because of the known relative enrichment of tetramer-positive cells within the liver (58). Indeed, although tetramer-positive cells were not detectable peripherally, we were able to sort and clone liver-infiltrating CD8<sup>+</sup> T cells specific to the NS3 1406 epitope on the day of transplantation. Total RNA was extracted from the peripherally reconstituted clone and the intrahepatic (pre-transplant) clone. The polymerase chain reaction (PCR) product was identified as an in-frame TCR that utilized TCRBV14, TCRBJ1S2, and TCRBC1. The junctional sequence was CASSLQGNNYGYT. (The end of VB14 is underlined and the start of JB1S2 is double underlined.) The CDR3 sequence was exactly the same in the peripheral reconstituted

clone and the intrahepatic clone (at both the nucleotide and amino acid level); thus, the clone expanded from the peripheral blood following antiviral therapy was originally present in the explanted liver.

In contrast, enumeration by soluble HLA-A2 tetramers revealed rapid decline in patients who developed progressive histologic recurrence (Fig. 6B); we excluded the presence of viral escape mutations as a potential cause for the changes in tetramer-specific frequencies by direct sequencing of the epitope coding region at various time points, in accordance with our previous findings.

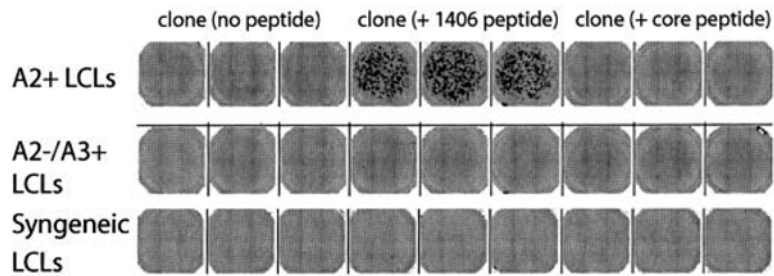
LT is performed with no regard to specific matching of donor-recipient MHC alleles, and this may serve as a barrier to the development of protective (i.e., antiviral) cell-mediated immunity directed against infected cells within the allograft. Since CD8<sup>+</sup> T cells recognize pathogen-derived peptides in the context of MHC class I molecules, recognition of intracellular infection requires the presence of CD8<sup>+</sup> T-cell populations capable of recognizing the infected allograft. Developing a comprehensive understanding of protective immunity to HCV will require an assessment of both recipient and allograft restricted CTLs. We recently demonstrated that receipt of hepatic allografts can select a unique population of recipient-derived cells capable of recognizing intracellular infection of HCV in the context of the donor HLA molecule (59).

As shown in Fig. 7, these HCV-specific CTLs become activated only in the presence of nonself and cognate viral peptide, indicating that these cells do not become functional in the periphery but only in the HCV-infected allograft. Importantly, these CTLs were not simply alloreactive since they did not bind irrelevant HLA-A2 tetramers that contained HIV gag peptide and did not respond when cocultured in an ELISPOT assay with HLA-A2-expressing LCLs alone (without cognate peptide). The *in vitro* specificity of these donor HLA allele-restricted CTLs suggest that these CTL clones could be exploited for adoptive immunotherapy in LT patients who



**Fig. 6.** (A) Reconstitution of HCV-specific cellular immunity in a patient with severe cholestatic HCV recurrence who responded to antiviral therapy (HCV RNA expressed as  $10^6$  copies/mL). *Top*, interferon- $\gamma$  (IFN- $\gamma$ ) ELISPOT responses to HCV recombinant proteins, viral load, and serum bilirubin. *Middle*, CD8 $^+$  T-cell responses to NS3 1073 tetramer. *Bottom*, amino acid sequence of NS3 1073 to 1081 epitope at four time points (HCV genotype 1a prototype sequence: CINGVCWTV). (B) HCV-specific immune responses in a patient with severe cholestatic HCV recurrence who failed to respond to antiviral therapy (HCV RNA expressed as  $10^6$  copies/mL). *Top*, IFN- $\gamma$  ELISPOT responses to HCV recombinant proteins, viral load, and serum bilirubin. *Middle*, CD8 $^+$  T cell responses to NS3 1073 tetramer. *Bottom*, amino acid sequence of NS3 1073 to 1081 epitope at four time points (HCV genotype 1a prototype sequence: CINGVCWTV); amino acid substitution (V for I at position 2 was detected but remained stable over time). (Reprinted from ref. 55, with permission.)





**Fig. 7.** HLA restriction of HCV-specific CTL clones isolated following liver transplantation; the clones were recipient derived but donor allele restricted. In the presence of cognate peptide (NS3 1406 epitope) and non-self HLA allele, CTLs produced IFN- $\gamma$  but not in the presence of self (recipient) HLA alleles. (Reprinted from ref. 58, with permission; copyright 2004, The American Association of Immunologists, Inc.)

develop severe recurrence of HCV infection within their allografts by specifically targeting infected donor organ tissues without triggering generalized alloimmunity against recipient tissues. Because of the unusual origin of these CTLs, i.e., across HLA barriers, we have further characterized their T-cell receptors (TCRs). We found that the TCRs of these HCV-reactive CTLs display high avidity for peptide, further supporting a potential immunotherapeutic role for these novel CTLs (60).

### NK CELLS AND LT FOR HCV

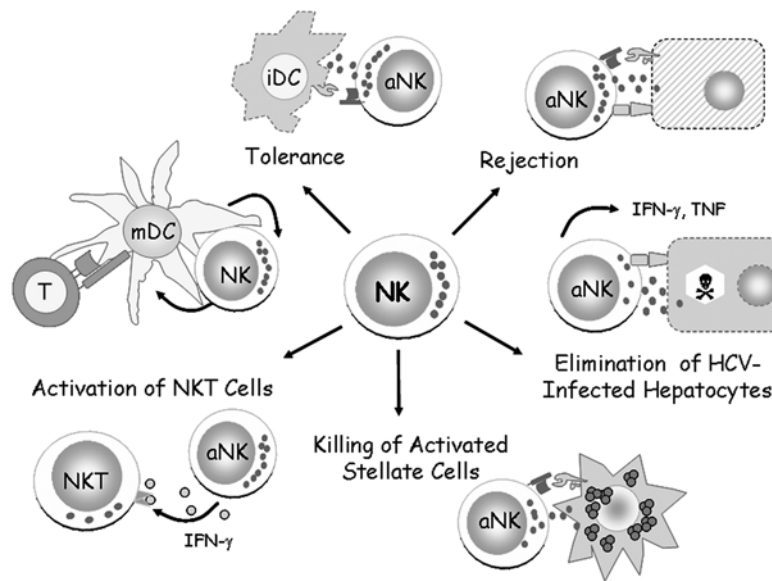
The role that NK cells play in determining the course of HCV infection in patients who have undergone LT for HCV-related cirrhosis is relatively unexplored. This population has the potential to affect outcomes post LT both favorably and/or adversely. NK cells, enriched in the liver (61), are critical for induction of antiviral immunity (62) and may therefore be important for early control of viral load at the time of infection of the donor organ. Our own preliminary data indicate that pre-LT peripheral NK cell levels stratifies patients at the risk of severe recurrence independently of viral levels, suggesting that control of HCV by recipient NK cells at the early stage of reinfection is important in determining subsequent outcome (63). Alternatively, NK cells may cause liver damage either directly through induction of apoptosis in infected hepatocytes (64) or indirectly through cytokine activation of CTLs and NKT cells, populations implicated in liver injury as well as protection in HCV infection. In addition, NK cells are actively involved in induction of both transplant tolerance (65) and rejection (66,67), cellular processes influencing outcome post LT for HCV-related cirrhosis. The frequency and severity of acute rejection episodes has been identified as an indicator of poor outcome post-LT for HCV cirrhosis (68). The occurrence of graft rejection despite the use of immunosuppressive drugs aimed at blocking T-cell activity indicates the involvement of other cells in this process. NK cells are the primary population involved in sensing non-self or altered MHC expression (69); thus, recipient NK alloreactivity may be the mediating factor of rejection. Furthermore, NK cell function does not appear to be affected by current clinical immunosuppressive agents (70,71); therefore NK cells may play an exaggerated role in determining outcome post-LT.

Hepatic stellate cell (HSC) activation is a pivotal step in the hepatic fibrogenic cascade preceding clinically apparent fibrosis. Early HSC activation, as measured by  $\alpha$ -smooth muscle actin staining, is predictive of subsequent development of histologically severe recurrence of HCV at 1 yr post-LT (72). Recent studies provide convincing data that NK cells, through induction of apoptosis of activated HSCs, are actively involved in protection from liver fibrosis (73,74). NK cells are reduced in HCV-infected cirrhotic livers and are activated by IFN- $\alpha$  and IFN- $\gamma$ , agents shown to ameliorate fibrosis, suggesting that NK cells in humans may also be an important antifibrotic population. Supporting this theory are recent data showing increased fibrosis progression during the first year after LT with fewer less HLA mismatches. (75). Furthermore, peripheral NK cell cytolytic function correlated inversely with liver fibrosis stage (76). Taken together, these studies suggest that functionally active NK cells may attenuate liver disease progression post-LT. Thus, hepatic NK cells represent a potentially destructive or a potentially protective population in the context of LT HCV infection (Fig. 8). Further studies are warranted to determine the role of these cells.

### CONCLUDING REMARKS AND OPEN QUESTIONS

Despite the significant advances in surgical techniques and transplant medical care, recurrence of HCV infection after LT represents a significant burden to the medical community, given that it is the leading indication for LT in the Western world, has nearly universal recurrence, an accelerated natural history, and that treatment regimens post-LT are only marginally effective at best. Our understanding of the role of the immune system is critical in the control of infection with HCV and likely explains the broad spectrum of allograft injury related to HCV infection, from mild histological abnormalities to development of cirrhosis in only a few years. Mechanisms of viral clearance remain incompletely understood. In the nontransplant setting, individuals who spontaneously clear HCV infection display vigorous HCV-specific cellular immune responses.

We have provided evidence that HCV-specific immunity correlates with improved outcome after LT. This understanding may allow for better means of identifying patients at risk for



**Fig. 8.** Natural killer (NK) cells play a central role in cellular processes influencing outcome post-LT for HCV infection. NK cells, through their interaction with mature and immature dendritic cells (mDC/iDC), have been implicated in the induction of both tolerance and rejection, processes closely linked to outcomes post-LT. Activated NKs (aNK) are directly involved in the elimination of virally infected hepatocytes and may therefore be important for early control of viral load in the setting of HCV infection of the donor liver. Their role in the control of activated stellate cells has implicated them as an important antifibrotic population. NK-derived cytokines drive activation of NKT-like cells, which in turn may eliminate virally infected cells and/or cause hepatocyte damage. IFN- $\gamma$ , interferon- $\gamma$ ; TNF, tumor necrosis factor.

severe recurrence and who would therefore benefit from antiviral therapy or immunotherapeutic approaches. Questions persist on how CD4<sup>+</sup> and/or CD8<sup>+</sup> T cells contribute to viral containment and allograft protection. This is of particular interest in the transplant setting, in which no regard to specific matching of donor-recipient MHC alleles exists. As a result, developing a comprehensive understanding of protective immunity to HCV will require an assessment of both recipient- and allograft-restricted CTLs.

Emerging data have indicated that NK cells play a central role in controlling HCV in the immunocompetent setting. Their role in mediating HCV replication post-LT and inducing fibrosis or protecting the allograft remains largely unexplored and represents an area of future research.

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