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Immunotherapy in 2020

Visions and Trends for Targeting Inflammatory Disease

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Editors: A. Radbruch | H.-D. Volk | K. Asadullah | W.-D. Doecke

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Preface

From 22 to 24 October 2006, internationally renowned scientists from the European Union and the United States met in Potsdam near Berlin, Germany, to discuss the future of chronic inflammatory disease treatment. The title of the symposium, organized by the Ernst Schering Foundation, was "Immunotherapy in 2020—Visions and Trends for Targeting Inflammatory Diseases".

The presentations covered the main mechanisms of immunoregulation such as peripheral and central tolerance, epigenetic programming, immunologic memory, and regulatory networks in inflammation as well as novel experimental and clinical approaches for targeting inflammation in autoimmunity and transplantation. Group discussions focused on the question of how recent findings in immunological research can lead to improved diagnostics, new drugs, and better therapies. In this regard, beside novel approaches, the individualization of immunomodulatory therapies and the establishment of reliable biomarkers for assessing the specific immune situation along with patient response to therapies were discussed.

In this volume, the symposium speakers give their view on current topics of immunopathophysiology and immunotherapy.

The chapter by Prof. Rikard Holmdahl (Section for Inflammation Research, Lund University, Sweden) deals with the genetic basis of

inflammatory diseases by means of gene polymorphisms and its implications for the finding of new therapies. He describes how, based on the proven genetic component of rheumatoid arthritis demonstrated by clinical studies, a search for susceptibility genes in mice models was performed, which led to the definition of novel pathways involved in the immunopathology of arthritis. Concluding from this, novel therapeutic approaches are proposed which are experimentally proven yet.

The posttranscriptional networks in inflammation are the focus of the contribution from Dr. Dimitris Kontoyiannis and colleagues (Biomedical Sciences Research Center, Athens, Greece). The biosynthesis of inflammatory mediators relies on the biogenesis as well as the utilization of their corresponding mRNAs. These latter "utilization steps" encompass posttranscriptional mechanisms that impose a series of flexible rate-limiting controls to modify the abundance of a mRNA as well as its rate of translation to proteins. The chapter deals with RNA-binding proteins that, for example, by binding to the AU-rich family of elements, stringently control the maturation, localization, turnover, and translation of mRNAs from inflammatory genes.

Prof. Andreas Radbruch and colleagues (German Rheumatism Research Center, Berlin, Germany) give an up-to-date overview on immunological memory, its role in chronic inflammation, and its dramatic impact on the limitations of current immunotherapies. Concerning the latter, the humoral memory—which is provided by long-living plasma cells—is discussed in particular. The immunologic memory is not sufficiently targeted by current immunotherapies that suppress ongoing immune activation. This is probably one of the main reasons frustrating the cure of diseases by therapies, except bone marrow transplantation. Therefore, targeting of the immunologic memory, which provides a consistent trigger of relapse, might be a major challenge for future immunotherapies.

Adoptive T cell therapies are used to substitute the non-sufficient immune response of the recipient, e.g., to virus infection or malignancy. The use of unselective allogeneic T cell therapies (e.g., donor lymphocyte infusion), however, is limited by major clinical complica-

tions such as graft-versus-host disease. Dr. Stephen Gottschalk and colleagues (Center for Cell and Gene Therapy, Baylor College of Medicine, Houston, Texas, USA) deal with the development of autologous antigen-specific T cell therapies for the treatment of different Epstein-Barr virus-associated malignancies. If effective, these strategies might have broad implications also for other indications, e.g., human tumors with defined antigens.

Interesting novel perspectives of a rather old approach, polyclonal antibody therapy, are given in the contribution by Drs. Roland Buelow and Wim van Schooten (Therapeutic Human Polyclonals Inc., Mountain View, California, USA). Despite their strong development in the last few years (e.g., humanization, novel antibody derivatives, and conjugates), monoclonal antibody therapies have principal limitations. Hence, their effectiveness especially in devastating diseases such as cancer and infections is often limited by the capacity of single monoclonal antibodies to trigger immune effector functions as well as by the adaptivity of the affected cells and the pathogens (e.g., mutation or loss of antigen expression). These limitations of mono-specific antibodies might be overcome by therapeutic oligoclonal (mixtures of monoclonals) and polyclonal antibody preparations. A major step forward for the latter is the generation of human polyclonal antisera in transgenic animals.

The chapter by Prof. Hans-Dieter Volk and colleagues (Institute of Medical Immunology, Charité Hospital, Berlin, Germany) deals with a major challenge for all immunomodulatory interventions, the individualization of immunotherapy. By means of immunosuppressive and anti-infective therapy in transplant patients and patients with autoimmune disease, the authors describe how the patients can be selected for special adjuvant therapies, how the most suitable immunosuppression is selected for the single patient, and how the effect of a specific immunomodulatory therapy can be monitored to allow its timely adjustment to be sufficient while minimizing the side effects.

All in all, the meeting was characterized by very intensive and fruitful discussions involving the concepts and thinking of researchers from academia, pharmaceutical industry and clinics. There was agreement that only through the close cooperation of everyone involved in this

research field a better understanding and finally a better immunotherapy for autoimmune diseases and allotransplantation can be achieved.

We would like to thank the Ernst Schering Foundation for sponsoring this meeting as well as all speakers and participants for their valuable input.

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Nature's Choice of Genes Controlling Chronic Inflammation

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Abstract. Inflammation is a physiological response that may go uncontrolled and thereby develop in a chronic way. This seems to happen in many common diseases of autoimmune, degenerative, or allergic character. Rheumatoid arthritis (RA) is by definition a chronic disease with an autoimmune inflammatory attack on diarthrodial cartilaginous joints. The development of new treatment neutralizing cytokines involved in the inflammatory attack has given relief and gives the promise of more effective treatment of already established disease. It is now time to set our eyes on a new vision to develop preventive and curative treatment based on knowledge of the unique and causative pathogenic mechanisms. To do this we believe it is important to identify the natural-selected polymorphisms that are associated with disease. These have proven to be extremely difficult to identify in complex diseases such as RA, but using animal models, this work is closer to reality. Animal models have recently been developed mimicking various aspects of the human disease. We will present an example in which a genetic polymorphism associated with the development of arthritis has

been identified. On the basis of this finding, a new pathway involving control of immune tolerance by reactive oxidative species has been identified and a new class of antiinflammatory agents activating the induced oxidative burst protein complex is suggested.

1 Chronic Versus Acute Inflammation as Disease Mechanisms

There is a growing insight that inflammatory mechanisms are crucial in the molecular pathogenesis of many common diseases (Fig. 1). This is obvious in allergic diseases, which are caused by an exaggerated and disproportionate response to foreign antigens. These antigens are only partly known in conditions such as bronchial asthma. In another allergic disease, celiac disease, not only has the causative allergen been identified but also parts of the molecular pathways leading to disease (Sollid 2002). Clearly these diseases also involve autoimmune reactivities as a component of their pathology (Sollid and Jabri 2005). A second group of diseases comprises degenerative diseases, in which aberrant molecular structures or precipitates accumulate in tissue creating an inflammatory response. Some examples are atherosclerosis (Hansson and Libby 2006), adult onset macula degeneration (Nozaki et al. 2006), and Alzheimer disease (Wyss-Coray 2006). A third group is autoimmune diseases, in which autoimmune reactivates are believed to be the driving forces in the pathogenesis. Some examples are rheumatoid arthritis (RA), multiple sclerosis (MS), and type I diabetes. In all these diseases the inflammatory response needs to progress in a chronic and uncontrolled way to be regarded as pathogenic.

2 Rheumatoid Arthritis

RA is classified using a set of seven different criteria and it is essential that the disease manifest for at least 6 weeks (Fig. 2). In practice, patients have had clinical manifest disease for at least 1 year before they get the diagnosis, and they are still described as early RA. The classification criteria reflect the present knowledge and indicate the type of underlying inflammatory mechanisms (Fig. 2). Naturally the statistical

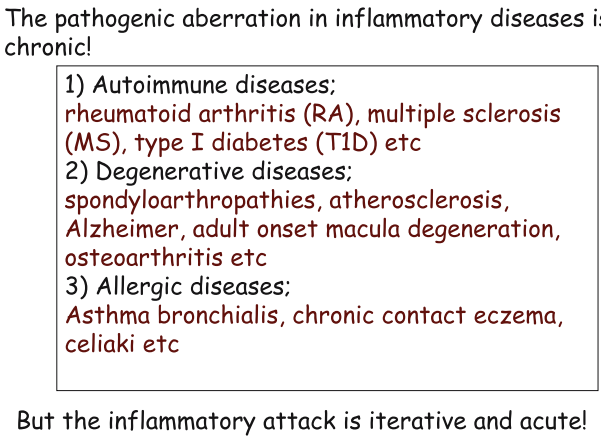

Fig. 1. Grouping of inflammatory diseases

occurrence of RA is dependent on the diagnostic criteria used, but it is believed to be relatively common, affecting 0.5%–1% of the Caucasian population, and occurs worldwide (Symmons et al. 2002). Women are about three times more often affected by RA as compared to men. Genetically, both X and Y chromosomes could be of importance, but also autosomal chromosomes, as all genes interact with the context shaped by gender. One such context-shaping effect is mediated by sex steroids. However, it is a complex influence; the female sex steroids (estrogens) potently suppress T cell-dependent autoimmune diseases such as experimental arthritis, and it is likely that this is also the case in humans (Jansson and Holmdahl 1998). The clinical onset of RA seems to be very variable along the lifespan, but has a peak around 40–50 years of age, when estrogen levels drop. A typical example of development of RA would be a woman at the age of 50 that has had arthritis symptoms for a year before she is classified as having classical RA (Fig. 3). Her disease did not start, however, at the time of the onset of these symptoms; it most likely started years before. From analysis of historic blood samples, it is believed that formation of rheumatoid factors predicts the development of RA (Aho et al. 1985), and more recently it has been shown that this is also the case with antibodies to citrullinated proteins

(Rantapaa-Dahlqvist et al. 2003), which show high specificity for RA (Schellekens et al. 1998).

Monozygotic twin studies, with a concordance rate of approximately 15% and the heritability estimated to be 60% (MacGregor et al. 2000), indicate a significant but not prominent genetic component. It has however, been very difficult to identify the major genes in this complex disease. Hypothesis-free linkage analysis has not been able to significantly pinpoint any gene region apart from the major histocompatibility complex (MHC) region (John et al. 2006).

If genetic analysis is difficult, it is even more problematic to identify the environmental factors. An example of these difficulties arises in the many studies on the role of estrogen, which is consumed by a large part of the population (Bijlsma and van den Brink 1992). Some studies show a dramatic protective effect and some no effect at all. A proper metaanalysis showed no significant effect. It is likely that we will find some of the major genes before we find the major environmental factors.

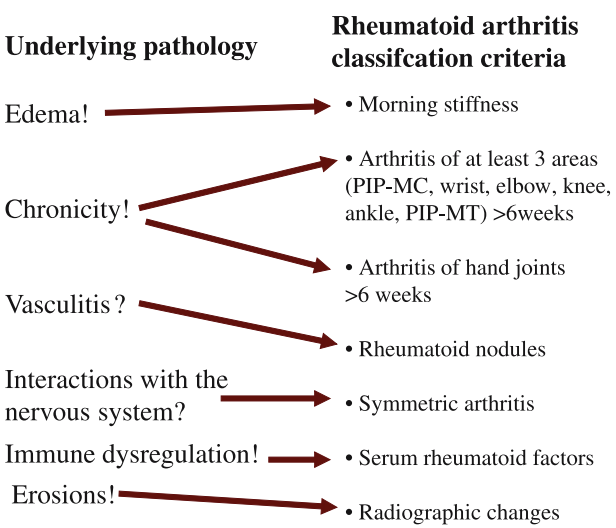

Fig. 2. The American Rheumatology Association classification criteria of RA and suggestions of the underlying pathology for each of the criteria

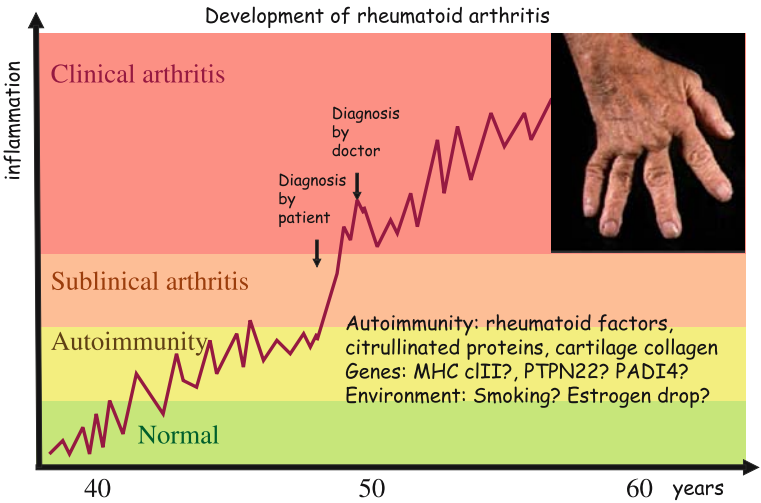

Fig. 3. A typical example of an untreated RA disease course. The disease activity is denoted "severity" on the *y* axis. Age is on the *x* axis. The curve represents an imaginary fluctuating disease activity of a woman developing RA. She is a smoker and carries an MHC haplotype expressing a shared epitope class II molecule. Maybe she has an RA-promoting PTPn22, PADI4, and other disease alleles. She develops rheumatoid factors and antibodies to citrullinated proteins starting even before symptoms of arthritis. When arthritis starts, it takes some time before she visits a rheumatologist and is given adequate treatment. The shown disease course is without effective treatment, as would have been the case some years ago, and she could expect a severe chronic relapsing disease and a premature death. Today she would have been given treatment, maybe with a combination of anti-TNFα and methotrexate, and the disease would have been less severe and less destructive

3 The MHC Region and Other RA Susceptibility Genes

An association between RA and the MHC region was observed a long time ago—before, in fact, the understanding of the fundamental function of MHC genes in the immune system (Stastny 1978). These data have subsequently been reproduced and refined in numerous studies in various populations. However, it has not yet been possible to identify the

responsible gene, although circumstantial evidence strongly supports the idea that polymorphisms in MHC class II genes play a role in RA. It could be shown that MHC haplotypes associated with RA contained MHC class II genes that shared a similar peptide-binding pocket, the so-called shared epitope (Gregersen et al. 1987; Jones et al. 2006). This has given rise to the belief that activation of MHC class II restricted autoreactive T cells, specific for an unknown antigen or antigens, is a critical pathogenic mechanism in RA. Preliminary identification of genes outside the MHC region supports this concept. A polymorphism in the *PTPN22* gene has been found to be associated with RA (Begovich et al. 2004), and the functional effect of the polymorphism is believed to lead to a weaker association of T cell receptor (TCR) signaling and thereby less efficient activation of regulatory T cells (Vang et al. 2005). Another suggested candidate is the *PADI4* gene, encoding the enzyme peptidylarginine deiminase 4, which mediates the citrullination of proteins forming neoepitopes (Suzuki et al. 2003). However, we expect that these candidate genes only represent the tip of an iceberg containing hundreds of interacting genes. Higher statistical power and more controlled systems, such as animal models, are clearly necessary for more rapid progress on understanding the genetic control of RA.

4 Gene Searching Using Animal Models for RA

There has been an important development of new animal models for RA, and different models in the arsenal available today are likely to reflect several subtypes of RA. For studies of genetic control, the selected animal model not only needs to mimic RA as much as possible but also be highly reproducible and show different penetrance in different inbred strains. A useful model is pristane-induced arthritis (PIA) (Vingsbo et al. 1996). Pristane is an alkane with adjuvant properties that induces severe and chronic arthritis after injection of 150 μl in the back skin of DA (dark agouti) rats (Fig. 4). It fulfills the classification criteria for RA and is highly reproducible, reaching almost 100% incidence in DA, whereas some other strains such as E3 are completely resistant. Genetic mapping of genetically segregating cohorts at the second filial generation (F_2) levels has revealed about 20 quantitative trait loci

Pristane induced arthritis (PIA)

Induced with 150 µl pristane subcutaneously
2-4 weeks later: onset of severe polyarthritis

RA criteria	PIA
Morning stiffness	nd
Arthritis >3 areas, > 6 weeks	+
Arthritis hand, > 6 weeks	+
Symmetric arthritis	+
Rheumatoid noduli	nd
Serum rheumatoid factors	+
Joint erosions	+

Fig. 4. The pristane-induced arthritis (PIA) model is induced by a subcutaneous injection of 150 µl pristane (see indicated molecular structure). A chronic relapsing disease will develop fulfilling the criteria for classical RA

(QTLs) associated with the development of arthritis (Vingsbo-Lundberg et al. 1998). One QTLs determined in this way was the Pia4 locus on chromosome 12, which was associated with arthritis severity both during the acute and chronic relapsing periods.

5 Positional Cloning of Ncf1

The next step was to positionally clone the underlying genes responsible for this effect. This was done using the congenic approach (Olofsson et al. 2003). Thus the chromosomal fragment from the E3 strain containing the putative gene mediating protection against severe arthritis was moved into the DA background through ten repetitive backcrosses. Once the new congenic strain, DA.PIA4, had been established, it was tested and was found to develop only mild arthritis as compared with the DA littermates. The next step was to minimize the congenic fragment. To do this, we screened for new recombinants, and when we discovered them they were bred as new subcongenic strains and subsequently tested for arthritis susceptibility. At last a congenic strain—having only two genes in the E3-derived fragment—was found to protect against arthritis, and after sequencing and functional testing, it could be concluded that the responsible gene was *Ncf1*.

During this long-term project, this was in fact our last candidate gene, and the reason we excluded this gene from our list of possible candidates was that the allele that was associated with arthritis was also associated with lower oxidative burst, a phenomenon we, at that time, believed would promote inflammation and arthritis. However, we could at that point clearly show that an allele of *Ncf1*—coding for a protein (also named p47phox) with a structural polymorphism—was associated with a lower oxidative burst and also with more severe arthritis. To investigate a possible connection between these two phenomena, we needed another mutation in the *Ncf1* gene. In the rat it seemed that there was an extensive polymorphism of *Ncf1*, even in the wild population, but no other candidate mutation with the potential to show the same effect was discovered. We then searched in the mouse. In parallel genetic mapping studies in our laboratory, we had in fact isolated a locus around the *Ncf1* gene on chromosome 5 associated with experimental autoim-

mune encephalomyelitis (EAE) (Karlsson et al. 2003). However, after establishing the congenic strain we excluded *Ncf1* as a candidate, and we found the *Ncf1* gene is not as polymorphic in the mouse as it is in the rat. However, thanks to the work of Huang et al. (2000), we identified a B6 strain at the Jackson laboratories carrying a spontaneous *Ncf1* mutation. This mutation was backcrossed to the collagen-induced arthritis (CIA)-susceptible B10.Q strain, and we found that it dramatically reduced the capacity to make an oxidative burst. Testing this new strain with CIA clearly showed a similar phenotype as in the rat, and a severe chronic arthritis developed (Hultqvist et al. 2004). In fact, these mice could develop arthritis spontaneously during their postpartum period, a period known to be very sensitive to arthritis relapses due to the drop of estrogen (Mattsson et al. 1991). Interestingly, the autoantibody response to type II collagen (CII) was raised in the *Ncf1*-mutated mice. Thus we concluded that we had positionally cloned *Ncf1* as associated with arthritis severity, and that the genetically controlled pathway involved regulation of reactive oxidative species (ROS) production.

6 Functional Studies of Ncf1

NCF1 (alias p47phox) is a protein component of the nicotinamide adenine dinucleotide phosphate, reduced (NADPH) oxidase (NOX) complex, which is an inducer of oxidative burst into the extracellular compartment (Fig. 5). Using the established rat and mouse congenic strains, we had unique tools to investigate the role of this gene. First we could show that the disease associated with the *Ncf1* allele operated before or during T cell activation. This was done by transferring MHC class II-restricted $CD4^+$ ab T cells between the DA and the DA.Pia4 rat (Olofsson et al. 2003). If the T cells originated from a DA.Pia4 donor, the protective E3 allele of *Ncf1* led to protection from arthritis in the recipient. Next we investigated whether the *Ncf1* gene was expressed in antigen-presenting cells or in the T cells. We found that several types of antigen-presenting cells, including macrophages and dendritic cells, expressed *Ncf1*, but not T cells. Thus, if T cell carries the information from the *Ncf1* gene, it needed to carry this information with a changed phenotype. To determine this phenotype we carefully investigated the

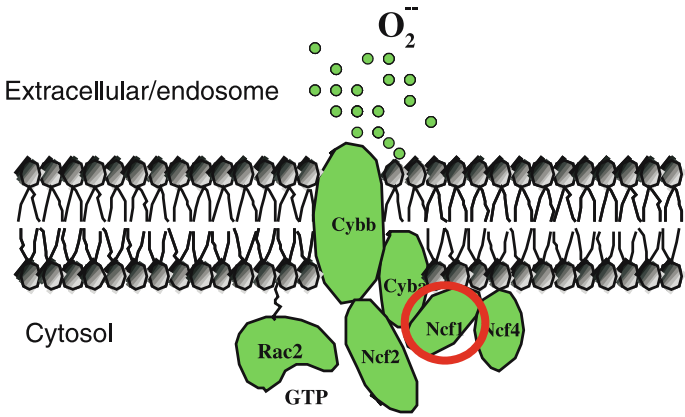

Fig. 5. The NADPH oxidase (NOX) complex showing the various subunits Ncf1 (p47phox), Ncf2 (p67phox), Ncf4 (40phox), Cybb (gp91), Cyba (gp20), and Rac2. When activated the complex releases oxygen radical anions to the outer space (extracellular or endosomal)

oxidative status of the T cells and found that T cells originating from DA rats had a lower number of thiol groups, i.e., free SH groups, on proteins in the cellular membrane as compared with T cells from DA.Pia4 rats (Gelderman et al. 2006). In contrast, no difference could be seen in the redox level of the cytosol. To determine whether the changed redox level in the cell membrane was responsible for the effect, we experimentally changed this by treating the cell in vitro with glutathione or with oxidized glutathione and then transferred them to nave recipients. The effect was clear: oxidation of the T cell membrane led to less arthritis and reduction of the T cell membrane to enhanced arthritis. Likewise, reduced T cells survived better in vivo and migrated to the to the joints. Thus we had identified at least one of the molecular pathways explaining the genetic effect. We suggest that T cells were educated, presumably by interacting with antigen-presenting cells, through exposure of ROS, leading to changed redox levels of their T cell membranes (Fig. 6). If correct, this finding will have implications not only for understanding arthritis, but also for T cell selection, tolerance, and immunity.

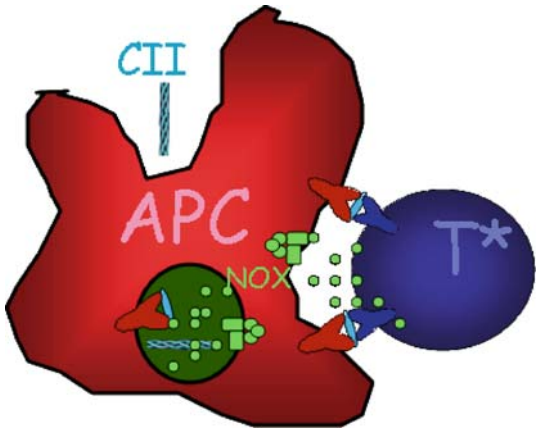

Fig. 6. A hypothetical model showing ROS as transmitters. An antigen-presenting cell (*APC*) could be a dendritic cell or a macrophage that express *NCF1* and have a functional NOX complex. The APC takes up type II collagen (*CII*) and processes it in the endosomes, and peptides are bound to the MHC class II molecules. Costimulatory agents will activate the NOX complex in the endosomal membrane and in the lipid rafts on the cell surface. They will oxidize the endosomal lumen as well as the synapse formed between the APC and the interacting antigen-specific T cell. Here the produced ROS will act as a transmitter produced by the APC and acting on the T cell surface

7 Ncf1 as a Pharmacologic Target

If activation of the *Ncf1*-containing NOX complex has such an important role in T cell autoimmunity and in regulating arthritis, it should serve as a possible target candidate for antiinflammatory treatment. Thus we developed a quite extensive program to identify substances that could activate the NOX complex and induce an enhanced oxidative burst capacity in vivo. As a prototype compound we selected synthetic phytol, a naturally occurring hydrocarbon (Hultqvist et al. 2006). Subcutaneous injection of phytol in DA rats led to a restoration of the low oxidative burst capacity within 24 h. One injection in fact gave a relatively long-lasting effect of 4–5 weeks. Importantly, treated rats developed an almost complete protection against arthritis.

To be of value for treating an already established chronic disease, the compound also needed to be tested in a relevant chronic phase of the disease. This is in fact seldom done, as there are few RA models with a chronic relapsing disease course. Normally, validation in the animal models is made only by preventing immune priming or possibly the onset of arthritis. However, the PIA model in the rat is truly chronic relapsing, which gave us a unique opportunity to test the chronic phase. The result was very promising: a single injection of phytol treatment stopped the progression of the disease for at least 4 weeks. Interestingly, phytol treatment of collagen-induced arthritis was also effective. In this model the autoimmune response could be evaluated, and phytol treatment led to a similar effect as a protective *Ncf1* allele; both the T cell-dependent autoantibody response to CII and delayed-type hypersensitive reactions were reduced, arguing that phytol affected the same T cell modulatory oxidative pathways as *Ncf1*.

8 Conclusion

Finding genes controlling complex diseases has not been an easy task, but the example of the positional cloning of *Ncf1* shows that it is possible. The *Ncf1* polymorphism is naturally selected and has profound effects on the control of both T cell autoimmunity and the development of chronic autoimmune inflammation as in arthritis. Isolation of the polymorphism in congenic strains also gives relevant in vivo tools to investigate the functional role of the gene in a complex contextual setting. We found that reactive oxidative species regulate T cell activation through oxidizing the thiol groups in the T cell membrane. We hypothesize that ROS are transmitters in the immunological synapse and regulate the activation threshold of T cell activation. The immune-activation outcome is thereby regulated by the involved antigen-presenting cell, and such a mechanism might be fundamental as an operative in all interactions between antigen-presenting cells and T cells.

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Targeting of Memory

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Abstract. Current therapeutic options that are based on immunosuppression do not provide a cure for the treatment of chronic inflammation. Though more efficient immunosuppression and the introduction of biologicals such as antibodies targeting cytokines have improved clinical outcomes, immunosuppressive therapy has to be continued to be efficient, thus enhancing the risk of adverse events and undesired side effects. Why can immunosuppression ameliorate, even stop, but not cure chronic inflammation? Is chronic inflammation perpetuated beyond suppression by mechanisms independent of the immune system, or is it perpetuated by components of the immune system which are resistant to a block of ongoing immune reactions? One such component of the immune system is immunological memory. This article will review the role of immunological memory in chronic inflammation, as far as we understand it today, and discuss implications for the development of novel therapeutic strategies aiming at a cure for diseases involving chronic inflammation.

1 Immunological Memory and Chronic Inflammation

Immunological memory is a hallmark of the adaptive immune response. It is dependent on a dialog between T and B lymphocytes recognizing the antigens. T helper (Th) lymphocytes provide the critical signals for the generation of memory B and T lymphocytes. The heterogeneity of memory lymphocytes resembles the functional diversity of lymphocytes in general, with regulatory, helper, and effector T lymphocytes; B lymphocytes expressing antigen-receptors, which are affinity-matured and class switched; and plasma cells, which secrete such antibodies (Lanzavecchia and Sallusto 2005; Kalia et al. 2006; Radbruch et al. 2006). Upon rechallenge with the antigen, memory B and T lymphocytes will react faster and their reaction will be functionally imprinted, i.e., independent of the physiological (and therapeutic influenceable) regulation of primary immune responses (Lohning et al. 2002). Apart from this reactive immunological memory, long-lived plasma cells, and probably also effector memory T cells, can secrete antibodies and cytokines, respectively, without proliferation, and thus are resistant to all therapies targeting proliferating cells, e.g., corticosteroids (Miller 1964; Brinkmann and Kristofic 1995), γ-irradiation (Slifka 1998; Grayson et al. 2002), and cyclophosphamide (Orme 1988; Hoyer et al. 2004).

Chronic inflammation is an essential pathogenetic element of many diseases such as rheumatoid arthritis (RA), type 1 diabetes, multiple sclerosis, systemic lupus erythematosus (SLE), spondyloarthropathies, asthma, psoriasis, and inflammatory bowel disease. Although variable, the genetic component of such diseases has been estimated to be not more than 30%, with, for instance, more than 30 different genes possibly being involved in RA. This genetic heterogeneity is reflected by the diversity of clinical progress and responsiveness to treatment.

Inflammatory diseases commonly progress through distinct phases, with long periods of subclinical disease progression into a relapse/remitting or chronic-progressive inflammatory, clinically apparent phenotype. In general, at diagnosis the disease has already progressed to the chronic phase. Retrospective analyses of patients with RA have shown a break of tolerance to self and the development of a humoral memory of secreted antibodies to self (rheumatoid factor) or modified self (citrullinated peptides) preceding treatment by up to 10 years. In com-

bination with genetic predisposition, e.g., the human leukocyte antigen (HLA)-DR4 "shared epitope," and environmental factors, e.g., tobacco smoking, the presence of such autoantibodies is a strong predictor of disease (Rantapaa-Dahlqvist et al. 2003; Klareskog et al. 2004). Currently, it can only be speculated that excessive immune reactions to pathogens might result in a break of tolerance and the generation of an immunological memory response to self in humans. Likewise, we have no information to date on how an acute, pathogen-driven inflammation is converted into a chronic inflammation which presumably is driven by self antigens. Current concepts are derived from animal models, demonstrating that such mechanisms could operate in principle. In particular, studies on the pathogenesis of the lupus-like disease in NZB/W mice have shown that an immunological memory of secreted antibodies specific for self antigens, e.g., DNA, is generated very early in ontogeny. Later in disease development, chronic immune reactions dominate, while the humoral memory for self persists. Moreover, it could be shown that this humoral memory is resistant to strong immunosuppression by cyclophosphamide, while the chronic immune reaction is completely abolished by this treatment (Hoyer et al. 2004).

A key question for assessing the role of immunological memory in the chronic phase of inflammation is whether or not (auto)immunity in the chronic phase is driven by (auto)antigen. The (auto)antigen-dependent case would be indicated by the reactivation of memory B and T cells, and their differentiation into effector cells, which in turn could be either short-lived or long-lived. The antigen-independent case would be indicated by the reactivation of memory B and T cells by pathogen-associated molecular patterns (PAMP) and/or cytokines. It has been elegantly demonstrated that memory B lymphocytes are readily activated into antibody-secreting cells by PAMP such as CpG-containing DNA and cytokines (Bernasconi et al. 2002). Whether or not these antibody-secreting cells have the potential to become long-lived remains to be shown. The antigen-independent case would also be indicated by the secretion of (auto)antibodies by long-lived plasma cells, i.e., the humoral memory. And for murine T memory cells, antigen-independent secretion of the cytokine interferon(IFN)-γ, a cytokine of key relevance for the regulation of inflammation, has been described (Yang et al. 2001).

Today we do not yet have clear proof that immunological memory is the missing target in our quest for a cure of chronic inflammation. Chronic inflammation persists through immunosuppression and provides secreted autoantibodies in the absence of the original antigenic trigger. It remains to be shown whether or not these autoantibodies suffice to perpetuate inflammation beyond immunosuppression. At present, the best evidence is provided by the experimental therapy of complete immunoablation in patients with autoimmune diseases such as SLE, juvenile idiopathic arthritis, or multiple sclerosis (Rosen et al. 2000; De Kleer et al. 2004; Jayne et al. 2004; Muraro et al. 2005; Tyndall and Saccardi 2005). Ablative protocols depleting memory, in particular long-lived, autoantibody-secreting plasma cells, have been curative in patients who had not responded to efficient immunosuppression. Their new immune system, generated from stem cells, was tolerant against those antigens to which they had reacted before.

2 Reactive Memory

2.1 T Cell Memory

Th cells, in particular T helper type 1 (Th1) and Th17 cells, are potent inducers of inflammation. This has been demonstrated in murine models of multiple sclerosis (Lafaille et al. 1997; Langrish et al. 2005), RA (Murphy et al. 2003; Maffia et al. 2004), and inflammatory bowel disease (Iqbal et al. 2002; Yen et al. 2006). Th1 cells, defined by the expression of IFN-γ, promote activation and recruitment of phagocytic cells and the B cell switch to complement-fixing IgG antibodies, such as IgG1 in humans. IFN-γ facilitates the homing of Th1 cells and B cells into inflamed tissues by inducing expression of the C-X-C motif chemokine receptor 3 (CXCR3) and its ligands CXCL-9, CXCL-10, and CXCL-11 (Nakajima et al. 2002; Rotondi et al. 2003; Muehlinghaus et al. 2005). Th17 cells secreting interleukin (IL)-17 activate fibroblasts, neutrophils, and epithelial cells to express a wide range of inflammatory mediators such as matrix metalloproteinases, CXCL-1, CXCL-8, IL-6, TNF-α, and IL-1β (Kolls and Linden 2004).

It has been amply demonstrated that Th1 and Th17 cells can induce inflammation in rodent models of inflammation. It is less clear, however,

whether they also drive inflammation in the chronic phase of inflammation. Memory Th cells constitute a significant proportion of the cellular infiltrate present in chronically inflamed tissue, such as the synovium of inflamed joints in RA (Thomas et al. 1992). In particular, terminally differentiated memory cells, i.e., CD45RA–/CD27– Th cells (Kohem et al. 1996), which probably had been repeatedly stimulated, are frequent in inflamed synovia. Functionally, many of these cells are imprinted to re-express IL-17 or IFN-γ. The levels of IL-17 and IFN-γ are enhanced in the inflamed joints (Ziolkowska et al. 2000), and many T cell clones derived from inflamed tissue of RA patients have a memory for the expression of IL-17 and IFN-γ (Morita et al. 1998; Aarvak et al. 1999).

As part of the reactive memory, Th memory cells could fuel a chronic inflammation, either upon reactivation by (auto)antigen, or independent of T cell receptor stimulation, by cytokines and ligands of pattern-recognition receptors, both provided by the micro environment in the lesion (Cope 2002; Brennan et al. 2006). The latter, antigen-independent activation of Th1 memory cells has been shown to induce them to secrete IFN-γ, even over extended periods of time (Yang et al. 2001). It is less clear if other cytokines can be expressed by Th memory cells activated independent of their antigen. Although in RA particular HLA class II alleles are associated with poor prognosis, i.e., a more progressive joint destruction (Calin et al. 1989), the contribution of antigen-dependent activation of memory Th cells to the perpetuation of inflammation remains unclear so far.

Another critical role for reactive memory Th cells in the pathogenesis of inflammation and its perpetuation is their essential help for the differentiation of B cells into long-lived plasma cells, secreting (auto)antibodies of enhanced affinity and switched isotype, at least in diseases with (auto)antibody-driven pathogenesis of chronic inflammation such as SLE. In the K/BxN murine model of RA, Th cells, despite their instrumental role in initiation of disease, are even dispensable later, in the chronic phase. In these mice, Th cells are required initially for the induction of autoreactive, presumably long-lived plasma cells that produce arthritogenic autoantibodies and drive the later stages of the disease, then are independent of further contributions of T cells (Korganow et al. 1999).

So far, therapeutic targeting of Th cells in the context of chronic inflammatory diseases has been rather discouraging. Systemic depletion of Th cells by antibodies directed against CD4 showed some initial clinical benefit (Horneff et al. 1991; Choy et al. 1996). However, the approach had to be abandoned due to the lack of efficacy, and concerns regarding the immunodeficiency induced. Immunomodulatory, nondepleting anti-CD3 and anti-CD4 antibodies aiming at the selective inhibition of pathogenic T cell responses and at the same time boosting the T cell-dependent regulatory mechanisms are under investigation (Nepom 2002; Panaccione et al. 2005). Future studies will reveal the potential of this approach, although expectations have been dampened by the lack of success using a superagonistic anti-CD28 antibody (Suntharalingam et al. 2006).

Apart from Th lymphocytes, the relevance of $CD8^+$ cytotoxic T cells for the initiation and, in particular, for the perpetuation of chronic inflammation remains elusive. The strong genetic predisposition of MHC class I antigen HLA-B27 for ankylosing spondylitis (Brewerton et al. 1973) points to an important role of the MHC class I-dependent $CD8^+$ cells in this disease. Murine models of chronic inflammatory diseases differ in their dependency on $CD8^+$ T cells. $CD8^+$ T cells mediate diabetes in the NOD mouse strain (DiLorenzo and Serreze 2005), while CD8 T cells are dispensable for the induction of collagen-induced arthritis, or are antiinflammatory (Ehinger et al. 2001; Taneja et al. 2002).

2.2 B Cell Memory

Memory B cells accumulate in inflamed tissue as a result of immigration and local expansion (Schroder et al. 1996). In the past few years, B cells have been receiving increasing attention as mediators and regulators of chronic inflammation. This interest has been essentially fuelled by the remarkable efficacy of B cell-depleting therapies in established RA and SLE. Rituximab, a chimerized monoclonal antibody recognizing CD20, a surface molecule that is expressed by various B cell subsets except plasma cells, has been successfully used in the treatment of SLE (Leandro et al. 2005; Smith et al. 2006) and RA (Cohen et al. 2006; Emery et al. 2006).

B cells can contribute to the development and perpetuation of chronic inflammation in multiple ways. Memory B cells are potent antigen-presenting cells of their cognate antigen, they secrete proinflammatory cytokines, and they can fuel immune responses with the aberrant production of pathogenic antibodies in particular autoantibodies upon differentiation into plasma cells. That several of those effects can play a role simultaneously in an inflammatory context has been demonstrated in lupus-prone MRL/*lpr* mice. In these mice, B cells contribute to the development of lethal immunopathology by the production of pathologic autoantibodies and by antibody-independent mechanisms. B cell-deficient mice (MRL-JHd) fail to develop autoimmune manifestations, e.g., vasculitis and nephritis (Chan et al. 1999), while mice in which the B cells are incapable of secreting antibodies (MRL/*lpr* mIgM) do develop lethal autoimmune manifestations even while mortality is reduced by 50% (Chan et al. 1999).

A possible explanation for the antibody-independent mechanisms of B cell action is their role as potent antigen-presenting cells. Memory B cells efficiently present antigen to Th cells in an inflammatory context via MHC class II, thereby activating Th cells to proliferate and release proinflammatory cytokines (Roosnek and Lanzavecchia 1991; Roth et al. 1996; Falcone et al. 1998). In particular they do express B7h, the ligand for inducible costimulator (ICOS), and thus can efficiently costimulate $ICOS^+$ Th cells, which have been shown to be essential for induction of chronic inflammation (Lohning et al. 2003).

However, just as memory T cells can be activated in an antigen-independent way, the same holds true for memory B cells. Human memory B cells express pattern-recognition receptors such as the Toll-like receptors 6 (TLR-6), -7, -9, and -10 that are absent or only weakly expressed by naïve B cells (Bernasconi et al. 2003). They display a lower threshold of activation compared to naïve B cells and do not require further T cell help for activation and differentiation into antibody-secreting cells. “Rheumatoid factors” are low-affinity autoreactive antibodies targeting autologous antibodies, and their presence indicates development of RA. Autoreactive B cells with the capability to produce such autoantibodies might be activated independent of T cell help by antibody–RNA or antibody–chromatin immune complexes crosslinking the antibody-specific B cell receptor and TLR-7, or TLR-9, respectively (Lead-

better et al. 2002; Lau et al. 2005). Furthermore, in combination with TLR ligand stimulation, cytokines might substitute for B cell receptor signaling, leading to a polyclonal activation of memory B cells and differentiation into antibody-secreting cells (Bernasconi et al. 2003).

3 Humoral Memory

Humoral immunity can be provided by long-lived antibody-secreting plasma cells that maintain protective antibody titers following termination of the immune reaction, or by short-lived antibody-secreting cells that are constantly generated in a chronic immune reaction (Radbruch et al. 2006). Antigen bound to secreted antibodies triggers a multitude of effector functions through the constant region of the antibody heavy chains. This can contribute essentially to immunopathology already in otherwise protective immune reactions, but even more so in autoreactive immune responses. Secreted autoreactive antibodies are one of the mechanisms with the potential to fuel chronicity of inflammation. Autoantibodies can block or stimulate signaling pathways upon binding and crosslinking of cell-surface receptors (Drachman 1994; Chistiakov 2003). Deposition of immune complexes, i.e., antibody-antigen complexes, and opsonization of cellular or extracellular matrix structures at the site of inflammation or initially unaffected tissue leads to activation of complement and Fc-receptor-bearing phagocytic cells. This results in the release of proinflammatory mediators and ultimately in destruction of the surrounding tissue and organ failure, e.g., kidney destruction in SLE. In addition, immune complexes represent a continuous source of antigen and costimulation for activation of more autoreactive B and T cells. Autoantibodies specific for particular autoantigens are a hallmark of many autoimmune diseases. Their specificities range from cell-surface molecules, e.g., antiacetylcholine receptor in myasthenia gravis (Drachman 1994) to intracellular and nuclear antigens, e.g., antinuclear antibodies in SLE, or low-affinity anti-IgG (rheumatoid factor) in RA. They also can be specific for products of inflammation such as citrullinated peptides. Specificity, affinity, isotype, and concentration of autoantibodies are valuable diagnostic tools with considerable prognostic value (Dorner and Hansen 2004). High titers of rheumatoid factor, and

antibodies recognizing citrullinated peptides are predictive for a more aggressive erosive disease course in RA (Vencovsky et al. 2003; Dorner and Hansen 2004; Machold et al. 2006).

Antibodies are produced by plasmablasts and by plasma cells that differentiate from any type of B cell upon antigenic stimulation and appropriate costimulatory signals (Radbruch et al. 2006). This differentiation step is fundamental; it is controlled by a switch of transcriptional programs regulated by paired box protein 5 (Pax5) in B cells, versus B lymphocyte-induced maturation protein 1 (Blimp1) and X-box binding protein 1 (XBP1) in antibody-secreting cells. Plasma cells are nondividing, have lost surface expression of MHC class II and antibody, as well as CD20, i.e., they are resistant to CD20-directed therapy with rituximab. The lifetime of plasma cells depends on their environment. Survival niches for plasma cells are found in the bone marrow, but also in inflamed tissue. Accordingly, plasma cells are enriched in chronically inflamed tissue such as the inflamed synovium of RA patients (Tsubaki et al. 2005) and the inflamed lamina propria of patients suffering from inflammatory bowel disease (Keren et al. 1987). Proinflammatory mediators present at sites of inflammation, such as TNF-α, IL-6, CXCL-12, and ligands for CD44, have been shown *in vitro* to support survival of plasma cells (Minges Wols et al. 2002; Cassese et al. 2003). In lupus-prone NZB/W mice, plasma cells that are generated early in the disease, before 12 weeks of age, form a pool of long-lived plasma cells and are resistant to immunosuppression by cyclophosphamide. These cells reside in the bone marrow and also in the spleen. Interestingly, later in the course of disease, such long-lived plasma cells no longer develop. Instead, short-lived plasmablasts and plasma cells are generated by chronic activation of B cells, a process that can be stopped by cyclophosphamide (Hoyer et al. 2004).

The coexistence of short-lived and long-lived plasma cells secreting autoreactive antibodies could also explain the differential effect of anti-CD20 therapy (rituximab) on titers of autoantibodies versus protective antibodies in patients with rheumatic diseases. The depletion of peripheral B cells, i.e., plasma cell precursors, should affect mainly the generation of short-lived plasma cells, while long-lived plasma cells should be resistant to rituximab. In RA patients, the titers of protective, tetanus or diphtheria-specific antibodies remain constant upon rituximab treat-

ment, while the titers of disease-related autoantibodies specific for citrullinated proteins and rheumatoid factor drop significantly to levels that might indicate the proportion of autoantibodies secreted by long-lived plasma cells (Cambridge et al. 2003; Edwards et al. 2004). It should be noted that long-lived plasma cells could also be indirectly targeted by rituximab, if their survival niches in inflamed tissue would be resolved by amelioration of inflammation. Only long-lived plasma cells of the bone marrow should be completely resistant to rituximab.

The exact role of autoantibodies provided by long-lived plasma cells in the pathogenesis of chronic inflammatory disorders remains to be elucidated, but the autoantibodies are an elegant explanation for the long periods of subclinical disease progression, refraction to immunosuppression, relapse upon termination of immunosuppression, and prevention of cure by currently available therapeutic strategies. Long-lived plasma cells memorize autoreactivity in the face of immunosuppression.

4 Current Immunosuppressive Therapies Do Not Provide a Cure

Today, therapeutic management of chronic inflammation relies on long-term immunosuppression. With more tailored regimens and combination therapies, patients suffering from chronic inflammatory disorders nowadays can expect clear-cut and long-lasting amelioration of disease symptoms, reduced morbidity, and attenuated or stopped disease progression, but no cure. Cessation of treatment ultimately will lead to relapse of the destructive inflammatory process. Traditional therapeutics include nonsteroidal antiinflammatory drugs (NSAID); immunosuppressive drugs such as the so-called disease-modifying drugs methotrexate (Chan and Cronstein 2002), sulfasalazine (Smedegard and Bjork 1995), and leflunomide (Fox et al. 1999); and glucocorticoids (Buttgereit et al. 2004). A prominent therapeutic strategy that stands for a more targeted therapy is the inhibition of proinflammatory cytokines using monoclonal antibodies or receptor–Ig fusion proteins (Fig. 1). The blocking of proinflammatory cytokines TNF-α (Scott and Kingsley 2006), IL-1 (Cohen 2004), and IL-6 (Nishimoto et al. 2004) that are abundantly expressed at the site of inflammation has shown efficacy in RA. In par-

ticular, TNF-α antagonists represent a major therapeutic advancement in treatment of RA and other chronic inflammatory diseases such as ankylosing spondylitis and Crohn's disease, and highlight the potential of this approach (Nepom 2002; Panaccione et al. 2005).

Though the proposed mechanisms of action are different for these therapeutic strategies, their target is the same, namely an ongoing immune reaction. They affect parameters such as proliferation, cell activa-

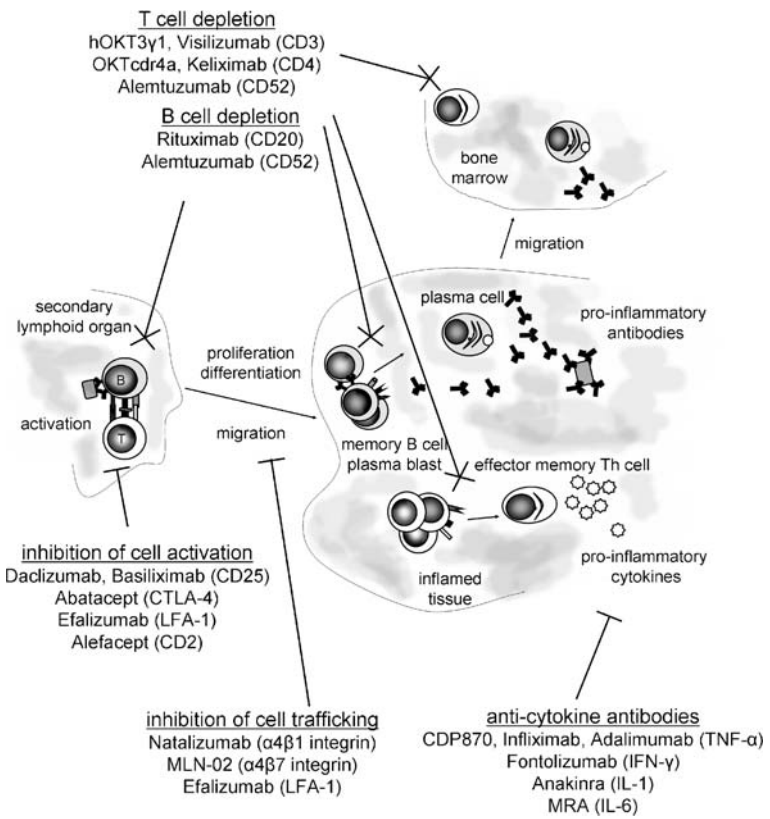

Fig. 1. Pathological mechanisms mediated by B and T cells are targets for therapeutic intervention. Selected biologicals and their target molecules are listed

tion, cytokine expression, and cell differentiation. Efficient systemic immunosuppression and immunomodulation are the bases of their efficacy, but also the reasons for their broad spectrum of adverse and side effects and impairment of immunocompetence. Their inability to cure may be due to either disease-driving mechanisms, which are independent of ongoing immune reactions, such as antigen-independent immune effector memory, as discussed above, or due to a resistance of chronic immune reactions to conventional immunosuppression. Alternative strategies of the future will aim at inhibition of migration and homing of proinflammatory cells, preventing their convention to reactivate and their presence in the target organ (Fig. 1). These targeted therapeutics will interfere with costimulatory requirements of advanced memory cells, e.g., ICOS, and they will aim at, as specifically as possible, the deletion of the cells driving disease, e.g., Th effector memory cells or autoreactive plasma cells (Nepom 2002; Panaccione et al. 2005).

5 Conclusion

The understanding of the mechanisms underlying chronic inflammation has improved over the last decades, and from it improved therapies have been developed. Still, those therapies do not provide a cure for chronic inflammatory diseases. This might be due to their insufficient interference with immunological memory. Proinflammatory immunological memory has been neglected so far as a prime candidate driving chronic inflammation. Therapeutic resolution of inflammation by suppression of ongoing pathogenic immune reactions may stop tissue destruction and associated immunopathology, and it may also result in the loss of survival niches for pathogenic memory cells resident in and dependent on the proinflammatory microenvironment of the inflamed tissue. However, resolution of inflammation will not affect memory cells that are located outside of the inflamed tissue, in particular long-lived pathogenic plasma cells and probably pathogenic effector memory T cells of the bone marrow as well (Di Rosa and Pabst 2005; Radbruch et al. 2006). The plasma cells will continue to provide pathogenic seed antibodies, a threshold and trigger of a relapse. It is a challenge for the develop-

ment of future therapeutic strategies to target memory cells from the bone marrow, specifically the pathogenic ones, if possible.

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Post-transcriptional Regulators in Inflammation: Exploring New Avenues in Biological Therapeutics

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Abstract. The biosynthesis of inflammatory mediators relies on controlling the biogenesis and utilization of their corresponding messenger RNAs (mRNAs). These latter "utilization steps" encompass post-transcriptional mechanisms that gradually and variably impose a series of flexible-rate limiting controls to modify the abundance of an mRNA and the rate of its translation to protein in response to environmental signals. Mechanistically, post-transcriptional machines comprise networks of RNA binding proteins (RBPs), which recognize, passively or inducibly, secondary or tertiary ribonucleotide structures located on their target RNAs. The outcome of these interactions is the stringent control of mRNA maturation, localization, turnover and translation. It is conceivable that if these post-transcriptional interactions fail, they may perturb cellular re-

sponses to provide the impetus for chronic disease. Such is the case of the signal-responsive mechanisms affecting inflammatory mRNAs containing the AU-rich family of elements (AREs), which are recognized by a specific subset of RBPs. Intense research in this area has yielded important insight on the specific signals and mechanisms affecting the utilization of ARE-containing mRNAs. Here, we indicate briefly the inflammatory relevance of ARE-related mechanisms to highlight their importance in pathophysiology and their potential in the development of future biological therapies.

1 Introduction

The post-transcriptional regulation of inflammatory gene expression appears to have evolved as a homeostatic mechanism that defines the spatiotemporal pattern of an elicited immune response whilst preserving the balance between inflammation and tissue preservation. Current concepts favour that inflammatory signals determine the biosynthetic rate of cytokines, chemokines growth factors and small peptides by targeting specific ribonucleotide structures located in the corresponding messenger RNAs (mRNAs). Although in many instances the actual ribonucleotide structures responding to these signals remain unresolved, many may be inferred by a specific primary sequence composition whose binding has been validated by functional assays (mobility shift assays, genetic/biochemical/immunochemical RBP:RNA interaction screens, effects on reporter mRNA fate, etc.). Such is the case for the AU-rich family of elements (AREs) that are located in the untranslated regions (UTRs) of many inflammatory mRNAs and that are recognized by a specific subset of RNA-binding proteins (ARE-binding proteins; ARE-BPs).

2 Features of AU-Rich Elements

Evidence for the inducible post-transcriptional regulation of inflammatory mRNAs was provided during the late 1980s. It was found that cytokine mRNAs [such as those encoding tumour necrosis factors (TNFs), interleukins (ILs), interferons (IFNs) and colony-stimulating factors

(CSFs)] were labile and displayed accumulation profiles that were discordant to the levels of the proteins induced in stimulated innate cells. This effect was attributed to small AU-rich sequences present in the 3′ UTRs of these mRNAs, which could promote mRNA decay even when they were placed in the context of stable mRNAs (Shaw and Kamen 1986). Additional studies on cytokine mRNAs injected into Xenopus oocytes indicated that these elements could also block translation of cytokine mRNAs (Kruys et al. 1989). These early studies paved the way for a large amount of data collected over the last 15 years on what these sequences are and how they can modulate mRNA turnover and translation in response to numerous stimuli and in different cellular responses.

AREs are composed of a variable number of copies of the AUUUA or UUAUUUAUU nonamers and were originally classified into three separate functional subgroups based on their primary sequence (class I discontinuous nonamers; class II continuous/overlapping nonamers; class III undefined AU-rich structures) and the mode of mRNA degradation that they impose (Chen and Shyu 1995). In subsequent years and with the development of bioinformatics, large-scale computational approaches were performed to reveal an 8% distribution of AREs in mRNAs transcribed from human genes (Khabar 2005). These analyses indicated that ARE-containing transcripts are involved in transient and important cellular responses ranging from development and haematopoiesis to immune responses and cancer, rendering the AREs "pathophysiologically-relevant" RNA signatures. Based on the data assembled in the form of the ARE-containing mRNA database (ARED) (Bakheet et al. 2006), the ARE classification scheme was expanded to include five additional ARE subsets in class II AREs (clusters I–V relating to the number of nonamers).

Most of the biochemical studies performed to date highlight the importance of AREs in mediating the destabilization of eukaryotic transcripts. Although still under intense investigation, current concepts favour that AREs promote the shortening of the poly-A tail (deadenylation) and/or removal of the 5′ cap (decapping) (Xu et al. 1997; Wilusz et al. 2001). Degradation may then proceed through the recruitment of 5′–3′ exonucleases or through the sequestration of a complex of 3′–5′ exonucleases called the exosome (Chen and Shyu 1995; Mukher-

jee et al. 2002; Stoecklin et al. 2006). Despite the increasing knowledge of the biochemistry underlying ARE-mediated decay, the biochemical mechanisms mediating the potential of some AREs to block translation have not been analysed to the same extent. Instead such mechanisms have been inferred from functional studies (see also Sect. 3).

Notably, the heterogeneity amongst the ARE subsets indicates that their respective functions towards mRNA turnover and translation may vary amongst the different transcripts. For example, in bacterial lipopolysaccharide (LPS)-stimulated monocytes and IL-1-stimulated glioblastoma cell lines, representative mRNAs possessing class I AREs had slower decay rates than those with class II AREs (Frevel et al. 2003; Tebo et al. 2003). Current concepts favour that this heterogeneity may reflect the different interactions with ARE-binding proteins and signalling cascades. If that hypothesis holds true, then one should expect that the level of AREs' functional complexity rises multi-fold in the context of a signal-and-tissue specific response. Until the end of the 1990s, the limitations of biochemical and unicellular studies addressing ARE-mediated effects could not reveal the full potential of ARE-dependent modulation and their role in physio-pathological dynamics. With the turn of the century, the field was to be rejuvenated by the power of mammalian genetics and transgenic technologies.

2.1 Assessing the In Vivo Importance of AREs in Inflammation

The first indications that the post-transcriptional regulation of cytokine mRNAs can play important roles in immune physiology came from studies correlating abnormal patterns of TNF expression with inflammatory disease. TNF plays a central role in various immune and inflammatory phenomena by eliciting differential signals in target cells ranging from cellular activation and proliferation to cytotoxicity and apoptosis. Biological anti-TNF therapies in chronic inflammatory diseases have been very successful in the clinic (Feldmann and Maini 2001), but are not without side-effects towards infection and autoimmunity, necessitating the discovery of alternative approaches. Early *in vitro* works have indicated that the presence of a conserved AU-rich element in the 3′ UTR of the TNF mRNA (now classified as a prototypical class II ARE) could affect the inducible expression of this molecule in innate

cells (Caput et al. 1986). Most intriguingly, the replacement of this 3′ UTR with that from the β-globin mRNA was sufficient to drive TNF overexpression in transgenic mice supporting the development of numerous pathologies (depending on the promoters used to drive expression of these transgenes) including arthritis and systemic and CNS inflammation (Douni et al. 1995; Kollias et al. 2002). At the same time, genetic analysis of lupus-prone mice indicated that a spontaneous dinucleotide disruption of the TNF 3′ ARE reduced the levels of TNF biosynthesis, supporting the development of autoimmune disease (Jacob and Tashman 1993); however, gene targeting in embryonic stem (ES) cells was meant to provide the definitive proof of the TNF3′ ARE function. The obligatory or conditional targeted deletion of this 69-bp element from the mouse TNF locus resulted in severe polyarthritic and inflammatory bowel disease (IBD) phenotypes with resemblance to the human conditions of Crohn's disease and rheumatoid arthritis (Kontoyiannis et al. 1999, 2001). In the absence of the ARE, the inducible production of TNF from mouse macrophages and lymphocytes was excessively prolonged, supporting a continuous state of innate activation as well as hypersensitivity to inflammatory agonists (Kontoyiannis et al. 1999, 2001, 2002). In addition, this small deletion was sufficient to drive the ectopic expression of TNF in cells that do not normally produce this cytokine (e.g. synovial fibroblasts), indicating that the AREs modulate both temporal and spatial parameters of mRNA expression (Kontoyiannis et al. 1999). In molecular terms, these phenomena were attributed to the increased stability of the TNF mRNA and the absence of translational silencing mechanisms (Kontoyiannis et al. 1999, 2001, 2002).

One important point that these studies revealed was that the AREs can respond both to immune-activating and immune-suppressive signals. For example, the absence of the TNF3′ ARE rendered this cytokine partially or totally unresponsive to the anti-inflammatory effects of corticosteroids, nonsteroidal anti-inflammatory drugs (NSAIDs) and natural antagonists such as IL-10 (Kontoyiannis et al. 1999, 2001). The inability of IL-10 to modulate TNF biosynthesis due to the absence of TNF3′ ARE appears as a key deterministic parameter for the development of inflammatory disease, as was exemplified in the case of TNF-induced IBD (Kontoyiannis et al. 2002). Whether IL-10 targets

the ARE-dependent modulation of TNF mRNA translation, stability or both is still a matter of debate, although signal- and species-specific variations may be responsible for these discrepancies. It is conceivable that other anti-inflammatory cues may also be affected as has been previously suggested for the IL-4 and IL-13 mediated suppression of TNF biosynthesis (Mijatovic et al. 1997).

The importance of class II AREs in the maintenance of homeostasis was also indicated in transgenic studies for the granulocyte-macrophage colony-stimulating factor (GM-CSF) mRNA (Houzet et al. 2001). In this case the early developmental expression of GM-CSF mRNA was permissive only when its ARE was deleted. This deletion had severe consequences for myelopoiesis manifested by the abnormal accumulation of myeloid progenitors, the increased proliferation of granulocytes and macrophages, and embryonic lethality, presumably due to an increase in GM-CSF mRNA stability and/or translation.

These results, however, should be cautiously considered and should not be generalized for all mRNAs containing AREs. This was exemplified in a third series of transgenic systems examining the effects of the c-*myc* 3′ UTR that possesses a different ARE signature from those found on the TNF and GM-CSF mRNAs. Despite numerous previous in vitro studies pointing towards the important role of c-*myc* ARE in the modulation of c-*myc* mRNA stability, its deletion from the corresponding c-*myc* encoding transgenes had nominal effects in their expression and mouse physiology (Langa et al. 2001). Thus, and as proposed earlier, the heterogeneity amongst different AREs may in fact indicate different ARE-mediated effects that can only be revealed in an in vivo context, as it occurs in the transgenic systems of ARE deletion described above.

As is clear from the studies on TNF mRNA in inflammation, AREs appear to respond to numerous signals to maintain the homoeostasis of a given response. Thus, the elucidation of "ARE signalosomes" is of high clinical and pharmacological importance; it reveals putative targets for pathology. In the following section we will indicate briefly the importance of specific signal transduction pathways targeting the AREs in inflammation that have been validated in transgenic systems.

2.2 Inflammatory ARE Signalosomes

Most of the current knowledge on signals affecting the post-transcriptional regulation of immune mRNAs is derived from studies on innate effector cells (e.g. macrophages). Apart from their importance as cellular mediators of inflammation, innate cells are excellent systems to study inflammatory gene expression due to their capacity to readily produce cytokines, chemokines and acute phase proteins in response to defined infectious activators and inflammatory signals. As the intricacies of Toll-like receptors (TLRs) and cytokine receptors [tumour necrosis factor receptors (TNFRs), IL-1 receptor (IL-1R) and IFN receptors (IFNRs)] are being unveiled, it is becoming clear that their signals target post-transcriptional modules. For example, the engagement of the classical TLR4 pathway on macrophages triggers positive changes in stability and the translation of TNF, IL-1, IL-3, IL-6, IL-8 and cyclo-oxygenase-2 (COX-2) mRNAs (Kracht and Saklatvala 2002).

In 1994 a class of pyridinyl imidazole compounds was identified that blocked these post-transcriptional changes, through binding to members of the serine/threonine family of p38 stress-activated protein kinases (kinases) (Lee and Young 1996). Through the use of such small molecule inhibitors and transgenic systems, the intense research that followed revealed that the p38α/β isoforms modulate the stability and the translation of cytokine mRNAs that contain AREs. This effect is primarily mediated through the p38-mediated activation of the downstream mitogen-activated protein kinase activated protein kinase 2 (MAPKAPK-2 or MK2) and to a lesser extent by MAPKAPK-3 (MK3) that target ARE-binding proteins (Gaestel 2006; see also Sect. 3). Mutant mice with a deficiency in MK2 are resistant to models of endotoxemia and collagen-induced arthritis, whereas they remain sensitive to intracellular pathogens, reciprocating states of TNF/TNFR deficiency (Kotlyarov et al. 1999; Lehner et al. 2002; Hegen et al. 2006). These effects have been partially attributed the lack of MK2 modulation on the ARE-dependent translation/stabilization of cytokine mRNAs such as those encoding TNF and IL-6 (Gaestel 2006; Neininger et al. 2002). The wealth of data on the p38/MK2 axis towards cytokine biosynthesis provided solid support for the development of new biological therapies

targeting these molecules in inflammatory disease with promising effects for rheumatoid arthritis, IBDs and lung inflammation (Saklatvala 2004; Peifer et al. 2006; Adcock et al. 2006). However, the p38/MK2 cascade appears to modulate a pleiotropy of cellular signals extending beyond their effects on cytokine biosynthesis (Gaestel 2006; Ashwell 2006). In addition they may cross-react with numerous additional cascades [such as the nuclear factor κB (NFκB)-related kinases and other MAPKs and stress-activated protein kinases (SAPKs); Karin 2005] and thus their respective blockade may lack the necessary "kinome" selectivity to target exclusively an anti-inflammatory effect, particularly in the case of chronic inflammation. Such an example has been provided in a genetic mouse model of Crohn's disease, where MK2 deficiency resulted in exacerbated inflammation in the intestinal mucosa, attributed to the lack of p38-mediated apoptotic control of infiltrating cells (Kontoyiannis et al. 2002).

Additional TLR-associated signals converging to AREs have been described and include c-Jun N-terminal kinase (JNK) isoforms, the MAP3 kinase Tpl2/Cot and the extracellular signal-regulated kinase 1/2 (ERK1/2) MAPK revealing additional signal-induced ARE-mediated effects such as nucleocytoplasmic mRNA transport (Kontoyiannis et al. 1999, 2001; Dumitru et al. 2000). Furthermore, ARE-related signalling cross-talks emanating from the concomitant engagement of inflammatory and anti- or co-inflammatory receptors [TLR to MAPK modules versus IL-10/IFNs to Janus kinase (JAK)/signal transducer and activator of transcription (STAT)/suppressors of cytokine signalling (SOCS) modules] have been described; whether these effects are directly targeting specific ARE-mediated processes or are interfering with the p38/MK pathway is currently unclear, although evidence for the latter has been provided in the case of IL-10 associated signals (Kontoyiannis et al. 2001; Rajasingh et al. 2006).

Overall the clinical exploitation of these signalling cascades provides the first line of evidence for the importance of post-transcriptional modules as putative therapeutic targets. To identify more targets of increased specificity, current research is focussing on the terminal acceptors of inflammatory signals, i.e. the RNA binding proteins that modulate mRNA turnover and translation.

3 Determinants of Fate: ARE-BPs

The RBP genes constitute of one of the largest gene families conserved across species, which may reflect the pleiotropy of their functions. It appears, however, that the determination of mRNA fate is the collective sum of numerous RBPs assembled in ribonucleoprotein particles (RNPs). The dynamics between RBPs and RNAs in RNPs are complex and difficult to monitor in the context of complex cellular response, e.g. the inflammatory response; however, they can be inferred by the effects of individual RBP components on their mRNA targets as reflected in the paradigm of ARE-dependent modulation. Numerous ARE-binding proteins (ARE-BPs) have been identified to date (Barreau et al. 2006); however, a few have been implicated in the control of ARE-containing mRNAs. Most of them have been associated with negative effects on mRNA fate, i.e. destabilization or translational silencing, whereas only one family—the Elavl/Hu—has been described as a positive regulator. These proteins contain different protein motifs through which they recognize RNA structures, suggesting that the AREs are "modular" in nature and consist of different binding sites. In the following sections we will focus on those ARE-BPs that have a proven involvement in inflammatory reactions, as has been suggested in studies using transgenic mammals.

3.1 Tristetraprolin Family

The tristetraprolin (TTP) family of CCCH tandem zinc-finger proteins is composed of four known members in mammals, three of which are highly conserved in humans and rodents (Zfp36/TTP, Zfp36L1/BRF1, Zfp36L2/BRF1). All members have the restricted capacity to recognize class II AREs and promote deadenylation-dependent destabilization of the transcripts they bind (Blackshear 2002). The prototype member of this family, TTP, is now known to affect the turnover of specific mRNA subsets. The generation of TTP-deficient mice, however, revealed that the functions of this molecule are primarily related to the haematopoietic and myeloid responses. These mice develop a systemic inflammatory syndrome with severe polyarticular arthritis and myeloid hyperplasia. The syndrome seemed to be due predominantly

to excess tumour necrosis factor-alpha (TNF-alpha) and GM-CSF, resulting from the increased stability of the TNF-alpha and GM-CSF mRNAs (Carrick et al. 2004). The molecular specifics of TTP functioning have been highlighted recently (Gaestel 2006). TTP is phosphorylated by MK2 in LPS-stimulated macrophages; this phosphorylation event appears to increase TTP protein stability and provide anchorage sites for binding to 14-3-3 proteins. This interaction prohibits the sequestration of TTP to its targets, thus inhibiting its destabilizing effect. Consequently the p38/MK2-dependent interaction of TTP to 14-3-3 is alleviated, presumably via protein phosphatases such as protein phosphatase 2A (PP2A), and TTP is activated to suppress the potentially harmful production of inflammatory mediators (Sun et al. 2006). In addition, the TTP mRNA and protein appear to be heavily regulated at the post-transcriptional (even by itself) and post-translational levels, as in the case of many of its target mRNAs (Brook et al. 2006; Hitti et al. 2006). These findings render TTP as the first RNA binding protein with a specific relevance to inflammatory responses and suggest that strategies aiming towards the augmentation of its functions (either by affecting its phosphorylation status or increasing its abundance) can have a clear clinical benefit in inflammatory conditions such as rheumatoid arthritis.

A similar mode of function has been recently described for the second family member, Zfp36L1/BRF1, that responds to protein kinase B (PKB) signals (Benjamin et al. 2006). Unfortunately, mice deficient for this molecule, die at early embryonic stages, thus prohibiting the assessment of the molecules' functions in inflammation (Stumpo et al. 2004; Ramos et al. 2004). However, it is highly likely that these proteins play similar roles—either in other cellular compartments (e.g. lymphocytes) or in response to additional inflammatory signalling cascades—which will be revealed in the future.

3.2 TIA-1/TIAR

TIA-1 and TIAR are closely related members of the RNA recognition motif (RRM) family of RNA-binding proteins binding to numerous mRNAs containing a U-rich motif. Both proteins inhibit the translation of TNF transcripts in macrophages. TIA-1 deficient mice are pheno-

typically normal, but in one specific genetic background they display mild symptoms of arthritis (Phillips et al. 2004; Piecyk et al. 2000). These mice also appear very sensitive to mouse models of endotoxemia (Piecyk et al. 2000). TIA-1 deficient macrophages overproduce TNF-alpha protein and the percentage of TNF transcripts found in polysomes is significantly increased, suggesting that TIA-1 functions as a translational silencer (Piecyk et al. 2000).

The mechanism of TIA-1 functions has been revealed in vitro, using cellular systems of oxidative stress-induced phosphorylation of eukaryotic initiation factor 2α (eIF2α), prohibiting the initiation of protein synthesis. Under these conditions, TIA-1 assembles with components of translation-initiation machinery which is directed at discrete cytoplasmic foci known as stress granules (SGs) (Anderson and Kedersha 2006). Stress granules are also rich in RNP complexes and thus are regarded as "decision making" sites where mRNAs are stalled prior to their destruction or translation. The recent demonstration that phosphorylated TTP is excluded from SGs whereas its non-phosphorylated form is present conforms to this notion and suggests that translational inhibition is indirectly linked to destabilization (Stoecklin and Anderson 2006).

Although these molecular events have not been clearly demonstrated in innate cells, due to the rapidity of their response, it is highly likely that they occur in a similar fashion. This is further exemplified in mice rendered deficient for both TIA-1 and TTP, where they develop a much-exacerbated form of arthritis compared the ones observed in either singly deficient mouse (Phillips et al. 2004). However, this result was accompanied with the puzzling observation that TTP/TIA-1 double-deficient macrophages produced very low amounts of TNF, whereas the major source of TNF overexpression was restricted to granulocytes. This points towards the existence of additional "silencer" molecules functioning in macrophages.

It is currently unclear whether a strategy aimed at augmenting TIA-1 functions could have a beneficial effect as an anti-inflammatory strategy; however, if TIA-1 is a requirement for "stalling" mRNAs in SGs and allows for their indirect tethering towards destabilization, then such an approach may have a wider application versus strategies affecting TTP.

3.3 AUF1

Historically, AUF was the first ARE-binding protein cloned, but most of the knowledge on this molecule does not directly relate to inflammation. AUF1 also belongs to the family of RRM-containing RBPs and exists as four different isoforms, p37, p40, p42 and p45, which result from differential splicing. AUF1 binds to numerous cytokine and growth-factor mRNAs in vitro, although the full spectrum of its affinities have not been determined. Interestingly both destabilizing and stabilizing roles for AUF1 have been suggested from the results of unicellular overexpression experiments (Wilson and Brewer 1999). The multiple isoforms of AUF1, however, may have different roles in different cell types and have not been individually analysed. Similarly to TTP, AUF1 is phosphorylated in vivo (Wilson et al. 2003), indicating a more prominent response to proliferating and stress signals. A very recent report describing the generation of an AUF1-deficient mouse revealed the potential implication of this ARE-BP also in the modulation of inflammatory cascades. AUF1-deficient mice show a mild growth-retardation phenotype but also appear relatively more sensitive to endotoxemia than wild-type controls (Lu et al. 2006). Although this effect is partially attributed to increases in TNF and IL-1β mRNA stability in macrophages, there is no clear cellular or molecular evidence that AUF1 acts in an anti-inflammatory fashion. Still, these findings render AUF1 as putative target for inflammatory disorders that need to be examined more thoroughly in the future.

3.4 Elavl1/HuR

HuR is the prototypical member of the *Elavl/Hu* family of RNA-binding proteins named after the lethal phenotypes of their homologue in *Drosophila* (embryonic lethal abnormal vision) and their appearance as specific tumour antigens in individuals with paraneoplastic neurological disorders (Hu antigens). In mammals the family is composed of the ubiquitously expressed HuR (HuA or Elavl1) and the neuronal-specific HuB, HuC and HuD. HuR has a prototypical RBP structure that includes two N-terminal RRMs with high affinity for a U-rich sequence (HuR binding motif, HBM), followed by a nucleocytoplasmic shuttling

sequence and a C-terminal RRM recognizing the poly-A tail (Fan and Steitz 1998; Fan and Steitz 1998; Lopez de Silanes et al. 2004). Although predominantly nuclear, HuR shuttles between the nucleus and the cytoplasm acting as an RNA adaptor. Numerous studies have indicated that the cytoplasmic HuR can stabilize specific mRNAs (Brennan and Steitz 2001). However, the constitutive and high expression of HuR and the wide distribution of HBM among numerous ARE/non-ARE-containing mRNAs indicate that HuR recognition may not be very discriminative, hence the mechanisms involved in inducing the specificity of its functions remain elusive. With respect to inflammation, studies on macrophage cell lines suggested that innate sensitizers increase the cytoplasmic binding of HuR to cytokine mRNAs supporting their stabilization (Brennan and Steitz 2001; Dean et al. 2004). Furthermore, genetic approaches have identified mouse strains with mutations in the HBM of inflammatory mRNAs that correlate with the development of autoimmunity (Di Marco et al. 2001). These observations suggest that the overt upregulation of HuR could support the hyper-activation of inflammatory mediators to drive ensuing inflammation. However, the search for HuR's role in inflammation has been obstructed by its predictive involvement in central developmental processes (Levadoux-Martin et al. 2003), which have been recently confirmed in HuR-deficient mice (V. Katsanou and D.L. Kontoyiannis, unpublished).

The positive role of HuR towards cytokine biosynthesis was recently challenged through in vivo systems of tetracycline-inducible and macrophage-specific overexpression in the mouse (Katsanou et al. 2005). These mice displayed reduced in vivo inflammatory responses in modelled endotoxemia and were resistant in modelled hepatitis. The anti-inflammatory effect of the transgenic HuR correlated with the reduced production of a specific set of inflammatory mediators, indicating that in vivo, HuR acts in a discriminative fashion. HuR was found to associate directly with TNF, COX-2 and transforming growth factor beta 1 (TGFβ1) mRNAs and indirectly with the IL-1β mRNA, consequently blocking the biosynthesis of the corresponding proteins in stimulated transgenic macrophages. The most surprising finding was that HuR overexpression reduced the translation of these inflammatory mRNAs, although it increased the stability of class II mRNAs (such as TNF and

COX-2 AREs), as has been previously suggested. The genetic elimination of TTP and TIA-1 in the context of HuR overexpression revealed a synergy between the corresponding functions of these RBPs towards the modulation of TNF mRNA translation; HuR required the functions of TIA-1 to inhibit the translation of TNF mRNA and this effect occurred even in the absence of TTP deficiency. By combining the current data on HuR, TTP and TIA-1, these studies postulate a functional hierarchy towards the modulation of mRNAs bearing class II AREs, where HuR is tethering such mRNAs towards TIA-1-mediated translational inhibition, which in turn tethers towards destabilization by TTP. This is also compatible with the inclusion of all three RBPs in stress granules and the fact that the HBM is distinct from the binding sites of other RBPs (Keene and Tenenbaum 2002; Lopez de Silanes et al. 2005).

The molecular details and the widespread representation for this "consequential tethering model" remain to be determined. Clues as to its necessity, however, are starting to come forth. The data provided from HuR transgenic studies indicate that the functions of this molecule are not definite but may be governed by the modular constitution of the ARE niche (i.e. the HBM with or without ARE of different class or cluster) in each independent mRNA species. For example, the TNF and COX-2 mRNAs that contain an HBM next to a class II- cluster III ARE and respond similarly to HuR in stimulated macrophages (increased stability, reduced translation) are targets for TTP and TIA-1. On the other hand, the TGFβ1 mRNA contains an HBM but does not contain a prototypical ARE; HuR overexpression reduces its translation and is not a target for TTP, although it is unknown whether or not it binds to TIA-1/TIA-R. This points towards a role for HuR in organizing the mRNAs in "functional clusters" required for an elicited response. This, in fact, has been proposed by Keene and Tenenbaum (2002) based on en masse, RBP-coupled RNA immunoprecipitation studies where it was demonstrated that Hu-containing RNPs contain functionally related mRNAs. We postulate that, at least in macrophages, HuR acts as a co-ordinator of downstream RBP associations that will be governed by the quantity and the intrinsic properties of a given mRNA subset, as well as the activation of specific RBPs in response to an external stimulus. Although this property of HuR remains to be validated in systems of HuR defi-

ciency, it reveals the increased potential of future therapeutic strategies aiming at the overexpression of this molecule in inflammation.

4 Concluding Remarks

This brief review aims to suggest to the prospective reader that post-transcriptional signalosomes and RBPs identify connections between genes and disease, and thus their analysis can provide better diagnostics and therapeutics. For the paradigm of inflammation, we postulate that targeting selective tissue-specific RNA:RBP associations in disease will provide better means for therapeutic intervention since a specific collection of functionally related pathogenic-effector products will be targeted instead of (1) a select few (e.g. antibodies to selected inflammatory mediators) that can have marginal effects on disease progression or (2) non-specifically too many (e.g. chemical inhibitors of receptors or intracellular signals affecting a wide spectrum of transcriptional, post-transcriptional and post-translational mechanisms) that current therapeutic schemes are focussed upon. The development of such therapeutic schemes, however, will have to rely on the outcome of the studies analysing RBPs in a tissue-specific context. For example, even though the analyses of ARE-mediated mechanisms in inflammation have made tremendous strides over the last few years, they is still quite limited. First, the mechanics of ARE-BP functions remain unresolved, especially in light of new data that indicate the interplay of these proteins with modulating micro-RNA subsets (Bhattacharyya et al. 2006; Jing et al. 2005). Second, and concerning inflammation, most of the current knowledge is derived from the analysis of innate cells in response to a limited number of signals. The molecular and cellular attributes of ARE-dependent modulation in other inflammatory effectors (i.e. lymphocytes, endothelia, etc.) and targets (e.g. epithelia, neurons, etc.) are largely unknown. It is almost certain, however, that the coupling conditional transgenic technologies to molecular and functional genomics' platforms will reveal the pathophysiological role for RBPs and signalling cascades in support of a novel class of biological therapeutics targeting post-transcriptional processes.

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Immunomodulatory Therapies: Challenges of Individualized Therapy Strategies

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Abstract. "Individualized therapy strategies" involve strategies that allow treatment to be guided by patient-specific conditions. For this, robust biomarkers are needed. Examples of biomarker-guided therapies already in use are the treatment of insulin-dependent diabetes (biomarker: blood glucose level) or the treatment of hypertension (biomarker: blood pressure). By contrast, most immunomodulatory therapies are given according to the patient's body weight or the patient's drug blood level rather than according to biomarkers indicating the patient's state of the immune system. Herein we report on new biomarker-guided studies in the immunosuppressive treatment of transplant patients and patients with autoimmune disease and we discuss its benefits and pitfalls.

1 Introduction

"Individualized therapy strategies" involve strategies that allow treatment to be guided by patient-specific conditions. To achieve these therapy strategies, two requirements need to be fulfilled: (1) subgroups with different response patterns have to be identified before the start of a particular treatment protocol to select the best therapy for the individual patient, (2) the therapeutic success/failure has to be monitored to adjust the therapy according to individual response as early as possible. To fulfill these criteria, robust biomarkers are needed.

Examples of individualized therapy strategies already in use are the medicinal treatment of diabetes or hypertension. In diabetic patients, the insulin dosage is adapted to the patient's blood glucose level. In the treatment of hypertension the dosage of the antihypertensive drug is adapted to the patient's blood pressure. By contrast, most immunomodulatory therapies are given according to the patient's body weight or the patient's drug blood level rather than according to biomarkers indicating the patient's state of the immune system.

To illustrate the first successful experiences with biomarker-guided studies, we will report on new biomarker-guided studies in the immunosuppressive treatment of transplant patients and patients with autoimmune disease and will discuss benefits and withdrawals. Concretely, three examples are presented and discussed:

1. Selecting transplant patients for an enhanced risk for cytomegalovirus (CMV) disease to improve antiviral management
2. Selecting the best immunosuppression for organ transplant patients
3. Monitoring the success of specific immunomodulatory therapies to select/adjust therapy in autoimmune patients

2 Selecting Transplant Patients on Enhanced Risk for CMV Disease to Improve Antiviral Management

CMV reactivation frequently occurs, but CMV disease only develops in cases of diminished T cell responses to control CMV reactivation (Quinnan et al. 1982). As immunosuppression in allotransplant patients so far does not discriminate between alloantigen specific and pathogen specific T cells, allotransplant patients are at high risk to develop life-threatening CMV disease (Fishman and Rubin 1998). Current standard treatment of CMV disease is antiviral therapy with ganciclovir. However, there is no correlation between initial viral load and response to ganciclovir, indicating that antiviral therapy alone may not be sufficient enough to control disease (Babel 2004).

Recently, Bunde et al. analyzed the T cells responses against the immunodominant CMV proteins pp65 and IE-1 in solid organ transplant patients and related them to the development of CMV disease (Bunde et al. 2005). This was done by usage of an epitope/HLA-independent flow cytometric method using protein spanning peptide pools of the pp65 and IE-1 protein (Kern et al. 1998). They could clearly demonstrate that, in contrast to pp65, the CMV IE-1 T cell response is associated with protection from CMV disease. The relevance of a strong IE-1-specific T cell response to control CMV disease development may be explained by the fact that following CMV reactivation—for instance by inflammation, stress, or some drugs—the IE-1 protein is the first protein expressed in monocyte precursor cells in the bone marrow (Stinski 1978; Döcke et al. 1994; Prösch et al. 1995). IE-1 protein-expressing precursor monocytes mature, become permissive to CMV replication, and migrate to the periphery, thus distributing the infectious virus (Waldman et al. 1995).

Consequences from these data are:

1. Antiviral strategies (e.g., long-term ganciclovir) should be accompanied by CMV-specific T cell monitoring.
2. Transplant patient with insufficient IE-1-specific T cell response are at enhanced risk for CMV disease, and their CMV load should be closely monitored to initiate preemptive antiviral therapy.

3. T cell-depleting strategies (e.g., Campath-1, ATG, but particularly anti-CD3) should be used very restrictive only if the CMV load is closely monitored.
4. Adoptive CMV targeting T cell therapy for severe CMV disease should include CMV IE-1-reactive cytotoxic T cells.

3 Selecting Best Immunosuppression for Organ Transplant Patients

Currently, standard immunosuppressive drugs suppress the immune system antigen independently. That is, alloantigen-specific as well as pathogen/tumor-specific responses are inhibited. Taken together with substrate specific actions, two types of side effects have to be taken care of when administering immunosuppressive drugs: inhibition of volitional immune responses and drug-specific side effects (for instance, renal dysfunction which may be caused by calcineurin inhibitors or rapamycin; post-transplant diabetes, which may be caused by FK506 or steroids). In order to minimize drug-specific side effects in allotransplant patients, standard treatment protocols usually contain combinations of various immunosuppressive drugs and, as mentioned earlier, the dosage is regulated by the patient's body weight or blood drug levels, respectively. However, except for the differentiation of high-risk patients, these protocols are used independently of patient-specific conditions. Therefore, among others, the following questions arise:

1. Does every patient need the same immunosuppression?
2. Which patients can be weaned from immunosuppression?

3.1 Does Every Patient Need the Same Immunosuppression?

The human T lymphocyte pool comprises everything from naïve T cells (including naïve alloreactive T cells), regulatory T cells, and memory T cells to environmental recall antigens and alloreactive memory T cells. By detecting alloreactive memory T cells using an interferon gamma (IFN-γ) ELISpot (enzyme-linked immunoabsorbent spot) assay, Nickel et al. recently showed that the occurrence of *pre*-transplant alloreactive

memory T cells in "naïve" transplant recipients predicts negative short- and long-term graft function under conventional immunosuppression (Nickel et al. 2004). Furthermore, high frequencies of alloreactive T cells *after* transplantation showed a poorer 1-year creatinine clearance, indicating insufficient control of alloreactivity (Nickel et al. 2004). Importantly, memory T cell frequencies to multiple HLA-specificities (that is, panel reactive T cells, PRT) do not correlate with panel reactive antibody titers (Andree et al. 2006).

These data indicated that patients with high frequencies of alloreactive memory T cells prior to transplantation may benefit from specific elimination/functional inhibition of these T cells. Elimination of T cells can be achieved by (monoclonal) antibodies such as antithymocyte globulin (ATG) or Campath. However, incomplete depletion of almost all memory T cells results in lymphopenia-derived proliferation of alloreactive T cells and complete depletion results in severe inhibition of anti-infectious and antitumor competence (Ernst et al. 1999). Regarding functional inhibition of alloreactive T cells, except for calcineurin inhibitors, standard immunosuppressive drugs rather inhibit naïve than memory T cells. Calcineurin inhibitors are very potent inhibitors of both naïve and memory T cells. Therefore they cannot be spared for only those patients with high frequencies of alloreactive memory T cells. Furthermore, as with complete depletion, complete functional inhibition of the (memory) T cell pool would result in severe inhibition of anti-infectious and antitumor competence. In contrast to the elimination of T cell subsets regardless of their antigen specificity, the first clinical approaches to *selectively* eliminate *alloreactive* memory T cells have been conducted and the results are encouraging (N. Babel, L. Gabdrakhmanova, M. Hammer, C. Rosenberger, G. Bold, J.S. Juergensen, C. Schoenmann, H.D. Volk, and P. Reinke, manuscript in preparation).

3.2 Which Patients Can Be Weaned of Immunosuppression?

For many reasons, drug weaning in transplant patients is of importance. These reasons include (1) the reduction of short and long-term complications of immunosuppression, (2) the prevention of drug-induced graft injury, (3) a reduction of costs of long-term immunosuppressive therapy, and (4) the reduction of psychological reasons/compliance problems.

Drug weaning in transplant patients is possible because in most patients the rejection risk decreases with time due to tolerance mechanisms that are, however, only poorly understood. The first weaning trials show successful drug reduction in part of the patients but rejection episodes in others. In order to successfully wean transplant patients from immunosuppression, we need biomarkers guiding our protocols.

The EU integrated project "Reprogramming the immune system for the establishment of tolerance" (*www.risetfp6.org*) addresses these issues with goals to develop assays that will:

1. Identify drug weaning failure and/or tolerance induction before graft injury to introduce therapeutic adjustment on time (failure of tolerance)
2. Detect developing tolerance (success of tolerance) early

Ideally, biomarkers (that is rejection markers/tolerance markers) taken from noninvasive methods, e.g., using blood or urine, should fulfill these requirements.

With respect to rejection markers, Kotsch et al. could recently demonstrate that renal transplant patients with enhanced urinary cytotoxic T cell marker expression like granulysin are at enhanced risk of acute rejection, indicating the need for prolonged/intensified immunosuppression (Kotsch et al. 2004). With respect to tolerance markers, B. Sawitzki et al. studied the molecular signature of tolerance in renal transplant animal models and in humans using complementary DNA (cDNA) microarray and reverse transcriptase PCR (RT-PCR) techniques; by analyzing blood, a distinct marker set may be able to distinguish putative tolerant patients where minimizing of immunosuppression might be possible (B. Sawitzki, A. Bushell, U. Steger, N. Jones, K. Risch, A. Siepert, M. Lehmann, I. Schmitt-Knosalla, K. Vogt, I. Gebuhr, K. Wood, and H.D. Volk, manuscript in preparation).

4 Monitoring Success of Specific Immunomodulatory Therapies to Select/Adjust Therapy in Autoimmune Patients

Multiple sclerosis (MS) is an autoimmune disease in which T cells may play a central role (Wandinger et al. 2003). Interferon-β (IFN-β)

has become a worldwide standard in MS treatment, and its efficacy is well documented even if the mechanisms by which it changes the clinical course of MS are fairly unclear (Wandinger et al. 2003). As with virtually every drug, IFN-β is not free from side effects, and some patients do not respond to IFN-β. However, the clinical differentiation of therapeutic responder from nonresponder patients takes 6 to 12 months, revealing the need for a marker (set) to distinguish responder from nonresponder patients very early after onset of IFN-β thera5spy or, even better, before onset of treatment. By using cDNA microarray analysis of peripheral mononuclear cells from MS patients treated with IFN-β, Wandinger et al. could demonstrate the upregulation of tumor necrosis factor (TNF)-related apoptosis inducing ligand (TRAIL) messenger RNA (mRNA) in all patients (Wandinger et al. 2003). Importantly, as early as 4 weeks after onset of IFN-β therapy, so-called first-year responders had a significant higher TRAIL upregulation compared to first-year nonresponders. In the case of the formation of neutralizing anti-IFN-β autoantibodies, initial upregulation of TRAIL was abrogated (Wandinger et al. 2003). Additionally, baseline soluble TRAIL in sera of responder patients was significantly higher than in nonresponder patients, indicating that, even prior to therapy, responders might be distinguishable from nonresponders (Wandinger et al. 2003).

Psoriasis is another autoimmune disease where T cells may play a central pathogenetic role (Philipp et al. 2006). Alefacept is a fusion protein made of two human lymphocyte function antigen 3 proteins (LFA-3, CD58) and the Fc part of a human immunoglobulin G1. Alefacept binds to the CD2 molecule on T cells, inhibits T cell activation, and induces selective apoptosis and depletion of memory/effector T cells (Miller et al. 1993; Majeau et al. 1994; Meier et al. 1995). Consequently, Alefacept therapy has been successfully used in psoriatic patients (Lebwohl et al. 2003). However, measured by a Psoriasis Area and Severity Index (PASI) score reduction of more than 50%, responders significantly separated from nonresponders as late as 9 weeks after onset of therapy. By analyzing mRNA expression in peripheral blood mononuclear cells (PBMC) from treated patients, Gube et al. found a distinct marker set predicting response as early as 3 weeks after onset of therapy (K. Gube, M. Friedrich, I. Gebuhr, S. Philipp, R. Sabat, W. Sterry, H.D. Volk, and B. Sawitzki, manuscript submitted).

These data demonstrate that biomarkers can help to define subgroups with different response patterns prior to the start of or very early after the beginning of a particular treatment protocol. By this method, non-responder patients can be kept from therapies that would not be effective and could cause drug-specific side effects (such as depression with INF-β or lymphopenia with Alefacept).

5 Summary

The data presented at the Ernst Schering Foundation Scientific Symposium in October 2006 demonstrate that the identification of subgroups with distinct response pattern to immunomodulatory therapy is possible. Furthermore, the first biomarker-guided clinical trials show promise towards the development of more individualized therapy strategies. However, further validation strategies are necessary. Figure 1 summarizes the approach toward a more individualized immune therapy.

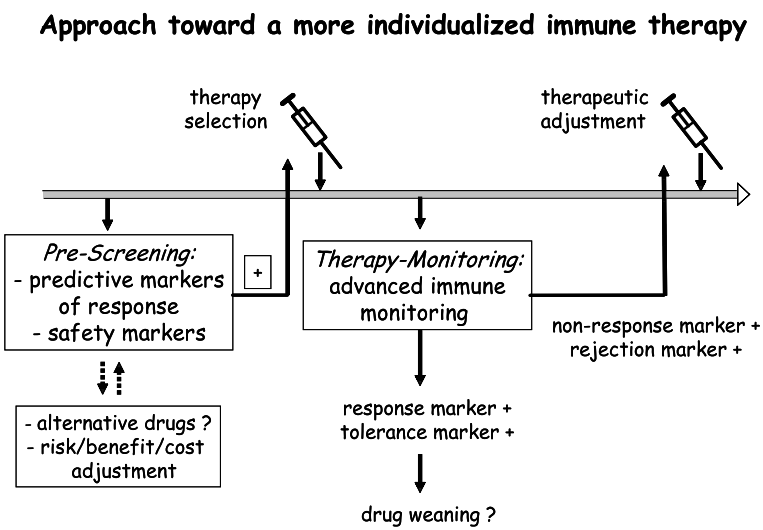

Fig. 1. Approach toward a more individualized immune therapy

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T Cell Therapies

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Abstract. T cell therapies are increasingly used for the treatment of malignancies and viral-associated diseases. Initial studies focused on the use of unmanipulated T cell populations after allogeneic stem cell transplantation. More recently, the use of antigen-specific T cells has been explored. This chapter reviews the clinical experience with polyclonal Epstein-Barr virus (EBV)-specific cytotoxic T cells (CTL) for the treatment of EBV-associated malignancies. Strategies on how to improve the antitumor activity of EBV-specific CTL are being discussed. If effective, these strategies will have broad implications for T cell therapies for a range of human tumors with defined antigens.

1 Introduction

The use of donor lymphocyte infusions (DLI) for the successful treatment of hematological malignancies such as chronic myelogenous leukemia after hematopoietic stem cell transplantation (HSCT) has demonstrated the curative potential of T cell therapies (Kolb et al. 1990, 2004). However, the use of DLI is limited by potentially fatal complications such as graft-versus-host disease (GVHD) that arises from the presence of alloreactive T cells. To overcome this limitation, strategies have been developed to generate antigen-specific T cell products that are devoid of alloreactivity.

Developing successful antigen-specific T cell therapies depends on the availability of specific antigens as targets and efficient methods for ex vivo T cell activation and expansion. Riddell et al. pioneered the use of antigen-specific T cells to prevent cytomegalovirus (CMV) reactivation in HSCT recipients (Riddell et al. 1992). Donor-derived CD8-positive T cell clones activated by coculture with CMV-infected, autologous fibroblasts and specific for the viral tegument proteins pp65 and pp150 proved safe and protected HSCT recipients against the reactivation of CMV (Riddell et al. 1992; Peggs et al. 2003). Since then, studies have been performed with other antigen-specific T cells, including Epstein-Barr virus (EBV)-specific T cells for the adoptive immunotherapy of EBV-associated diseases, melanoma antigen recognized by T cells (MART-1)-specific or glycoprotein 100 (gp100)-specific T cells for melanoma, and adenoviral-specific T cells (Rooney et al. 1998; Bollard et al. 2004a; Straathof et al. 2005a; Yee et al. 2002; Morgan et al. 2006). The results of these clinical trials indicate that antigen-specific T cells are safe, and produce antiviral and antitumor effects. However, T cells did not expand significantly in vivo unless the recipient's lymphoid compartment was depleted, as seen after HSCT, and only persisted if the infused T cell product contained CD4 T helper cells. Moreover, T cell escape mutants were observed when clonal or oligoclonal T cell lines were infused (Yee et al. 2002; Gottschalk et al. 2001). These results imply that therapeutic T cell products should target multiple antigens, and contain CD8-positive as well as CD4-positive T cells. In addition, robust *in vivo* T cell expansion most likely requires a lymphodepleted environment and/or the presence of antigen. In this chapter we

will highlight the successes and challenges of T cell therapies using EBV-associated malignancies as a model system.

2 EBV-Associated Malignancies

EBV is a latent γ-herpesvirus, and more than 90% of the world's population is EBV-positive. During primary infection, EBV establishes lifelong latency in the memory B cell compartment, and the number of latently infected B cells within an individual remains stable over years (Cohen 2000). Healthy individuals mount a vigorous humoral and cellular immune response to primary infection (Rickinson and Kieff 2001). Although EBV-specific antibodies neutralize virus infectivity, the cellular immune response, consisting of CD4-positive and CD8-positive T cells, is essential for controlling primary and latent EBV infection.

All EBV-associated malignancies are associated with the virus' latent cycle (Hsu and Glaser 2000). In type I latency, which is found in EBV-positive Burkitt's lymphoma, only Epstein-Barr nuclear antigen 1 (EBNA1), EBV-encoded small nuclear RNAs (EBERs), and the BamHI-A rightward transcripts (BARTs) are expressed. Type II latency, characterized by EBNA1, latent membrane proteins (LMP1, LMP2), EBERs, and BARTs expression, is found in EBV-positive Hodgkin's disease, nasopharyngeal carcinoma (NPC), and peripheral T/natural killer (NK) cell lymphomas. While malignancies associated with type I and II latency occur in individuals with minimal or no immune dysfunction, type III latency is associated with malignancies in severely immunocompromised patients. It is characterized by the expression of the entire array of EBV latency genes, being EBNAs 1, 2, 3A, 3B, 3C, leader protein (LP), LMP1, LMP2, EBERs, and BARTs. This pattern of gene expression is found in EBV-associated lymphoproliferative disease (EBV-LPD) after HSCT or solid organ transplant (SOT), and in EBV-associated lymphomas occurring in patients with congenital immunodeficiency or human immunodeficiency virus (HIV) infection. In addition, type III latency is found in lymphoblastoid cell lines (LCL), which can be readily prepared by infecting B cells in vitro with EBV and are instrumental in the generation of EBV-specific cytotoxic T lymphocytes (CTL) for the prophylaxis and therapy of EBV-LPD.

3 Adoptive Immunotherapy for EBV Latency Type II Malignancies

3.1 Adoptive Immunotherapy for EBV-LPD After HSCT

Unmanipulated donor T cells have been used to treat HSCT recipients with established EBV-LPD with variable success, likely reflecting differences in EBV-specific T cell precursor frequencies in the infused T cell lines and/or a better outcome with early diagnosis and treatment (Papadopoulos et al. 1994; Lucas et al. 1998; Porter et al. 1994; Heslop et al. 1994; O'Reilly et al. 1997). Moreover, the use of donor T cells is limited by GVHD, a potential life-threatening complication. Two strategies have been developed to reduce the risk of GVHD. One strategy discussed in Sect. 5 relies on the transduction of T cells with a 'suicide gene' so that cell death can be induced if GVHD develops. The second strategy to prevent GVHD after donor T cell infusion is to infuse EBV-specific T cell lines, which lack alloreactivity. EBV-specific T cells can readily be generated from EBV-seropositive donors and the generated T cell lines are polyclonal and contain not only CD8-positive but also CD4-positive EBV-specific T cells (Rooney et al. 1998; Heslop et al. 1996). We have administered donor-derived EBV-specific CTL as prophylaxis or therapy for EBV-LPD in high-risk HSCT recipients. Infused CTL (1) were safe and induced no significant GVHD, (2) expanded by several orders of magnitude *in vivo*, (3) survived for up to 7 years after infusion, and (4) reduced the high virus load that was present in about 20% of patients at the time of infusion (Rooney et al. 1998; Heslop et al. 1996). EBV-specific CTL also appeared to prevent development of EBV-LPD, since none of 60 patients who received prophylactic CTL developed this malignancy, compared with 11.5% of controls. Six of seven patients who received CTL as treatment for EBV-LPD achieved complete remissions (Rooney et al. 1998; Pakakasama et al. 2004). The patient who did not respond illustrates one of the problems of immunotherapy: mutation of CTL target epitopes on tumor cells allowing escape from T cell recognition. The therapy failure was caused by a deletion in the EBV-derived EBNA3B gene in the tumor that removed immunodominant epitopes, thereby causing tumor-cell resistance to CTL killing (Gottschalk et al. 2001).

3.2 Adoptive Immunotherapy for EBV-LPD After SOT

The success of donor-derived EBV-specific CTL as prophylaxis and treatment of EBV-LPD after HSCT has resulted in the development of adoptive immunotherapy strategies for EBV-LPD after SOT. Since the majority of EBV-LPD after SOT are of recipient origin and donors are not HLA matched, the use of donor-derived EBV-specific T cells is of limited value. Therefore the use of autologous or partially HLA-matched EBV-specific CTL has been explored (Savoldo et al. 2006; Haque et al. 2002, 1998; Khanna et al. 1999). These studies demonstrated that infused EBV-specific CTL (1) did not cause graft rejection, (2) increased EBV-specific cellular immune responses *in vivo*, and (3) had antiviral and antitumor effects. However, in contrast to HSCT recipients, the infused EBV-specific CTL persisted only transiently and did not expand significantly, which may indicate that CTL do not persist because of ongoing immunosuppression and that CTL expansion is limited in patients who have a lymphocyte compartment close to or at steady state.

4 Adoptive Immunotherapy for EBV Latency Type III Malignancies

Hodgkin's disease and NPC are associated with EBV latency type II. In contrast to EBV-LPD, only a limited number of EBV-derived antigens, EBNA1, LMP1, and LMP2, are present in EBV-positive Hodgkin's disease and NPC. Nevertheless, the viral antigens provide targets for the adoptive immunotherapy with EBV-specific CTL.

4.1 Hodgkin's Disease

Autologous EBV-specific CTL have been given to patients with EBV-positive Hodgkin's disease with multiple relapses or with minimal residual disease after autologous HSCT (Bollard et al. 2004a; Roskrow et al. 1998). No immediate toxicities were seen. Infused CTL localized to a malignant pleural effusion in one patient and were detected at the tumor site of another patient at autopsy. Immunological studies showed an increase of LMP2-specific and EBV-specific cellular immunity after

CTL infusion, and gene-marked CTL were detected for up to 12 months. Eight patients with advanced disease remained alive for 2–20 months after CTL infusion. One patient with stable disease received an allogeneic HSCT and is in remission 4½ years after CTL infusion. Two patients are in complete remission 9–27 months after CTL infusion. These results indicate that infused EBV-specific CTL in Hodgkin's disease patients (1) are well tolerated, (2) persist for up to 12 months after infusion, (3) enhance EBV-specific immunity, and (4) localize to tumor sites.

4.2 Nasopharyngeal Carcinoma

Three groups of investigators have reported on the use of autologous EBV-specific CTL for the adoptive immunotherapy of patients with recurrent/refractory NPC (Straathof et al. 2005a; Chua et al. 2001; Comoli et al. 2005). In one study, four NPC patients with advanced disease were infused and an increase in EBV-specific CTL precursor frequency was observed, as well as a reduction in plasma EBV-DNA levels. No decrease in tumor size was observed; however, all patients had a large tumor burden (Chua et al. 2001). In the second study, ten patients were treated with EBV-specific CTL and in six of the ten patients control of disease progression was achieved (Comoli et al. 2005). Our group has evaluated the use of EBV-specific CTL in 13 patients; 7 of them had recurrent/refractory disease, while 6 had advanced-stage disease at presentation but were in remission at the time of CTL infusion. Of the 7 patients with recurrent disease, 2 patients are in biopsy-proven complete remission (CR) after CTL, 1 had a partial response for 12 months, 2 had stable disease, and 2 had no response (Straathof et al. 2005a).

The clinical experience with EBV-specific CTL for Hodgkin's disease and NPC indicates that EBV is a legitimate target for T cell targeted therapies. However, EBV-specific CTL were less effective for the treatment of Hodgkin's disease and NPC than for the treatment of EBV-LPD. One explanation for this failure is that EBV-specific CTL generated with LCL are dominated by clones reactive to EBV proteins not expressed in Hodgkin's disease or NPC. Furthermore, EBV-specific CTL did not expand significantly after infusion, indicating that CTL expansion may be limited in patients in which the lymphocyte compartment

is close to steady state. In addition, EBV-specific CTL might be inhibited at the tumor site by immune-evasion strategies employed by the malignant cells of Hodgkin's disease and NPC. Thus, improvement of EBV-specific CTL therapy for Hodgkin's disease and NPC will require strategies to (1) expand CTL specific for EBV proteins expressed in these malignancies, (2) create a favorable environment for T cell expansion in vivo, and (3) genetically modify CTL to render them resistant to the inhibitory tumor environment.

5 Improving T Cell Therapies for EBV-Associated Malignancies

5.1 LMP-1-Specific and LMP2-Specific T Cells

In Hodgkin's disease and NPC, three EBV proteins are expressed: EBNA1, LMP1, and LMP2. Of these, only LMP1 and LMP2 are good targets for adoptive immunotherapy approaches, since EBNA1 is mainly presented on MHC class II molecules. Several groups have developed strategies to generate LMP1-specific and LMP2-specific CTL with autologous dendritic cells (DC) as antigen-presenting cells (APC) expressing LMP2, functionally inactive LMP1 or an "LMP1-LMP2 polyepitope" (Gahn et al. 2001; Gottschalk et al. 2003; Duraiswamy et al. 2004). We have developed a "polyclonal CTL expansion protocol" in which LMP2-specific CTL are initially activated with DC expressing LMP2, and subsequently expanded with LMP2 overexpressing LCL (Bollard et al. 2004b). The safety and efficacy of LMP2-specific CTL for EBV-positive Hodgkin's and non-Hodgkin's lymphoma are currently being evaluated in a phase I clinical trial (Bollard et al. 2005). So far 14 patients have been infused, and we have observed an increase in the frequency of infused CTL in 8 out of 10 evaluable patients. In addition, LMP2-specific CTL were detected at tumor sites. Out of 8 patients with detectable disease at the time of CTL infusion, 5 had clinical responses. These results indicate that LMP2-specific CTL are safe, accumulate at tumor sites, and have antitumor activity.

5.2 Lymphodepletion

EBV-specific CTL expanded *in vivo* by orders of magnitude only in HSCT recipients, indicating that the CTL expansion rate may be lim-

ited in patients in which the lymphocyte compartment is close to steady state. Dudley et al. reported the use of fludarabine and cyclophosphamide to create a proliferative environment prior T cell transfer (Dudley et al. 2002; Dudley and Rosenberg 2003). Selective expansion of infused T cells might also be obtained by using monoclonal antibodies (MAbs) to deplete the lymphoid compartment. We are currently evaluating MAbs directed to the common leukocyte antigen CD45. In murine studies administration of CD45 MAbs depleted all leukocyte lineages (Wulf et al. 2003). This depletion was prolonged only in lymphoid lineages, as neutrophils began to recover 48 h after injection. By contrast, marrow progenitor cells, which express CD45 at low levels, were spared. For our clinical studies, we have chosen a pair of rat IgG1 antibodies that can fix human complement and induce antibody-dependent cellular cytotoxicity, while having a short half-life that permits rapid subsequent infusion of the CTL. CD45 MAbs were initially evaluated in the clinic as part of an ablative preparative regimen for a stem cell allograft. The MAbs were well tolerated at a dose of 400 μg/kg per day for 4 days, producing more than 95% depletion of peripheral blood lymphocytes, predominantly T cells and NK cells (Krance et al. 2003). They reduced neutrophil counts by more than 90%, but, as anticipated, marrow sampling showed retention of $CD34^+$ progenitor cells, followed by partial neutrophil recovery within 48 h of the last dose of antibody. We are currently evaluating the use of CD45 MAbs in phase I clinical trials to transiently lymphodeplete patients with EBV-positive malignancies prior to infusion of EBV-specific or LMP2-specific CTL. In several patients we have observed enhanced T cell expansion after infusion in comparison to CTL infusions in the same patients without lymphodepletion (Louis et al. 2006).

5.3 Genetic Modification

5.3.1 Rendering CTL Resistant to the Immunosuppressive Tumor Environment

In vivo EBV-specific T cells often encounter an immunosuppressive environment created by tumor cells. This includes the presence of inhibitory cytokines secreted by tumor cells, such as transforming growth

factor β (TGF-β) and/or apoptosis-inducing molecules such as Fas ligand (FasL), which are present on the cell surface of tumor cells. In preclinical studies, EBV-specific CTL have been genetically modified to render them resistant to TGF-β by expression of a dominant negative TGF-β type II receptor (Bollard et al. 2002). In addition, EBV-specific CTL expressing a small interfering RNA (siRNA) against Fas were shown to be resistant to FasL-induced apoptosis (Dotti et al. 2005). While these "proof of principle" studies were performed *ex vivo*, current efforts are focused on showing in preclinical animal models and/or phase I clinical trials that these genetic modifications translate into greater antitumor activity of adoptively transferred EBV-specific CTL *in vivo*.

5.3.2 Genetic Safety Switches

Genetic safety switches to selectively kill infused T cells to prevent serious side effects will make current T cell therapies, such as DLI, safer. In addition, safety switches will also reduce the risk:benefit ratio of evaluating genetically modified T cells in clinical trials. Several genetic safety switches have been developed, and the most widely used "switch" takes advantage of the herpes simplex virus derived thymidine kinase (HSV-tk), which phosphorylates acyclovir, valacyclovir or ganciclovir to toxic nucleosides. In a phase I clinical trial, donor-derived T cells transduced with the HSV-*tk* gene were infused into HSCT recipients (Bonini et al. 1997). Six patients developed GVHD and four had a complete resolution of GVHD after ganciclovir treatment. One drawback of this approach is the inherent immunogenicity of HSV-*tk* (Berger et al. 2006). Therefore, genetic safety switches using nonimmunogenic human components such as CD20, inducible Fas, or caspase, have been developed and successfully tested in preclinical animal models (Straathof et al. 2005b; Serafini et al. 2004; Thomis et al. 2001).

5.3.3 Expanding the Use of EBV-Specific CTL to EBV-Negative Malignancies

EBV-specific CTL are an attractive "T cell therapy platform" to target other malignancies since they (1) can be generated reliably from EBV-

seropositive donors for clinical use, and (2) have an excellent safety record in clinical studies. One strategy to target EBV-specific CTL to non-EBV antigens is to express chimeric antigen receptors (CARs) on EBV-specific CTL (Pule et al. 2003; Eshhar et al. 1993). CARs are fusions between an antigen-recognizing ectodomain and a signaling endodomain, most commonly connecting the antigen-recognition properties of a monoclonal antibody with the endodomain of the CD3-ζ chain of the T cell receptor. CARs recognize tumor cells in an MHC-unrestricted manner and are therefore immune to some of the major mechanisms by which tumors avoid MHC-restricted T cell recognition, such as downregulation of HLA class I molecules and defects in antigen processing. We have shown in preclinical studies that EBV-specific CTL expressing CARs specific for antigens such as GD2a, CD30, and HER2, kill autologous EBV-positive target cells as well tumor cells expressing the CAR-specific antigen (Rossig et al. 2002). A clinical trial using EBV-specific CTL expressing GD2a-specific CARs is currently being conducted.

6 Conclusions

T cell therapies are increasingly used for the treatment of malignancies and viral-associated diseases. The adoptive immunotherapy with EBV-specific CTL is an effective strategy after HSCT to reconstitute EBV-specific immunity and prevent or treat EBV-LPD. For other EBV-associated malignancies, the use of EBV-specific CTL is so far less effective; however, the results are sufficiently encouraging to justify continued exploration of this approach. New strategies are being developed to enhance the antitumor activity of EBV-specific CTL by targeting CTL to subdominant EBV antigens, and by genetically modifying CTL to render them resistant to the immunosuppressive tumor environment. If effective, these strategies will have broad implications for T cell therapies for a range of human tumors with defined antigens.

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The Future of Antibody Therapy

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Abstract. Antibodies have been used successfully as therapeutics for over 100 years. The successful development of therapeutic human(ized) monoclonal antibodies (mAbs) in the last 20 years has demonstrated the potency of mAbs but also revealed some of their limitations. Studies in animals and humans demonstrated that it is possible to overcome some of these limitations using mixtures of mAbs or polyclonal antibody (pAb) preparations. pAbs from human and animal plasma are efficacious and safe therapeutics for the treatment of many diseases. Novel technologies are being developed for the production of human pAbs in genetically engineered animals. Immunization of such animals should allow the production of effective and safe high-titer antibody preparations for the treatment of infectious diseases, cancer, and autoimmunity.

1 Historical Perspective

Antibodies have been used successfully as drugs since the 1890s, when it was found that polyclonal antiserum taken from animals could treat life-threatening infections in humans (Casadevall and Scharff 1994, 1995; Buchwald and Pirofski 2003). By the 1920s, "serum therapy" had gained widespread use in treating many infectious diseases including pneumonia, meningitis, scarlet fever, whooping cough, anthrax, botulism, tetanus, diphtheria, measles, mumps, and chickenpox. However, this use was ultimately limited by a side effect called serum sickness, an unwanted immune response mounted against the nonhuman proteins within the antiserum. The introduction of the antibiotics sulfonamide, in 1935, and penicillin, in 1942, further diminished use of serum therapy for treating many infectious diseases. Even with these limitations, animal-derived polyclonal therapy continued to be used to treat numerous diseases, and is still used today as standard care for treating botulinum toxin exposure, venomous bites, certain drug overdoses, and immune suppression in organ graft recipients.

A significant advance in antibody research occurred in 1975 when Kohler and Milstein described a technology to prepare and produce monoclonal antibodies (mAbs) in vitro (Kohler and Milstein 1975). For the first time, researchers and clinicians were able to replicate and harness the therapeutic powers of single antibodies created by the immune system.

Although mAbs promised to be biotechnology's "magic bullet," by the early 1990s all but one of 49 tested in clinical trials had failed. The first generation of mouse-derived monoclonals suffered serious side effects due to an unwanted immune reaction in humans referred to as a "human anti-mouse antibody response" (HAMA) (Badger et al. 1987; Khazaeli et al. 1994; Lee et al. 1998). This response, characterized by fever, chills, arthralgia and life-threatening anaphylaxis was identical to the serum sickness observed some 50 years earlier with animal antisera. To realize the full promise of monoclonals, scientists needed a technology to "humanize" animal-derived antibodies and make them less immunogenic and safer.

Advances in genetic engineering in the late 1980s provided the technology to humanize mouse-derived mAbs including chimerization,

complementarity-determining region (CDR) grafting, display libraries, and human immunoglobulin transgenic mice (Adams and Weiner 2005; Wu and Senter 2005; Hoogenboom 2005; Lonberg 2005; Holliger and Hudson 2005). These approaches have created substantially human mAbs that have proven themselves effective in human patients with minimal side effects and long half-lives. The humanization of monoclonals has been the springboard for launching antibodies as a clinically acceptable class of therapeutics. Even though major pharmaceutical firms were initially reluctant to adopt mAb therapy, most have now one or more mAbs in clinical studies, clearly demonstrating that antibody therapy has come of age. Currently, approximately 150 humanized mAbs are being tested in clinical trials, and 18 antibody drugs were approved by the FDA in the last 10 years, generating revenue in excess of US $10 billion in 2005.

The potential future of mAbs and their derivatives (single chain antibodies, antibody fragments, conjugates with toxins/radionucleotides/enzymes) has been reviewed in a number of recent publications (Adams and Weiner 2005; Wu and Senter 2005; Hoogenboom 2005; Lonberg 2005; Holliger and Hudson 2005). Here we will contemplate the limitations of currently used monoclonal and polyclonal antibody therapies and discuss novel developments of therapeutic oligoclonal (mixtures of monoclonals) and polyclonal antibody preparations.

2 Limitations of Current Monoclonal and Polyclonal Antibody Therapy

Today, 18 mAb therapies have been approved for human use and a large number of mAbs are in clinical development for cancer and inflammatory diseases. For these indications, development of therapeutic mAbs has a high success rate, and roughly 20% of mAbs entering clinical trial obtained approval from the FDA (Reichert et al. 2005; Reichert and Dewitz 2006). In contrast, mAbs as anti-infective agents have been shown to be more difficult to develop. Out of 46 anti-infective mAbs tested in the clinic, only one, palivizumab, was approved as a prophylaxis of respiratory syncytial virus infection in high-risk pediatric patients (Reichert and Dewitz 2006).

Antibodies mediate protection by a variety of mechanisms, including blockade of receptor–ligand interactions, agglutination and immobilization, viral and toxin neutralization, antibody-mediated cellular cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC), and opsonization. Because mAbs are limited to binding a single epitope on a single antigen, they are ideal for some functions but less optimal for others. MAbs are best suited for blocking ligand docking to a receptor. For example, treatment of rheumatoid arthritis by blocking a critical inflammatory cytokine, tumor necrosis factor (TNF)-α, with infliximab ameliorates disease by suppression of the inflammatory response (Finckh et al. 2006). Likewise, antagonizing CD25, the interleukin (IL)-2 receptor, with basiliximab or daclizumab has also proven effective in treating allograft rejection (Church 2003). In such cases, the monoclonal drug is effective because it blocks the cytokine from docking and stimulating its receptor. The narrow specificity of monoclonals is also exploited by the clot-busting drug abciximab, which is active only against the pro-thrombic factor IIb/IIIb (Ringleb 2006).

Although human mAb drugs have been used to treat cancer, the effectiveness of such therapies is limited by the capacity of single mAbs to trigger immune effector functions that lead to target cell death. Many anti-tumor mAbs have been shown to mediate ADCC in vitro, but the relevance of this mechanism of action to clinical efficacy has not been proven. On the other hand, several groups have recently shown that the efficacy of rituximab is substantially greater in patients with high responder' Fc-receptor polymorphism (Cartron et al. 2002; Weng and Levy 2003). These findings indicate that interaction between the antibody Fc domain and the Fc-receptor underlie at least some of the clinical benefit of rituximab, and imply the importance of ADCC.

Similarly, the ability of anti-tumor mAbs to elicit CDC is limited by the low density of some target molecules on the surface of cancer cells. Since cancer is a disease characterized by a heterogeneous population of cells, it is rare to find a single target that is expressed in high abundance on all cancer cells. Monoclonal therapies such as rituximab, which targets CD20, and trastuzumab, which targets HER2, are effective in targeting some cells, but tumors expressing lower levels of the target protein escape from the monoclonal therapy (Golay et al. 2001; Mina and Sledage 2006).

An Important effector mechanism of mAbs appears to be perturbation of signaling events that promote proliferation and survival of target cells. Some mAbs work by physically blocking the interaction between growth factor and its receptor, others by sterically hindering the receptor from assuming a conformation necessary for signaling (Sunada et al. 1986; Li et al. 2005). Virtually every clinically effective, unconjugated mAb perturbs signaling that promotes the proliferation and survival of the targeted cell population (Adams and Weiner 2005).

To compensate for the lack of potency of mAbs, countless methods have been devised to use monoclonals as targeting molecules that carry toxic payloads such as conjugated radioisotopes, toxins, and prodrug-converting enzymes (Wu and Senter 2005). While these approaches marginally increase the potency of monoclonals, they add significant manufacturing and quality-control costs and increase the regulatory hurdle by adding serious nonspecific toxicity. More recently, manipulations of Fc-domain structure have been described that customize antibody clearance and interaction with cellular Fc-receptors (Shields et al. 2001; Ghetie and Ward 2000; Umana et al. 1999). These modifications appear to increase the potency of monoclonals in in vitro assays, but so far there is no evidence that such modifications will increase clinical efficacy.

Currently, most polyclonal antibody (pAb) preparations for human use are derived from human plasma (Casadevall et al. 2004). Such polyclonals (intravenous immunoglobulin, IVIg) are used to prevent and treat many virus infections including rabies, cytomegalovirus, measles, rubella, hepatitis A and B, and varicella; IVIg therapy is indicated for post-exposure prophylaxis, and treatment of acute infections in immunosuppressed patients and newborns. However, despite its clinical success, the supply of IVIg is limited and large quantities must be administered because of the product's low specific activity (in general, neutralizing titers are 1:4 to 1:8). In contrast, polyclonal antisera taken from hyperimmunized animals have high specific activity but upon repeated usage in humans inflict significant side effects. Despite these limitations pAbs from hyperimmunized sheep, horses, and rabbits are the treatment of choice for venoms, bacterial toxins, and organ rejection (Casadevall et al. 2004; Casadevall 2002; Burton et al. 2006; Okum et al. 2006; Knight et al. 2006; Webster et al. 2006; Rainey and Young 2004). Spi-

der or snake venoms contain a large number of diverse poisonous compounds that cannot be neutralized by a single mAb. Similarly, a number of bacteria produce several virulence factors that cannot be neutralized with a single antibody. In addition, it has been demonstrated that complexation of low molecular weight compounds, like botulinum neurotoxin or interleukins, with one or two mAbs actually prevents clearance and prolongs the half-life of the low molecular weight compound (Lu et al. 1995; Tomlinson and Zitener 1993; Finkelman et al. 1993; Sato et al. 1993; Debets and Savelkoul 1994; Mihara et al. 1991; Klein and Brailly 1995; Montero-Julian et al. 1994; Heremans et al. 1992; May et al. 1993). The minimum number of antibodies in an antigen–antibody complex that triggers rapid clearance of an antigen appears to be three. Therefore, effective elimination of venoms and toxins requires oligoclonal or polyclonal antibody preparations.

Another frequently used animal-derived pAb preparation comprises anti-T cell globulins (ATG) prepared in horses and rabbits (Burton et al. 2006; Okum et al. 2006; Knight et al. 2006; Webster et al. 2006). For the last 40 years rabbit ATG has been used for immunosuppression in organ graft recipients, and over 200,000 patients have been treated worldwide. The excellent safety profile of Thymoglobulin (Genzyme, Cambridge, MA) and Fresenius ATG (Fresenius Biotech, Graefelfing, Germany) demonstrates that rabbit IgG can be used safely in immunocompromised individuals.

Polyclonal antibodies from animals are also considered safe drugs if used nonchronically in healthy persons. Recently, the FDA approved Thymoglobulin and DigiFab (Protherics, Nashville, TN), polyclonal antibody preparations from rabbits and sheep, respectively. In addition, ten polyclonal antibody preparations from human plasma were approved in the last 5 years.

3 Overcoming Limitations of mAb Therapy

Devastating diseases such as cancer and infections with virulent pathogens are difficult to treat due to their complexity, multi-factorial etiology, and adaptivity. Therapies such as monoclonals directed against singularly defined targets fail when those targets change, evolve, and mu-

tate. Such adaptive evolution is the bane of mono-specific drugs, which are quickly circumvented by resistant strains.

A well known example is Staphylococcus, which causes an ever increasing number of infections resistant to currently available antibiotics. Staphylococci elaborate a broad range of virulence factors that enable the organism to colonize, infect, and eventually cause disease in a variety of host tissues. Among these virulence factors are several adhesion molecules (MSCRAMMs) (Foster and Hook 1998; Simpson et al. 2003; Wann et al. 2000), capsular polysaccharides (O'Riordan and Lee 2004), extracellular toxins, hemolysins, superantigens (Ladhani 2003; Novick 2003; Schafer and Sheil 1995), protein A (Gomez et al. 2004), mediators of antibiotic resistance (Hiramatsu 2001; Srinivasan et al. 2002), proteases (Dubin 2002), lipases (Gotz et al. 1998), and formation of biofilm (Fowler et al. 2001; Rhode et al. 2001). While interference with any single one of these virulence factors results in some protection in animal models, effective therapy of infected humans most likely requires interference on several levels.

In cancer, development of multi-drug resistance is a common phenomenon (Vincent 2006; Fojo and Menefee 2005; Tulpule 2005; Vasir and Labhasetwar 2005; Vidal et al. 2004). With regards to the expression of particular cancer associated cell surface antigens there is a wide variation in expression levels between cancer cells of different patients (Golay et al. 2001). Cells expressing low levels of target antigen frequently escape mAb therapy and eventually rebound. For example, rituximab treatment has shown impressive remission rates in low-grade B cell lymphoma, but all patients develop resistance. For this reason, combination therapies of CD20 with chemotherapy are in use to prolong response rates (Hillmen 2004; Chow et al. 2002; Czuczman et al. 1999; Czuczman 1999; Vose et al. 2001).

One possible solution to address these issues is antibody therapy with a mixture of a limited number of mAbs (an oligoclonal antibody preparation, oAb) or with a polyclonal antibody preparation from serum (mixture of many antibodies, pAb). Such antibody preparations will be specific for several epitopes of one or several target molecules. Binding of antibodies to several epitopes of one or several antigens will increase the concentration of surface bound antibody and trigger antibody-depen-

dent effector functions more effectively than a single monoclonal. As a consequence, cells with low expression levels of target antigens may be eliminated effectively. In addition, oAbs or pAbs may allow elimination of heterogeneous cell populations and reduce selection of escape mutations.

The synergistic effect of mixtures of mAbs has been demonstrated in clinical trials, where rituximab (anti-CD20) therapy was combined with anti-CD19 or anti-CD22 therapy (Sapra and Allen 2004; Herrera et al. 2003; Stein et al. 2004; Leonard et al. 2005). Similarly, the potency of pAbs is well documented. Currently, polyclonal antibodies for human therapy are produced in horses (botulinum, snake venom), rabbits (anti-thymocyte), and humans (CMV, vaccinia, botulinum, HepA, and HepB). Recently a number of in vivo and in vitro studies elucidating the mechanism of action of ATG (anti-thymocyte rabbit globulin: Thymoglobulin) have been performed (Michallet et al. 2003; Preville et al. 2001; Bonnefoy-Berard and Revillard 1996; Bonnefoy-Berard et al. 1994; Beiras-Fernandez et al. 2006). The results demonstrated that ATG eliminated not only T cells but also B cells and macrophages through complement fixation, ADCC, and induction of apoptosis. In addition, it stimulated proliferation of regulatory T cells. A clinical trial comparing Thymoglobulin with basiliximab (a monoclonal anti-CD25 antibody) for induction immunosuppression in cadaveric renal transplant recipients demonstrated 2.8 times lower acute rejection frequency in the ATG arm at a mean follow up of 9.8+/-3.9 months, while adverse events were similar in both groups (Brennan et al. 2006).

4 Limitations of Oligoclonal Antibody Therapy

Even though oAb (mixture of a few mAbs) therapy appears to be an attractive strategy to overcome some of the limitations of mAb therapy, there are several stumbling blocks in the realization of this approach. In particular, these impediments are the cost of development of several mAbs for the same indication, increased immunogenicity, lower safety, and difficult repeat dosing due to different pharmacokinetics and pharmacodynamics of mAbs.

The manufacturing of oAbs requires production of several mAbs contained in the mixture. The development and manufacturing of mAbs is labor intensive and, therefore, costly. In order to minimize production cost it has been proposed to combine cell lines expressing different mAbs in a single fermentor. A recent publication described the production of such an oligoclonal antibody preparation by co-cultivation of transfected CHO cell lines expressing various mAbs against Rhesus factor (Wiberg et al. 2006). Comparison of several independent production runs demonstrated consistent levels of individual mAbs in the culture broth. However, even though recombinant DNA encoding the various mAbs was integrated into the genome of CHO cells site-specifically using the FRT/Flp-In recombinase system, expression of individual mAbs differed substantially, with an up to 30-fold difference between lowest and highest producers. Currently, it is unclear if the observed differences in mAb expression are due to variation in transcription or in protein translation. These results suggest that production of oligoclonal antibody preparations will require fermentation of individual mAb-producing cell lines and subsequent mixing.

Due to the limited number of mAbs in oligoclonal antibody preparations, each individual antibody in the mixture should be efficacious and safe. Therefore, efficacy and safety has to be demonstrated for each individual antibody as well as for the oAb mixture. As a consequence, the cost of preclinical development of oAbs will be substantially higher and longer than the development of a single mAb.

Safety of mAb therapy is dependent on mechanism-dependent and mechanism-independent toxicities. Mechanism-independent toxicities relate to the occasional hypersensitivity reactions caused by a protein containing xenogeneic sequences and contaminants. Humanization of animal-derived mAbs allowed the generation of chimeric and humanized antibodies which cause sensitization of a low number of treated patients. Nevertheless, the immunogenicity problem is not eliminated with removal of all xenogeneic sequences (Pendley et al. 2003; Hwang and Foote 2005). The idiotype of a mAb, even a so-called fully human antibody, may be recognized as foreign by the immune system of certain patients. Currently available data indicate that even therapy with fully human mAb results in immune responses in up to 15% of treated patients. Sensitization of patients results in the formation of human anti-

mAb antibodies that neutralize and/or eliminate circulating mAb, making it difficult or impossible to achieve therapeutic levels. In the worst case, hypersensitivity is sufficiently severe to require aggressive treatment of symptoms and discontinuation of therapy. Based on the observation that idiotypes of mAbs cause sensitization in certain patients it appears likely that oAbs containing several mAbs will cause sensitization of a higher number of patients than single mAb therapy.

Mechanism-dependent toxicities result from binding of the antibody to its target. Examples include cardiac toxicity occurring during trastuzumab antibody therapy of breast cancer, because heart tissue expresses low levels of anti-Her2/neu (Slamon et al. 2001). Treatment with rituximab can cause a profound first-dose toxicity related to the rapid lysis of normal and malignant B cells expressing CD20 (Byrd et al. 1999). Cetuximab therapy causes significant skin eruptions that are based on epidermal growth factor receptor (EGFR) expression in the skin (Robert et al. 2001; Herbst and Langer 2002). Bevacizumab, targeting vascular endothelial growth factor (VEGF) can induce hypertension, bleeding, thrombosis, or proteinuria (Hurwitz et al. 2004). Anti-CD3 antibodies or agonistic anti-CD28 antibodies can cause a severe cytokine release of activated cells. Based on these observations, it appears likely that mechanism-dependent toxicity of oAbs will be more frequent than such toxicities of single mAbs.

Besides differences in immunogenicity, mAbs display substantially different pharmacokinetics and pharmacodynamics (Lobo et al. 2004). The half-life of currently approved mAbs differs between 0.3 and 27 days. To complicate things further the pharmacokinetics of a particular mAb can vary substantially between patients. For example, the half-life of trastuzumab has been shown to vary between 2.7 and 10 days. These differences are influenced by a number of factors including the target antigen, binding-affinity, Fc-receptor binding, and immunogenicity. Therefore, mAbs in an oAb preparation are expected to display different pharmacokinetics and pharmacodynamics, and repeat dosing of oAbs to maintain a consistent effective antibody concentration of each individual antibody in a patient will be difficult.

The clinical development of antibody therapies requires a demonstration of safety and efficacy in human patients. Due to differences in toxicity and pharmacokinetics, it is expected that the safety of each in-

dividual mAb needs to be demonstrated in humans. The medical need of oAb therapy implies that mAb therapy in a particular patient population is rather ineffective. Therefore, a demonstration of efficacy of each individual mAb in the oAb mixture will be difficult and require large clinical studies.

Taken together, oAb therapy is expected to be more effective than mAb therapies, but in all likelihood also more expensive, more immunogenic, and less safe.

5 The Future of Antibody Therapy: Polyclonal Antibodies

Like oAbs, pAbs from human or animal serum have been shown to be effective because they can address multiple targets over heterogeneous cell populations and therefore have expanded utility in treating diseases not effectively treated today with mAbs. Currently, polyclonal antibodies for human therapy are produced in horses (botulinum, snake venom), rabbits (anti-thymocyte), and humans (i.e., CMV, vaccinia, botulinum, HepA, and HepB). Polyclonals purified from human plasma have low potency, and large amounts of pAb are required to treat and/or prevent human diseases effectively. On the other hand, pAb produced from animals have high titers (100- to 1,000-fold higher than human plasma-derived specialty IVIg preparations) and manufacturing costs are similar to mAbs. Due to high antigen-specific antibody titers, dosing of animal-derived IgG in hyperimmunized animals is similar to mAb dosing requirement (0.1–3 mg/kg). A significant advantage of pAbs is their lack of immunogenicity. Repeated administration of polyclonal antibody preparations derived from human plasma (IVIg) did not result in detectable anti-idiotypic antibody responses or other anti-human responses (Andresen et al. 2000). Similarly, anti-idiotypic antibodies could not be detected in patients treated with a rabbit-derived pAb (Thymoglobulin) (Regan et al. 1997). This is probably due to the low concentration of each individual antibody expressing one idiotype in the pAb.

To produce large quantities of safe, nonimmunogenic polyclonal antibodies of high avidity, it is necessary to move from human production to genetically engineered animals. The development of transgenic

mice that produce human antibodies was a breakthrough and permitted the production of human antibodies of a desired specificity (Lonberg 2005). However, to produce commercial levels of human polyclonal antisera from transgenic animals, it is necessary to use a larger species such as cows, pigs, chickens, or rabbits.

The selected animal must produce sufficient volumes of antiserum to meet supply requirements. In addition, the animals must have a short breeding cycle to enable rapid scale-up and to allow the use of a large number of animals for antibody production. To obtain high batch-to-batch consistency of antibody specificities and titers, it is necessary to harvest sera for a limited period of time after immunization (a few weeks) and to pool sera from a large number of animals (>100). Rabbits have a gestation period of 28 days, conceive with every mating, have an average litter size of eight, and reach sexual maturity at the age of 5 months. As a consequence, a pair of sexually mature rabbits can be expanded to more than 500 animals within 1 year, corresponding to a biomass of 2,500 kg (approximately the mass of three mature cows). These characteristics allow the production of polyclonal antibodies by using a large number of rabbits per batch. In contrast, the long generation times of large farm animals (e.g., 2.5 year for cows), and the small number of offspring per conception, necessitate the extended use of a limited number of these larger animals for antibody production. The use of an extended bleeding schedule, coupled with the necessity of booster injections to maintain sufficient levels of specific antibodies, will result in variation of antibody specificities over time and in a tendency to oligoclonality.

The animal should be of a size and temperament to allow for ease of handling and maintenance of proper sanitation. Optimally, the animal should be an accepted source of GMP-produced therapeutics by the FDA. Currently, rabbits are the only species that meet these criteria. Several contractors in the United States and Europe currently produce GMP polyclonal antibodies from rabbits. The products include, for example, Thymoglobulin and Fresenius ATG, which are sold in the United States and Europe and consist of clinical-grade rabbit polyclonal preparations produced under GMP conditions. The use of these products in over 200,000 patients worldwide has demonstrated the excellent safety profile of therapeutic rabbit IgG in humans.

Based in part on the perceived notion that the cost of therapeutic polyclonals will be lower with the large blood volumes of cows and pigs, these animals are being used for genetic engineering. However, it is important to understand that the costs of the downstream manufacturing activities for therapeutic antibodies (purification, sterility, quality control, and final fill) are very similar regardless of the antibody source, and these constitute more than 90% of the final manufacturing cost.

For the effective expression of a human antibody repertoire in a farm animal it is important to consider species-specific aspects of B cell development and antibody diversification. Generally, all vertebrates start the creation of the primary antibody repertoire by recombining V, D, and J gene segments. In mice and humans this step results in considerable diversity as hundreds of VDJ genes are randomly recombined and genes are imprecisely joined together. In most other vertebrates, including rabbits, chickens, and cows, this first step of VDJ recombination does not lead to significant diversity because only a limited number of V genes are employed. To enhance diversity of the primary repertoire, these animals use a second step to modify antigen-binding regions through templated and/or nontemplated (hypermutation) mutational processes. The process of gene conversion transfers sequence information encoded (i.e., templated nucleotide substitutions) in upstream V genes to the rearranged exons (Fig. 1). A rearranged V gene undergoes several gene-conversion events during B cell development, resulting in changes to each of the antigen-binding sites or CDRs. Even though these processes are different between rabbits and humans, both mechanisms create a similar magnitude of antibody diversity (Knight and Crane 1994; Lanning et al. 2000; Weill and Reynaud 1996).

It is notable that gene conversion of endogenous antibody genes is not used for the generation of the antibody repertoire in humans and rodents. Based on these differences, one may speculate that the placement of an entire human immunoglobulin locus into a gene-converting animal may not result in sufficient antibody diversification for the production of high-titer, high-affinity antibodies, since neither gene conversion nor recombination/hypermutation will occur in an efficient manner.

Another important difference to consider is in immunophysiology. In mice and humans the fetal liver, omentum, and bone marrow serve as primary sites for B cell development, and the process of immunoglob-

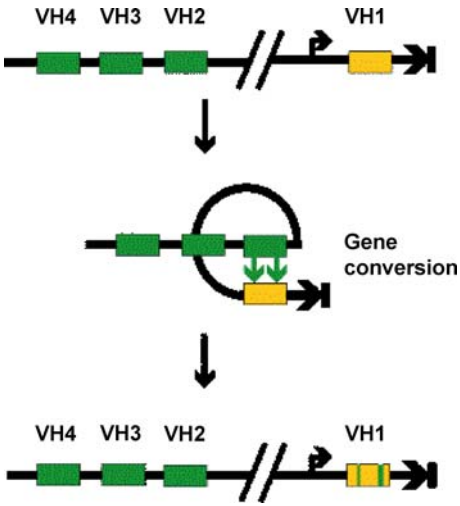
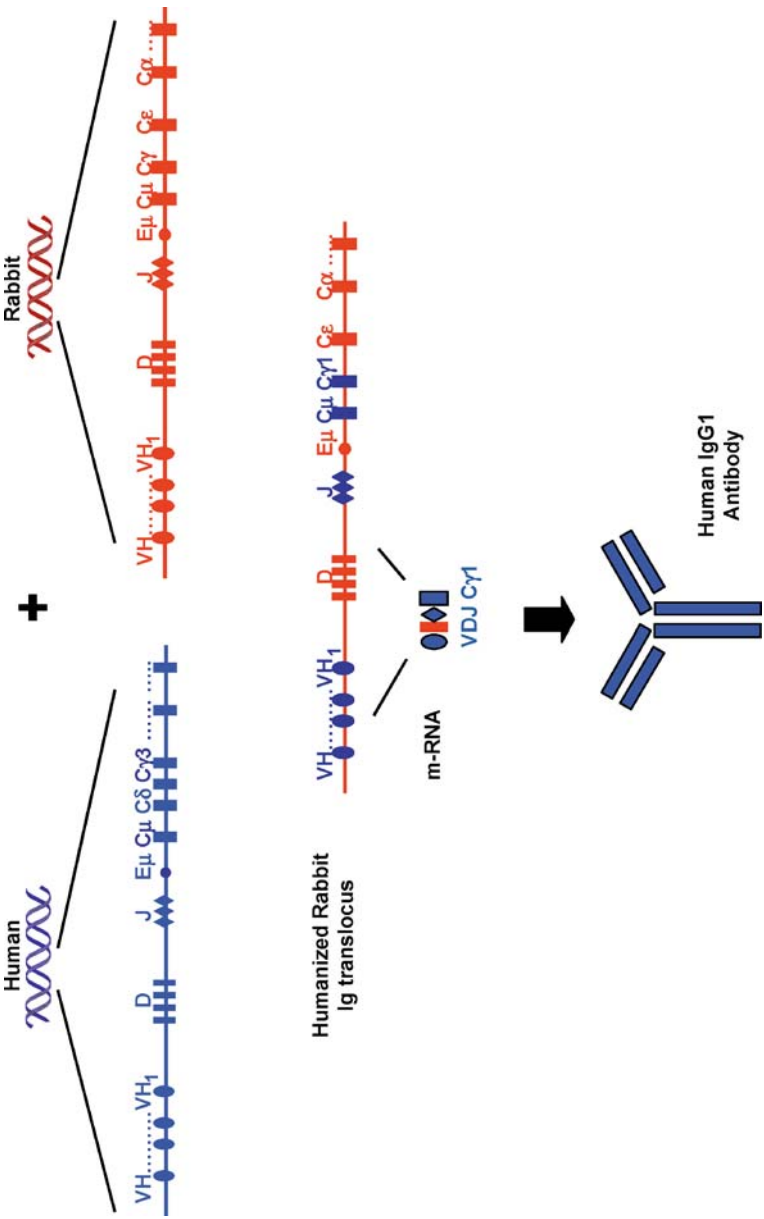


Fig. 1. Schematic representation of gene conversion. In gene conversion, DNA sequences from upstream V gene segments are introduced into the rearranged V gene. Homology between V genes is required for gene conversion to occur. Due to conserved framework regions, most changes are in the CDR regions of the rearranged V gene. Therefore gene conversion mimics "CDR grafting"

ulin gene rearrangement appears to occur throughout life. In rabbits diversification by gene conversion occurs in the appendix and other gut-associated lymphoid tissue, in chickens it occurs in the bursa of Fabricius, and in cows in spleen. Rearrangement of Ig genes stops in the chicken at hatching and diminishes after birth in rabbits, cows, and sheep.

Several companies are currently working on the generation of genetically engineered large animals for the production of human polyclonal antibodies: Hematech on cows, Revivicor on pigs, Origen on chicken, and Therapeutic Human Polyclonals (THP) on rabbits.

Fig. 2. Schematic representation of THP's approach to express human antibodies in animals. Only coding regions in the rabbit immunoglobulin gene were replaced with the corresponding human gene elements


Human
Rabbit
Humanized Rabbit
Ig translocus
m-RNA
VDJ Cγ1
Human IgG1
Antibody

Hematech (a wholly owned subsidiary of Kirin Brewery Co.) generated transgenic cows by nuclear transfer cloning of bovine primary fetal fibroblast transfected with a human artificial chromosome. Similar to observations in mice, the expression of human immunoglobulin in wild-type cows was low (<1 μg/ml) (Kuroiwa et al. 2002). This may be because the human immunoglobulin locus cannot compete effectively with the cow's endogenous loci. One may speculate that this is due to the fact that cows generate a diverse antibody repertoire by gene conversion. In the meantime cows with inactivated immunoglobulin heavy chain loci have been generated (Kuroiwa et al. 2004), but, as of today, expression levels of human antibodies in knockout cows have not been reported.

A different approach has been developed by THP for the genetic engineering of rabbits. This novel approach is based on the humanization of rabbit immunoglobulin loci through replacement of protein coding rabbit DNA sequences with corresponding human counterparts while leaving the endogenous regulatory and antibody-production machinery intact (Fig. 2).

This human–animal translocus is expected to be a substrate for enzymes involved in DNA repair by gene conversion to support production and diversification of high-titer, high-affinity antibodies in gene-converting animals. The rabbit is particularly well suited for this approach because B cell immunology and antibody production in rabbits have been extensively characterized.

THP has validated this approach by successfully creating transgenic rabbits that produce humanized light chains and heavy chains. In these animals IgG levels of up to 2 mg/ml have been observed.

6 Conclusion

Antibodies have been used successfully as therapeutics for over 100 years. The successful development of therapeutic human(ized) mAbs in the last 20 years has demonstrated the potency of mAbs but also revealed some of their limitations. Studies in animals and humans demonstrated that it is possible to overcome some of these limitations using mixtures of antibodies. Combination therapy with several mAbs appears

to be limited by the high cost of development of several mAbs for the same indication. In addition, such mixtures are expected to be more immunogenic and less safe than mAbs. Differences in pharmacokinetics and pharmacodynamics of individual mAbs make repeat dosing of such mixtures difficult. Such limitations can be overcome using pAbs.

Novel technologies are being developed for the production of human pAbs in genetically engineered animals. Immunization of such animals should allow the production of effective and safe high-titer antibody preparations for the treatment of infectious diseases, cancer, and autoimmunity.

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