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Apoptosis and Its Relevance to Autoimmunity

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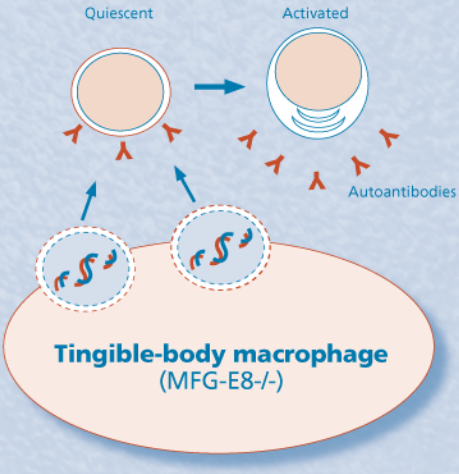

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A.N. Theofilopoulos *La Jolla, Calif.*

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Contents

Preface

Over the last 15 years, apoptosis has moved from a peripheral circumscribed interest amongst a small group of scientists to the mainstream of modern biology and a highly prominent and, in some cases, dominant focus of medical research. This is particularly true in the field of immunology where more than 10 billion cells are turned over each day and cell death is a necessary part of immune tolerance and contraction following immune activation.

In this volume of *Current Directions in Autoimmunity* on Apoptosis, contributors discuss the three major areas of apoptosis research: Extrinsic Death Receptor Pathways, Intrinsic Death Pathways, and the mechanisms responsible for Apoptotic Cell Clearance. In each of these sections, the proteins and signal transduction pathways are delineated and genetic alterations that lead to autoimmune diseases are described. Although most cell death abnormalities have been associated with systemic autoimmune disorders such as lupus, it is evident that regulation of cell death is pertinent to disease expression in many organ-specific diseases as well.

The precise understanding of how molecular defects in apoptotic pathways lead to different diseases provides innovative directions in autoimmunity research that will ultimately facilitate the development of new classes of disease-modifying agents.

Sincere thanks is given to the outstanding contributors of this volume for their time and effort.

Keith B. Elkon
Seattle, Wash.

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Death Receptor Signaling and Its Function in the Immune System

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Abstract

Death receptors belong to the TNF (tumor necrosis factor)/NGF (nerve growth factor) receptor superfamily. Signaling via death receptors plays a distinct role, e.g. in the immune system, where it contributes to regulation of the adaptive immune response in various ways, most notably by triggering activation-induced cell death (AICD) of T cells. Thus, dysregulation of death receptor signaling, either allowing too much or too little apoptosis, can lead to autoimmune disorders and also impacts on tumorigenesis or other diseases. In this chapter we address components, molecular mechanisms and regulation of death receptor signaling with particular focus on CD95 (APO-1, Fas). We discuss the role of death receptor-mediated AICD in regulation of the adaptive immune response against foreign and self antigens in comparison to cytokine deprivation-mediated death by neglect. Finally, the contribution of dysregulated death receptor/ligand systems to autoimmune diseases such as diabetes, multiple sclerosis and Hashimoto's thyroiditis is discussed.

Death Receptors

Death receptor signaling plays a distinct role, e.g. in the immune system, where it contributes to the regulation of the adaptive immune response but also in other physiological and pathophysiological states such as development, differentiation and tumorigenesis. Death receptors belong to the TNF (tumor necrosis factor)/NGF (nerve growth factor) receptor superfamily. Members of this family are type I transmembrane receptors, contain 1–5 cysteine-rich domains in their extracellular domain and an 80 amino acid death domain (DD) in the cytoplasmic tail which is essential for transduction of the death signal.

Six members of this death receptor subfamily are known so far (fig. 1), namely TNF-R1 (tumor necrosis factor receptor 1; also known as CD120a), CD95

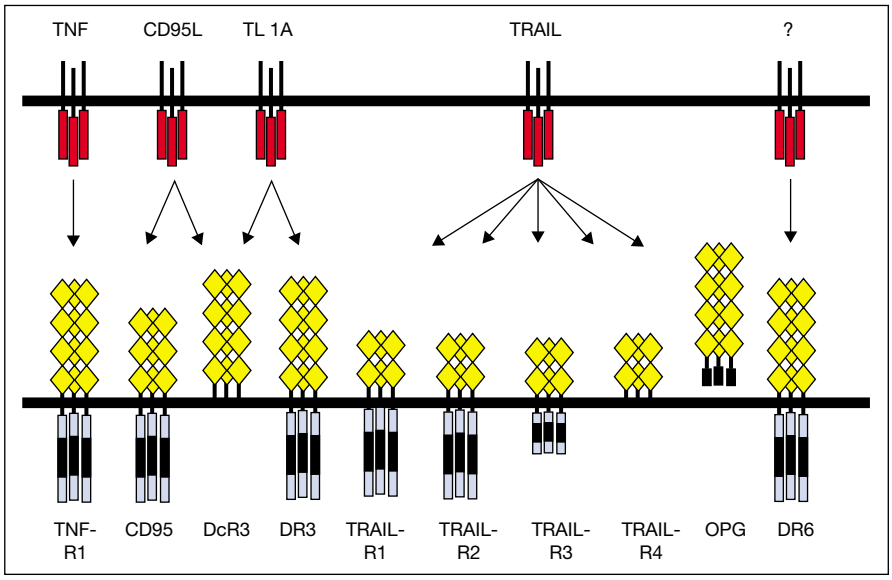

Fig. 1. Death receptors (DRs), decoy receptors (DcRs), death ligands and their interactions. Members of the death receptors are characterized by extracellular cysteine-rich domains (depicted as diamonds) and by an intracellular death domain.

(also known as APO-1 and Fas), DR3 (also known as APO-3, LARD, TRAMP and WSL1), TRAIL-R1 (TNF-related apoptosis-inducing ligand-receptor 1; also known as APO-2 and DR4), TRAIL-R2 (also known as DR5, KILLER and TRICK2), and DR6 [1]. In addition, the ectodysplasin-A receptor (EDA-R) and the nerve growth factor receptor (NGF-R, p75, NTR) are sometimes referred to as death receptors, because they contain cytoplasmic regions similar to death domains. However, their death domains show striking structural and functional differences from the classical death domain and no binding to FADD or TRADD has been detected.

Death Ligands

Death receptors are activated by their natural ligands which have co-evolved as members of the TNF family (fig. 1). Except for lymphotoxin-α (LT-α) they are type II transmembrane proteins, and all death ligands form homotrimers or trimeric complexes of higher order such as hexamers. TNF-α, CD95L (CD178, FasL, APO-1L) and TRAIL (APO-2L) have also been reported to exist in a soluble form, cleaved from the membrane by metalloproteases. The

effect of cleavage from the cell membrane for the function of death ligands has been controversially discussed. It has been reported that membrane-bound CD95L kills human peripheral blood cells, whereas soluble CD95L blocks this killing. However, others have reported that a form of soluble CD95L can mediate cell death with similar efficiency as the membrane-bound form, and it has been proposed that efficient killing is dependent on the target cell type [2].

At least one ligand is known for every death receptor, except for DR6. For some ligands multiple receptors have been reported, some of which do not belong to the death receptor families but all are members of the TNF-R/NGF-R superfamily. TNF-α and LT-α bind to TNF-R1 (and the non-death receptor TNF-R2), TRAIL is the ligand for TRAIL-R1 and TRAIL-R2 (and the decoy receptors TRAIL-R3/R4 and OPG), whereas CD95L (and LIGHT) bind to CD95 (fig. 1).

Decoy Receptors

In addition to death receptors, so-called decoy receptors (DcRs) were found which also bind the same ligands of the TNF superfamily. To date, four decoy receptors have been characterized, namely DcR3 (decoy receptor 3) binding CD95L, TRAIL-R3 (DcR1) and TRAIL-R4 (DcR2) binding TRAIL, and finally OPG (osteoprotegerin). These receptors either lack functional intracellular death domains such as in the case of DcR3 or are even found as soluble receptors like OPG and are therefore unable to transmit an intracellular signal. Thus, DcRs, by sequestration of death ligands, may prevent signal transduction from death receptors. However, the caveat remains that such functions of DcRs have so far only been shown in overexpression studies.

CD95 System

The CD95 death receptor is the best characterized member among the death receptors. CD95 is a widely expressed glycosylated cell surface molecule which can also occur in a soluble form generated by differential splicing. It was shown that CD95 receptors are expressed on cells as preassociated trimers by interaction of their PLAD (pre-ligand binding assembly domain). Expression of the CD95 gene is enhanced by IFN-γ and TNF and by activation of lymphocytes [3]. Naturally occurring mutations of the CD95 gene cause complex disorders of the immune system in mice, manifested as lymphoadenopathy and autoimmunity, symptoms on the MRL background that are similar to those of systemic lupus erythrematosus (SLE). In *lpr* (lymphoproliferation) mice, a splicing defect results in greatly decreased expression of CD95. In lpr^{cg} (allelic

to *lpr*) mice, a point mutation in the DD of CD95 abolishes transmission of the apoptotic signal. However, CD95 ablation in lymphoid as well as nonlymphoid tissue is necessary to generate lymphoproliferative disease, as selective inactivation of CD95 in lymphocytes led to severe lymphopenia over time [4].

CD95-mediated apoptosis is triggered by its natural ligand, CD95L, or by agonistic antibodies. CD95L is expressed in a far more restricted way than the receptor, e.g. on activated T cells, at immune-privileged sites, on certain tumors or upon post-ischemic reperfusion. CD95L can also be found on killer cell-derived vesicles. In *gld* (generalized lymphoproliferative disease) mice, a point mutation in the carboxy-terminus of CD95L impairs its ability to interact successfully with its receptor. The symptoms from the disease arising from *gld* are essentially the same as in *lpr* mice [3].

CD95 Signaling

Upon stimulation of CD95 with its corresponding ligand, CD95L, signaling is either induced by conformational changes of preformed death receptor trimers or, alternatively, by formation of multimeric complexes. Triggering of CD95 leads to formation of a protein complex within seconds. This so-called death-inducing signaling complex (DISC) contains the adaptor FADD/Mort1, procaspase-8a and procaspase-8b, procaspase-10, CAP3 (a molecule that contains the N-terminal death effector domains of caspase-8) and c-FLIP (fig. 2a). FADD/Mort1 which, like CD95, contains a DD binds via homophilic interaction to the DD of the receptor and recruits, via its death effector domain (DED), two isoforms of procaspase-8 and procaspase-10 to the DISC. Recruitment to the DISC leads to autoproteolytic activation of caspase-8 and caspase-10. The prodomain of caspase-8 remains at the DISC whereas active caspase-8 dissociates from the DISC as an active heterotetramer consisting of two p10 and two p18 subunits starting the execution phase of apoptosis by initiating the caspase cascade [3, 5].

Recently, a refined model of the proximal steps of CD95 signaling for certain cell types (type I cells, see below) was proposed, involving (1) formation of CD95 microaggregates; (2) DISC formation; (3) formation of large CD95 surface clusters and (4) actin filament dependent internalization of activated CD95 [5]. Membrane lipid rafts frequently constitute scaffolds for large signaling complexes such as the T cell receptor/CD3 complex. With respect to the CD95 signaling complex, reports are highly conflicting and so far there is no indication that these controversial data might be due to cell type or species specific differences. In Jurkat cells it has been suggested that CD95 is constitutively localized to lipid rafts together with all its cytoplasmic signaling components and caspase-3,

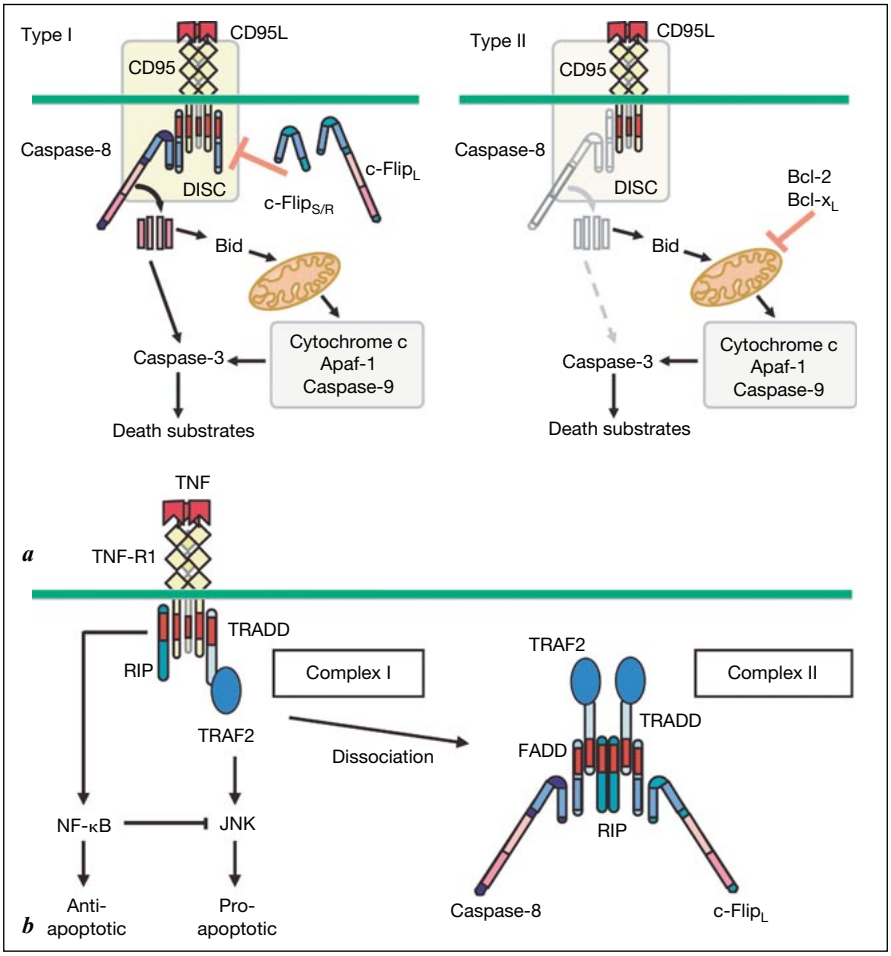

Fig. 2. Schematic representation of the CD95 and the TNF signaling pathways. ***a*** CD95 signaling in CD95 type I or type II cells. ***b*** Two TNF-R1 signaling complexes: complex I is formed at the membrane triggering NF-κB signaling. Complex II (traddosome) dissociates from the receptor and signals apoptosis.

which, by the same group, was proposed to be a component of the DISC [6]. In contrast, others showed, using the same cell line, that CD95 localizes to lipid rafts only upon triggering of the T cell receptor, whereas another cell line displayed constitutive raft localization of CD95 [7]. In murine cell lines and primary thymocytes, a portion of CD95 constitutively localized to lipid rafts, but neither FADD nor caspase-8 were recruited to rafts upon stimulation, suggesting that apoptosis signaling by CD95 occurs through the non-raft fraction [8].

Triggering of CD95 has also been reported to have costimulatory effects under certain conditions. It has been proposed that one mechanism of costimulation could be activation of the NF-κB pathway. Possible links to NF-κB signaling are caspase-8, c-FLIP and RIP as discussed below.

Components of the DISC

Caspase-8/Caspase-10

Caspases comprise a family of cysteine proteases which specifically cleave proteins after an aspartate residue. Caspases are produced as procaspases (zymogenes) and are activated by proteolytic cleavage. Active enzymes are heterotetrameric complexes of two large subunits and two small subunits. The genes encoding caspase-8, caspase-10 and c-FLIP are located on human chromosome 2q33–34 in a cluster of 200 kb, suggesting that they arose from gene duplication. Interestingly, there is no caspase-10 gene in mice. Both, caspase-8 and caspase-10 contain two tandem DEDs in their N-terminus and a C-terminal caspase domain. Upon stimulation, they are recruited to the DISC, where autoproteolytic cleavage occurs, ultimately leading to release of the catalytically active tetramer. Using caspase inhibitors such as zVAD-fmk or c-$FLIP_L$ it has been demonstrated that caspase-8 has at least two different catalytic activities, one initial at the DISC, required for full auto-activation and not inhibitable by zVAD-fmk or c-$FLIP_L$ and the full catalytic activity of the heterotetramer which can be blocked by zVAD-fmk. While multiple isoforms of caspase-8 and caspase-10 have been described, only caspase-8a and caspase-8b, and caspase-10a, caspase-10c and caspase-10d could be detected on protein level.

Recruitment of caspase-10 to the DISC and its activation was reported in the case of CD95, TRAIL-R1 and TRAIL-R2 stimulation. However, it remains controversial if caspase-10 can trigger cell death in the absence of caspase-8. Thus, it might have other yet elusive roles. Importantly, caspase-10 does not appear to be essential for DR-mediated apoptosis, as cell lines deficient in caspase-10 are susceptible to CD95 triggering and the caspase-10 gene is missing in mice. Several knock-out and transgenic mice underscore the central role of the DISC-associated molecules FADD and caspase-8 in signaling via death receptors. FADD and caspase-8 knock-out mice are lethal at embryonic day 11. They show cardiac failure and abdominal haemorrhage. Due to embryonic lethality *FADD*−/− chimeric mice were constructed. In thymocytes and fibroblasts of these mice, CD95-mediated apoptosis was completely blocked. Conditional ablation of caspase-8 in mice revealed that it is indeed essential for CD95-mediated apoptosis in liver cells, thymocytes and T cells. In addition, it was proposed that caspase-8 also serves nonapoptotic functions, as deletion of

caspase-8 severely impairs hemopoietic progenitor function. T cell-specific deletion of caspase-8 revealed reduced T cell numbers, impaired T cell activation and increased susceptibility to viral infections. Recently, it has been shown that caspase-8 deficiency in humans and mice abolishes activation of NF-κB upon antigen receptor stimulation. Caspase-8 has been proposed to activate NF-κB by causing the IKK (inhibitor of NF-κB kinase) complex to associate with the Bcl10-MALT1 (mucosa-associated lymphatic tissue) adaptor complex [9].

c-FLIP

Activation of caspase-8 at the DISC can be counteracted by FLIP proteins which, like caspase-8, contain tandem DEDs. The first discovered member was v-FLIP (viral FLICE-like inhibitory protein) expressed by γ-herpesvirus.

Thirteen different mRNAs for cellular FLIP (c-FLIP, also known as FLAME-1, I-FLICE, CASPER, CASH, MRIT, CLARP and Usurpin) have been reported; however, so far only three, namely c-$FLIP_S$, c-$FLIP_L$ and, recently, c-$FLIP_R$ were detected on protein level. The short splice variant c-$FLIP_S$ confers resistance to CD95-mediated apoptosis in primary human T cells upon costimulation and is likely to contribute to the CD95 resistance of freshly activated T cells. c-$FLIP_S$ and c-$FLIP_R$ contain only tandem DEDs and are thus structurally similar to v-FLIP. c-$FLIP_L$ contains not only tandem DEDs but is structurally homolog to caspase-8, containing a protease-like domain in which several amino acids, including the cysteine of the active site, are mutated. It is found to be cleaved at the DISC but because c-$FLIP_L$ is not an active caspase, the cleavage is not reciprocated [10]. The role of c-$FLIP_L$ is controversially discussed. Caspase-8 activation at the DISC is inhibited at two different cleavage steps by splice variants of c-FLIP. A detailed analysis of the domains of c-$FLIP_L$ revealed that its p10 subunit contributes to the first cleavage step of caspase-8 and therefore may provide a scaffold for caspase-8 activation [10]. In a cell-free system heterodimers of caspase-8 and $FLIP_L$ show higher caspase activity and might constitute the unit that catalyzes caspase-8 processing at the DISC [11]. High expression levels of c-$FLIP_L$ might still allow for this scaffold function, but also act anti-apoptotic by preventing further caspase-8 processing. c-FLIP isoforms are characterized by a short half-life time. It has been reported previously that mechanisms of differential upregulation or degradation of c-FLIP isoforms might be important for the modulation of apoptosis sensitivity at the DISC level [1].

Mice deficient in c-FLIP die at embryonic day 10.5 most probably due to cardiac failure resembling the phenotype of *caspase-8*−/− and *FADD*−/− mice. These similarities suggest that for heart development a functional interplay between the three DISC components FADD, caspase-8 and c-FLIP is absolutely

required. However, the question arises whether this interplay requires a signal from a – known or unknown – death receptor or a different type of receptor. Moreover, it remains elusive whether the signal required for heart development is associated with regulation of apoptosis or reflects a novel role for the three molecules involved. Mice carrying a T cell-specific v-FLIP-E8 transgene show strongly reduced thymocyte numbers, although thymocytes of these mice are resistant towards CD95-mediated apoptosis [10]. The reduction in thymocyte numbers seems to be independent of the CD95-system since it was also observed in a *CD95*−/− background. Interestingly, the thymic phenotype resembles that of T cells from FADD-dominant negative transgenic mice, suggesting that another death receptor system distinct from the CD95-system is critically involved in thymocyte selection [10]. Another v-FLIP transgenic mouse expressing v-FLIP-MC159 under control of the huCD2-enhancer displayed impaired CD8 T cell responses and defective memory formation. Mice expressing human c-$FLIP_S$ under control of the proximal lck-promoter show decreased T cell proliferation, similar to the v-FLIP-MC159 transgenic mice. However, in contrast to the latter, the memory T cell pool was increased in c-$FLIP_S$ transgenic mice. Furthermore, two different mouse models have been described overexpressing c-$FLIP_L$ in a T cell-specific manner. In one model, c-$FLIP_L$ transgenic mice have an increased proliferative response to stimulation via the T cell receptor. In addition, they display a TH2 cell (T helper cell 2) cytokine bias and are more susceptible to allergic airway inflammation. The second model, expressing c-$FLIP_L$ under a similar promoter displays a fundamentally different phenotype: Here, overexpression of c-$FLIP_L$ leads to reduced proliferation upon triggering of the T cell receptor and concomitant reduced cytokine production. Moreover, a mild thymic phenotype was observed with reduced cell numbers and reduced positive selection. Multiple reasons might account for these different phenotypes, such as different expression levels or time points due to different promoter elements used. In addition, it has been shown that, despite the high degree of structural similarity, v-FLIP and c-$FLIP_S$ may act differently. Thus, a more refined analysis is warranted to elucidate the role of c-FLIP in vivo.

Recently, CD95 has been shown to trigger the NF-κB pathway [12, 13]. However, the role of c-FLIP in linking the CD95 to NF-κB pathway has been controversially discussed. c-$FLIP_S$ and c-$FLIP_L$ were shown to block CD95L-induced NF-κB activation [12] as well as to induce NF-κB activation [14].

Further Reported DISC Proteins

PEA-15 (phosphoprotein enriched in astrocytes – 15 kDa), also known as PED (phosphoprotein enriched in diabetes) was characterized as a DED-containing protein that has been demonstrated to have a role in modulating

apoptosis at the DISC level in astrocytes, neural progenitor cells and glioma cells. PEA-15 was shown to inhibit CD95-mediated apoptosis, TRAIL-mediated apoptosis and, in certain cell types, TNF-mediated apoptosis.

RIP (receptor-interacting protein) was described as a DD-containing protein interacting with CD95. In addition to its presence in the DISC, considerable amounts of RIP were shown in association with nonstimulated CD95. Thus, it does not strictly fulfill the criteria of being a component of the DISC. RIP has been implicated in CD95-dependent NF-κB activation, particularly upon high expression of c-$FLIP_L$ and, in the absence of caspase-8, in CD95-mediated necrotic cell death [12, 15].

Besides the above mentioned proteins several other proteins were reported to directly interact with DISC proteins, namely Daxx, Fap-1, FLASH, DAP3, FAF-1 and others. Endogenous binding and functional roles of many of these proteins are unclear [for review, see 5].

Two Types of CD95 Signaling

Two pathways of CD95 signaling were described by our laboratory, distinguishable by the amount of DISC formation upon triggering of the receptor (fig. 2a). In type I cells, following CD95 stimulation, strong DISC formation is observed directly leading to efficient caspase-8 activation. Sufficient amounts of caspase-8 are activated to directly activate downstream effector caspases such as caspase-3 further activating caspase-6 and caspase-7 which all cleave intracellular targets such as PARP (poly ADP-ribose polymerase) and ICAD, the inhibitor of CAD (caspase-activated DNAse) ultimately leading to cell death. In contrast, in type II cells hardly any DISC formation is observed and only little active caspase-8 is formed. These cells depend on an amplification loop via the mitochondria. Apoptosis in type II cells and strong activation of caspases is dependent on cleavage of the BH3-only pro-apoptotic Bcl-2 homologue Bid which leads to aggregation of Bax or Bak. This aggregation leads to loss of the mitochondrial membrane potential ($\Delta\Psi m$) and to the release of pro-apoptotic molecules from the mitochondria such as cytochrome c and SMAC/Diablo. Cytochrome c in concert with APAF-1 (apoptosis-activating factor-1) and caspase-9 form the apoptosome in which caspase-9 is activated and subsequently activates effector caspases such as caspase-3.

Type II cells can be protected at the mitochondrial level by high expression of Bcl-x_L and Bcl-2. Another step to mediate apoptosis inhibition is to modulate IAP (inhibitor of apoptosis protein) expression which interferes with SMAC/Diablo. The difference between the two CD95 signaling pathways on the molecular level remains elusive. In addition to the differences described

above, it has been suggested that CD95 is constitutively localized to lipid rafts only in type I cells. It has also been proposed that actin-dependent internalization is confined to type I cells. Finally, type I and type II cells can be distinguished based on their differential sensitivity to different recombinant CD95 ligands [2]. However, in vivo, there is not always a clear distinction between the different CD95 types. Nevertheless, thymocytes are reported to be CD95 type I whereas hepatocytes are CD95 type II cells. In primary human T cells a switch from CD95 type II to CD95 type I has been observed upon stimulation [1].

TNF-R1 Signaling

TNF-R1 signaling (fig. 2b) differs from that via CD95 and TRAIL-R. However, also a conserved extracellular domain was characterized that mediates specific ligand-independent assembly of receptor trimers called PLAD. In most instances, TNF-R1 signaling results in NF-κB activation. However, cell death can be triggered by TNF-R1 under conditions of protein synthesis block or NF-κB inhibition [16]. TNF-R1 stimulation has recently been proposed to result in the formation of two signaling complexes [7]. Complex I is formed at the membrane comprising the following proteins: TNF-R1, its ligand TNF, RIP, the adaptor protein TRADD (TNF-R-associated death domain protein) and TRAF2 (TNF-R-associated factor 2). Complex I is proposed to trigger the NF-κB pathway via RIP by recruitment of the IKK complex mediating IκB degradation and to activate JNK through a TRAF-dependent mechanism involving MEKK1. This complex was reported to translocate to the cytosol where FADD, procaspase-8 and -10, c-$FLIP_L$ and c-$FLIP_S$ are recruited to form the so-called complex II (traddosome) [7]. Activation of procaspase-8 takes place at complex II and is followed by activation of downstream death signaling. In this model the switch between survival and death depends on the ability of NF-κB activation at complex I and on the efficiency of complex II formation, caspase-8 activation and the amount of c-FLIP, that blocks caspase-8 activation at complex II [for review, see 7]. Although this model provides an elegant mechanism of life versus death decisions, it needs further experimental confirmation.

Recent studies indicate that following TNF-binding, TNF-R1 translocates to lipid rafts. In lipid rafts, TNF-R1 and RIP are ubiquitinated resulting in their degradation by the proteasome pathway. Interfering with lipid raft organization not only abolishes ubiquitylation, but also switches TNF-R signaling from pro-survival NF-κB activation to apoptosis, indicating that lipids rafts are crucial for the outcome of TNF-activated signaling pathways [7].

Further Death Receptor Complexes

Signaling of apoptosis by other members of the death receptor subfamily seems to follow similar basic rules. Receptor oligomerization is triggered by binding of their corresponding ligands leading to a conformational change. Upon triggering by their respective ligands TRAIL-R1, TRAIL-R2 and CD95 form a DISC with similar composition comprising the adaptor FADD, caspase-8, caspase-10 and c-FLIP.

DR3 and DR6 signaling pathways are less characterized. These receptors seem to be connected to survival signals. RIP and TRADD are recruited to the receptor complex and DR3 and DR6 promote activation of NF-κB leading to the expression of survival genes [17].

Activation-Induced Cell Death

The adaptive immune response to antigens is characterized as a multistep process: upon encounter of antigen, T cells become activated, differentiate into effector cells and undergo clonal expansion. Following the peak of an immune response, the majority of activated, antigen-specific T cells need to be eliminated in order to maintain homeostasis of the T cell population. Elimination of T cells during the termination phase occurs through apoptosis, which is mainly induced via two mechanisms: (1) death by neglect caused by cytokine withdrawal, and (2) activation-induced cell death (AICD) via death receptor engagement. The role for each of these two mechanisms for peripheral T cell death remains largely elusive [1].

Activated T cells express both CD95 and CD95L and are sensitive to CD95-mediated apoptosis indicating that they are able to undergo suicide or fratricide to terminate the immune response [18]. TCR-triggered CD95-mediated apoptosis is also found in Jurkat T cells in vitro and a single TCR-activated T cell in the absence of costimulation may autonomously decide to die by apoptosis employing, at least in part, the CD95 pathway. These results suggest a minimal model in which TCR-induced death in activated T cells involves CD95/CD95L-mediated suicide. In vivo, the situation is less clear and it is conceivable that CD95L could also be provided by inflamed tissue [19]. However, the CD95/CD95L system is not the only death system which plays a role in deletion of peripheral T cells. Thus, it has been suggested that late after triggering of the TCR in vitro, TNF-R2 and TNF dominate over the CD95/CD95L system [19].

Death by neglect is induced by cytokine deprivation, which occurs during the termination phase of an immune response. If antigen is successfully cleared

by the immune system, T cell stimulation and costimulation become limited, IL-2 levels decrease and T cells suffer from cytokine deprivation. Death by neglect can be inhibited by the addition of any common γ-chain cytokines or by overexpression of anti-apoptotic Bcl-2 family members, but death still occurs in *lpr* or *gld* mice, suggesting that it is independent of death receptor triggering [20]. Release of the BH3-only-Bcl-3 homolog Bim from the dynein motor complex has recently been implicated to be one of the main mechanisms to trigger death by neglect. Bim-deficient mice were shown to be resistant towards death by neglect similar to mice overexpressing Bcl-2. Another proposed mechanism involves reactive oxygen species. Although it is possible that Bim and death receptor-mediated AICD interact at some level to mediate peripheral deletion, different mechanisms might be engaged depending on the way of antigen presentation, for example, the presence of costimulatory proteins, the antigen-presenting cell and, importantly, the amount of antigen and its persistence [19]. In summary, death by neglect may be dominant in the removal of antigen-specific T cells in the downphase of an immune response when clearance of low amounts of antigen leads to cytokine deprivation. In contrast, death receptor-dependent AICD mainly appears to contribute to the removal of T cells in the presence of high antigen amounts or when antigen persists. Thus, AICD may play a role in chronic infections and probably helps to ensure peripheral tolerance by the removal of T cells specific for self antigen [1].

The role of AICD in different T cell subsets has only recently become of interest and thus far remains largely elusive. However, it has been suggested that T helper cells 1 (TH1) preferentially express CD95L and use the CD95 pathway for AICD, whereas TH2 cells are relatively resistant towards death receptor-mediated AICD and express only minor amounts of CD95L. However, they express high amounts of TRAIL and are able to kill TH1 cells in vitro in a TRAIL-dependent manner [21]. Furthermore, our laboratory has recently found that CD4+CD25highFoxP3+ regulatory T cells (Treg) are highly sensitive to CD95-induced cell death, while they are resistant to TCR-mediated AICD [35]. Finally, 'helpless' CD8+ T cells triggered in the absence of CD4+ helper cells are sensitive to TRAIL-mediated killing [22].

Role of Death Receptors in Central and Peripheral Tolerance

Regulation of the generation and function of the lymphocyte repertoire is crucial to prevent autoimmunity. Since formation of the T cell receptor (TCR) repertoire by recombination is a random process, the 'useful' thymocytes need to be selected. It has been proposed that CD95-induced apoptosis might be involved in clonal deletion of thymocytes that are highly reactive to antigens

expressed in the thymus. However, mice deficient in components of the CD95/CD95L system do not display alterations in the TCR repertoire, suggesting that it is not involved in negative selection. The TRAIL system has also recently been implicated in regulating negative selection. However, also for this death receptor system conflicting data exist. Bcl-2 has also been reported to rescue thymocytes from negative selection which is consistent with the report that mice deficient in Bim display a defect in negative selection, suggesting that death receptors are not involved in this process [23, 24].

Negative selection in the thymus is not totally efficient, and a number of mechanisms in the periphery exist that ensure tolerance, such as anergy, ignorance, regulatory T cells and apoptosis. In the periphery, it is conceivable that T cells encounter self antigen multiple times, as it cannot be easily cleared. Adoptive transfer experiments with TCR transgenic T cells carrying either CD95 mutations or overexpressing Bcl-2 and recipients expressing the cognate antigen revealed that deletion of such auto-reactive T cells is CD95-dependent and is not prevented by Bcl-2 overexpression. In line with this observation, *lpr* or *gld* mice contain autoreactive T cells that expand in vivo even though thymic selection appears to be normal. Thus, AICD seems to be important for the establishment of self tolerance in the periphery [1, 25].

Considering the important role of death receptors regulating immune responses and function, it is not surprising that a complicated network of regulation of these systems has evolved.

Principles of Death Receptor-Mediated Apoptosis in Autoimmunity

Recent evidence has suggested death receptor-mediated apoptosis as a possible key player in the pathogenesis of several autoimmune disorders [26]. Both increased and decreased sensitivity to death receptor-mediated apoptosis may be involved in autoimmunity. Whereas apoptosis defects may be responsible for ineffective deletion of autoreactive lymphocytes by AICD during the down phase of an immune response, excessive apoptosis contributes to the destruction of target tissue in the affected organs. Genetic alterations in components of death receptor pathways as well as cytokine-driven dysregulation of such components have been reported to contribute to autoimmune diseases.

Alterations in death receptor-mediated apoptosis may have opposing effects in different autoimmune diseases: increased survival and resistance of target tissue towards apoptosis is in the focus of therapy in several organ-specific autoimmune diseases including multiple sclerosis (MS), Hashimoto's thyroditis (HT) and insulin-dependent diabetes mellitus (IDDM) [26]. In contrast, resistance

of epithelial cells to TNF-induced apoptosis in Crohn's disease may be crucial in maintaining a sustained autoimmune response [27].

Here we focus on emerging paradigms of death receptor-mediated apoptosis as common denominators in various organ-specific autoimmune diseases.

Genetic Alterations in Death Receptor Pathways

Unlike most organ-specific autoimmune diseases, the autoimmune lymphoproliferative syndrome (ALPS) is typically observed in relatively rare cases during childhood. Patients with ALPS clinically present a non-malignant accumulation of lymphocytes in lymphoid organs, hyper-gammaglobulinemia, autoantibody production, glomerulonephritis and arthritis. Resistance of lymphocytes towards CD95L-mediated apoptosis and towards AICD is an obligatory criteria for the diagnosis of ALPS and is thought to allow lymphocyte accumulation and autoantibody production [28]. Mutations in CD95, CD95L, caspase-10 and other still unknown alterations of the CD95 pathway may constitute the molecular basis of the disease and define subtypes of ALPS. However, CD95/CD95L-independent defects are thought to trigger the onset of the disease, as parents with the same mutation as their children are often reported to be disease-free. Similarly, *lpr* mice (retroviral insert in the CD95 gene) mice or *gld* mice (mutation in CD95L) only develop autoimmune disease in susceptible mouse strains. Recent reports further support a close relation between genetic alterations in the CD95 pathway and autoimmunity. Children with caspase-8 mutations present clinically with the association of ALPS-like lymphocyte accumulation and immunodeficiency [29].

Resistance of Autoaggressive Lymphocytes to Death Receptor-Mediated Apoptosis

Genomic alterations in death receptor genes may also be involved in autoimmune diseases which are thought to involve multiple genetic defects. Polymorphism in the CD95 gene [30] has been reported to be associated with female susceptibility to MS. Although such genetic studies often fail to combine genetic alterations with a clear pathogenetic mechanism, they may reflect the emerging paradigm of death receptor dysfunction in autoimmunity. Further work is needed to test if such polymorphisms may contribute to the relative resistance of T cells towards CD95-mediated apoptosis in MS. A combination of sustained T cell activation and reduced sensitivity of T cells towards CD95-mediated apoptosis has been suggested as a pathogenetic factor in MS. Similarly, the presence of autoreactive T cells may reflect a defect in the clonal deletion of harmful B and T cells in other autoimmune diseases like IDDM [26].

Death Receptor-Mediated Apoptosis in Target Tissues

Organ-specific autoimmunity leads to cell death in target tissues. Although a variety of mechanisms have been proposed to account for tissue destruction, several reports indicate that death receptor triggering is one major mechanism. In MS, oligodendrocytes have been described to express high levels of CD95. Infiltrating T cells, macrophages, microglial cells and astrocytes express CD95L and may trigger excessive CD95-mediated apoptosis of oligodendrocytes [26]. Similarly, in IDDM β-cells upregulate CD95 and were suggested to be killed during insulitis by CD95L-positive, autoreactive T cells [26], although this is currently debated. Furthermore, infiltrating lymphocytes do not necessarily confer cell death of target tissue by direct killing. In HT, T cells approaching thyroid follicles are highly sensitive to CD95 stimulation and undergo apoptosis when interacting with CD95L-positive thyroid follicular cells. It has been proposed that thyrocytes kill themselves by autocrine/ paracrine apoptosis [26] and a similar mechanism may account for deletion of acinar cells in Sjogren's syndrome [31]. Whereas normal thyrocytes express CD95L and only very low amounts of CD95, HT thyrocytes strongly express both CD95 and CD95L during active phases of the disease. Of note, normal thyrocytes, β cells and oligodendrocytes do not express significant levels of CD95. Upregulation of CD95 and sensitization of target tissue cells towards apoptosis is a crucial step for CD95-mediated apoptosis of tissue cells which is thought to depend on infiltrating cells and their inflammatory cytokines [26].

Modulation of Death Receptor-Mediated Apoptosis in Autoimmunity

Typically, lymphocyte infiltrates in autoimmune diseases such as MS, IDDM or HT are dominated by autoreactive TH1 cells. TH1 cells produce IFN-γ and IL-1β which in turn not only induce upregulation of CD95 on β cells or oligodendrocytes, but also stimulate production of other inflammatory mediators like nitric oxide (NO) or TNF-α [26]. However, disease-specific factors should also be taken into account. In IDDM, high glucose itself induces CD95 upregulation and β cell apoptosis and in MS, brain cell death may also involve TRAIL-mediated apoptosis [32]. Moreover, uncontrolled expansion of autoaggressive lymphocytes may not only be explained by relative apoptosis-resistance of self-reactive cells. As mentioned above, autoreactive T cells are still highly CD95L-sensitive in HT. Other mechanisms such as defects in survival or immunosuppressive function of CD4+CD25highFoxP3+ Treg may contribute to an uncontrolled expansion of autoaggressive lymphocytes. Reduced numbers of Treg are observed in myasthenia gravis, and a reduction of suppressive Treg function has been reported in MS [33, 34], RA and other autoimmune disorders. Furthermore, we observed a high sensitivity towards CD95L-mediated apoptosis

of unstimulated, constitutively CD95-positive Treg both in human and mice [35]. Accordingly, Treg may be modulated by the CD95/CD95L system which further highlights the versatility of potential death receptor modulation in autoimmunity.

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Inherited and Acquired Death Receptor Defects in Human Autoimmune Lymphoproliferative Syndrome

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Abstract

The death receptor Fas/TNFRSF6 is a key player in lymphocyte apoptosis induction. Patients lacking a functional Fas/TNFRSF6 receptor develop a chronic lymphoproliferation termed Autoimmune LymphoProliferative Syndrome (ALPS), characterized by a benign tumoral syndrome, autoimmune cytopenias, hyperglobulinemia (G and A) and accumulation of TCRαβ CD4−CD8− cells (called double-negative, or DN, T cells). Inherited mutations in the *TNFRSF6* gene are responsible for most ALPS cases (ALPS-I). *Caspase 10* gene mutations are found in a few of the remaining cases (ALPS-II). In a third group of patients (ALPS-III), somatic mosaicism of *Fas/TNFRSF6* mutations as found in sporadic cases. Consequences of this finding will be discussed in terms of functional and molecular diagnosis as well as in the understanding of the pathophysiological basis of ALPS.

Control of lymphocytes homeostasis is essential to ensure efficient immune responses and prevent autoimmunity. Expansions followed by contractions of the lymphocytes pool are the basis of adaptive immune responses, and apoptosis is a crucial cellular modus operandi of the contraction phases. The death receptor Fas is a key player in lymphocyte apoptosis induction and patients lacking a functional Fas receptor develop a chronic lymphoproliferation termed autoimmune lymphoproliferative syndrome (ALPS). In rare instances, defects of the Fas signaling pathway have been associated with ALPS. Although these defects with familial history are usually caused by inherited mutations of

the corresponding genes, somatic mosaicism of these Fas mutations were also found in sporadic cases of ALPS.

Fas Signaling and Apoptosis

The death receptors delineate a subfamily of the tumor necrosis factor receptor (TNF-R) family that includes five receptors containing a similar intracellular 'death domain' (DD). These receptors are TNF-R1 (TNFRSF1A) [1], Fas/APO-1/CD95 (TNFRSF-6) [2, 3], TRAMP/DR3/WSL1/APO-3/LARD (TNFRSF25) [4–8], TRAIL-R1/DR4/APO-2 (TNFRSF10A) [9], TRAIL-R2/DR5/Trick/Killer (TNFRSF10B) [10–15] and DR6 (TNFRSF21) [16]. Fas is a prototypical member with an 80 amino acid residues intracellular DD. This DD is the functional link between extracellular signals provided by ligands of the TNF family and the apoptotic machinery governed by the caspases (fig. 1). Adaptor molecules, such as the Fas-associated death domain (FADD) protein, belonging to an emerging family containing a 'death effector domain' (DED) [17], enable formation of a multimolecular complex called death-inducing signaling complex (DISC) [18]. Other cytoplasmic proteins were reported to interact with Fas such as Daxx [19], RIP [20] and FAF [21], but their roles in Fas-induced apoptosis remain a matter of debate.

DISC formation differs between cell types in ways that affect the efficiency of Fas signaling [22]. In recently activated T cells or 'type II' cell lines the DISC forms inefficiently. Fas molecules are not associated with glycosphingolipid-enriched microdomains, called rafts, and strong cross-linking of Fas is required to induce apoptosis. On the contrary, in restimulated primary T cells or in 'type I' cells, Fas is associated with lipid rafts. The DISC forms efficiently and moderate Fas-cross-linking can trigger apoptosis. Recently, signaling protein oligomeric transduction structures (termed SPOTS) have been characterized upon Fas ligation by agonistic antibodies [23]. Formation of these structures requires intact Fas DD and FADD, but is independent of caspase activity. The procaspase-8 and procaspase-10 are then activated, probably after forced oligomerization in SPOTS. This caspase activity is required for the following steps that are capping of Fas and internalization [24]. Production of large amounts of activated caspase-8 and caspase-10, in type I cells, promotes activation of various downstream caspases, including caspase-3, caspase-6 and caspase-7, and then triggers apoptosis. In such cells the Fas-induced apoptosis cannot be inhibited by Bcl-2 or Bcl-xl. In type II cells, the low amount of activated caspase-8 and caspase-10 allows the cleavage of Bid, a pro-apoptotic member of the BH-3 only proteins family, but not of caspase-3. Truncated Bid (tBid) molecules then complex with and inhibits

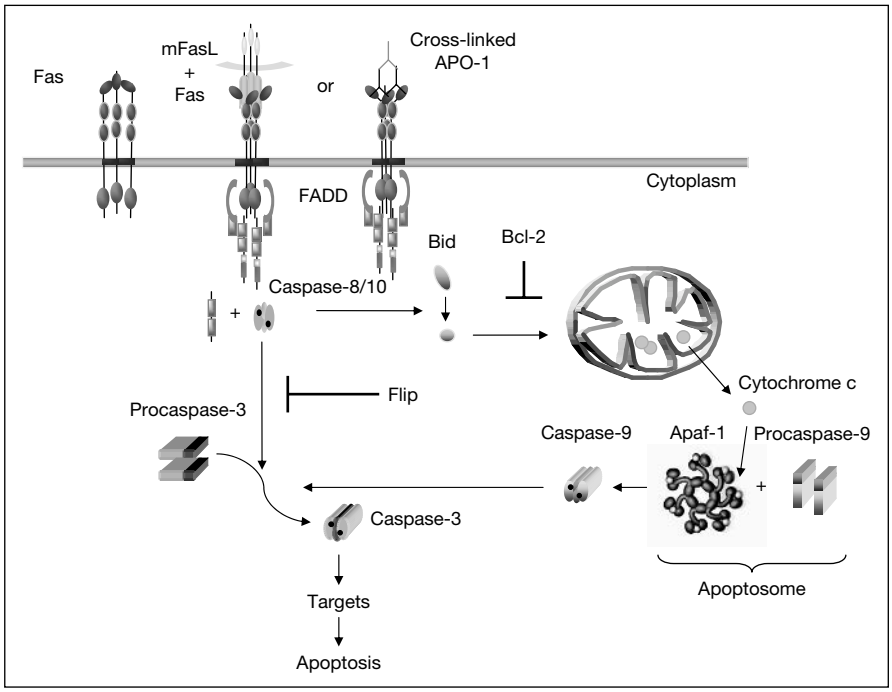

Fig. 1. Fas signaling pathway. Fas is self-trimerized through interactions of the amino-terminal domain termed pre-ligand-associating domain (PLAD). Upon interaction with membrane FasL (mFasL), homophilic interactions of death domains allow the association of Fas with the cellular adapter called FADD. FADD contains another domain called the death effector domain allowing interactions with procaspase-8 (Mach/Flice) and 10 (Flice-2) in a death-inducing signaling complex (DISC), thereby connecting Fas to a proapoptotic pathway. The FasL/Fas interaction can be mimicked in vitro by the use of cross-linked agonistic anti-Fas monoclonal antibody.

Bcl-2 in the outer mitochondrial membrane, thereby activating a mitochondrial-dependent cell death pathway.

Another key player in the DISC is the molecule called Flip [25]. The cFlip gene encodes, like its viral homolog vFlip, proteins that are structurally similar to caspase-8 and caspase-10. Alternative splicing gives rises to two isoforms. The short isoform, $Flip_S$, like vFlip, consists of two DEDs and can inhibit death receptor signaling by competing the recruitment of caspase-8. The long isoform, $Flip_L$, is a caspase-8 like molecule composed of two DEDs and small and large subunits that lack enzymatic activity. $Flip_L$ has a dual role. Low amount of $Flip_L$ is required to enable a full DISC activity. However when present in large amount it inhibits the caspases activation [26]. A defect of the DISC component

like FADD, caspase-8, caspase-10 or $Flip_L$ profoundly blocks apoptosis induced by death receptors. In contrast, defects of down stream molecules affect only partially this 'extrinsic' pathway of apoptosis.

Mouse Models of Fas Signaling Defect

Defects of Fas-induced apoptosis were first described in natural mouse mutant strains termed *lpr* (for lymphoproliferation) [27]. Adult MRL/*lpr* mice develop splenomegaly and adenopathy as well as hyperimmunoglobulinemia (hyper Ig), anti-nuclear antibody and nephritis. They accumulate CD4−, CD8− TCR $\alpha\beta^+$ T cells called double-negative (DN) T cells in peripheral lymphoid organs. The *lpr* strain carries a retrotransposon insertion within the *fas* gene, leading to an almost complete defect of Fas expression. It is proposed that *lpr* CD8+ T cells cannot be killed following stimulation by self-antigen. Consequently, they modulate the co-receptor, and accumulate as IL-10 secreting anergic cells [28, 29]. Other natural mutants developing the same phenotype were also described, i.e. the lpr^{cg} and *gld* mice [30]. In the lpr^{cg} strain, a missense mutation within the Fas DD allows the expression of a nonfunctional protein. In the *gld* mouse, a missense mutation in the extracellular domain of FasL abrogates its interaction with Fas. The lymphoproliferative syndrome develops in all homozygous animals whereas autoimmune manifestations depend on genetic backgrounds, suggesting the involvement of modifier genes [31, 32].

Several engineered animal models of Fas deficiency have been generated. Fas or FasL-deficient mice develop a severe lymphoproliferative syndrome, earlier than the corresponding natural mutants [33, 34]. Interestingly, a conditional Fas KO model underscored the role of nonlymphoid Fas-deficient cells in the onset of the lymphoproliferative disease [35]. Surprisingly, FADD or caspase-8-deficient mice [36–38], as well as transgenic mice expressing a dominant-negative form of FADD [39–41] or a DISC inhibitor, such as CrmA [42], p35 baculovirus protein [43] or Flip [44], do not develop a *lpr*-like syndrome in spite of a profound impairment of apoptosis induced by Fas or Trail-R. This is consistent with the observations in humans that autoimmune lymphoproliferative syndromes are found associated with Fas or caspase-10 deficiency, whilst caspase-8 deficiency rather leads to combined immunodeficiency (see below).

The Autoimmune Lymphoproliferative Syndrome

Biological and Clinical Presentation

In 1967, Canale and Smith [45] reported a condition characterized by non-malignant lymphadenopathy associated with autoimmune features in children.

Lymphocyte phenotyping of ALPS patients revealed the presence in high proportion of unusual polyclonal TCRαβ CD4−, CD8− (called double-negative or DN) lymphocytes. By analogy with the *lpr* model, defects of the Fas pathway were identified in ALPS patients [46–48].

Lymphocyte counts are variably increased, reflecting the intensity of the lymphoproliferative syndrome [49]. Chronic generalized lymphocyte activation was demonstrated by the presence of high levels of HLA-DR expression on peripheral CD3 T cells as well as by the presence of high levels activation markers such as soluble interleukine-2 receptor, soluble CD30 and soluble Fas-ligand in sera of ALPS patients [49, 50]. DN T cells are detected in excess in the blood of most patients with ALPS and may account for 1–60% of T cell counts [46, 51]. Human DN T cells exhibit a phenotype of antigen-experienced cytotoxic T cells (TCRαβ(high), CD2+, CD5+, CD27++, CD28+, CD57+, CD45RA+ RO−CD31+, CD62Ldull, CXCR-5−, perforin+) [52]. Detailed CD45 expression analysis on human DN T cells confirmed strong similarities with their murine counterparts, which express the B220 marker. Human DN T cells are CD31+ but in contrast to recent thymic emigrants [53] they have a very low content of T cell recombination circle (TREC) [Rieux-Laucat, unpubl. data]. A striking feature of ALPS-Ia consists in overproduction of IL-10 by DN T cells along with reduced IL-12 production by monocytes [54]. This is likely a secondary regulatory event attempting to counterbalance the persistence and activation of autoimmune clones. This is consistent with the observation made in a mouse model where IL-10 was found to exacerbate autoimmune manifestations [29].

Polyclonal hyper IgG and A is a very frequent finding while the level of serum IgM is usually reduced. However, in rare cases hypo Ig has been described [55]. Polyclonal B cell lymphocytosis with expansion of CD5+ B cells is also a characteristic finding [51].

In lymph nodes, paracortical areas are hyperplastic and contain many large lymphocytes with numerous mitoses. Many of those cells do express the Ki-67 antigen (indicative of active proliferation) and markers associated with cytotoxicity, such as perforin and CD57. The majority of the paracortical cells were DN cells (fig. 2), i.e. TCRαβ CD3+ CD45RO− CD45RA+. T and B cells accumulate in paracortical areas while the overall architecture of lymphoid organs is preserved [49, 56]. Apoptotic cells are often seen [49], suggesting the activation of compensatory apoptotic pathways. In addition, there is also excessive B lymphocyte accumulation with plasmocytosis in some patients but not all. In the spleen, expansion involves the red pulp and to a lesser extent the white pulp. Lymphoid cells in the red pulp are similar to the ones observed in paracortical areas of lymph node. Periarteriolar sheets are also enlarged. DN T cells can also infiltrate the liver at the level of the portal tracts and sinusoids.

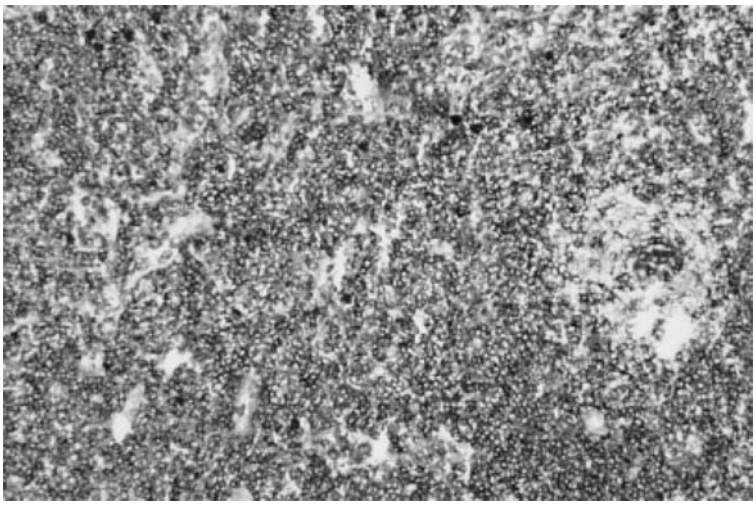

Fig. 2. Immunohistology of lymph node from ALPS-Ia patient. Immunohistochemical labeling for CD3 (dark grey) CD4 and CD8 (light grey) showing typical expansion of double-negative (DN) T cells in the paracortex.

Clinically, two forms of the disease can be described, a rare severe form and a frequent milder form.

The severe form is generally associated with a complete Fas deficiency (see below) and a massive proliferation starting at birth, suggesting a process that had started in the prenatal period [46, 49]. This active lymphoproliferation can cause massive lymphadenopathy, splenomegaly and hepatomegaly (not associated with liver dysfunction) (fig. 3). In this setting, hyperlymphocytosis was noted with a very high proportion (up to 70%) of DN T cells. If not treated by bone marrow transplantation (see below) [57], this condition is lethal [58]. Autoimmune manifestations are marginal in this condition but could have been underestimated considering the severity of the proliferative syndrome.

The milder form of the ALPS is the most frequent one. Onset of symptoms occurs in early childhood (0 to 5 years, at 2.3 years on average) [55]. However, onset in adulthood is occasionally observed. Splenomegaly fluctuates in a given patient or from patient to patient and splenectomy is often performed because of discomfort or hypersplenism. Lymph node enlargement is multifocal and their size fluctuates with time [59]. Sometimes, blood lymphocytosis or increased adenopathy (in number and in size) are observed after splenectomy. In contrast, a paradoxical decrease in lymph node has been observed during viral infection [45]. Hepatomegaly, when observed, is mild and is not associated

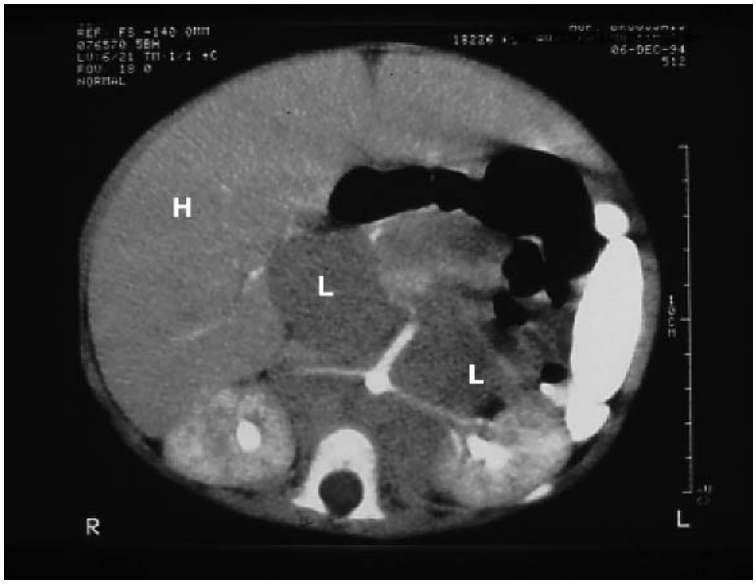

Fig. 3. Abdominal CT scan in an ALPS-0 condition. Hepatomegaly and massive lymph node enlargements are visualized (splenectomy was performed at 3 months because of hypersplenism). L = Lymph nodes; H = hepatomegaly.

with liver dysfunction. The lymphoproliferation may also involve the thymus, which is enlarged as visualized by computed tomographic studies [59].

Autoimmune manifestations are found in about 70% of the patients [51, 55]. Age at onset varies considerably in contrast to the lymphoproliferative syndrome. The most common autoimmune manifestations involve hematological lineages leading to anemia, thrombocytopenia and neutropenia and are associated with corresponding autoantibodies. A similar spectrum of symptoms may be seen in patients with Evan's syndrome [60]. Indeed, in a small cohort of ES patients, a functional Fas defect together with increased proportion of DN T cells was documented [61]. Hemolytic anemia is the most frequent one and has been found associated with dyserythropoiesis in two cases [62]. Other autoimmune manifestations can be observed such as glomerulonephritis, Guillain-Barre syndrome, uveitis, arthritis, hepatitis and diabetes [50, 55, 63]. Autoimmune manifestations involving the skin include urticarial rashes, vasculitis as well as linear IgA disease [64]. Autoimmune basis is suspected in some cases of seizure, autism, ovarian failure and mucosal ulceration [65]. In contrast to *lpr* mice, typical lupus was never detected in Fas-deficient patients. Autoantibodies towards cardiolipin, smooth muscle, nuclear antigens as well as rheumatoid factor are commonly detected, but anti-DNA antibodies were never

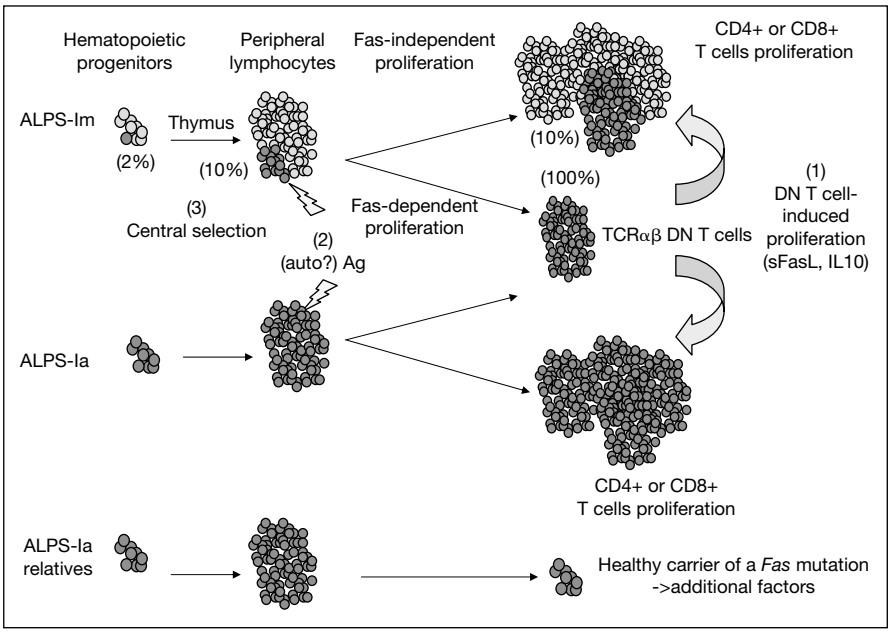

Fig. 4. Lymphoproliferation in ALPS-Ia and ALPS-Im. Fas-deficient cells (dark) and Fas-proficient cells (light), co-exist in ALPS-Im patients, showing the somatic mosaicism. In ALPS-Ia patients or in their clinically unaffected relatives, all cells are mutated and exhibit a Fas-mediated apoptosis defect. Unsolved questions are: (1) What is the role of the DN T cells, or of the cells that generate them, in the induction and the persistence of ALPS? (2) Is Fas only controlling proliferation towards self-antigen? (3) Is the central tolerance preserved in ALPS? Normal T cell homeostasis in healthy carriers of Fas mutations suggests the existence additional factors, which can either protect these carriers or activate onset of the disease in patients.

seen. Of note, autoimmunity appears to be always associated with autoantibodies, although direct pathogenic intervention of T cells in some of the autoimmune process cannot be excluded. Failure to thrive is a frequent symptom in children, but splenectomy could reverse it in a number of cases. By a long-term follow-up of a number of patients, it has been possible to determine that there is a significant reduction of lymphoproliferation in a number of them over time [55]. Nevertheless, Fas mutations represent a significant risk factor for malignancy. Liver carcinoma (in 1 patient with hepatitis C infection) and multiple thyroid and breast adenomas together with basal cell carcinomas in another one were reported [48].

A study performed on a cohort of ALPS patients and relatives showed that the risk of non-Hodgkin as well as Hodgkin lymphoma in carriers of heterozygous

Fas mutation was, respectively, 14 and 51 times higher than expected [66]. In this study, the average age of lymphoma occurrence was 28 years. This observation is in accordance with the description of somatic Fas mutations in both children and adult leukemia and lymphomas [62, 67–69]. However, as discussed below, somatic mutations of Fas, most likely occurring in hematopoietic progenitor, are also associated with classical benign ALPS [70].

Treatment indications depend on the type and severity of the symptoms. In many patients, the clinical status does not require any treatment. Splenectomy is often performed because of discomfort and hypersplenism. But sometimes it is also required because of protracted autoimmunity toward blood cells [50, 55]. In some patients autoimmunity tends to be severe, requiring aggressive immunosuppressive regimens including steroids and cyclophosphamide [49]. The anti-folate drug Fansidar®, or a combination of pyrimethamine and sulphadoxine, were found effective in some cases [71], especially for the lymproliferative manifestations.

In 2 severe cases, characterized by progression of lymphoproliferation in spite of chemotherapy including cyclophosphamide, vincristine and prednisone, bone marrow transplantation was performed from an unrelated donor in 1 case [72] and in a haploidentical situation in the other [57]. In both cases, bone marrow transplantation led to correction of the Fas deficiency and to disappearance of clinical and biological manifestations.

Molecular Basis of the ALPS

Based on the molecular defect, ALPS can be subdivided into at least five subtypes. Complete expression defects, associated with a severe form of the disease, are termed ALPS-0 to underline the complete absence of Fas, at least on lymphocytes. In this regard, the ALPS-0 is similar to the Fas-null mice. ALPS-Ia defines functional Fas deficiencies (with slightly diminished or normal Fas expression). They are associated with heterozygous dominant mutations of Fas. ALPS-Ib is used for the FasL defect; it may be inappropriate, since the phenotype of the unique described case is dissimilar to other ALPS patients. ALPS-II is used to describe defects of the Fas signaling pathway such as defects of caspase-10. In contrast, the caspase-8 deficiency may not be classified as ALPS since it is rather associated with combined immunodeficiency. Finally, ALPS-III describes patients presenting with ALPS symptoms but with a normal in vitro Fas-induced apoptosis. In a recent work, somatic heterozygous dominant Fas mutations have been identified in a group of ALPS-III patients. It was proposed to call these cases ALPS-Im to refer to the mosaicism observed in these cases.

ALPS-0: Complete Expression Defect of Fas

ALPS caused by complete Fas deficiency (ALPS-0) are consequences of homozygous null mutations. Three cases of homozygous mutations have been reported [46, 58, 73]. Given that heterozygous parents were healthy, it was proposed that these mutations were recessive [29, 58]. However, observation of another unpublished family with ALPS-0 does not support this conclusion. In that family, whereas the heterozygous mother is healthy, the heterozygous father presented with symptoms of classical ALPS-Ia [Rieux-Laucat et al., unpubl. obs.]. Importantly, cells from both parents exhibited a Fas-induced apoptosis defect of the same magnitude. The child who received both mutated alleles is presenting with complete Fas deficiency and typical ALPS-0. From this example, it can be suggested that most, if not all, mutations are dominant, and that when homozygous, they lead to a more severe phenotype. In one family, three siblings were compound heterozygotes and developed a moderate form of ALPS. One mutation resulted in an amino acid substitution in the extracellular domain [74]. It is unclear whether this modified Fas molecule has an impaired function.

ALPS-Ia: Partial Functional Fas Deficiency

ALPS-Ia is the consequence of heterozygous dominant *Fas* mutations and more than seventy ALPS-Ia patients are described [49, 52, 55, 56, 58, 62, 65, 73–83]. Mutant Fas molecules exert a transdominant negative effect on wild-type molecules [80, 84]. Seventy percent of the identified mutations affect the intracellular domain (ICD) and most of those mutations are localized within the DD. ICD mutants result in reduced FADD binding and caspase recruitment much greater than a 50% reduction as predicted in a 1:1 non-cooperative interaction between Fas and FADD [84]. This implies cooperativity between Fas subunits in the recruitment of FADD, consistent with the presence of only 1 of 8 normal trimers in this setting. One third of mutations affect the extracellular domain (ECD) of Fas. The PLAD model explains how ECD mutants resulting from nonsense mutations can affect triggering of apoptosis. Although unable to anchor the membrane, they can still complex with wild type Fas through the N-terminal domain [85]. The identification of a mutation leading to a stop codon at position +12 [Rieux-Laucat et al., unpubl. obs.], suggest that the signal peptide and the very first residues of Fas can constitute a functional PLAD. Alternatively, it can be expected that such a short mutant would not be expressed, thus leading to haplo-insufficiency. This last hypothesis is consistent with experiments performed on thymocytes from heterozygous Fas KO mice,

which exhibit a reduced Fas-induced apoptosis [86]. Missense mutations in ECD result in expression of an abnormal Fas molecule, most likely incapable of an interaction with FasL.

Of note, ALPS-Ia patients may have clinically unaffected relatives with the same apoptotic defects (and same mutations) [55, 79, 80]. Thus, from a functional point of view, mutations are fully penetrant. On the contrary, the clinical penetrance is partial since only 70% of Fas mutation carriers will develop the disease. This clinical penetrance is highest for ICD missense mutations, reaching 90%. Mutations leading to ICD truncation have a clinical penetrance of roughly 75%. Finally, the clinical penetrance dropped to 30% for ECD mutations. This is the unique genotype-phenotype correlation in ALPS. No other correlation could be found between the type of mutation, magnitude of the functional defect and severity of the syndrome. The partial clinical penetrance strongly indicates that a second event should be associated to Fas mutations in order to cause an overt ALPS. Genetic rather than environmental factors, like in *lpr* mice, likely influence the ALPS expression, and account for variable penetrance of some mutations. Interestingly, mutations of both Fas alleles (either in homozygous or in compound heterozygous) are associated with full clinical penetrance. Similarly, in mice, symptoms develop only in homozygous animals. However, it was reported that double-heterozygous *lpr*^{cg}/*gld* animals develop a mild lymphoproliferative disease [87]. These findings suggest that defects in the Fas/FasL pathway can synergize and direct development of the syndrome.

ALPS-Ib: A Unique Example of Human FasL Deficiency

A unique case of dominant FasL mutation was found in a screen of 75 patients presenting with features of systemic lupus erythematous along with chronic lymphoproliferation [88]. This case was defined as ALPS-Ib despite the lack of some biological and clinical criteria found in ALPS (DN T cells and splenomegaly were absent). Of note, inheritance of this mutation could not be proven. The phenotype of lymphoproliferation and *gld* mice being identical, one could predict an occurrence of FasL mutations in humans as frequent as Fas mutations. The absence of inherited FasL mutations in ALPS suggest either that FasL is more important for human development than it is in mice (thus, a FasL defect would not be compatible with life) or that a FasL defect would cause a completely different disease. This could be an immune deficiency (if FasL is involved in triggering cell proliferation), or other severe disease (related to potential extra-hematopoietic manifestations) masking the diagnosis of ALPS. Alternatively, a potential unforeseen second ligand in humans could lead to an absence of phenotype.

ALPS-II: Defect of the Death Receptors Signaling Pathway

Lymphocytes from ALPS-II patients exhibit abnormal lymphocyte Fas-mediated apoptosis, but normal Fas expression and sequence. Lenardo's group has reported the occurrence of caspase-10 mutations associated with ALPS in two families [89]. Moreover, apoptosis triggered by other DRs (TNFRSF1, TNFRSF25 and TNFRSF10A and B) is also impaired in caspase-10 deficiency, strongly indicating that besides caspase-8, the related caspase-10 is also integrated into the DISC upon death receptors engagement. Accordingly, recent work confirmed the key role of caspase-10 in Fas-induced apoptosis [90, 91] and its capacity to cleave Bid and trigger the caspase cascade activation [92]. Importantly, the V410I mutation identified in a patient with ALPS-II was found at the heterozygous state with a high frequency in the Danish population [93, Vonarbourg et al., unpubl. data], questioning its involvement in the onset of the disease. However, this polymorphic mutation has not been found at the homozygous state in a healthy patient. Regardless of the relevance of V410I, the dominant caspase-10 mutation has been validated and underscores the physiological role of this caspase in the cascade leading to apoptosis.

A homozygous caspase-8 mutation has been described in two siblings from a consanguineous family. They presented with lymphoproliferative syndrome associated with clinical and biological immunodeficiency [94]. The defect in activation of T and B lymphocytes found in this situation suggested that caspase-8 is involved in early TCR and BCR signaling. Recently, Su et al. [95] showed that caspase-8 deficiency in humans and mice specifically hinders activation of the transcription factor NF-κB after stimulation through antigen receptors, Fc receptors, or Toll-like receptor-4 (TLR4) in T, B and natural killer cells, thereby explaining the phenotype of combined immunodeficiency (CID). Consequently, the classification of the caspase-8 deficiency as ALPS-IIb (OMIM#607271) is perhaps inappropriate.

ALPS-III: ALPS without in vitro Fas Functional Defect

We have investigated more than 50 patients who presented with lymphoproliferation and autoimmunity but normal in vitro Fas-induced apoptosis as well as normal FasL gene (see ALPS-Ib). Therefore, this type of ALPS remained enigmatic until recently. Retrospectively, we observed that most cases were sporadic and that the diagnostic criteria for ALPS were met in only 20% of these patients. We thus pursued the hypothesis that an increased proportion of DN T cells (above 2%) reflected the accumulation of cells that carry a Fas deficiency. Purification of the DN T cells led to the identification of heterozygous dominant

Fas mutations [70]. Whereas these mutations were found in 100% of the DN T cells, they were detected in only a fraction (10–20%) of other cell subsets such as CD4+ or CD8+ T cells, B cells, NK cells, monocytes or granulocytes. In one case, the mutation was found in 1% of the CD34+ progenitor-enriched population of the spleen. In contrast, these Fas mutations remained undetectable in epithelial cells from the mouth or skin and thus were somatic rather than germline mutations. They most likely occurred during embryonic or fetal development. We proposed to term these cases ALPS-Im in order to underline the somatic mosaicism observed in these patients. Analyses of in vitro-activated T cells revealed that the mutant T cells (including DN as well as CD4+ and CD8+ T cells) exhibited a paradoxical spontaneous apoptosis in culture. Therefore, mutant cells were absent when the functional assay was performed, thus explaining the normal in vitro Fas-induced apoptosis in ALPS-Im patients [70]. In conclusion, a subgroup of patients with ALPS-III carries somatic Fas mutations in hematopoietic cells in the absence of any malignant condition. These patients are probably at risk for lymphomas, like those with inherited mutations. Whether the progeny of ALPS-Im patients will be at risk to develop an ALPS depends on the extent of the mosaicism and the presence of mutation in germ cells. In future work, it will be of interest to decipher the mechanisms by which a fraction of mutant T cells can trigger an overt ALPS in mosaic patients, and to determine whether all sporadic cases of ALPS with normal in vitro Fas-induced apoptosis, are caused by somatic mosaicism of Fas mutations or of other genes involved in the Fas pathway, such as FasL, FADD and caspase-10.

Pathophysiological Mechanisms in ALPS

The characteristic feature of ALPS with Fas deficiency is chronic lymphoproliferation. The initial explanation was that Fas-deficient cells accumulate because of apoptosis resistance. However, if it fits with a progressive development of the disease in aging mice, it cannot explain the early onset of ALPS in children or in neonates. In addition, the frequent observation of spontaneous apoptosis of T cells in ALPS (either DN T cells or single positive CD4 or CD8) supports a dynamic generation/removal process of DN T cells. In ALPS condition, active proliferation cause a production of DN T cells which is faster than the removal by compensatory apoptotic mechanisms. Importantly, the proportion of DN T cells stays steady over time in a given patient (without treatment), but may vary from patient to patient. Modifier genes encoding products involved in apoptosis might thus control expression of the disease. DN T cells have been shown to secrete high amounts of soluble FasL (sFasL) and IL10. It was also shown that sFasL can potentially inhibit the cytotoxic activity of the

membrane form (mFasL) [96]. This could theoretically explain the generalized proliferation observed in ALPS-Im in spite of the low proportion of mutant cells (fig. 4). In addition, IL-10 can enhance the proliferation of autoreactive B cells [29] and induce regulatory T cell apoptosis [97]. These combined effects could potentially account for the onset of autoimmunity as observed in the majority of ALPS patients. It thus remains to explain why some ALPS-Ia patients do not develop autoimmune manifestations, and why responses to exogenous antigen (such as vaccination as well as viral or bacterial infections) are normally controlled. This suggests that the Fas pathway is controlling AICD during chronic exposure to auto antigens but not to exogenous antigen (fig. 4). It was proposed that chronically stimulated T cells downmodulate CD28 and therefore become more sensitive to FasL-induced cell death [98]. Fas-deficient T cells escape cell death, proliferate and can activate Fas-deficient B cells. In physiological setting, FasL expressing T cells kill normal autoimmune B cell clones, likely because chronic exposure to antigens no longer induce protecting signals from cell death. Fas deficient B cells will thus escape this regulatory process and further proliferate. CD40L/CD40 T/B interaction can induce plasmocyte differentiation and isotype class switch, thereby explaining the hyper-IgG and A found in more than 80% of ALPS patients. Interestingly, correction of Fas only in T cells enables to control both lymphoproliferation and autoimmunity [99]. Therefore, the Fas pathway deficiency creates a defect in peripheral tolerance. Defects of central tolerance of T cells in *lpr* mice remain a matter of controversy [100, 101]. The increased proportion of mutant cells in the periphery as compared to hematopoietic progenitors in one ALPS-Im patient supports a role for Fas during lymphoid and myeloid development [70].

Autoimmune features vary from strain to strain carrying the lpr mutation. These manifestations are moderate in C57BL6 or 129 mice [86]. This important finding shows that Fas mutations are a predisposing factor for the onset of autoimmunity, but that the latter requires other genetic susceptibility factors. Loci encoding some of them have been recently mapped [32]. Further genetic studies of mice should thus lead to the identification of other important gene products in the control of autoimmunity. Similarly, in humans, several modifier gene products might be involved, and might affect T cell proliferation or apoptosis, as well as elimination of auto-reactive cells.

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Tumor Necrosis Factor Ligand-Receptor Superfamily and Arthritis

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Abstract

The current studies of apoptosis in rheumatoid arthritis (RA) suggest that the TNF ligand-receptor superfamily (TNFRsF) molecules, downstream pathways (activation of pro-apoptosis or anti-apoptosis pathway), cell types (lymphocytes and synovial fibroblast), and the mechanism that triggers apoptosis (tolerance induction-related, downmodulation of inflammation-related, or DNA damage-related) all exhibit a capability to determine the induction or prevention of RA. This series of defects at different levels and in different cells have been shown to lead to T cell and synovial hyperproliferation, defective apoptosis, excessive apoptosis, or bone erosion. In this chapter, we summarize the available knowledge of the regulation of TNFRsF and their likely pathogenic roles in RA to help identify candidate target cells and target molecules for delivery of gene constructs to modulate apoptosis to prevent the development of RA in both humans and mice.

The members of the TNF ligand-receptor superfamily (TNFRsF) are intimately involved in the regulation of the proliferation and death of immune cells and are of particular interest in relation to their role in the genesis of arthritis and autoimmune disease. The number of receptors, and their corresponding ligands that are recognized as members of the TNFRsF has increased rapidly, and the biological functions of these molecules are now being revealed (table 1). Many therapeutic strategies that target members of the TNFRsF, and their signaling pathways, have been proposed for the treatment of arthritis and autoimmune disease.

Table 1. Summary of the major TNF ligand-receptor superfamily members that are associated with autoimmune disease

Ligand	Receptor	Functions and connection with autoimmune disease
TNF-α	TNFR1 (CD120a) TNFR2 (CD120b)	– increase in human RA patients – TNF-α transgenic mice develop arthritis spontaneously promotes apoptosis but also inflammatory response promotes apoptosis but also inflammatory response
FasL (CD95L, or CD178)	Fas (CD95)	mutation leads to generalized autoimmune disease in *gld* mice mutation leads to generalized autoimmune disease in *lpr* mice
TRAIL (Apo-2L)	TRAIL-R1 (DR4) TRAIL-R2 (DR5) TRAIL-R3 (DcR1) TRAIL-R4 (DcR2) DcR3	– blockade leads to CIA and EAE – upregulation inhibited the development of CIA induction leads to apoptosis or activation of NF-κB and JNK induction leads to apoptosis or activation of NF-κB and JNK does not have a death domain and mainly inhibit apoptosis has only a partial death domain and does not induce apoptosis can bind to FasL and prevent the FasL-mediated apoptosis
TWEAK	DR3 (TWEAK-R, WSL-1, TRAMP, LARD)	induces apoptosis and costimulation of T cells deficiency leads to abnormal thymocyte negative selection
Unidentified	DR6	deficiency leads to increased T cell proliferation and altered Th1 and Th2 response
BAFF (Blys, THANK, TALL-1, zTNF4)	TACI BAFF-R BCMA	– supports the survival and proliferation of activated B cells – Blys transgenic mice develop lupus-like autoimmune disease inhibits B cell activation deficiency lead to B cell maturation defects does not appear to be crucial for B cell activation
RANKL	RANK	induction of osteoclast formation
OX40L	OX40 (CD134)	expressed in RA synovial sublining cells – expressed on T lymphocytes in synovial fluid from RA patients – in vivo treatment of IL-1R−/− arthritis mice with an anti-OX40 antibody suppressed the development of spontaneous arthritis
4-1BBL	4-1BB (CD137)	s4-1BBL were significantly higher in RA patients – s4-1BBL were significantly higher in RA patients – agonistic anti-CD137 treatment of DBA/1 mice inhibited humoral immune response and prevented the development of collagen-induced arthritis

Table 1. (continued)

Ligand	Receptor	Functions and connection with autoimmune disease
GITR	GITRL	removal of GITR-expressing T cells or administration of a monoclonal antibody to GITR produced organ-specific autoimmune disease in otherwise normal mice constitutively expressed on retinal pigment epithelium and in high levels on photoreceptor inner segments

Regulation of Cell Proliferation and Apoptosis by TNFRsF

Structurally, the members of TNFRsF share several common features (fig. 1). All members of the receptor family are type I transmembrane proteins with conserved cysteine-rich repeats and a certain degree of homology in the extracellular domain. A subgroup of receptor family members contains a conserved 'death domain', which is responsible for transducing an apoptosis signal in the cytoplasmic domain. All members of the ligand family are type II transmembrane proteins, which can be expressed in both a membrane-bound and a secreted form. The oligomerization of the receptor as a consequence of interaction with its ligand is required to deliver a functional signal. An immune cell may express either the receptor or ligand alone or express paired receptors and ligands on the cell surface simultaneously. Therefore, the interaction between the receptor and ligand in immune cells can be associated with both autocrine and paracrine responses.

Functionally, the members of the TNFRsF can deliver signals leading to either apoptosis or survival and proliferation. For each TNFRsF member, the differentiation between cell death and proliferation depends upon the type of cell and its functional status. The receptors of the superfamily share signal transduction pathways in common with the apoptosis signal transduction being mediated through the Fas-associated death domain (FADD)/caspase-8 cascade, and the stimulatory signal transduction being mediated primarily by TNF receptor-associated factors (TRAFs) and the NF-κB pathway (fig. 2). The balanced expression of the death receptors and the survival receptors on immune cells and their appropriate signaling maintains the homeostasis of the immune system and also regulates death or survival of tissue targets of autoimmune attacks.

Regulation of Apoptosis by Bcl-2 Family Members

The family of *Bcl-2*-related proteins also functions as a regulator of apoptosis (fig. 3). These molecules share homology at four conserved Bcl-2

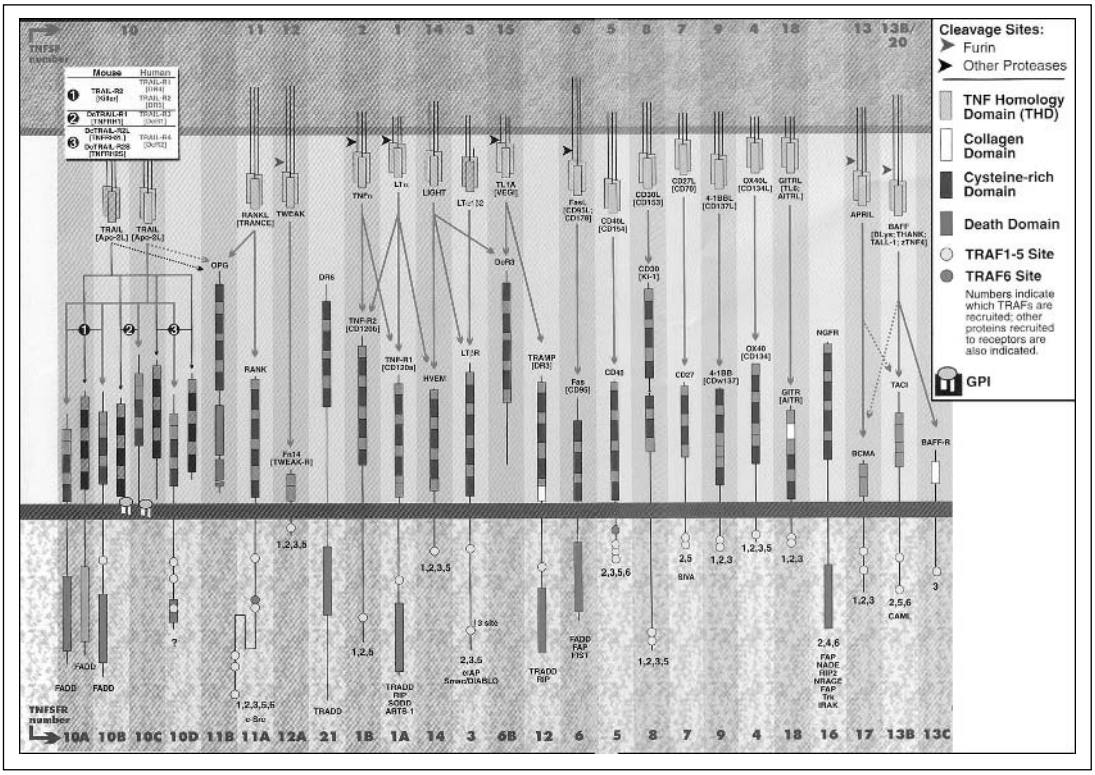

Fig. 1. The TNF superfamily of ligands and receptors. The extracellular region of the TNFRsF receptors carries two to six repeats of a cysteine-rich subdomain that have approximately 25% homology. For example, Fas has three cysteine-rich subdomains, and TNFR1 has four such subdomains. The intracytoplasmic death domain is shown by the rectangular box, and the different TRAF sites are shown by different color-coated circles. This figure has been modified and adapted from the ALEXIS Immunology catalog (copyright permission granted by the ALEXIS Platform, San Diego, Calif., USA and from Koopman WJ: Apoptosis and autoimmune disease, chap 26,; in Mountz JD, Zhou T (eds): Arthritis and Allied Conditions, ed 15. Baltimore, Lippincott Williams & Wilkins).

homology (BH1-4) domain regions, which control the ability of these proteins to dimerize and affect apoptosis. The conserved domains BH1, BH2, and BH3 participate in the formation of various dimer pairs as well as the regulation of cell death. The Bcl-2 family includes the death antagonists Bcl-2, Bcl-X_L, Mcl-1, and A1, as well as the proapoptotic molecules Bax, Bcl-X_s, Bak, Bik, Bid, Bim, and Bad. The overall ratio of the death agonists to antagonists determines the susceptibility to a death stimulus. Bcl-X_L, Bcl-2, and Bax also can form

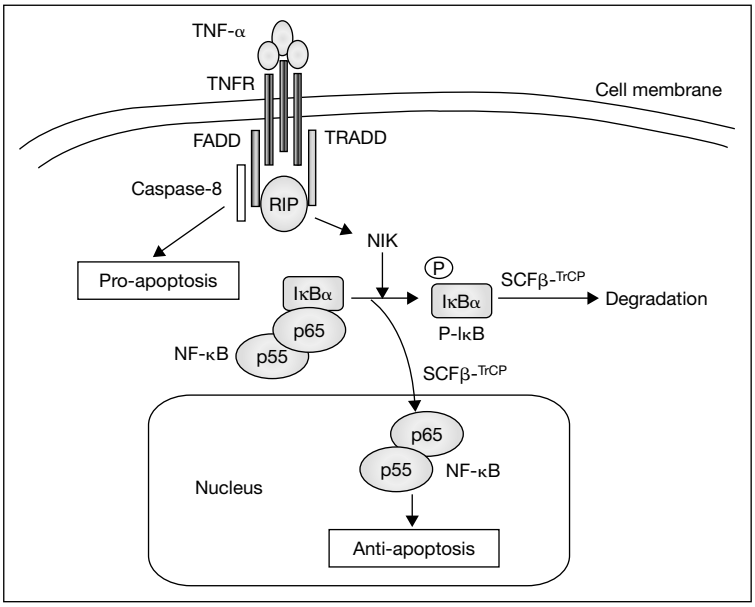

Fig. 2. Apoptosis signaling pathway. Apoptosis signaling by a death inducing signaling complex (DISC) was mediated by binding of the Fas-associated death domain (FADD) and Fas-like IL-1-converting enzyme (FLICE), otherwise known as caspase-8, to Fas. The formation of the DISC depends upon cross-linking of the Fas molecule. This leads to production of active caspase 8. In the case of tumor necrosis factor 1, the first molecule to bind to DISC is the TNF receptor apoptosis death domain (TRADD), followed by assembly of the same molecules as described for Fas. TNF receptor signaling by TNF-α acts through TRAF-2 and Rip to activate NF-κB inhibitor kinase (NIK). This phosphorylates IκBα and IκBβ chains. The phosphorylated IκBα and IκBβ are rapidly degraded in the proteasome. The NF-κB 40-kDa and the NF-κB 65-kDa dimer undergo nuclear translocation which is guided by nuclear localization. Nuclear translocation of NF-κB leads to upregulation of a number of genes including anti-apoptosis genes (adapted with copyright permission from Koopman WJ: Apoptosis and autoimmune disease, chap 26,; in Mountz JD, Zhou T (eds): Arthritis and Allied Conditions, ed 15. Baltimore, Lippincott Williams & Wilkins).

ion-conductive pores in artificial membranes. Bcl-2 and Bcl-X_L display a reciprocal pattern of expression during lymphocyte development.

Bid and Bad possess the minimal death domain BH3, and the phosphorylation of Bad at Ser-112, Ser-136, and Ser-155 residue connects proximal survival signals to the Bcl-2 family. In contrast, the dephosphorylated (active) form of Bad binds to pro-survival Bcl-2 family members at the mitochondria. The binding of Bad to pro-survival Bcl-2 proteins is followed by the oligomerization of the proapoptotic Bcl-2 proteins Bax and Bak, which results in

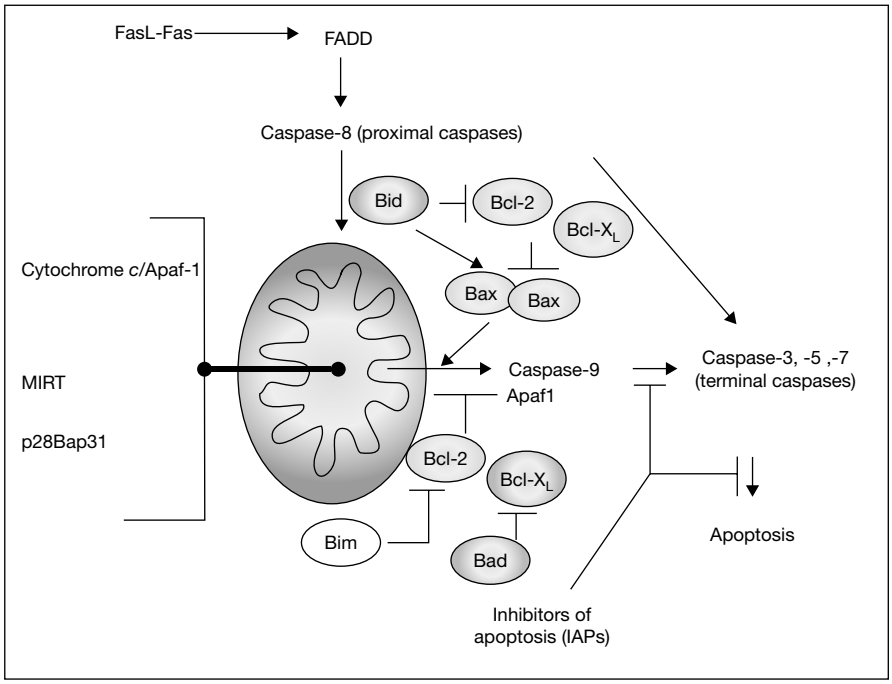

Fig. 3. Regulation of apoptosis by Bcl-2 family members. Protein-protein interactions between Bcl-2 family members regulate apoptosis function. Bax can form homodimers or heterodimers with either Bcl-2 or Bcl-X_L. Formation of Bax homodimers promotes cell death where Bax heterodimerization with either Bcl-2 or Bcl-X_L blocks cell death. Bad, a proapoptotic Bcl-2 family member, heterodimerizes with Bcl-2 and Bcl-X_L and promotes cell death. Bid can be directly activated by Fas and promote cell death. Members of the Bcl-2 family have been suggested to play a role in mitochondrial ion channel formation and promote the release of cytochrome *c* for mitochondria (adapted with copyright permission from Koopman WJ: Apoptosis and autoimmune disease, chap 26,; in Mountz JD, Zhou T (eds): Arthritis and Allied Conditions, ed 15. Baltimore, Lippincott Williams & Wilkins).

mitochondrial dysfunction, cytochrome *c* release, caspase activation, and apoptotic cell death.

Regulation of Apoptosis of Rheumatoid Arthritis Synovial Fibroblasts

It has been reported that the synovial fibroblasts that undergo hyperplasia in patients with rheumatoid arthritis (RA) have several dysregulations in Fas and Fas

ligand expression, apoptosis function, and the expression of other apoptosis molecules, such as p53. Analysis of fresh synovial tissue sections from patients with RA reveals higher apoptosis of the type A (macrophage-like) synovial lining cells with little apoptosis of type B (fibroblast-like) synovial cells. Synovial fibroblasts have been demonstrated to be sensitive to apoptosis in a human T cell leukemia virus 1 (HTLV-1) *tax* transgenic mouse model when high levels of anti-Fas antibody were injected intra-articularly [1]. These and similar experiments were carried out by using a novel anti-Fas monoclonal antibody including RK-8 and HFE-7A [2], which can cross-link Fas and induce apoptosis in some strains of mice without causing significant liver toxicity. Transfection of human Fas ligand into RA synovial fibroblasts that were transplanted into SCID mice also resulted in the induction of apoptosis. TNF-α has been shown to either inhibit or facilitate Fas signaling in human RA synovial fibroblasts. To date, the studies indicate that Fas apoptosis signaling may be defective in human synovial fibroblasts and that this signaling can be modulated by other cytokines, such as TNF-α and transforming growth factor-β (TGF-β), that are present in abundance in the joint tissue.

The TNF-Related Apoptosis-Inducing Ligand (TRAIL)-Mediated Apoptosis

TRAIL, another member of the TNF superfamily, has an apoptosis-inducing activity that is equivalent to that of TNF-α and Fas ligand. TRAIL has been of particular interest in the development of therapeutics because it differs from TNF-α and Fas ligand in that it induces apoptosis of tumor cells preferentially, with little or no effect on normal cells. At least five receptors for TRAIL have been identified, two of which, DR4 (TRAIL-R1) and DR5 (TRAIL-R2), are capable of transducing an apoptosis signal whereas the other three (TRAIL-R3, TRAIL-R4 and OPG) serve as decoy receptors to block TRAIL-mediated apoptosis. All five receptors for TRAIL share significant homology in their extracellular ligand-binding domain.

Recent studies indicate that defective TRAIL-mediated apoptosis has the potential to contribute to the development of autoimmune disease. Blockade of TRAIL-mediated apoptosis with the soluble receptor, DR5, has been shown to significantly increase the susceptibility of mice to collagen-induced arthritis and experimental allergic encephalitis. DR5 is of great interest as it is selectively expressed by abnormally proliferating pathogenic cells, and selective targeting of DR5 may induce apoptosis of autoimmune cells.

A novel anti-human DR5 antibody can induce apoptosis of RA synovial fibroblasts [3]. Synovial fibroblast cells isolated from patients with RA, but not those isolated from patients with OA, expressed high levels of DR5 similar to

most malignantly transformed tissues and cells. In contrast, the expression of other death receptors such as DR4, did not differ between RA and OA synovial cells. DR5-mediated apoptosis was highly selective for the RA synovial cells as all primary RA synovial cells but not OA synovial cells were susceptible to anti-DR5-mediated apoptosis. In contrast, there was no difference in Fas-mediated apoptosis between the two types of synovial cells. In vitro treatment of RA synovial cells with anti-DR5 strongly inhibited the production of matrix metalloproteinases (MMPs) induced by pro-inflammatory cytokines. In a human RA synovial cell xenograft model, treatment with anti-DR5 effectively inhibited hyper-proliferation of RA synovial cells and completely prevented the bone erosion and cartilage destruction induced by RA synovial cells. These results indicate that increased DR5 expression and susceptibility to DR5-mediated apoptosis are characteristic of the proliferating synovial cells in RA and suggest that specific targeting of DR5 on RA synovial cells with an agonistic anti-DR5 antibody may be a potential therapy for RA.

Signaling Pathways of Apoptosis in Rheumatoid Arthritis Synovial Fibroblasts

The signaling pathway for Fas in synovial fibroblasts has not been studied extensively, but several observations indicate that Fas signaling is downregulated. Fas apoptosis has been shown to involve the Jun kinase and the AP-1 pathways, as well as ceramide signaling. Other investigators have reported that the Jun kinase pathway is a critical MAPK pathway for IL-1-induced collagenase gene expression in synoviocytes and in joint arthritis, suggesting that Jun kinase is an important potential therapeutic target for RA [4].

Another pathway of growth regulation in RA synovial fibroblasts involves TNF-receptor signaling. TNF-receptor signaling can activate a potent anti-inflammatory pathway by NF-κB nuclear translocation. A mutant form of IκB also has been used in human RA synovial cell lines to block nuclear translocation of NF-κB in response to TNF-α [5]. This leads to unopposed activity of the proapoptotic pathway and high apoptosis of human RA synovial cells. The *inhibitor of apoptosis* (*IAP*) gene products play an evolutionarily conserved role in regulating programmed cell death in diverse species ranging from insects to humans. Human XIAP, cIAP1, and cIAP2 directly inhibit caspase 3, 6, and 7. The IAPs also can block cytochrome *c*-induced activation of pro-caspase-9 and inhibit Fas-mediated apoptosis. The murine homologue of the human X-linked IAP, called miap, has been mapped to the X chromosome. We previously [6] showed that XIAP was upregulated by TNF-α in rheumatoid arthritis synovial fibroblasts (RASF), and this upregulation was inhibited using a dominant negative form of IκB plus TNF-α XIAP is an inhibitor of apoptosis after TNF-α

signaling since transfection of primary RASF with an XIAP antisense adenovirus promoted to apoptosis of RASF. Therefore, XIAP is a TNF-α-inducible specific inhibitor of apoptosis in RASF. This and other modulators of the TNF receptor or the Fas apoptosis pathway may be therapeutically beneficial in facilitating apoptosis of synovial tissue in patients with RA.

An important pathway of apoptosis resistance for some synovial cells appears to be expression of mutant p53 [7]. It was hypothesized that free radical production associated with the highly oxidative metabolism present in the inflammatory synovium may lead to mutations of the p53 tumor-suppressor gene. Overexpression of p53 has been shown to result in significant apoptosis of human and rabbit synovial cells in culture. Furthermore, intra-articular injection of an Ad-p53 vector resulted in a significant reduction in leukocytic infiltrate and extensive and rapid induction of synovial apoptosis in the rabbit knee without affecting cartilage metabolism. Thus, p53 may be a critical regulator of fibroblast-like synovial cell proliferation, apoptosis, and invasiveness. Abnormalities of p53 function might contribute to synovial lining expansion and joint destruction in RA.

Akt Regulation of Fas Apoptosis of RASF

TNFR signaling also has been shown to activate PI 3-kinase and Akt, also known as protein kinase B, in HepG2 liver cells and U937 cells. Akt is a serine-threonine protein kinase that is regulated by PIP3 and has been implicated in signaling survival in a wide variety of cells, including fibroblastic, epithelial, and neuronal cells as well as T cells. Akt was first recognized as an anti-apoptosis factor during analysis of signaling by insulin-like growth factor-1 (IGF-1), which promotes the survival of cerebellar neurons. We have observed previously that the levels of phosphorylated Akt (P-Akt) are higher in RASF than in OASF [8]. The levels of P-Akt and Akt kinase activity were increased by stimulation of primary RASF with TNF-α. Treatment of RASF with the phosphatidylinositol (PI) 3-kinase inhibitor, wortmannin, plus TNF-α resulted in apoptosis of 60% of RASF within 24 h. This pro-apoptosis effect was specific for Akt, since equivalent levels of apoptosis were observed upon TNF-α treatment of RASF transfected with AdAkt-dominant-negative (DN) and with AdPTEN, which opposes the action of Akt. We propose that, under pathologic conditions observed in RASF, there is increased activation of Akt which inhibits the apoptosis pathway leading accumulation of the RASF [8]. It was first proposed that oncogenes may result in increased proliferation or dysregulated growth of cells associated with autoimmune disease. Elevated expressions of oncogenes were first observed in RASF with the reports of increased expression of *ras* and *myc*, and the association of oncogene expression with invasiveness of synovium.

TNFRsF as Regulators of T Cells in RA

Death Domain Receptors in T Cells and Autoimmunity

The immunological functions of most members of the TNF receptor and ligand family have been identified. All of the receptors with an apoptosis-inducing function are involved in the downmodulation of the immune response. One of the most important functions of the death receptors is to mediate the AICD of immune cells. AICD is a highly regulated event that involves several apoptosis signaling molecules, including Fas and the TNF-R, which are expressed on different cell types including B cells, T cells, and macrophages [9]. The identification of mutations of the murine *fas* and *fas ligand* genes over a decade ago indicated that the products of these genes are a pair of receptor/ligand molecules that are specialized to carry out the function of apoptosis. Moreover, the identification of mutated *fas* and *fasL* genes in the autoimmune *lpr/lpr* and *gld/gld* strains of mice, respectively, provided the first evidence that autoimmune disease could result from defective apoptosis of immune cells. A major function of Fas-mediated apoptosis is in the AICD of T cells, which is critical for maintenance of peripheral T cell tolerance. On activation, T cells express increased levels of both Fas and Fas ligand, which enables apoptosis to occur in an autocrine fashion. Studies using a T cell receptor transgenic mouse model have demonstrated that failure of Fas-mediated apoptosis results in increased production of self-reactive T cells in the periphery and decreased clonal deletion of superantigen-stimulated T cells. Other biological functions of Fas-mediated apoptosis include: (1) a role in the immunosurveillance function of cytotoxic T cells and natural killer (NK) cells, both of which express high levels of Fas ligand and mediate apoptosis of virally infected host cells, and (2) a role in immunoprivilege, in which overexpression of Fas ligand by the immunoprivileged tissues and organs prevents attack by T cells and other inflammatory cells.

Defective apoptosis or a reduction in the levels of apoptosis of autoimmune T cells may lead to the development of autoimmune disease, and it was logical to assume that manipulation of the levels of Fas would be an effective therapeutic strategy. The feasibility of this approach has been limited to date, however, by the severe liver toxicity associated with administration of both soluble Fas ligand and agonistic anti-Fas antibodies. Strategies to avoid toxicity have included the local delivery of the *fasL* genes and the development of monoclonal antibodies that target Fas and induce effects on inflammatory cells selectively, thereby bypassing the associated potential for liver toxicity.

T cell apoptosis is associated with sequential activation of the caspases, which cleave after Asp residues, beginning with caspase 8 (fig. 3). Caspases are expressed constitutively in most cells, residing in the cytosol as a single-chain proenzyme. These are activated to fully functional proteases by a first proteolytic

cleavage that divides the chain into large and small caspase subunits and a second cleavage that removes the N-terminal domain (prodomain). Inefficient activation of caspase-8 results in direct activation of Bid, a proapoptotic member of the Bcl-2 family, and the C-terminal fragment acts on mitochondria, triggering cytochrome *c* release. The released cytochrome *c* binds to apoptotic protease-activating factor-1 (Apaf-1), which self-associates and binds to and activates caspase-9. This is associated with a decrease in inner mitochondria membrane potential corresponding to the opening of the inner membrane permeability transition (PT) pore complex and loss of the ability to take up certain dyes. In immune cells in which this mitochondrial amplification loop is important, anti-apoptosis Bcl-2 family members can suppress *Fas*-induced apoptosis. Bcl-2 and Bcl-X_L act to prevent cytochrome *c* release and thus interfere with this pathway. Activated caspase-9 and caspase-8 then act on the terminal caspases 3, 5, and 7 that are activated immediately before cell death. Strong signaling through the Fas receptor can lead directly to high levels of activated caspase-8 activity and immediate activation of terminal caspases 3, 6, and 7, resulting in death of the cell.

Regulation of T-cell Apoptosis in Rheumatoid Synovium

Initial investigations of apoptosis in rheumatoid synovium indicated that apoptotic cells were confined to the synovial lining layer and that the infiltrating T cells express high levels of Bcl-2 and FLIP exhibited high resistance to apoptosis [10]. In other studies, either lower expression of Bcl-2 in the synovial fluid T cells, or no significant difference in Bcl-2 expression, in synovial tissue T cells of patients with RA compared with those of osteoarthritis (OA) and reactive arthritis has been reported [11]. Many studies support the concept that T cells in the synovium of patients with RA are activated and correspondingly express increased levels of Fas and Fas ligand, and tend to undergo Fas-mediated apoptosis. The question remains, however, as to whether this upregulation of apoptosis is sufficient to effectively eliminate the T cells that promote the inflammatory disease.

APC-FasL Eliminated Peripheral T Cells and Ameliorated Murine Arthritis Induced by Mycoplasma pulmonis *Infection*

We have shown that elimination of autoreactive T cells in FasL mutant B6-*gld/gld* mice using FasL cell therapy was effective in ameliorating arthritis induced by an infectious pathogen, mycoplasma [12]. However, this treatment itself did not induce synoviocyte apoptosis and did not completely prevent the infected mice from the development of arthritis. We infected adult B6-*gld/gld* mice with a moderate intravenous dose of *M. pulmonis* and observed both acute periarticular inflammation and severe, chronic, erosive arthritis in B6-*gld/gld* mice. Similar

inoculation of congenic wild-type B6-+/+ mice resulted in a self-limiting acute-phase inflammatory response without leading to the development of arthritis. We further used a novel FasL gene delivery strategy in which antigen-presenting cells (APC) isolated from Fas mutant *lpr* mice (*lpr*-APC) were transfected ex vivo with an adenovirus (Ad) vector (AdCMVLoxpFasL + AxCANCre) to bypass the induction of liver toxicity. Treatment of mice with *lpr*-APC-AdFasL reduced the degree of synovial inflammation and arthritis induced by *M. pulmonis* infection at both the early and chronic phases of the response to the infection (fig. 4). In associated with this, there was a significantly reduced number of PBMC and the abnormal CD3+B220+ T cells in the lymph node, but there was no enhanced induction of apoptosis in synovial fibroblast in *lpr*-APC-AdFasL-treated mice compared to *lpr*-APC-AdLacZ-treated mice. These results indicate that the combination of a genetic defect and an environment trigger can contribute to the onset and development of certain forms of arthritis, and that defective downmodulation of the T cell response by Fas/FasL-mediated apoptosis can be an important pathogenic factor in this process. These results further suggest that effective elimination of autoimmune lymphocytes and proliferating synoviocytes are both important to prevent the development of arthritis induced by mycoplasma infection.

TNFRsF that Serve as Co-stimulatory Molecules in RA

OX40 and RA

In contrast to the death receptors, the survival receptors of the TNFRSF function as co-stimulatory molecules to stimulate activation and proliferation of immune cells. Co-stimulatory molecules including OX40 and 4-1BB are expressed at low levels on naïve T cells and are inducible. OX40 has been proposed to be an inducible ligand important in TNF-α-inducing T cell activation in murine arthritis model and blocking the interaction of OX40 with its ligand suppressed the spontaneous development of autoimmune arthritis the IL-1Ra(−/−) arthritis model [13]. T lymphocytes in synovial fluid and synovial tissue from RA patients have been shown to express OX40, while OX40L was expressed on sublining cells in synovial tissue. These results suggest that ligation of OX40 with OX40 ligand may play an important pathogenic role to activate CD4+ T cells during the development of arthritis.

We have recently discovered that the BXD2 recombinant inbred strain of mice develops increased rheumatoid factor by 6 months of age and 90% of the mice develop spontaneous erosive arthritis by 1 year of age [14]. We have further identified that BXD2 mice develop an erosive arthritis associated with development of synovial fibroblasts capable of producing RANK ligand (RANKL) that

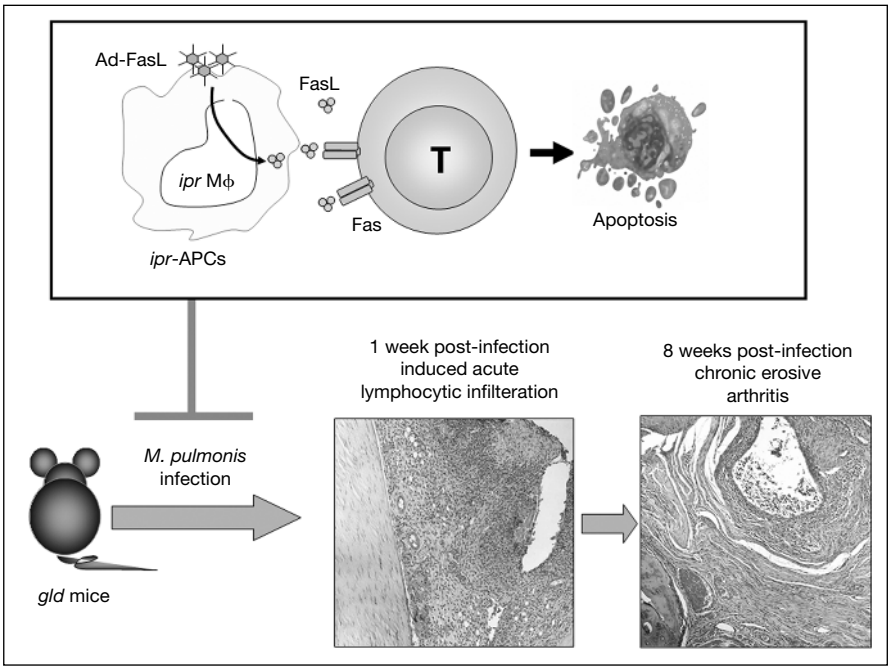

Fig. 4. Development of post-*M. pulmonis* infection-induced acute lymphocytic infiltration and chronic arthritis in *gld* mice was markedly ameliorated by *lpr*-APC-AdFasL treatment. Infection of FasL defective *gld* mice with *M. pulmonis* resulted in severe acute (1 week postinfection) lymphocytic infiltration and chronic (8 weeks postinfection) destructive arthritis and development of aggressive synovial fibroblast that eroded and invaded joint cartilage and bone. The development of *M. pulmonis*-induced arthritis in *gld* mice was significantly inhibited by simultaneous treatment of these mice with an antigen-presenting cell (APC)-FasL cell gene therapy strategy. Adenovirus was transfected into APCs derived from *lpr* mice, which lacked expression of Fas and therefore, would not undergo autocrine apoptosis. These APCs could then be transferred in vivo into mice and survive for at least 2 weeks. Systemic administration of *lpr*-APC-AdFasL is found to migrate primarily to the lymphoid organs of these mice and mediate apoptosis.

induces development of osteoclasts [15]. To determine if CD4 T cell and synovial fibroblasts interaction can result in increased RANKL production, CD4 T cells and mouse synovial fibroblasts cell lines (MSF) from B6 mice were compared to co-cultured T cells in BXD2 MSF. There was increased RANKL production by BXD2 synovial fibroblasts, compared to B6 synovial fibroblasts cultured with BXD2 T cells compared to B6 T cells, respectively. This increased RANKL production was enhanced and co-culture of BXD2 synovial fibroblasts with BXD2 T cells stimulated by anti-CD3, but there is less enhancement of B6 synovial fibroblasts co-cultured with B6 CD4 T cells. To determine if the expression of

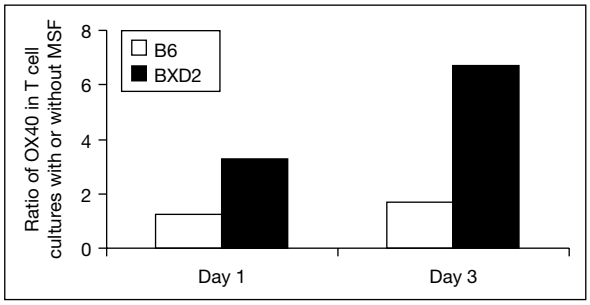

Fig. 5. MSF-induced increased expression of OX40 in T cells. MSFs were isolated from 2-month-old B6 and BXD2 mice. Early passages of MSFs from each strain were co-cultured at 37°C with T cells isolated from the isogenic strain of mice. The suspension cells (T cells) were separated from MSFs at the indicated time point. RNA was isolated from T cells. The expression of OX40 in T cells was determined using a real-time PCR (RT-PCR) method. The results represent the ratio of OX40 in T cell cultures with or without MSF for B6 and BXD2 mice.

OX40 is upregulated on T cells from BXD2 and B6 mice by interaction with synovial fibroblasts, T cells were co-cultured for 24 or 72 h in the presence of mouse synovial fibroblasts (MSF). At days 1 and 3, there was a 2- to 4-fold increase in the expression of OX40 mRNA in T cells from BXD2 mice co-cultured with BXD2 MSF compared to the expression of OX40 mRNA in T cells from C57BL/6 mice (B6) after co-culture with B6 MSF (fig. 5). These results provide evidence that the interaction of T cells from BXD2 mice with BXD2 mouse synovial fibroblasts is associated with the upregulation of OX40 in the BXD2 T cells.

4-1BB and Arthritis

The T cell costimulatory receptor 4-1BB (CD137) is induced when T cells receive antigen-specific signals. 4-1BB signaling is TRAF2-dependent and can act independently of CD28 signaling. A major effect of 4-1BB appears to be on T cell survival. 4-1BB engagement prevents activation-induced cell death in T cells, and in vivo administration of agonistic anti-4-1BB during superantigen treatment of mice prolonged T cell survival, with greater effects on CD8 T cells. This effect of 4-1BBL on T cell survival is dependent on NF-κB activation, which in turn induces Bcl-X_L and Bfl-1, as well as of cyclins D2 and E, and inhibited expression of the cyclin-dependent kinase (cdk) inhibitor p27kip1 [16]. Additionally, 4-1BB signaling has been shown to interfere with activation and function of CD4+CD25+ regulatory T cells in induced tolerance to experimental autoimmune thyroiditis [17].

Despite these findings, engagement of the 4-1BB costimulatory molecule has been shown to inhibit and reverse the autoimmune process in collagen-induced

arthritis and establishes lasting disease resistance. In the presence of 4-1BB ligation and Toll-like receptor 3 (TLR)3 and/or TLR4 triggering, CD8 T cell clonal expansion and survival was profoundly augmented. These rescued CD8 T cells suppressed CD4 T cell proliferation via a TGF-β-dependent mechanism. Thus, 4-1BB and TLR ligands induce survival of specific effector CD8 T cells with suppressive recall potential, which may explain the possible role of 4-1BB activation in prevention of autoimmune disease.

4-1BB was not expressed on T cells of BXD2 mice or B6 mice (unpubl. data). After 2 and 3 days of culture of BXD2 T cells with BXD2 MSF, there was high expression of 4-1BB in the T cells, compared to unstimulated T cells, or to B6 T cell stimulated with B6 MSF. Our results on BXD2 mice suggest that upregulation of OX40 and perhaps also 4-1BB may play an important retro-regulatory mechanism by which MSF can activate T cells, which in turn may induce other factors such as IL-17 enhance RANKL production (see below).

Role of TNFRsF RANK and RANKL in Bone Erosion

RANKL and RASF

Osteoclasts have been shown to develop adjacent to bone in rheumatoid arthritis, and are thought to cause of bone erosions [18]. It has been shown that receptor activator of NF-κB ligand (RANKL; also known as ODF, OPGL [osteoprotegerin ligand], TRANCE and TNFsF11) is essential for the induction of macrophages/monocytes to develop into mature osteoclasts. During normal bone metabolism, RANKL is expressed by the stromal cells and osteoblast precursor cells of the bone marrow and is one of the factors that couple osteoclast formation and osteoblast formation such that bone turnover is balanced appropriately. In patients with RA, both the T cells and the synovial fibroblasts have been found to produce RANKL, and it has been proposed that this promotes osteoclast development. In the TNF-α transgenic mouse model of arthritis, osteoprotegerin, a decoy receptor of RANKL, protects against joint erosions suggesting that the production of RANKL plays a pathogenic role in the development of the erosions.

Analysis of RANKL and Osteoclast Development in BXD2 Mice

We recently identified that in the joints of BXD2 mice, there are large numbers of osteoclasts that reside in close proximity to the macrophages and synovial fibroblasts in the active erosion sites [15]. Mouse synovial fibroblasts (MSFs) from the joint of BXD2 mice, but not control B6 or D2 mice, constitutively produce high levels of RANKL. Co-culture of BXD2 MSF with BXD2 macrophages resulted in the development of osteoclasts that are capable of resorbing bone as demonstrated by the osteoclast resorption pit assay. Constitutively enhanced

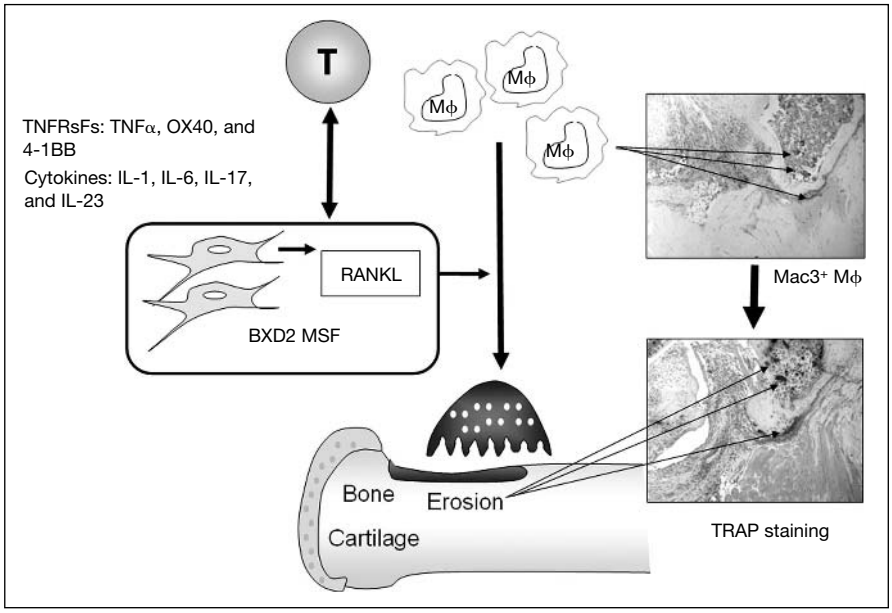

Fig. 6. A proposed model for T cell/MSF interaction to induce bone erosion in the BXD2 arthritis mouse model. We have recently identified that the BXD2 strain of mice develop an erosive arthritis associated with development of synovial fibroblasts capable of producing RANK ligand (RANKL) that induces development of osteoclasts. The possible T cell factors that may potentiate the induction of RANKL from BXD2 MSFs included several TNFsF proteins including TNF-α, OX40, and 4-1BB as well as several proinflammatory cytokines including IL-1, IL-6, IL-17, and IL-23. The representative bone erosion associated with macrophage derived osteoclasts is shown. Paraffin-embedded sections of the hind paws of 6-mo-old BXD2 mice were stained with immunoperoxidase labeled with antibody against mouse Mac-3 (dark staining upper panel) or tartrate-resistant acid phosphatase (TRAP) (lower panel).

expression by the isolated BXD2 MSF indicates that these cells do not require external signals for RANKL production. We [6] have shown increased NF-κB nuclear translocation in synovial fibroblasts from patients with RA. Increased systemic circulation of macrophages that are c-Fms-positive and express RANK has been observed in inflammatory disease [19]. It has been proposed that through the action of chemokines, these macrophages migrate from their origin in the bone marrow to the site of inflammation in the synovium. Macrophages are present throughout the hyperplastic lesion in the synovium of BXD2 mice, and are present at the interface between the site of synovial fibroblast hyperplasia and the bone, and that these macrophages likely develop into osteoclasts at this site (fig. 6).

Regulation of RANKL by TNF-α, IL-1β, IL-17 and IL-23

Upregulation of RANKL in bone marrow stroma has been shown to be regulated by many cytokines, including TNF-α and IL-1β. IL-17 is a T cell-derived, proinflammatory cytokine that is involved in the development of various inflammatory diseases. RASF exhibit increased RANKL production after stimulation with IL-17 (fig. 6). IL-17 expression is greatly enhanced in IL-1Ra(−/−) mice [20], suggesting that IL-17 activity is involved in the pathogenesis of the arthritis that develops in these mice. IL-23 is a heterodimeric cytokine composed of a unique p19 subunit, and a common p40 subunit shared with IL-12. IL-12 is important for the development of T helper (Th)1 cells that are essential for host defense and tumor suppression. In contrast, IL-23 does not promote the development of interferon-γ-producing Th1 cells, but is one of the important factors required for the expansion of a pathogenic CD4+ T cell population, which is characterized by the production of IL-17, IL-6, and TNF-α [21]. CD4 T cells and mouse synovial fibroblasts cell lines from B6 mice were compared to co-cultured T cells in BXD2 mouse synovial fibroblasts. There was increased RANK ligand production by BXD2 synovial fibroblasts, compared to B6 synovial fibroblasts cultured with BXD2 T cells compared to B6 T cells, respectively. This increased RANK ligand production was enhanced and co-culture of BXD2 synovial fibroblasts with BXD2 T cells stimulated by anti-CD3, but there is less enhancement of B6 synovial fibroblasts co-cultured with B6 CD4 T cells (fig. 6). This production of RANK ligand could be further enhanced by addition of IL-23 through co-culture. Blocking of IL-17 with anti-IL-17 did not completely this upregulation of RANK ligand. These results suggest that proinflammatory cytokines produce by T cells may play an important role in modulating RANK ligand production by BXD2 MSF.

Acknowledgements

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Mitochondria, Apoptosis and Autoimmunity

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Abstract

A functional immune system is dependent on the generation and selection of a lymphocyte repertoire that is sufficiently diverse to respond to innumerable foreign antigens yet be adequately self-tolerant to avoid the development of autoimmunity. Programmed cell death by a process known as apoptosis is responsible for negative selection of nonreactive leukocyte precursors and autoreactive thymocytes, killing of infected and transformed cells by cytotoxic lymphocytes and deletion of superfluous activated lymphocytes by activation-induced cell death (AICD) and peripheral deletion at the termination of an immune response. Mitochondrial respiration is required to meet the energy requirements of activated and proliferating peripheral lymphocytes. Several mitochondrial proteins have been implicated as regulators of apoptosis in the immune system that are required for prevention of autoimmunity. Recent discoveries have shed light on mitochondrial functions as they relate to cell death, including caspase-dependent and -independent apoptosis, mitochondrial death substrates and events that disable mitochondrial functions during apoptosis. These discoveries, taken with reports that the specific manner by which a cell dies greatly impacts on the nature of subsequent immune responses, highlight an exciting era of research on mitochondrial function and its role in apoptosis and the effects on immune responses.

Apoptosis in the Immune System

The immune system is constantly changing and adapting to defend the body from pathogens and potentially dangerous cells. The ability to mount a response requires production of large numbers of lymphocytes with a sufficiently diverse repertoire but devoid of autoreactive clones. Generation of these lymphocyte populations requires exquisite control over the selection of appropriately reactive

cells and deletion of those that are either nonreactive or excessively responsive to self-antigen. Further, vast numbers of cells produced during an immune response must subsequently be removed to maintain homeostasis. Immune cells that are no longer required are generally removed by activating apoptotic pathways. Failure to remove infected cells, potentially tumorigenic cells or cells that are no longer required, can result in lymphoproliferative disorders, autoimmune diseases, persistent infection or cancer.

Apoptosis was originally defined by the morphological features of a dying cell, including overall cell shrinkage, formation of membrane blebs, nuclear condensation and chromatin margination [1]. The underlying cellular events leading to the morphological changes during apoptosis include DNA fragmentation, as seen by oligonucleosomal ladders in gels of genomic DNA, loss of asymmetry in the plasma membrane resulting in exposure of phosphatidylserine (PS), loss of mitochondrial function and release of mitochondrial proteins into the cytoplasm. Apoptotic cells display a number of signals on the external surface of the plasma membrane indicating to macrophages, dendritic cells and nonprofessional phagocytes, such as neighboring epithelial cells, to engulf the dying cell [2]. In this way, the vast numbers of cells produced during an immune response can be readily removed, often without detection at a physical level.

It was originally thought that the purpose for death by apoptosis, in which the plasma membrane retains its integrity until well after the dying cell was engulfed, was simply to avoid spillage of cellular contents that would undoubtedly lead to a disseminated inflammatory response which would then lead to the generation of autoreactive lymphocytes following inappropriate display of self-antigens. However, in addition to allowing for efficient disposal, it is now apparent that death by apoptosis and the interactions between dying cells and phagocytes play a much greater role than originally suspected in the generation of immune cell repertoires, regulation of immune responses and maintenance of self-tolerance. Rapid and efficient removal of apoptotic cells avoids secondary necrosis, thereby tempering the inflammatory response, and defects in uptake have been implicated as a causal factor in the development of autoimmune disorders, such as systemic lupus erythematosus (SLE) [2, 3]. Uptake of apoptotic cells has also been proposed to enhance cross-priming of antigen displayed in the context of MHC class I, further influencing decisions toward tolerance following engulfment of apoptotic cells and/or apoptotic bodies.

Apoptosis in the Regulation of Immune Responses

Most healthy cells display very little PS on the outer leaflet of the plasma membrane. Loss of PS asymmetry (known as PS-flip) results in exposure of PS

on the outer leaflet of the plasma membrane and is considered a hallmark of apoptotic death. In addition to providing a convenient research marker for apoptosis, PS-flip contributes to the recognition of apoptotic cells by phagocytes expressing the PS receptor (PSR). PSR is a member of recognition receptors that bind signals displayed by apoptotic cells and facilitates uptake of apoptotic cells. Engagement of PSR has the additional effect of stimulating secretion of immune suppressive cytokines IL-10 and TGF-β by both de novo synthesis and release from intracellular stores. In fact, dying lymphocytes themselves have been shown to secrete IL-10 while in the death throes of apoptosis. Therefore, the underlying mechanisms leading to death by apoptosis contribute to the removal of dangerous cells while tempering a commensurate inflammatory responses and maintaining a suitably self-tolerant but functional peripheral lymphocyte pool.

Historically, it was thought that PS displayed on apoptotic cells triggered macrophage recognition and phagocytosis. It is now clear that several receptor/ligand interactions and opsonins are involved in this process. Engagement of CD36, αvβ3 and αvβ5 integrins, CD14 and CD68 caused tethering but little internalization. Engagement of the PSR does not trigger binding or phagocytosis on its own, but stimulates internalization of dead/dying cells that are already tethered by other receptors [2].

In the thymus, the MER surface receptor kinase has been shown to be critical for the removal of apoptotic cells. Mice with a targeted disruption of MER (MER−/−) displayed normal susceptibility to apoptosis induced by the glucocorticoid, dexamethasone, but there were increased numbers of thymocyte corpses [4]. MER−/− mice also had pronounced lupus-like defects, thus supporting the notion that apoptosis and engulfment are vital to select and maintain a self-tolerant immune cell repertoire [4, 5].

The involvement of apoptosis in immune cell regulation was firmly established when it was discovered that the underlying defects in the *lpr* (lymphoproliferative) and *gld* (generalized lymphoproliferative disorder) mouse strains were mapped to mutations in the Fas and Fas-ligand genes, respectively. Corresponding mutations in the human Fas and Fas-ligand genes are associated with autoimmune lymphoproliferative syndrome (ALPS) Type Ia and Ib, respectively (see [6] and chapters by Fas and coworkers and Rieux-Lacat, this vol.). The syndromes in both mice and humans show a diversity of phenotypic penetrance as a function of genetic background, but are characterized by splenomegaly and lymphadenopathy with an accumulation of double negative (CD4−CD8−) T cells and the presence of antinuclear and anti-DNA autoantibodies characteristic of patients with SLE.

It now appears that death by apoptosis may serve a more subtle function in that apoptotic cells present their contents to antigen-presenting cells in a

different way than necrotic cells, which release or expose their cellular contents. The first demonstration that apoptotic cells have a qualitatively different effect on the immune responses came from experiments in which cells dying by apoptosis or necrosis were fed to dendritic cells (DC) [7]. Engulfment of cells induced to die by apoptosis resulted in better antigen presentation by DC in the context of both MHC class I and class II, but DC that were exposed to necrotic cells showed a greater activation. This may reflect a tempered response by DC when only apoptotic cells are present, whereas necrotic cells are likely perceived as a danger signal that triggers a more profound immune response.

Many of the processes governing cell death, including generation of the apoptotic signals to phagocytes and the biochemical events within the dying cell will all impact on the resulting immune response. Conversely, disruption of biochemical events during death and engulfment may lead to situations in which tolerance is broken and autoimmunity results.

Pathways to Apoptosis

Generally, the morphological features that define apoptosis are orchestrated by a family of cysteine proteases known as caspases [8]. The caspase protease family can be divided into two genetically and functionally distinct groups: the initiators and executioners. Initiator caspases are characterized by a long pro-domain that serves as an interaction domain allowing intermolecular homotypic interactions with adaptor proteins. Initiator caspases (caspase-8, -9, -10 and -2) are activated by association in an activation complex whereas the executioner caspases are activated by removal of their short pro-domain and intramolecular, interchain cleavage that allows the formation of an $(\alpha\beta)_2$ tetramer [9]. Caspases share a preference for cleavage of substrates at the C-terminal side of aspartate residues, and include amongst their protein substrates, other caspases, with initiator caspases causing the proteolytic activation of the executioner caspases in a protease cascade. With one exception, the only proteases known to cleave and activate executioner caspases are other caspases. The exception is the serine protease, granzyme B, a key cytolytic effector protease expressed by cytotoxic lymphocytes [10].

Initiation of the caspase-dependent death pathways can be divided conveniently into two categories, the intrinsic and extrinsic pathways engaged by intracellular and extracellular stimuli, respectively (fig. 1). The extrinsic pathway is characterized by death induced by ligands of the tumor necrosis family (e.g. TNF-α, Fas-ligand (CD95L; CD178) and TRAIL) upon engagement of their corresponding receptors (i.e. TNFR1, Fas (CD95) and DR4/5) which triggers recruitment of adaptor molecules that facilitate the association and activation of caspase-8 (and possibly caspase-10).

The intrinsic pathway is engaged in response to an intracellular stress or disruption to a cellular process. The signals that trigger the intrinsic pathway are many and varied, including, but not limited to, genotoxic stress and growth factor withdrawal or transfer of granzyme B from cytotoxic lymphocytes, and most rely on the transmission of a biochemical death signal via the mitochondria, centrally placed in this pathway. During apoptosis, the mitochondrial outer membrane becomes permeable releasing several soluble proteins from the mitochondrial intermembrane space, including cytochrome *c* and SMAC/Diablo. After release, cytochrome *c* interacts with the cytosolic adaptor protein, APAF-1 and dATP to form a complex known as the apoptosome which forms the foundation upon which caspase-9 is recruited and activated [11]. Caspase-9, although assuming an active conformation at this point, is not yet free to perform its apoptotic duties; it is held inactive by members of the inhibitor of apoptosis protein (IAP) family [12], predominantly the X-linked IAP (XIAP), with possible involvement of c-IAP1 and c-IAP2. The inhibitory effect of XIAP is repressed by SMAC/Diablo [13, 14], a resident mitochondrial protein released coordinately with cytochrome *c*, although not necessarily in a mechanistically identical fashion. Repression of IAP by SMAC/Diablo targets the former for degradation by the proteasome and allows 'release' of active caspase-9, which then cleaves and activates caspase-3. Caspase-3 can also be inhibited by XIAP, an inhibition that is overcome by SMAC/Diablo. Activation of the executioner caspases (caspase-3, -6 and -7) then results in the generation of cellular hallmarks and morphological features that have come to define apoptosis.

Although the intrinsic and extrinsic pathways are often drawn in parallel, there are many points of crosstalk and potential for feedback and/or amplification. In the intrinsic pathway, the mitochondrion figures prominently and therefore 'normal' mitochondrial function and activities of resident mitochondrial proteins are central to affecting death processes. It now appears that pathways utilized and the biochemical events leading to death will impact on the efficiency of clearance of a deleterious or undesirable cell population, such as autoreactive thymocytes and the events during death can impact on the nature of an immune response in the periphery.

Modulation of Mitochondrial Events in Apoptosis – the Bcl-2 Proteins

Modulation of mitochondrial events during apoptosis is regulated in large part by the Bcl-2 proteins. This protein family consists of members related by the BH (Bcl-2 homology) domains and are divided into two groups, one with activities leading toward engagement of apoptotic process and the other that

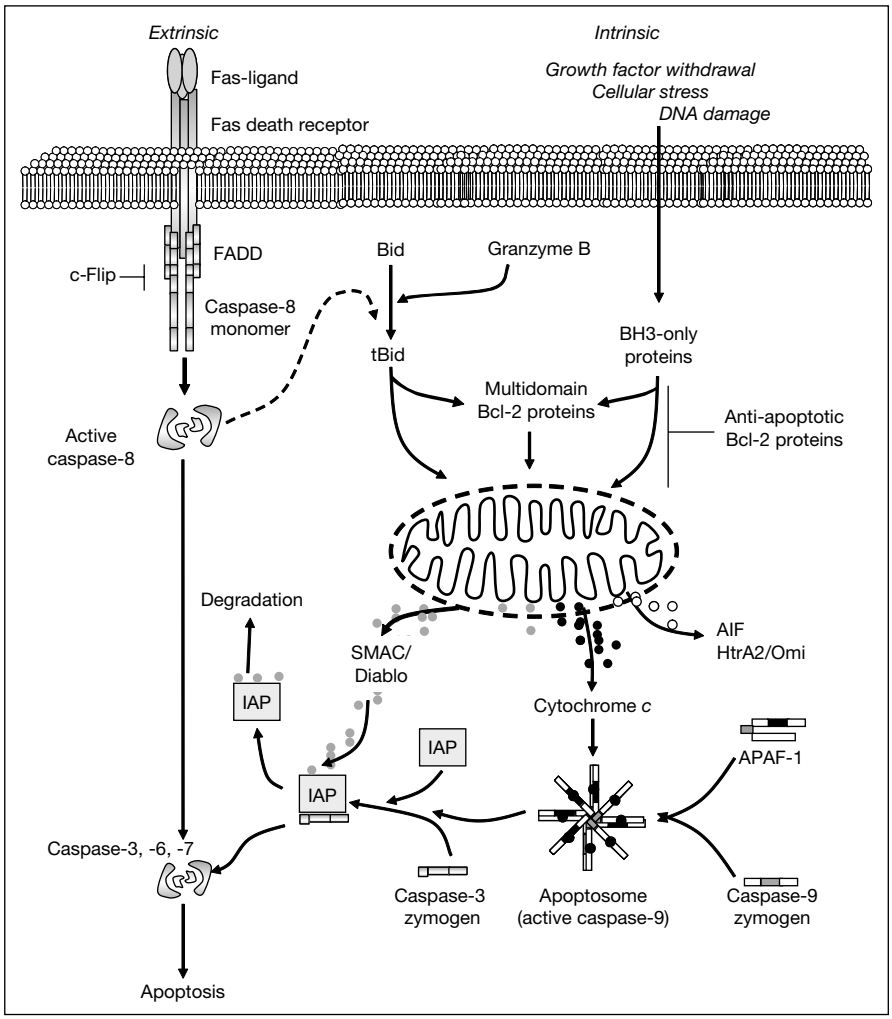

Fig. 1. General pathways of apoptosis. In the intrinsic pathway, transmission of stress signals is mediated by the BH3-only members of the Bcl-2 protein family. BH3-only proteins function with the pro-apoptotic multidomain (BH1–3) Bcl-2 proteins to effect release of mitochondrial proteins. The concerted action of the pro-apoptotic Bcl-2 proteins is countered by the pro-survival members of this protein family (fig. 2). Upon release into the cytosol cytochrome *c* is recruited into the apoptosome with APAF-1 and dATP resulting in recruitment and activation caspase-9. Release of active caspase-9 is facilitated when XIAP inhibition of caspase-9 (and possibly caspase-3) is overcome by SMAC/Diablo, which is released from mitochondria concurrently with cytochrome *c*. Activated caspase-9 cleaves and activates caspase-3 and the executioner caspases which bring about the end stages of the death program. Release of additional mitochondrial proteins, AIF and HtrA2/Omi mediate nuclear events during death that are not dependent on the presence of active caspases, although

presents an opposing, pro-survival activity (fig. 2). Bcl-2, the prototypic family member was discovered in at t(14:18) translocation in follicular B-cell lymphomas which placed the Bcl-2 gene under the regulation of the immunoglobulin heavy chain promoter and resulted in inappropriate Bcl-2 overexpression and survival of B cells that should not have escaped selection [15]. Survival of immature and potentially self-reactive B cells into the periphery leads to two undesirable scenarios: escape of B cells capable of surviving in the absence of appropriate growth signals and survival of potentially self-reactive clones. A role for Bcl-2 proteins in T cell selection was proposed shortly after the elucidation of its role in B cell maturation and selection, however it was discovered that inappropriate overexpression of Bcl-2 during T cell development, although central deletion was reduced, overexpression did not adversely affect the establishment of self-tolerance during thymocyte selection [16]. As additional Bcl-2 proteins were discovered, our understanding of their involvement in apoptosis in the immune system has also increased, with family members involved in virtually every stage of lymphocyte selection, development, activation and deletion. Members of this protein family have emerged as key regulators of lymphocyte apoptosis and therefore on events that influence the prevention or development of autoimmunity [16].

The first members of the Bcl-2 family were discovered based on an ability to protect cells from various apoptotic stimuli, but it was soon discovered that some members of this growing protein family, although they share regions of significant homology, appeared to be necessary to promote apoptosis. As the family grew, it became evident that the family could be subdivided into two groups, one promoting cell death and the other opposing the action of the first and thus protecting from apoptosis. The pro-apoptotic group is further subdivided based on structural and functional criteria. The BH3-only proteins contain only the third BH-domain and appear to function as sensors of intracellular stress. BH3-only proteins, upon activation, function in concert with a member of the second subgroup BH1-3 proteins (fig. 2) [16].

Many of the stressors that stimulate the BH3-only proteins are encountered under physiological conditions common during lymphocyte development

release of AIF appears to require caspase activity. The extrinsic, death receptor, pathway is triggered by caspase-8 recruitment to the death inducing signaling complex (DISC) which forms when a death receptor is engaged by its ligand. The nature of the DISC and the mechanism by which caspase-8 becomes activated is dependent on the specific death receptor. Active caspase-8 has been shown to cleave the BH3-only protein, Bid, to engage the intrinsic pathway or, alternatively to cleave and activate caspase-3 directly. The latter of which is not inhibitable by Bcl-2.

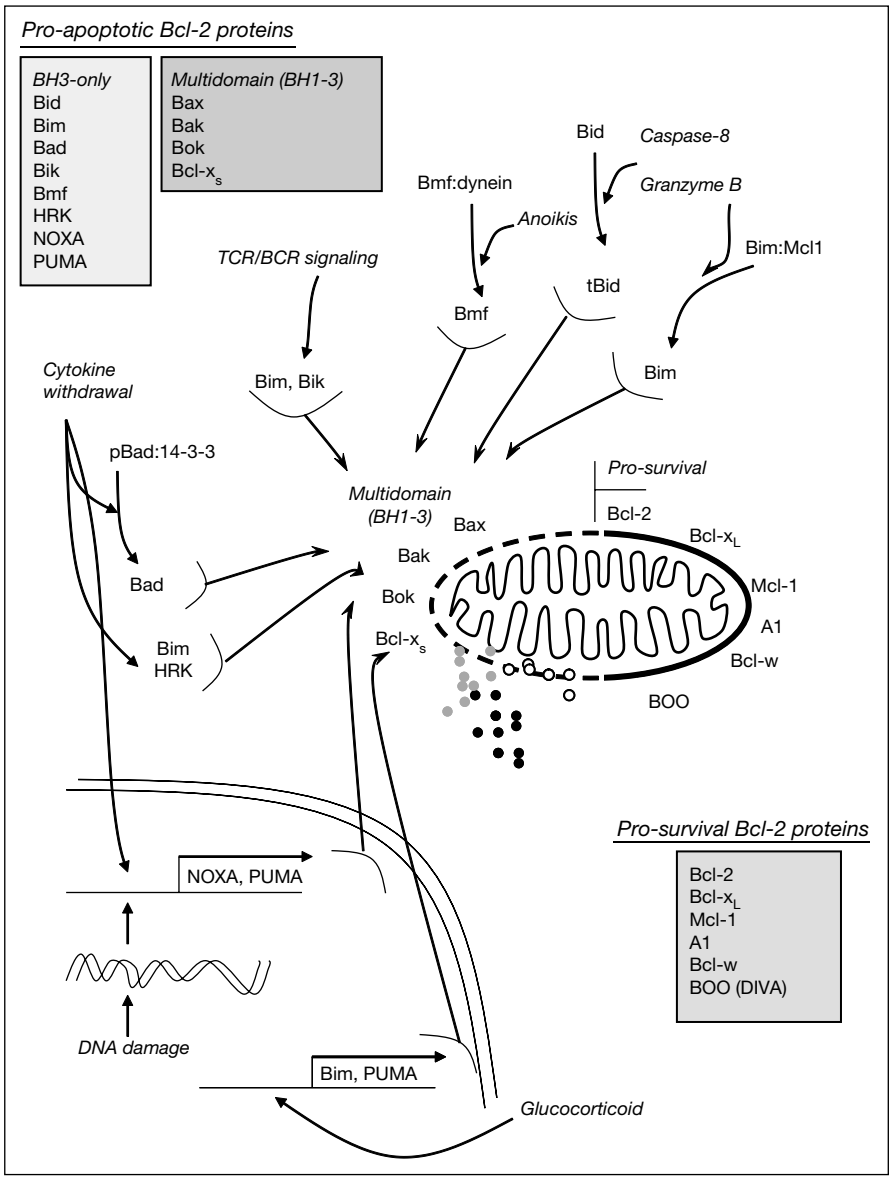

Fig. 2. The activities of the three groups of the Bcl-2 protein family. BH3-only proteins serve as sensors of cellular stress, which includes growth factor (cytokine) withdrawal, detachment from substrata, encounter with cytotoxic proteases (e.g. granzyme B or caspase-8 vs. Bid), glucocorticoid by immature thymocytes, genotoxic stress (e.g. DNA-damaging chemotherapeutics) and antigen receptor engagement in T and B cells. After activation, BH3-only proteins interact with multidomain (Bax-like) proteins that then integrate or associate

and function. For example, glucocorticoid, an effective inducer of death in thymocytes, has been shown to induce expression of Bim and PUMA [16]. Similarly, cytokine withdrawal results in the removal of signaling events and trigger Bim and Hrk while inducing expression of PUMA and NOXA under certain conditions. Bim, along with Bmf, have been implicated as sensors of anoikis, caused by cell detachment and disruption of integrin signaling and disturbance of the actin cytosleleton. Disruption of the cytoskeleton in this way causes Bmf dissociation from the dynein light chain of the myosin V motor complex. Cytokine withdrawal has also been shown to result in the reduced phosphorylation of pBad causing Bad to dissociate from 14-3-3 proteins and migrate to the mitochondria. Phospho-Bad has also been found in the mitochondrial intermembrane space in a complex with the enzyme glucokinase, which normally converts glucose to glucose 6-phosphate, effectively trapping glucose in the cell for use in glycolysis. In this context, dephopshorylation of Bad (whether by direct dephosporylation or inhibition of Bad-kinases) during glucose deprivation is a trigger for Bad activation.

Bid is currently the only member of this group known to be activated by cleavage. The cytotoxic protease granzyme B and the initiator caspase, caspase-8, both have proteolytic activity against Bid, albeit at different cleavage sites. The C-terminal fragment of cleaved Bid, known as tBid, is the apoptotically active fragment, although a role for the N-terminal fragment has not been ruled out [17].

Genotoxic stress (such as that induced by DNA damaging chemotherapeutic agents failed mismatch repair or during recombination during lymphocyte development) induces PUMA and NOXA by a p53-responsive mechanism [18] and p53 itself has recently been demonstrated to possess an activity similar to that of the BH3-only proteins [19]. Importantly, signaling through the T cell and B cell antigen receptors in autoreactive clones, which is the hallmark of apoptosis induced during negative selection, causes Bim and Bik-dependent apoptosis. Clearly, dysfunction of any of these proteins can have profound impact on the susceptibility of immature and self-reactive lymphocytes to physiological death stimuli, thus creating an environment conducive to the development of autoimmunity. It is worth noting that the effects of the BH3-only proteins in the immune system are often more prominent in conjunction with cell cycle

with the mitochondrial membrane which results in release of apoptogenic proteins. The concerted action of BH3-only and Bax-like proteins is inhibited by members of the pro-survival family members. It is important to note that not all members of each group is capable of substituting for all members in its group and not all pro-survival members confer protection for all members of the pro-apoptotic groups.

defects. For a comprehensive review of BH3-only proteins in the immune system see reference [16] and the chapter by Hughes et al. in this volume.

Each of the BH3-only proteins is capable of translocating to the mitochondria and acting in concert with a member of the multidomain group to trigger release of mitochondrial proteins. The multidomain group includes Bax, Bak, Bok and Bcl-x_s. Recent evidence suggests that 'activated' BH3-only proteins trigger monomeric Bax (and by extension its relatives) to form openings in the mitochondrial outer membrane of a size sufficient to allow passage of macromolecular complexes [20]. Formation of Bax membrane pores is dependent on the lipid composition of the outer membrane, with cardiolipin as a key component [21]. Interestingly, cardiolipin is primarily found in specific regions of the outer membrane and apparently absent from contact points between the outer and inner membranes, which may serve to restrict permeabilization by Bax to the outer membrane, thus providing access only to proteins of the intermembrane space.

Bax and Bak appear to have overlapping or compensatory roles in apoptosis and their importance has been clearly established from studies using mice with targeted disruption of both Bax and Bak genes. Initial characterization showed a severe lymphadenopathy and splenomegaly among other developmental disturbances. To limit the investigation into the role of Bax/Bak in thymocyte selection, hematopoietic stem cells from Bax−/−Bak−/− mice were used to reconstitute RAG1−/− recipient animals [22]. Bax−/−Bak−/− recipients displayed significant increase in the percentage of CD3+CD4−CD8− double-negative thymocytes. Further, thymocytes from these animals displayed an overall resistance to apoptosis induced by glucocorticoid and anti-CD3 stimulation relative to wild type counterparts. Consistent with the thymocyte profile (i.e. high double-negative population) indicative of a defect in negative selection, thymocytes from these mice were also able to survive much longer than wild-type controls ex vivo and were largely resistant to cytokine withdrawal. The phenotype of Bax−/−Bak−/− reconstituted mice with respect to defects in thymic and lymphoid development is similar to that observed for knockout mice lacking the BH3-only protein, Bim, suggesting that Bim and Bax/Bak function in a common pathway as critical mediators of apoptosis in negative thymocyte selection.

Further evidence to support a major role for Bcl-2 proteins in thymic development comes from studies of IL-7 receptor alpha chain knockout mice, IL-7Rα−/−. IL-7 is required for normal T development and withdrawal, by depletion of IL-7 or disruption of the receptor, leads to thymocyte apoptosis due to cytokine withdrawal that is modulated by the Bcl-2 proteins. IL-7 has been shown to induce the expression of Bcl-2 and in the presence of IL-7R signaling, Bad is rendered inactive by phosphorylation and sequestration in complex with 14-3-3 proteins. Removal of IL-7 results in downregulation of Bcl-2 and activation of Bad which functions with Bax to induce apoptosis. Studies in mice with

targeted disruption of both Bax and IL-7Rα genes showed that deficiency of Bax partially rescued thymocytes from a deficiency in IL-7Rα [23]. It will be interesting to learn if the Bax−/−Bak−/− double knockout will completely rescue thymocytes devoid of IL-7R signaling.

Bcl-2 sequesters BH3 domains of the BH3-only proteins, thereby preventing BH3 motifs from inducing the conformational changes necessary for oligomerization of Bax/Bak in the outer mitochondrial membrane [20]. The Bcl-2 domain responsible for interacting with BH3 domains has been targeted for therapeutic potential. Small molecule inhibitors were designed based on the BH3-binding region of Bcl-x_L and used to target Bcl-2 in a region that prevents sequestration of BH3-only proteins, thus sensitizing cells to apoptotic stimuli [24]. While not directly apoptotic when delivered alone, one of these BH3 mimetopes was used successfully in a small cell lung cancer xenograft model and found to sensitize cells to apoptotic stimuli that were previously blocked by Bcl-2. It is important to note that this inhibitor was not apoptotic by itself and still required a trigger to induce death.

The follicular lymphoma cell line, FL5.12, is dependent on IL-3 to survive, however, when these cells overexpress Bcl-x_L, they are resistant to IL-3-withdrawal. It is interesting that Bcl-x_L expressing FL5.12 cells do not continue to grow and proliferate as they do in the presence of IL3, but they undergo a reduction in size and downregulate most cellular functions leading toward a senescent like phenotype that can be rescued upon re-introduction of IL-3 [25]. While it remains arguable as to the actual function a cell might carry out once it has become senescent, in the context of immune cell selection and repertoires, etc., space itself may be an important consideration in the body's ability to mount an appropriate immune response. Therefore, a cell that is capable of surviving in the absence of growth factors, even if it is incapable of performing basic functions, such as proliferation, may impact the potential for additional responses, simply by occupying the space required to expand another responding lymphocyte pool.

Electron Transport

Mitochondria are key organelles in all organisms that rely on aerobic respiration. They appear to have arisen from the symbiotic relationship between an α-protobacteria and an early amitochondriate eukaryotic cell – although the identity of the early players and the nature of the first union or if this union ever took place remains open to scientific and philosophic debate. Mitochondria consist of two lipid bi-layers, of which the inner membrane may be derived from the protobacteria and the outer membrane may be derived from the eukaryotic cell

(fig. 3). The inner membrane encapsulates the matrix, which houses such activities as the Krebs cycle (also known as the tricarboxylic acid; TCA) cycle, detoxification of reactive oxygen species (ROS) and production of proteins encoded by mitochondrial specific DNA. The mitochondrial inner membrane contains the complexes of the electron transport chain that generates a proton based transmembrane potential ($\Delta\Psi m$) that fuels the generation of ATP by ATP synthase. The compartment formed between the two mitochondrial membranes, known as the intermembrane space, houses proteins such as cytochrome *c* which transfers electrons from electron transport chain complex III to complex IV in the electron transport chain and creatine kinase which participates in creatine metabolism. The outer and inner membrane also contain protein complexes required for the transport of nuclear encoded proteins to their respective compartments (TIMs and TOMs) and translocation of metabolites such as ADP/ATP (VDAC and ANT). Functionally, mitochondria are also involved in cellular processes such as metabolism of amino acids, steroids and fatty acids, and have been proposed to act as a storage sink for intracellular calcium and maintain pH all of which may impact on cellular stress and viability [26].

Therefore, in addition to promoting caspase activation (as discussed in figures 1 and 2), release of proteins from mitochondria during apoptosis is likely to impact on vital survival functions carried out by mitochondria. For example, loss of cytochrome *c* would likely impair its ability to transfer electrons from complex III to complex IV thereby impairing ability of mitochondria to generate $\Delta\Psi m$, resulting in loss of ATP production. Indeed it has been proposed that in cases where caspase activation is inhibited, loss of mitochondrial function as a consequence of release of proteins from the intermembrane space may result in death of the cell by a nonapoptotic program as a consequence of passive loss of ATP or an active process mediated by other mitochondrial proteins such as AIF or EndoG. Recent evidence, however, suggests that loss of mitochondrial functions may be an active process rather than the passive loss of function originally proposed.

The electron transport chain is responsible for generating and maintaining the proton gradient across the inner mitochondrial membrane that is measured as $\Delta\Psi m$. $\Delta\Psi m$ is important for production and transport of ATP as well as transport of mitochondrial proteins and metabolites. Loss of $\Delta\Psi m$ is indicative of death by apoptosis and a relationship between $\Delta\Psi m$ drop and death has recently been established. In an unbiased search for caspase-3 substrates, p75 (NDUSF1) was identified [27]. p75 is a 75-kDa subunit of the respiratory complex I, which, with coenzyme Q, transfers electrons to complex III (fig. 3). Cleavage of p75 by caspase-3 is a destructive event that impairs electron transport at one of the two earliest entry points (fig. 4). Use of mutants of p75 (p75(D255A)) rendered uncleavable by caspase-3, shed new light on the

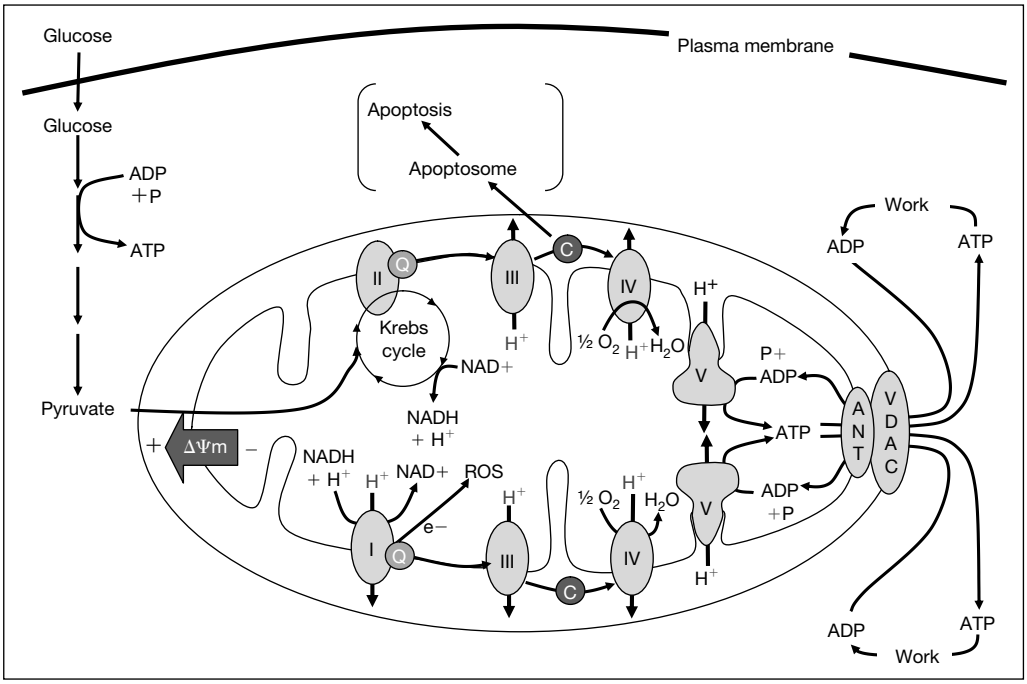

Fig. 3. Mitochondrial respiration. Energy in the form of ATP is produced by complex series of events in which pyruvate, the final product of glycolysis, is transferred to the mitochondrial matrix where it fuels the Kreb's (TCA) cycle. As part of the Krebs cycle, succinate is converted to fumarate by complex II resulting in the production of an electron that is transported along the electron transport chain, first by coenzyme Q to complex II and then by cytochrome *c* to complex IV. A proton gradient of approximately −180 mV ($\Delta\Psi$m), produced as a consequence of transfer of electrons along the electron transport chain is then used by complex V to convert ADP and free phosphate to ATP. ATP is exported by the adenine nucleotide transporter (ANT) and voltage-dependent anion channel (VDAC) to release ATP into the cytosol to fulfill cellular requirements. Reactive oxygen species are produced primarily as a byproduct of the reactions involving electron transport from complex I to complex III. Release of cytochrome *c* without concurrent termination of the early events in electron transport could therefore result in continued ROS production until the late stages of death at which point mitochondrial function fails terminally. C = Cytochrome *c*; Q = coenzyme Q; I–IV refer to the respective respiratory complexes.

importance of suppressing mitochondrial activity during caspase-dependent apoptosis. Cells expressing p75(D255A) showed sustained $\Delta\Psi$m and ATP levels during the response to apoptotic stimuli. ROS production was reduced, which suggests that ROS production is a byproduct of caspase-dependent

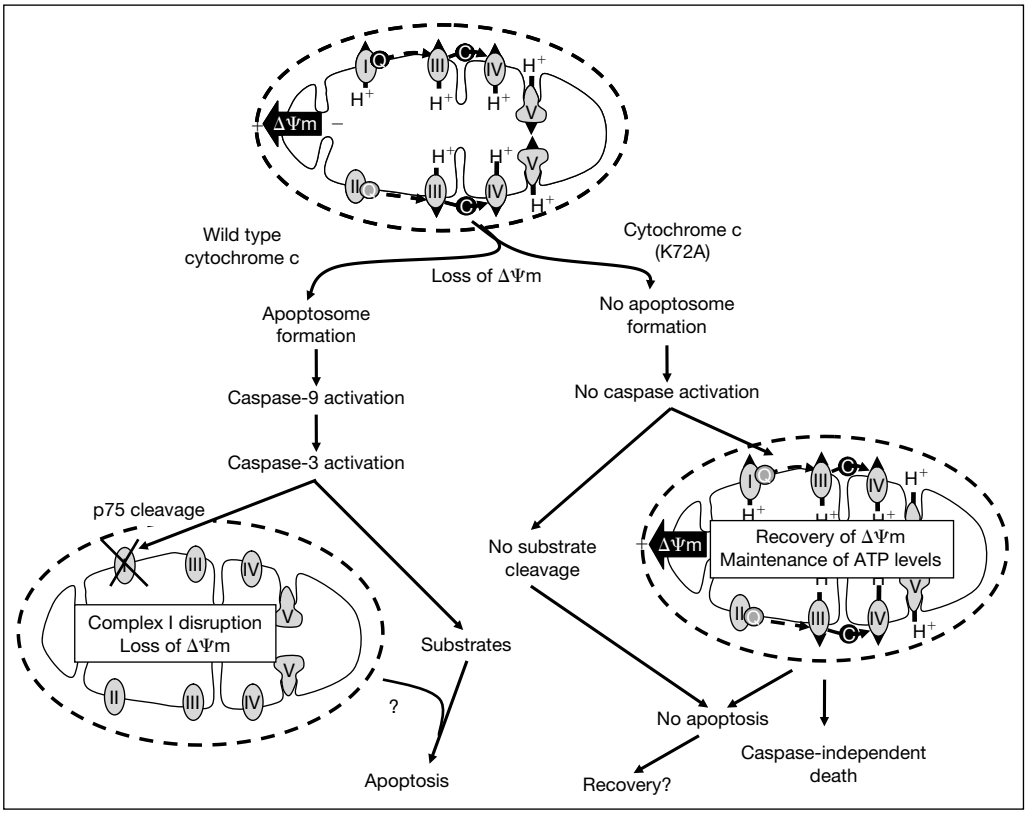

Fig. 4. Release of cytochrome *c* results in caspase-9 activation following formation of the apoptosome. Active caspase-9 cleaves and activates caspase-3 which then, among its apoptotic substrates, feeds back on the mitochondria where it cleaves p75, a component of the respiratory complex I. Cleavage of p75 is proposed to terminate the activity of complex I, thereby diminishing ATP production, attenuating ROS production and ensuring a sustained drop in $\Delta\Psi m$ and cessation of survival functions as the dying cell enters the final stages of apoptosis in preparation for engulfment. Mutant cytochrome *c* which retains electron transport activity but is unable to form a functional apoptosome cannot initiate the apoptotic program. Release of a cytochrome *c* mutant unable to form a functional apoptosome has allowed the dissection of cytochrome *c*'s apparently disparate death functions and established a clear role for cytochrome *c* as an active player in thymocyte and lymphocyte apoptosis.

apoptosis events rather than a causal factor. As expected, other caspase-mediated events were not affected, such as cytochrome *c* release and DNA fragmentation, however membrane events were significantly delayed. There was a sustained asymmetry of the plasma membrane and delayed breach of plasma membrane integrity indicating delayed progression to secondary necrosis.

Although it has not yet been formally established, there are profound implications of this discovery with respect to the regulation of immune responses. Taken with the observation that cells dying by necrosis elicit a differential effect on APC activation compared with apoptotic cells, it is a distinct possibility that caspase-dependent attenuation of mitochondrial function contribute to the signals that influence APC activation. At a simplistic level, the delay of PS-flip will also delay recognition of a dying cell by phagocytes, which in turn will determine the degree to which a dying cell is allowed to progress down the death pathway before being engulfed and therefore will determine the manner in which it is perceived by the phagocyte.

Due to the indispensable involvement of cytochrome *c* in respiration, simple ablation of the cytochrome *c* gene only demonstrated that cytochrome *c* was required for progression of apoptosis in cells utilizing glycolysis as the major source of ATP. Mutation of a critical lysine residue (K73A) abrogated the ability of cytochrome *c* to interact with APAF-1, but left the electron transport functions intact [28]. The inability of released cytochrome *c* to form a functional apoptosome has allowed the dissection of the former's life and death functions (fig. 4). Mice carrying cytochrome c KA/KA displayed severe lymphoid disturbances including depletion of double positive (CD4+CD8+) thymocytes, marrow pre-B cells, and splenic B and T cells. The lymphoid deficiencies in the KA/KA mice were found to be the result of external factors because reconstitution of recipient mice by adoptive transfer of KA/KA cells resulted in a phenotype similar to Bim−/− and Bax−/−Bak−/− knockouts, notably lymphadenopathy and splenomegaly. Activation-induced cell death appeared to be relatively normal following LCMV challenge, but some T cells showed an enhanced resistance to secondary stimulation with anti-CD3, which is an indicator of a pre-disposition to development or persistence of autoreactive clones. Further data from the KA/KA mice suggest that stress induced thymocyte death proceeds in a manner that is independent of apoptosome formation but is still caspase-dependent and a function of APAF-1 monomers.

An interesting additional note to the release of mitochondrial proteins is that it has been reported that AIF and EndoG release is dependent on active caspases, whereas cytochrome *c*, SMAC/Diablo and HtrA2 is not [29]. This opens the possibility of selective release of proteins from the intermembrane space. Given that the macromolecular pore formed by Bax should be sufficiently large to allow passage of both AIF and EndoG, there may be other forces to overcome to effect their release, such as cleavage of an adaptor protein or disruption of a mitochondrial complex. There is a strong likelihood that a cell that is not capable of fully engaging the execution phase of apoptosis may be capable of recovering its mitochondrial function. This may bear on the survival of peripheral

T lymphocytes during the period of activation during which caspase-8 activity appears to be necessary for lymphocyte function, without the induction of apoptosis.

Mitochondrial Metabolism in Life and Death Decisions

There is emerging evidence showing the involvement of additional mitochondrial proteins and metabolic pathways impacting life and death decisions. In resting lymphocytes, little energy is required to meet cellular demands. However, upon activation by encounter with antigen or nonrestricted (polyclonal) triggering of the TCR, metabolic requirements escalate rapidly and mitochondrial activity responds accordingly to meet new demands.

Transcription and translation of cytokines and effector proteins is rapidly ramped up as is the replicative machinery necessary to drive clonal expansion. With the new activation and proliferation state comes an increased demand for energy that is met, in large part, by a greater dependence on aerobic processes governed by mitochondrial events such as the TCA cycle and electron transport/oxidative phosphorylation. Increased mitochondrial energy output comes with a commensurate increase in the byproducts of energy generated in this way. Under normal physiological conditions, production of ROS may serve as a feedback signal indicating the output of the complex I-associated entry into the electron transport chain. Elevated ROS are often observed during apoptosis, thought to be a consequence of mitochondrial dysfunction during death. In AICD of peripheral T lymphocytes, ROS produced as a byproduct of heightened mitochondrial activity during lymphocyte activation and proliferation, was proposed to be the triggering factor of AICD. A mechanism whereby ROS initiates apoptosis is not known, but elevated ROS have been postulated to induce the expression of Fas-ligand and initiate the Fas-mediated apoptosis pathway. This latter model is consistent with early reports showing the necessity for Fas: Fas-ligand interactions and the associated death pathway for AICD of peripheral T cells.

Further linking mitochondrial functions with life and death decisions, the TCA cycle enzymes succinate dehydrogenase and fumarate hydrolase, have both been implicated as tumor suppressors. SDH is a multimeric enzyme that catalyzes the conversion of succinate to fumarate in the mitochondrial matrix. Inhibition or dysfunction of SDH results in the accumulation of cytosolic succinate that HIF-α prolyl hydroxylases, which in turn prevents the de-stabilization of HIF-α by the von Hippel-Lindau gene product (pVHL) [30]. Therefore, increased cytosolic succinate levels leads to a sustained stabilization and activation of HIF-α. HIF-α then induces expression of genes responsible for angiogenesis and

glyocolysis, all of which contribute to survival and growth of transformed cells into tumors. These recent data showing a connection between the TCA cycle and tumorigenesis is currently outside the realm of autoimmunity, however, any affect on a cellular process that confers altered growth and survival advantages under suboptimal conditions has the potential to affect the death of lymphocytes, whether in the progression of lymphoid tumors or development of autoimmunity. This example illustrates how mitochondrial function, and in this case dysfunction, communicates signals to the cytosol and nucleus which in turn have a profound impact on life and death decisions.

It is interesting to note that thymus specific ablation of the VHL gene resulted in reduced thymic cellularity corresponding to a significant reduction in the number of CD4+CD8+ double-positive, CD4+ and CD8+ single-positive subsets [31]. The decreased numbers of thymocytes was attributed to increased apoptosis that is triggered in a HIF-dependent manner. Caspase-8 was implicated as a key effector in this model, as a caspase-8 inhibitor restored numbers of VHL null thymocytes. It is not yet clear how the apparently apoptotgenic effects of HIF1α in the thymus can be reconciled with the apoptosis inhibitory activity observed in SDH mutants. It is possible that physiologically occurring hypoxic microenvironments in the thymus rely on HIF activity for some vital function during thymocyte development.

Conclusion

As the mechanisms of apoptosis become more clearly defined, another subtle subtext is also emerging: that the biochemical and cellular events that occur during cell death have a profound impact on the manner in which dying cells interact with their environment. Therefore, how an immune cell dies influences the regulation of positive and negative selection, peripheral lymphocyte deletion and the regulation of immune responses as they contribute to tolerance or autoimmunity.

Disclaimer/Apologies

It has not been possible to cite comprehensively all of the primary literature for the many thousands of research reports that have led to discoveries discussed in this chapter. We apologize to the countless numbers of researchers whose invaluable contributions to this explosive area of research have not been cited directly.

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Role of Bim and other Bcl-2 Family Members in Autoimmune and Degenerative Diseases

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Abstract

Apoptosis is essential for the development, function and homeostasis of the immune system. Experiments with transgenic and gene knock-out mice have shown that defects in the control of apoptosis in the hematopoietic system can promote the development of autoimmunity or hematological malignancy. In contrast, excessive apoptosis of normally long-lived hemopoietic cells can lead to lymphopenia and immunodeficiency. In mammals, cell death in response to developmental cues and many cell stress signals is regulated by the opposing factions of the Bcl-2 family of proteins. In particular, the pro-apoptotic subgroup called BH3-only proteins, which includes Bim, is critical in the initiation of apoptosis in response to many death stimuli. Bim has been found to be an important regulator of the negative selection of B lymphocytes in the bone marrow and of T lymphocytes both in the thymus and the periphery. Mice lacking Bim accumulate self-reactive lymphocytes, develop autoantibodies and on certain genetic backgrounds succumb to SLE-like autoimmune disease. Abnormalities in Bim expression and the thymic deletion of auto-reactive lymphocytes have also been implicated as a component of the complex, polygenic predisposition to autoimmune diabetes seen in NOD mice. Bim is also an essential regulator of T lymphocyte apoptosis during the termination of an immune response. This chapter focuses on the role of Bim in the development and function of the immune system and its potential role in autoimmunity. Degenerative disorders due to increased apoptosis mediated by Bim are also discussed.

Introduction

All metazoan organisms have developed the mechanism of programmed cell death, or apoptosis, for the destruction and removal of redundant, damaged or infected cells [1, 2]. Apoptosis is required for normal tissue morphogenesis,

maintenance of homeostasis and development and function of an effective immune system. Abnormalities in the cell death pathway have been implicated as a cause or contributing factor in autoimmune disease, cancer, immunodeficiency and certain other degenerative diseases [2]. This chapter focuses on Bim, a pro-apoptotic member of the Bcl-2 protein family, that is essential for initiation of developmentally programmed cell death and stress-induced apoptosis [3]. The role of abnormalities in Bim function in autoimmunity, defects in immune system function and degenerative diseases will also be covered.

Overview of the Molecular Control of Apoptosis

Apoptotic cell death is characterized by cellular destruction by aspartate-specific cysteine proteases, called caspases [4]. Through proteolysis, caspases destroy vital cellular proteins, such as lamin or gelsolin, and activate enzymes that degrade DNA (CAD) or promote cell demolition by some other process [4]. According to their structure and function, caspases can be divided into two groups: effector caspases (caspase-3, -6 and -7), which cleave several hundred cellular substrates, and the initiator caspases which activate the effector caspases from their inactive zymogen state [4]. In mammals two distinct but ultimately converging apoptosis signaling pathways control the activation of caspases [5]. These are the Bcl-2 protein family-regulated pathway (also known as the stress, mitochondrial or intrinsic pathway) and the death receptor (also called extrinsic) pathway (fig. 1).

The death receptor pathway is triggered when so called death receptors, members of the tumor necrosis factor receptor (TNF-R) family with an intracellular death domain (DD, such as Fas/APO-1/CD95 or TNF-R1, on the cell surface are engaged by their ligands [2]. Upon activation, death receptors recruit the adaptor protein FADD (Fas-associated death domain) via homotypic interaction directly (or, in the case of TNF-R1, indirectly via another adaptor, TRADD) to its death domain. FADD then binds via its death effector domain (DED) to one of the two DEDs in pro-caspase-8. Aggregation promotes activation and proteolytic processing of pro-caspase-8 molecules and this then allows activation of effector caspases. In some cell types, including lymphocytes, this pathway only converges with the Bcl-2-family-regulated pathway at the level of effector caspase activation. However, in some other cell types, such as hepatocytes, caspase-8 proteolytically activates the pro-apoptotic Bcl-2 family member Bid, which then engages the Bcl-2-regulated apoptotic pathway (see below). This appears to act as a critical amplification loop in these cells to generate sufficient caspase activity for apoptosis to proceed [6].

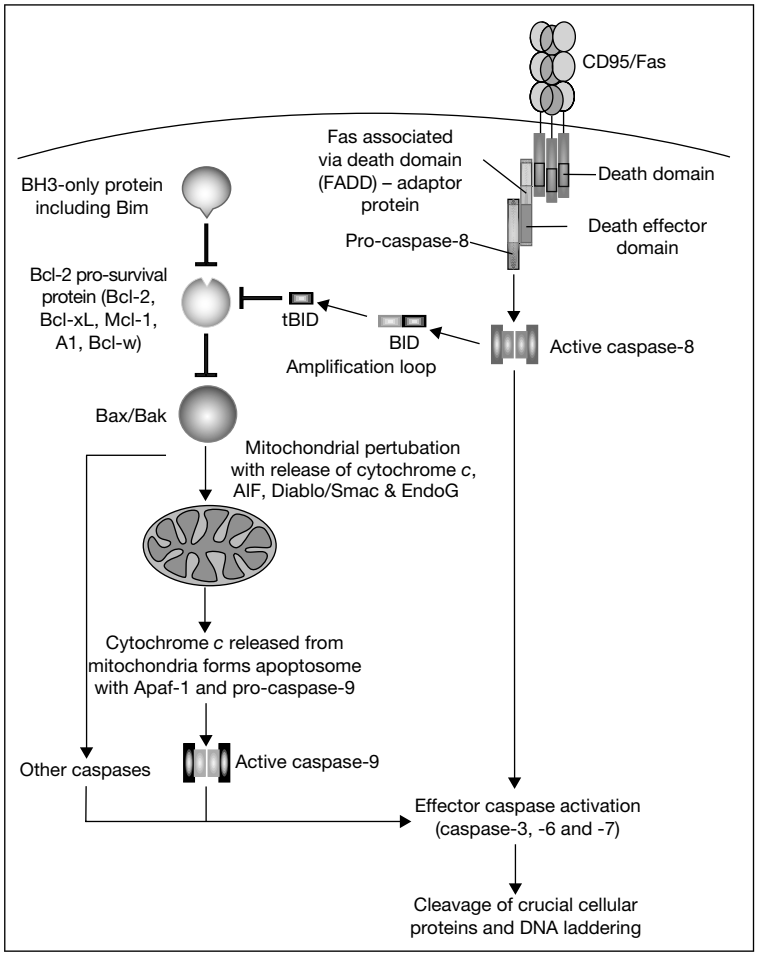

Fig. 1. Two major pathways to apoptosis. Apoptosis is mediated either by the death receptor pathway (extrinsic) or by the Bcl-2 family-regulated pathway (intrinsic). Both pathways converge on the activation of effector caspases (see text).

The Bcl-2-regulated apoptotic pathway is activated by a broad range of stimuli, including developmental cues, cytokine withdrawal and many cytotoxic drugs. The decision of whether a cell will survive or undergo apoptosis is made by the interaction of the various pro- and anti-apoptotic members of the Bcl-2 protein family [2].

The Bcl-2 protein family consists of approximately twenty members that are related to each other by four regions of significant amino acid sequence

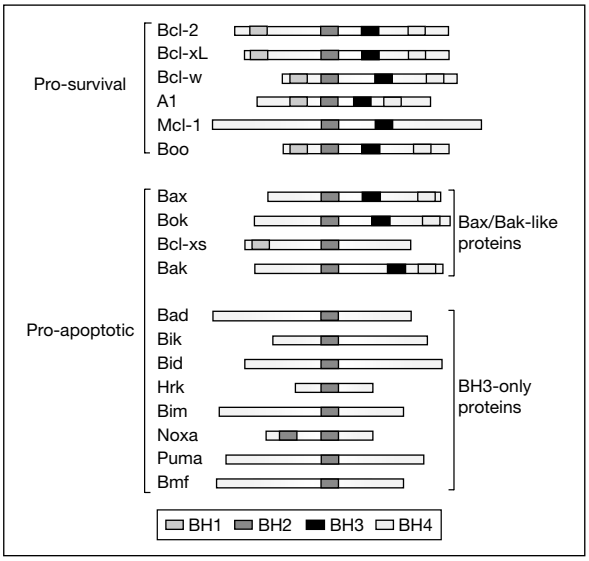

Fig. 2. The Bcl-2 family of apoptosis regulators. The Bcl-2 family is composed of pro-survival and pro-apoptotic members which contain one to four of the Bcl-2 homology (BH) signature domains.

similarity – so-called Bcl-2 homology domains (BH1–4) (fig. 2). According to function, family members can be broadly divided into pro-survival and pro-apoptotic groups [2]. Pro-survival Bcl-2 family members all share 4 (or at least 3) BH regions and include Bcl-2 itself as well as Bcl-xL, Bcl-w, Mcl-1 and A1/Bfl1. The pro-apoptotic members can be further subdivided according to their structure and function into two sub-families: the Bax/Bak-like proteins (also including Bok/Mtd, Bcl-xS) which have 3 or at least 2 BH regions, and the BH3 only proteins, which share with each other and the Bcl-2 family at large only the BH3 domain. The interactions between the different sub-groups of the Bcl-2 protein family in the control of apoptosis are described in more detail below.

BH3-Only Proteins and the Control of Apoptosis

BH3-only proteins include Bad, Bik (also known as Blk or Nbk), Hrk (also known as DP5), Bid, Bim (also known as BOD), Noxa, Puma (also known as BBC3) and Bmf. These proteins act as sensors of developmental cues and a broad range of cellular stress signals [3]. The pro-apoptotic activity of BH3-only proteins can be regulated by several transcriptional and post-translational

mechanisms [7]. For example, upon DNA damage Puma and Noxa are transcriptionally activated by the tumor suppressor p53, whereas cytokine withdrawal induces Bim expression through the transcription factor FOXO3A. Bad, Bid and Bim can also be regulated post-translationally through phosphorylation or proteolysis which affects their subcellular localization and interaction with other Bcl-2 family members [3, 7]. When activated by apoptotic stimuli BH3-only proteins translocate to intracellular membranes, such as to the cytosolic aspect of the mitochondria and endoplasmic reticulum, where they bind to Bcl-2-like pro-survival family members. This interaction occurs by insertion of the α-helix formed by the BH3 region of BH3-only proteins into the hydrophobic groove formed by the BH1, -2 and -3 regions of the pro-survival proteins. Studies with *bax*/*bak* double knock-out mice have demonstrated that developmentally programmed and stress-induced apoptosis [8, 9] as well as cell death triggered by enforced expression of BH3-only proteins requires the multi-BH domain containing pro-apoptotic Bcl-2 family members Bax and Bak [10, 11]. This demonstrates that BH3-only proteins are essential for apoptosis initiation whereas Bax/Bak are required at a point further downstream. Also, since *bax* and *bak* single knock-out mice are essentially normal (with the exception of the sterility of *bax*−/− males) Bax and Bak appear to have largely overlapping functions.

How BH3-only proteins, Bax/Bak-like proteins and Bcl-2-like pro-survival proteins interact to control apoptosis is still unclear. It has been proposed that BH3-only proteins directly activate Bax/Bak and that Bcl-2-like proteins promote survival by acting as a sink for the BH3-only proteins. This model appears unlikely to us because no high affinity (direct) binding of BH3-only proteins to Bax/Bak-like proteins could be demonstrated so far. We prefer the model that Bcl-2-like pro-survival proteins keep Bax/Bak in check in healthy cells and that BH3-only proteins initiate apoptosis by binding to Bcl-2-like proteins thereby freeing Bax/Bak. Since BH3-only proteins differ in their affinity for different pro-survival Bcl-2 family members [12], it is possible that apoptotic stimuli must activate an appropriate set of BH3-only proteins to overcome the protective effect of the Bcl-2-like pro-survival proteins. Differences in expression of pro-survival proteins may thus explain cell type specific differences in responses to cytotoxic stimuli.

The mechanisms by which activated Bax/Bak trigger apoptosis are also still not fully understood. Bax/Bak activation causes mitochondrial release of cytochrome *c* and other apoptogenic proteins (e.g. AIF, Diablo/Smac, EndoG). Cytochrome *c* together with the adaptor protein Apaf-1 then recruits and oligomerizes pro-caspase-9 to form the 'apoptosome'. This promotes autocatalytic activation of caspase-9, which in turn activates effector caspase-3, -6 and -7, initiating proteolysis and cellular destruction. Studies with *Apaf-1* and *caspase-9* knock-out mice have shown that developmentally programmed cell

death can occur in the absence of the apoptosome [13]. Effector caspase activity was still evident in these cells (albeit at much lower levels than in wt cells) and synthetic caspase inhibitors could delay mitochondrial release of cytochrome *c* [13, 14]. It is therefore possible that caspases acting upstream of the mitochondria can initiate apoptosis and that the apoptosome functions as a very important amplification loop in apoptosis signaling. Alternatively, the death seen in the absence of the apoptosome may be mediated by a caspase-independent mechanism, such as Bax/Bak-induced mitochondrial release of death inducing molecules. Bax/Bak have been postulated to form channels in the outer mitochondrial membrane and to modulate mitochondrial channel proteins, but it is also possible that they can directly activate certain initiator caspases. Since apoptosis signaling appears to proceed not through a strictly linear pathway, but seems to be composed of inter-connected amplification loops, it is possible that more than one of the proposed models is in fact correct.

Bim

Bim (Bcl-2-Interacting mediator of cell death), one of the pro-apoptotic BH3-only proteins, is essential for initiating apoptosis in response to many physiological or experimentally applied death stimuli in a range of cell types, including hematopoietic cells, neurons, the developing kidney and melanocytic stem cells. Two groups independently discovered Bim in1998: one by screening a λ-phage expression library from a mouse thymic lymphoma using Bcl-2 as bait [15] and the other by screening of a yeast two-hybrid library from ovarian tissue using Mcl-1 as bait [16]. Alternative splicing gives rise to three main isoforms of Bim, BimEL, BimL and BimS (extra-long, long and short) [15], with BimEL being the most abundant, followed by BimL [17]. Several additional isoforms (also resulting from alternative splicing) have been described, but it is not clear yet which form(s) is/are critical for cell death. Bim is expressed in many cell types, including most lymphoid cell types, epithelial cells, male and female germ cells and certain neuronal populations [17].

The pro-apoptotic activity of Bim is controlled by both transcriptional and post-translational mechanisms. Induction of Bim transcription has been demonstrated following growth factor withdrawal and other cytotoxic stimuli. For example, increased levels of Bim transcription has been demonstrated in neurons following growth factor withdrawal, transient ischemia or status epilepticus and in hematopoietic cells following growth factor withdrawal or antigen receptor stimulation [18–21]. It has been reported that members of the c-Jun N-terminal kinase (JNK) family through activation of AP1 transcription factors mediate induction of Bim expression in growth factor-deprived neurons but this could not

be reproduced [22] by another group, which proposed that JNK activates BimEL through direct phosphorylation [18]. Cytokine withdrawal in hematopoietic cells has been shown to increase Bim mRNA and protein expression through activation of the forkhead-like transcription factor FOXO3A (also known as FKHRL1) [20].

Post-translational modifications thought to be important in the control of Bim include phosphorylation and ubiquitination. ERK-mediated phosphorylation of Bim in hematopoietic cells has been shown to increase ubiquitination and proteosomal degradation of Bim and to thereby promote survival of cells receiving cytokine support [23–25]. A reduction in Bim ubiquitination in osteoclasts has been demonstrated following growth factor withdrawal and this was associated with reduced proteosomal degradation of Bim, Bim accumulation and increased cell death.

In healthy cells, Bim can be sequestered from its Bcl-2-like pro-survival relatives by binding to the microtubular dynein motor complex via dynein light chain (LC) 8 [26]. Following certain apoptotic stimuli, such as treatment with the anti-cancer drug taxol, Bim is released and able to translocate to bind its pro-survival relatives. The relative importance of these different mechanisms of transcriptional and post-translational control of Bim function is unclear and can probably be tested most reliably by generating Bim mutant (knock-in) mice in which critical regulatory regions are removed.

It has been postulated that upon activation, Bim initiates apoptosis by binding to and neutralising pro-survival Bcl-2 family proteins and/or by direct activation of Bax/Bak. We believe that the second scenario is unlikely because no direct binding of Bim to Bax/Bak with physiologically relevant affinity could be demonstrated so far. Interestingly, unlike some BH3-only proteins (e.g. Noxa or Bik), Bim has been shown to be able to bind to all pro-survival Bcl-2 family members [12], consistent with the observation that its loss produces a more profound phenotype in knock-out mice than loss of some of its relatives [27].

Bim Is Critical for Apoptosis and Homeostasis in the Lymphoid and Myeloid Compartment

Bim is expressed in many hematopoietic cell types and is vital for the maintenance of homeostasis in the hematopoietic system. Bim knock-out mice have abnormally increased numbers of lymphoid and myeloid cells [28]. In young *bim*−/− mice, mature T cells (CD4+8− and CD4−8+), B cells, granulocytes and monocytes numbers in peripheral lymphoid tissues are increased 2- to 4-fold compared to wt animals.

With age, *bim*−/− mice develop progressive lymphadenopathy and splenomegaly and accumulate IgG-secreting plasma cells [28]. This is associated

with hypergammaglobulinemia with increased levels of IgG (10-fold), IgM (3-fold) and IgA (3-fold) and high titres of autoantibodies to nuclear antigens (e.g. dsDNA, histones, smRNP), similar to human systemic lupus erythematosus (SLE). On a mixed genetic background (C57BL/6 × 129/Sv), but not on an inbred C57BL/6 background, older *bim*−/− mice succumb to a fatal systemic autoimmune disease mainly in the form of a diffuse proliferative glomerulonephritis with immune complex deposition. Some animals also develop histological evidence of cardiac infarction and vasculitis. By one year of age ~55% of *bim*−/− and even ~35% of *bim*+/− mice die from this disease, indicating a gene dosage effect.

Consistent with findings from in vivo analysis of *bim* knockout mice, *bim*−/− lymphocytes are resistant to a range of apoptotic stimuli in vitro. Immature *bim*−/− CD4+8+ thymocytes are resistant to cell death induced by cytokine withdrawal or treatment with ionomycin, which deregulates calcium flux, or the anti-cancer drug taxol, which stabilizes microtubules [28]. Under these conditions Bim-deficient thymocytes survive 10–30 times better than wild-type cells, whilst *bim*+/− cells have an intermediate phenotype. Loss of Bim provides only minor protection from apoptosis following treatment with dexamethasone or γ-radiation and has no effect on cell death triggered by phorbol 12-myristate 13-acetate (PMA) or Fas ligand [28]. Similar resistance to apoptotic stimuli was observed in *bim*−/− pre-B cells, mature T and B cells [28] and granulocytes [29].

These results demonstrate that Bim is an important limiting factor in controlling the initiation of apoptosis in lymphoid and myeloid cells in response to cytokine withdrawal and calcium flux, but is dispensable for other apoptotic stimuli. This is indicated by the increasing resistance to cell death seen with progressive reduction in the level of Bim expression from *bim*+/− to *bim*−/−. This is reinforced by the observation that removal of Bim expression provided similar protection from cell death following cytokine withdrawal or treatment with ionomycin as Bcl-2 overexpression, which is able to protect cells from all Bcl-2 regulated ('intrinsic') apoptotic stimuli [30].

Experiments involving crosses between *bcl-2*−/− and *bim*−/− mice have provided further insight into the critical opposing roles that these two proteins play in controlling lymphocyte homeostasis. Due to increased apoptosis, *bcl-2*−/− mice have abnormally low numbers of lymphoid and myeloid cells and these cells are abnormally sensitive to many apoptotic stimuli [31, 32]. Remarkably, removal of a single allele of *bim* (*bcl-2*−/− *bim*+/−) restores levels of all hematopoietic cell populations substantially [33]. When both alleles of *bim* are removed (*bcl-2*−/− *bim*−/−) both lymphoid and myeloid cell numbers are increased, close to the levels seen in a *bim*−/− mouse.

In summary, the absence of Bcl-2 increases the likelihood of cells dying due to the unopposed action of Bim, whilst concomitant removal of Bim abrogates

this effect. Therefore the relative balance of these two opposing proteins appears to be important in deciding which way the balance is tipped between the cell living or dying. Other Bcl-2 family members, in particular the pro-survival protein Mcl-1 [34] and the pro-apoptotic BH3-only protein Puma [35, 36], have also been found to be important regulators of lymphoid and myeloid cell apoptosis.

Bim and Thymocyte Selection

T cells are derived from immature progenitors that migrate to the thymus for development and selection. During development in the thymus more than 90% of T cells undergo programmed cell death [37]. This occurs through a process of positive and negative selection, which only allows cells with functional but not self-reactive T cell receptors (TCR) to mature and leave the thymus.

T cell survival during the first stages of thymic development (CD3–4−8− pro-T cells) is dependent on continued exposure to IL-7. In mice lacking IL-7, IL-7Rα chain or the common γ chain (γc), required for signaling by IL-2, -4, -7, -9 and -15, levels of immature and mature T and B lymphocytes are significantly reduced [38, 39]. In contrast mice expressing an IL-7 transgene develop progressive lymphadenopathy [40]. IL-7 stimulation has been shown to increase expression of Bcl-2 in lymphoid cells and expression of a bcl-2 transgene can restore normal numbers of lymphocytes in *IL-7Rα*−/− mice indicating that this cell death is controlled by the Bcl-2 family of proteins [41, 42]. Removal of Bim increased thymocyte numbers, but clearly not to normal levels, and restored near normal numbers of mature T cells in IL-7Rα−/− mice [43]. The rescue was less complete than when Bcl-2 is over-expressed, indicating that Bim is probably one of several pro-apoptotic proteins involved in thymocyte apoptosis induced by the absence of IL-7 signaling.

Developing thymocytes first undergo TCRβ and then TCRα chain gene rearrangement to form an MHC-restricted TCR [44]. TCRβ is initially expressed in combination with the invariant pTα chain and the CD3 protein complex as the so-called pre-TCR. Pre-TCR signaling is required for development from the CD3–4–8− pro-T to the immature CD4+8+ stage and loss of pTα results in developmental arrest and apoptosis, at a similar stage to that seen in *scid*, *rag-1*−/−, *rag-2*−/− and CD3ε−/− mice. The apoptosis of cells in the absence of pre-TCR signaling was not affected by Bcl-2 overexpression [45] but could be reduced by blocking death receptor signaling with a dominant negative inhibitor of FADD [46]. Since FADD-DN expression inhibited this death only partially, it remains possible that it also involves the action of pro-apoptotic Bcl-2 family members that are not potently opposed by Bcl-2.

This hypothesis can be tested by generating mice that lack components of the pre-TCR or rag-1/2 plus one or several BH3-only proteins (e.g. Bim or Puma).

Once the TCRβ chain is expressed, pre-TCR signaling activates TCRα gene rearrangement in late stage pro-T (CD4–8−25–44−) and then immature CD4+8+ thymocytes. Cell survival during TCRα chain gene rearrangement appears to be maintained by up-regulation of Bcl-xL expression, under the control of the steroid transcription factor RORγ and transcription factors that act in the *wnt* signaling pathway [47].

Immature CD4+8+ thymocytes expressing a TCR that binds with low affinity to MHC molecules incorporating self-antigen-derived peptides are positively selected for differentiation into mature T cells, which then emigrate into peripheral lymphoid organs. Thymocytes lacking TCRα/β molecules and those expressing a TCR that does not interact with self-peptide/MHC complexes fail to receive a survival signal and die by neglect. This death can be inhibited by Bcl-2 overexpression [45, 48], demonstrating that it occurs by the 'intrinsic' apoptotic pathway, but it is presently unclear which BH3-only protein triggers it. Bim appears to be a good candidate but so far this has not been tested in TCR transgenic mouse models.

While expression of a TCR that binds with low affinity to self-peptide/MHC promotes positive selection, expression of a high affinity TCR causes apoptotic death of developing thymocytes. This death can be inhibited by over-expression of Bcl-2 [45, 48] but not by inhibition of death receptor signaling (FADD-DN or loss of caspase-8) [49, 50], indicating that it is mediated by the 'intrinsic' pathway. Experiments with knock-out mice have shown that Bim [51, 52] and Bax/Bak [9] are essential for killing of autoreactive thymocytes. High affinity TCR stimulation, for example by anti-CD3 antibodies, kills ~80–90% of wt CD4+8+ thymocytes both within the whole animal or in culture, but essentially all *Bim*−/− thymocytes survive this treatment [51, 52].

Bim−/− mice have also been shown to have impaired deletion of thymocytes in vivo. When *Bim*−/− mice are crossed with transgenic mice expressing a male antigen HY/class I MHC-specific αβTCR, loss of both alleles of *Bim* significantly impaired the deletion of autoreactive CD4+8+ thymocytes in male mice [51]. Moreover, loss of Bim protected thymocytes against apoptosis induced by super-antigens, either endogenously expressed Mtv9, or experimentally applied to fetal thymic organ cultures [51, 52]. Collectively, these results demonstrate that Bim is essential for apoptosis of autoreactive thymocytes. Increased Bim (particularly BimEL) expression and increased association of BimEL with Bcl-xL (the most highly expressed pro-survival protein in CD4+8+ thymocytes) has been observed in response to TCR stimulation. However, which mechanisms induce Bim expression during negative selection is presently not known.

Defects in Bim induction in deletion of autoreactive thymocytes has been implicated in certain models of autoimmune disease, in particular autoimmune diabetes in non-obese diabetic (NOD) mice. Immature CD4+8+ as well as semi-mature (intermediate between CD4+8+ and CD4+8− or CD4–8+) thymocytes of NOD mice have been found in some [53–55], but not all [56], studies to be relatively resistant to cell death induced by TCR crosslinking and to have defects in thymic deletion.

A recent study has examined gene expression during negative selection, comparing NOD with control thymocytes [55]. Mice in this study were transgenic for a TCR with high affinity for hen egg lysozyme (HEL) peptide 46–61 bound to I-Ak (3A9 TCR) and also contained transgenes expressing HEL in different cell types and at different levels. Double transgenic mice were backcrossed onto the MHC congenic backgrounds NOD.*H2k* (contains all autoimmune susceptibility loci of NOD except the H-2 locus) or as a control B10.BR (carries diabetes resistance alleles at most non-MHC loci). This allowed the comparison of gene expression in response to negative selection in thymocytes with and without non-MHC NOD autoimmune susceptibility genes. Thymocytes from these mice were then sorted and gene expression analyzed by microarray. *Bim* and *Nur77* expression were dramatically increased early in B10.BR thymocytes (CD4+CD8lowCD69+1G12+) undergoing negative selection. However, only small increases in Bim and Nur77 expression were seen in NOD.*H2k* thymocytes. These were the only differences seen in gene expression between the B10.Br and NOD strains despite looking at multiple candidates. This indicates that NOD mice have a selective defect in the induction of *Bim* in thymocytes triggered with self-antigens. This is thought to contribute to the increased resistance of immature and semi-mature NOD thymocytes to apoptosis and their defect in the deletion of autoreactive thymocytes. Genetic analysis revealed that the defect in thymocyte negative selection in NOD.*H2k* mice is associated with four chromosomal regions, interestingly including one on chromosome 2, which is linked to the *bim* gene. This indicates that the resistance to thymic deletion in NOD mice is a complex polygenic trait, which may be due, at least in part, to defects in the *bim* gene or defects in genes that are required for *bim* induction in response to TCR ligation.

Role of Bim in Peripheral Deletion of Autoreactive T Cells

Bim has also been shown to be important in the maintenance of peripheral T cell tolerance. This has been demonstrated in a TCR transgenic model of cross-tolerance in which naïve autoreactive CD8+ T cells are deleted after dendritic cells cross-present their cognate (self)-antigen to them. The deletion of

these autoreactive CD8+ T cells can be prevented to a similar extent by the over-expression of Bcl-2 or the removal of Bim [57]. Although earlier reports suggested that the death receptor pathway was important in the deletion of these autoreactive CD8+ T cells following the cross-presentation of self antigen [58], this now does not seem to be the case [57].

The Bcl-2 family has so far not been shown to be important in controlling the peripheral deletion of autoreactive CD4+ T cells. When naive 3A9 TCR-transgenic (HEL-specific) CD4+ T cells were adoptively transferred into transgenic mice expressing soluble hen egg lysozyme (HEL) in their serum, Bcl-2 overexpression failed to inhibit T cell deletion in vivo [59]. The deletion was, however, significantly impaired, by the lack of functional Fas (*lpr* mice) or Fas ligand (*gld* mice) in T cells themselves, indicating that autocrine activation of the death receptor pathway might be more important in this cell type. This model differs from that used to study CD8+ T cells where self-antigen was expressed in a tissue-restricted manner [57] in that the self-antigen was circulating and therefore ubiquitous. Therefore the signaling pathways controlling the peripheral deletion of autoreactive T cells might depend on the T cell type as well as other factors, such as the affinity of TCR/MHC interactions and the quantity, nature and expression pattern of the self-antigen [57].

Role of Bim in B Cell Development

Bim expression is seen in B cells at all stages of development [17, 60]. Like pro-T cells, the earliest forms of B cell precursors, the pro-B cells, depend on IL-7R signaling for cell survival and sustained differentiation. The cell death that occurs in the absence of IL-7R signaling appears to be predominantly initiated by Bim, as in the absence of Bim, pro- and pre-B cells have similar survival in vitro whether IL-7 is present or not [60]. However, although the absence of Bim could promote the survival of these B cell precursors deprived of IL-7, these cells were unable to undergo further differentiation. Interestingly, *IL-7Rα*−/− mice have very low levels pro-, pre-, immature and mature B cells whether Bim is present or not [60]. Similar observations were made in the B lineage of *IL-7Rα*−/− mice expressing a *bcl-2* transgene [61]. Therefore, unlike in early T cell precursors in which IL-7 is mostly required for cell survival, in developing B cells IL-7 also appears to be critical for differentiation and proliferation [44].

During B cell maturation, the genes encoding the immunoglobulin heavy (HC) and light chain (LC) genes must be rearranged in frame to produce a functioning B cell receptor (BCR). This begins with Ig HC rearrangement at the pro-B cell stage and subsequent expression of the pre-BCR composed of the IgH HC with the λ5 and Vpre-B surrogate light chains. Pre-BCR signaling allows cell

survival and differentiation into the pre-B cells stage. IgLCs are then rearranged at the pre-B cell stage to create a functioning BCR.

Failure to productively rearrange the Ig heavy and light chain genes results in apoptosis which is most likely under the control of the Bcl-2 family. B cells in *rag-1−/−*, *rag-2−/−* and *scid* mutant mice, which are unable to rearrange Ig genes segments, do not progress past the pro-B cell stage. Over-expression of Bcl-2 or Bcl-xL in these mice prevents the deletion of the pro-B cells and thereby promotes accumulation of B lineage cells lacking a BCR [45, 62, 63].

Once B cells reach the immature B cell stage of development (sIgMhi IgDlo) their survival is no longer dependant upon IL-7 signaling but requires BAFF, a member of the TNF family [64]. BAFF signaling appears to promote survival of transitional and mature B cells via NF-κB dependent upregulation of *bcl-2* expression [65]. It will be interesting to examine whether loss of Bim or another BH3-only protein can promote accumulation of B cells in mice lacking functional BAFF or BAFF receptor.

Sustained survival of mature B cells requires continued BCR expression and signaling. Removal of BCR expression in mature B cells by inducible Ig gene deletion causes a rapid reduction in B cell numbers, and this deletion appears to involve the Bcl-2-regulated (intrinsic) apoptotic pathway [66].

Role of Bim in the Deletion of Autoreactive B Cells

The stochastic nature of assembling genes encoding the BCR entails the risk that autoreactive B cells are created. B cells expressing a BCR with high affinity for self-antigen must be modified by further gene rearrangement (receptor editing), silenced (anergy) or deleted to prevent the development of autoimmune disease.

BCR-ligation-induced deletion of both immature and mature resting B cells was shown not to require the death receptor pathway [67, 68]. This cell death can, however, be inhibited by over-expression of Bcl-2 or Bcl-xL [68, 69]. Furthermore, in an anti-HEL Ig/HEL double transgenic mouse model of B cell tolerance loss of Bim was found to inhibit the deletion of autoreactive B cells in vivo [69]. These findings are consistent with the observation that *bim−/−* mice have a dramatic accumulation of plasma cells with age and an associated increase in serum immunoglogulins levels, auto-antibodies and a SLE-like fatal autoimmune disease (the latter only on a mixed C57BL/6x129Sv, but not on an inbred C57BL/6 background) [28].

Loss of Bim expression does not prevent immature B cell killing following BCR ligation in vitro as effectively as does Bcl-2 overexpression, indicating

that additional BH3-only proteins might have a partially overlapping role. Bik has been shown to be up-regulated upon BCR cross-linking in certain human B lymphoma-derived cell lines [70], but *bik*−/− B cells were found to respond normally to BCR crosslinking [27]. Moreover, *bim*−/− mice with a transgenic system of B cell tolerance (anti-HEL Ig/HEL; see above) have a similar increase in the number of autoreactive B cells [69] as anti-HEL Ig/HEL doubly transgenic mice also expressing a B cell restricted *bcl-2* transgene [71]. This indicates that Bim is the major initiator of BCR cross-linking-induced apoptosis of autoreactive B cells.

Interestingly, although the anti-HEL Ig/HEL doubly transgenic *bim*−/− mice accumulated abnormal numbers of autoreactive B cells, systemic tolerance appeared to be maintained, possibly due to induction of B cell anergy [69]. This is consistent with the observation that in *bim*−/− mice, peripheral T cell tolerance can be maintained despite the abnormalities in thymic and peripheral negative selection of autoreactive T cells [51]. This may indicate that immunological tolerance is safeguarded by several processes, including apoptotic death, anergy and developmental arrest of autoreactive T and B cells. Perhaps, full-blown autoimmune disease only occurs when two or more of these processes are severely impaired.

Role of Bim in Termination of Immune Responses

An acute immune response to infection with a pathogen is characterized by activation, proliferation and acquisition of effector functions of foreign antigen-specific T and B lymphocytes. These processes are critically dependent on inflammation-associated cytokines and they generally lead to elimination of the invading pathogen. Although a so-called memory population persists long-term, the immune response is downregulated, involving the death of the vast majority of responding lymphocytes. The shut-down of the lymphocyte response is critical to maintain lymphocyte homeostasis, to prevent the non-specific tissue damage that prolonged immune responses could cause and to minimize the risk of autoimmunity [72].

The signaling pathways controlling T cell death during shut-down of an immune response have been a matter of some debate. Whilst the death receptor pathway is required for the apoptosis of cultured T cells following repeated ligation of the TCR (activation induced cell death) [73], several groups have demonstrated that this pathway is not required for T cell death during immune response shut-down [5, 74, 75]. Members of the Bcl-2-family do, however, appear to be critical regulators of this process.

Two groups have demonstrated that following injection of the superantigen *Staphylococcus* enterotoxin B (SEB), both CD4+ and CD8+ antigen-specific T cells expressing a *bcl-2* transgene survive significantly longer in vivo and in vitro than normal T cells [30, 74].

It has also been shown that the primary pro-apoptotic Bcl-2-family member initiating apoptosis in T cells during the termination of an immune response is Bim [74]. T cells from *bim*−/− mice injected with SEB are largely protected from the apoptosis that occurs during the immune response shut-down that occurs between 5–10 days after injection [74]. In fact, while there was a slight reduction in the numbers of SEB-reactive CD4+ and CD8+ T cells in the lymph nodes of *bim*−/− mice over the twenty-days after SEB injection, no reduction was seen over this period in the spleen. By comparison, SEB-specific CD4+ and CD8+ T cell numbers had returned to pre-stimulation levels by approximately day seven in wild-type mice. Interestingly, the levels of Bcl-2 and Bcl-xL were found to fall in T cells at the peak of the immune response, just before they begin to die in vivo [76]. This fall in Bcl-2 and Bcl-xL would significantly change the balance between Bim and Bcl-2-like pro-survival proteins within the cell and might constitute the trigger to initiate apoptosis.

More recent experiments have studied the kinetics of the T cell response in wild-type (wt), *bim*−/− and *lpr* mice following infection with herpes simplex virus (HSV) [75]. The proliferation of HSV-specific cytotoxic CD8+ T cells was similar in mice of all genotypes, and all mice were able to efficiently clear the virus. However, whilst the number of HSV-specific CD8+ T cells in the spleens of wt and *lpr* mice peaked at seven days before falling to almost baseline levels, *bim*−/− mice continued to accumulate splenic HSV-specific CD8+ T cells due to impaired apoptosis [75]. Remarkably, the CD8+ T cell numbers in the spleens of *bim*−/− mice had not fallen significantly even at four weeks following viral infection.

These experiments indicate that Bim is critical for the induction of apoptosis of both CD4+ and CD8+ T cells during the termination of an acute immune response. Given that Bim is required for the apoptosis of T cells in culture following IL-2 or IL-7 withdrawal [28], it is likely that T cells die during shut-down of an acute immune response due to the declining levels of essential cytokines [72].

It is theoretically possible that death receptors do play a role in the death of T cells during immune responses to certain pathogens. Since the death receptor pathway is triggered in an autocrine and/or paracrine manner when activated T cell blasts are re-stimulated through their TCR, we speculate that persistent pathogens, such as plasmodia, could trigger this process. This may be a mechanism to prevent excessive tissue destruction as a consequence of sustained T cell activation and may serve to establish long-term co-habitation of a

pathogen within its host. It is also possible that in certain immune responses, death receptors and the Bcl-2-regulated apoptotic pathway, in particular through Bim induction, cooperate in the killing of no longer needed activated T and B lymphocytes.

Bim and Degenerative Disease

Excess apoptosis can be a feature of certain degenerative diseases and it is possible that Bim or other BH3-only proteins play a role in such disorders. Loss of Bcl-2 expression results in increased levels of apoptosis and degeneration in a range of tissues. Mice lacking Bcl-2 appear normal at birth but fail to thrive, develop short ears and their thymus and spleens degenerate; later, their coats turn gray and they all die from renal failure due to polycystic kidney disease within 4–16 weeks (time of death is influenced by genetic background) [31, 32, 77]. Increased cell death can be observed within the degenerating tissues of *bcl-2−/−* mice and their lymphocytes are abnormally susceptible to a range of cytotoxic stimuli both in vivo and in vitro.

Within the tissues in which Bcl-2 acts as the critical guardian against apoptosis, Bim appears to act as the dominant initiator of cell death. Loss of Bim expression prevents the abnormal cell death and degenerative disorders in Bcl-2-deficient mice [33]. Strikingly, removal of even a single allele of *bim* (in *bcl-2−/−bim+/−* mice) prevents polycystic kidney disease and restores normal growth in *bcl-2−/−* mice. As outlined above, these mice also have significantly higher numbers of lymphoid and myeloid cells compared to *bcl-2−/−* mice and the sensitivity of these cells to certain apoptotic stimuli, such as cytokine withdrawal, was found to be intermediate between that of *bcl-2−/−* and wt cells. Removal of both alleles of *bim* in *bcl-2−/−bim−/−* mice almost completely prevents melanocyte stem cell death, and thus graying of the mice, and lymphoid and myeloid cell numbers are even increased above those of wt mice, similar to the levels seen in a *bim−/−* mice.

Therefore it is the interaction between Bim and Bcl-2 and their relative amounts that controls the fate of this broad range of cell types. In the absence of Bcl-2, unopposed Bim triggers unscheduled apoptosis (presumably by acting on other pro-survival proteins expressed in these cells) and causes degenerative disorders. Some experiments have been performed to determine whether blocking Bim expression or function might be a useful therapeutic tool in the treatment of certain degenerative disorders. *Lurcher* mice, which have a semidominant mutation in the gene encoding the δ2 glutamate receptor, develop ataxia due to the abnormal apoptosis of cerebellar Purkinje cells and most afferent granule neurones [78]. The death of the granule neurones but not

that of the Purkinje cells can be prevented by loss of Bax [79] and both of these cell types are also known to express Bim [80]. However, loss of Bim had no effect on the *Lurcher* phenotype, indicating that Bim does not play a limiting role in the development of this degenerative disease [81]. *Bim*−/− mice have also been crossed with mice lacking *Pkd1*, the most commonly mutated gene in human autosomal dominant polycystic kidney disease [82]. Heterozygous *Pkd1*+/− mice have been reported to develop late onset renal cysts but the removal of Bim in these mice did not appear to alter this phenotype [Bouillet et al., in preparation].

As mentioned above, loss of Bim was, however, able to alleviate the immunodeficiency caused by IL-7Rα deficiency [43, 60]. Collectively, these results indicate that deregulated activation of Bim and/or perhaps some other BH3-only proteins may play a contributory or even causative role in certain degenerative diseases. Other degenerative diseases do, however, not require Bim, perhaps because cell death in these disorders proceeds through other apoptotic pathways or may be of a non-apoptotic nature.

Conclusion

Bim, a BH3-only member of the Bcl-2 protein family, is a critical initiator of developmentally programmed cell death and stress-induced apoptosis in a broad range of cell types. In the absence of its opposing pro-survival relative Bcl-2, Bim is able to cause the degeneration of a range of tissues by causing excessive apoptosis. Targeting the action of Bim and/or other BH3-only proteins might therefore be a therapeutic option in the management of certain degenerative disorders.

Bim has also been found to be important in the developmentally programmed cell death and selection that occur during lymphopoiesis and in maintaining peripheral tolerance and terminating immune responses. Loss of Bim in gene knock-out mice has been shown to affect all these aspects of lymphocyte and immune function and has been associated with the development of autoimmune disease in some mouse models. Furthermore abnormalities of Bim expression and negative selection of thymocytes might be one of the multiple genetic factors underlying the complex polygenic trait of susceptibility to autoimmune disease.

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Mitochondria, Cell Death, and B Cell Tolerance

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Abstract

To prevent autoimmunity, it is critical that tolerance mechanisms block autoantibody production from self-reactive B cells. B cell tolerance is maintained through mechanisms that can reversibly or irreversibly silence autoreactive B cells. Of these mechanisms, those that lead to B cell death offer the most reliable form of tolerance to prevent autoimmunity. In many cases, death of autoreactive B cells is regulated by the cell intrinsic, or mitochondrial pathway of cell death. The pro-apoptotic Bcl-2 family proteins, Bak, Bax, and Bim have been shown to be required for disruption of mitochondria and intrinsic cell death of self-reactive B cells whereas the anti-apoptotic Bcl-2, Bcl-xL, and Mcl-1 can prevent cell death by interfering with the action of Bax and Bak. Bcl-2 and Bcl-xL have also been shown to regulate the autophagic cell death pathway that may also play a role in B cell tolerance. Even after mitochondrial disruption, mechanisms exist that may impede activation of caspases and death of autoreactive B cells. Together, understanding of cell death mechanisms and how they may affect B cell tolerance has made significant recent advances and it is now important to incorporate alternate and post-mitochondrial cell death mechanisms into B cell tolerance models.

B cell tolerance is maintained through a series of tolerance checkpoints. Of these checkpoints, those that lead to B cell death offer the only irreversible form of tolerance to prevent autoantibody production and disease. Many instances of B cell death in the regulation of tolerance are mediated through the mitochondrial pathway of cell death and are regulated by members of the Bcl-2 family. Mitochondria, thus act as central players in both metabolic and cell death pathways that are critical in the maintenance of B cell tolerance. Tolerance is

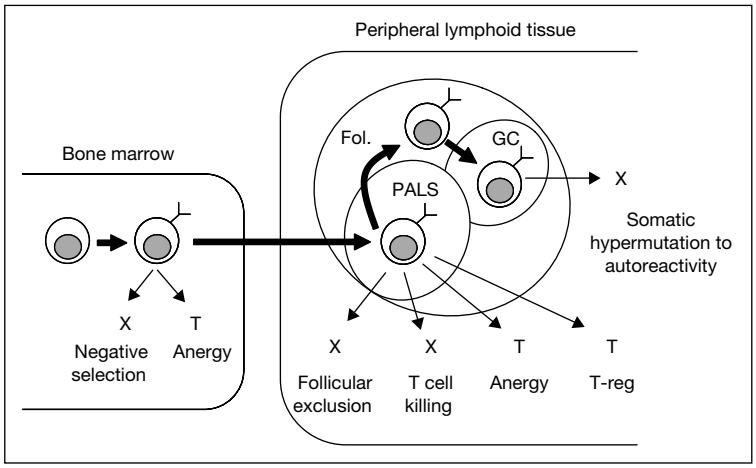

Fig. 1. B cell checkpoints for B cell death or tolerance. B cells are rendered tolerant by programmed cell death (X) or mechanisms that maintain cell viability but repress B cell function (T). In the bone marrow during B cell development, B cells that rearrange to generate BCR specific for self-antigen undergo receptor editing. If this fails to alter B cell specificity away from self, B cells are eliminated by negative selection if BCR are of too high avidity for self-antigen or are rendered anergic if BCR avidity to self-antigen is lower. In the periphery, self-reactive B cells are eliminated in the periartieolar sheath (PALS) by follicular exclusion or by T cells upon presentation of self-antigen. B cells can remain viable yet remain functionally impaired by anergy or by Treg cells. B cells can enter the follicle (Fol.) and germinal center (GC) where they can undero cell death after somatic mutation to gain autoreactivity.

maintained and autoreactive B cells are eliminated by changes in expression of Bcl-2 family members, changes in cell metabolism, and possibly non-apoptotic pathways of cell death, such as autophagy. In addition, recent findings have shown that even after mitochondrial release of cytochrome *c*, kinase cascades can prevent cell death, possibly allowing autoreactive B cells an opportunity to produce autoantibodies and promote disease. This review summarizes recent advances in the study of how mitochondrial and postmitochondrial mechanisms can inhibit B cell apoptosis and how this may contribute to autoimmunity.

B cell tolerance checkpoints can lead to death of autoreactive B cells or can act to prevent B cell activation and autoantibody secretion (fig. 1) [1]. Tolerance checkpoints that do not cause cell death include induction of B cell non-responsiveness, anergy, or control of B cell activation by regulatory T cells (Tregs) [2, 3]. In each case, tolerant B cells remain in place within the immune repertoire. While setting a high threshold for initiating tolerance-induced cell death and retaining tolerant cells may prevent large holes in the antibody repertoire, these cells pose tremendous risk for subsequent autoantibody production

and development of autoimmunity. Anergic B cells can indeed be stimulated to recover from anergy and begin to produce autoantibodies under a variety of circumstances. Anergic B cells can be stimulated to reverse their state of anergy upon long-term separation from antigen [4], repeated stimulation via toll-like receptors by LPS or CpG DNA [4–6], or upon stimulation with activated T cells [2, 7]. Indeed a critical element to retain B cell anergy in some model systems is the inability of tolerant B cells to provide T cell co-stimulation when presenting antigen because anergy of B7.2 transgenic B cells is rapidly reversed and antibody production is potently induced by specific T cells [8].

To prevent autoantibody production and autoimmunity, therefore, the only certain pathway of cell tolerance is through the elimination, or programmed cell death, of the autoreactive cells. Death of autoreactive B cells can occur in a number of tolerance checkpoints (fig. 1). In development, B cells with high avidity self-reactive antigen receptors after V(D)J rearrangement and potential receptor editing undergo developmental arrest and elimination [9–11]. In the periphery, B cells that bind self-antigen fail to upregulate the chemokine receptor CXCR5 to exit T cell zones and migrate into B cell follicles. If T cell help is not available to promote B cell survival and activation, B cells that are excluded from follicles undergo programmed cell death [12, 13]. Self-reactive B cells that survive follicular exclusion and present antigen to T cells also risk being induced to undergo programmed cell death by those T cells via the Fas death receptor [14]. In addition, B cells that gain self-reactivity or have increased self-reactivity after somatic hypermutation in germinal centers will also be eliminated if sufficient T cell help is not present [15, 16]. If these death-promoting B cell tolerance checkpoints are prevented, autoimmunity can ensue [17]. Thus, it is of critical importance to understand how B cell death tolerance checkpoints are regulated and how they can be circumvented in the development of autoimmunity.

Mitochondrial Control of B Cell Elimination

There are two primary mechanisms of cell death that play critical roles in elimination of autoreactive B cells, the cell intrinsic and cell extrinsic pathways. In the cell intrinsic pathway, a key point in the regulation of cell death is the disruption of the outer mitochondrial membrane and release of cytochrome *c* [18] and other pro-apoptotic proteins from the mitochondrial intermembrane space [19]. Once released, cytochrome *c* can interact with Apaf-1, dATP, and caspase 9 in a structure called the apoptosome. This leads to activation of caspase 9 and the proteolytic caspase cascade. Together, mitochondrial dysfunction and caspase activation lead to intrinsic cell death. Expression of the

anti-apoptotic protein Bcl-2 is sufficient to prevent disruption of mitochondria and release of mitochondrial intermembrane contents [20, 21], thereby inhibiting both the consequences of impaired mitochondrial function and the effects of caspase activation. As a consequence of preventing death of autoreactive cells, Bcl-2 family proteins have been shown to be key regulators of autoimmunity. Even after release of cytochrome *c*, however, specific proteins and kinases can prevent caspase activation to inhibit cell death and these may also contribute to autoimmunity. In the extrinsic pathway of B cell death, death receptors on otherwise healthy B cells are triggered and generally cause the cell to commit to die at the point of death receptor complex formation. Each pathway plays an important role in maintaining B cell tolerance and their disruption can lead to autoantibody production. This review will discuss the role of the intrinsic cell death pathway in B cell tolerance and autoantibody production.

Bcl-2 Family of Proteins

After initial discovery as an oncogene in follicular lymphoma [22–24], it quickly became clear that Bcl-2 was unlike other oncogenes in that it did not appear to promote cell cycle, but rather promoted oncogenic transformation by inhibiting cell death [25–27]. In this way, slow accumulation of cells that failed to undergo programmed cell death lead to an indolent and chemotherapy resistant cancer. In the years since the identification of Bcl-2, a series of studies using homology to Bcl-2 and physical interactions have identified a large number of Bcl-2-related proteins. Bcl-2 family proteins are cytosolic or associated with intracellular membranes, including outer mitochondrial membrane and endoplasmic reticulum, in healthy cells and concentrate in intracellular membranes during cell death [27]. This physical association with mitochondria highlighted the role that mitochondria may play in cell death and the role of Bcl-2 family proteins with these mitochondrial cell death pathways.

Based on their function and the presence of four distinct Bcl-2 Homology domains (BH regions 1–4), these proteins fall into three classes (fig. 2). Members of the first class, the anti-apoptotic sub-family, have multiple BH domains and are characterized by Bcl-2 itself. The second class of Bcl-2-related proteins are the multi-BH domain pro-apoptotic sub-family. This subfamily consists of just three members, Bax [28], Bak [29, 30], and Bok [31]. Each has contains BH1–3 and can promote apoptosis when expressed. Of these three family members, Bax and Bak are ubiquitously expressed and are present in all hematopoietic cells. Bok, in contrast, is restricted to reproductive tissues and has no known role in immune homeostasis [27, 31]. The final subfamily is a diverse group of proteins that share homology only through the presence of a BH3 domain, the

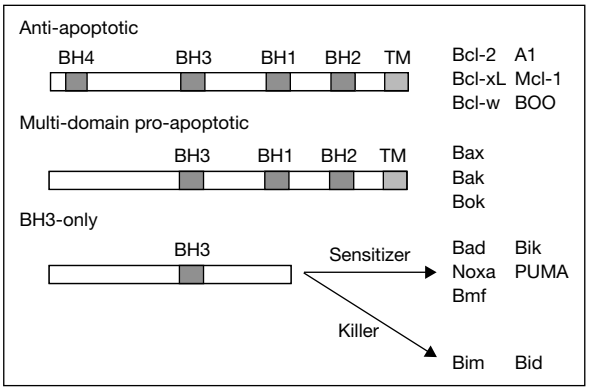

Fig. 2. Bcl-2 family. The Bcl-2 family can be subdivided based on function and the presence of Bcl-2 homology regions (BH) 1–4 and a transmembrane region (TM). The BH3-only family can be further divided into sensitizers or killer proteins.

BH3-only proteins. These proteins play critical roles to initiate cell death in response to a wide variety of stimuli [32]. It has become clear in recent years that BH3-only proteins can be further divided into two categories, those proteins that sensitize to death and those that stimulate cell death [33, 34].

Bcl-2 Family and Autoantibody Production

The role that members of the Bcl-2 family play in inhibition of cell death and the development of B cell autoimmunity has been made clear through a number of experimental models. Very early on in the study of Bcl-2, it was observed that Bcl-2 may regulate autoimmunity in addition to follicular lymphoma. Transgenic mice with B cell specific expression of Bcl-2 developed anti-nuclear, anti-histone, and anti-ds DNA autoantibodies and glomerulonephritis with age [35]. There is not a wholesale loss of B cell tolerance in Bcl-2 transgenic mice, however, as autoantibodies appear to preferentially be specific for nuclear antigens as anti-IgG rheumatoid factor autoantibodies are not increased with Bcl-2 expression [36]. In addition, the autoantibody response in Bcl-2 transgenic mice has since been shown to be variable and fail to lead to glomerunephritis on some backgrounds [37]. A variety of studies have analyzed the mechanism of this autoantibody production and generally come to the conclusion that failed elimination of B cells that mutate to gain anti-DNA specificity in germinal cells is a key source of autoantibodies in Bcl-2 transgenic mice [38–40]. Developmental negative selection of Bcl-2

transgenic B cells appears to occur normally to both model autoantigens, such as hen egg lysozyme [41], and endogenous autoantigens, such as dsDNA [39]. In contrast, despite the similar anti-apoptotic functions of Bcl-2 and Bcl-xL [42], transgenic expression of Bcl-xL was sufficient to allow autoreactive B cells to resist negative selection [43]. These autoreactive B cells were, nevertheless, tolerized by anergy and did not produce autoantibodies, demonstrating the multiple levels of regulation over B cell tolerance.

Similar to how overexpression of anti-apoptotic Bcl-2 family members can lead to autoantibody production and autoimmunity, reduced expression of multi-BH domain pro-apoptotic sub-family members can also inhibit lymphocyte death and potentially lead to autoimmunity. Deficiency in Bax, while not sufficient to cause B cell autoimmunity, did lead to a mild combined B and T cell lymphoid hyperplasia [44]. The functionally related pro-apoptotic protein, Bak, had no observable phenotype when eliminated by gene targeting [45]. Together, however, double-deficiency for Bax and Bak lead to a dramatic lymphoid phenotype [45, 46]. Due to the high lethality and variety of developmental and homeostatic defects in Bax−/−Bak−/− mice [45], bone marrow chimeras were made by transplanting Bax−/−Bak−/− bone marrow into irradiated Rag1−/− recipients [46]. Analysis of intact Bax−/−Bak−/− mice and bone marrow chimeras made with Bax−/−Bak−/− bone marrow showed massive accumulation of peripheral B and T lymphocytes with splenomegally and lymphadenopathy. A large fraction of these accumulated peripheral lymphocytes were phenotypically similar to memory cells. Many T cells had increased CD44 and decreased CD62L expression. Similarly, many B cells appeared to have undergone immunoglobulin class switch and were B220bright yet IgD– [45]. While B cell-mediated autoimmunity caused by deficiency in Bax and Bak has yet to be thoroughly examined, lymphoid infiltrates into both kidney glomeruli and liver [45] suggest that combined Bax and Bak deficiency leads to autoimmunity. Furthermore, analysis of specific T cell developmental and peripheral responses demonstrated that many aspects of T cell selection require Bax or Bak. In particular, thymocyte cell death by neglect and death by negative selection as well peripheral T cell death by neglect and death in clonal contraction following immune responses were defective in the absence of Bax and Bak [46]. Bak and Bax-deficient T cell did, however, retain their susceptibility to the Fas death receptor. Members of the Bcl-2 family, therefore, are critical regulators of lymphocyte cell death and homeostasis yet do not regulate death receptor-induced apoptosis in lymphocytes.

Deficiency of BH3-only proteins can also have significant impact on B cell tolerance [32]. In particular, the BH3-only protein Bim has been shown to be required to maintain lymphoid homeostasis and prevent autoimmunity [47]. Bim is constitutively expressed in both B and T cells and associates with the

anti-apoptotic Bcl-2 family members, Bcl-2, Bcl-xL, and Mcl-1 on the outer mitochondrial membrane [48, 49] and appears to preferentially bind Mcl-1 [49, 50]. This is in contrast to Bim localization primarily to LC8 and dynein motor complexes in fibroblasts [51]. Regulation of Bim localization in the basal, non-apoptotic state, therefore, is a subject that will require clarification in future studies. Bim expression can be further induced by the stress kinase JNK [52, 53] or by FOXO [54, 55] transcription factors. Bim-deficient mice displayed a variety of lymphoid defects including lymphocytosis, lymphoid hyperplasia, increased frequency of IgM and IgG antibody forming cells, glomerulonephritis, and IgG immune complex deposits on glomeruli [47]. Similar to Bax−/−Bak−/− lymphocytes, Bim−/− T and B cells resisted a variety of death stimuli, including death by neglect, corticosteroids, and irradiation, but remained sensitive to killing via the Fas death receptor. A series of experiments have now shown that Bim is induced by antigen receptor signaling and is required for efficient T [56–58] and B cell [59, 60] negative selection. In addition, Bim is induced in follicularly excluded B cells and has been implicated in their death [61] as well as in clonal contraction of T cells following an immune response [62, 63].

These data show that expression levels of both anti-apoptotic and pro-apoptotic Bcl-2 family members play critical roles in B cell homeostasis and prevention of autoimmunity. Consistent with this finding, stimuli that regulate B cell survival and autoimmunity are often potent regulators of Bcl-2 family member expression. As described above, BCR stimulation can induce Bim [59, 60]. The pro-apoptotic BH3-only protein, Bik, is also induced by BCR [64], although its role in B cell apoptosis is less well established. BCR stimulation also leads to induction of the anti-apoptotic proteins, Bcl-2 and A1, in a PLCγ-dependent manner [65]. The balance of these pro- and anti-apoptotic proteins then sets the threshold for B cell apoptosis in activation and tolerance.

In addition, cytokines such as BAFF and IL-7 are important regulators of Bcl-2 family gene expression in B cells. BAFF is critical to promote B cell survival [66]. Increased BAFF availability has been proposed to keep Bim in check in self-reactive B cells that survive follicular exclusion [61]. The BAFF receptors, BAFF-R and BCMA, may prevent Bim-induced toxicity by inducing the expression of anti-apoptotic Bcl-2 family member such as Bcl-2 [67, 68], Bcl-xL [68], A1 [69], and Mcl-1 [70]. In contrast, the inhibitory BAFF inhibitory, TACI, promotes apoptosis and is required to prevent autoimmunity [71], possibly by antagonizing BAFF-R induction of Bcl-2 and Bcl-xL [68]. IL-7 is required for B cell development in mice [72] and has been shown to be a potent inducer of Bcl-2 [73, 74] and Mcl-1 [50]. When either BAFF or IL-7 are not available or are antagonized, the loss of expression of anti-apoptotic Bcl-2 family members lowers the threshold for cell death and may lead to apoptosis.

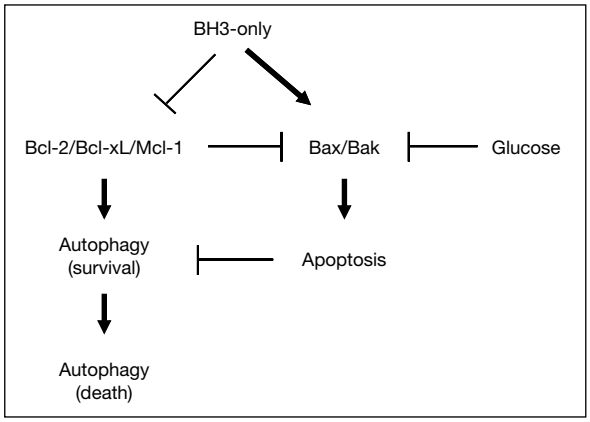

Fig. 3. Mechanism of Bcl-2 family function. Cell death is initiated by BH3-only proteins which act to either inhibit anti-apoptotic Bcl-2 family members (such as Bcl-2, Bcl-xL, or Mcl-1) or activate pro-apoptotic Bcl-2 family members (such as Bax or Bak). Anti-apoptotic Bcl-2 family proteins inhibit pro-apoptotic Bcl-2 family proteins. Glucose metabolism can also inhibit Bax activation. Bax and Bak are required to initiate mitochondrial dysfunction, caspase activation, and apoptosis. In contrast, Bcl-2 and Bcl-xL can promote autophagy. Caspases activated in apoptosis can, in turn, suppress autophagy. If autophagy becomes too excessive, cells will die a type II, or autophagic, cell death.

In contrast, enhanced BAFF or IL-7 levels can increase cell survival and contribute to autoimmunity [75–79].

Bcl-2 Proteins Regulate Mitochondrial Integrity and Release of Cytochrome *c*

How changes in Bcl-2 family member expression affect mitochondrial homeostasis are beginning to become clear. That the different expression levels of Bcl-2 family proteins affects cell fate and B cell autoimmunity has led to a model of Bcl-2 function in which anti- and pro-apoptotic proteins have opposing and mutually inhibitory functions. One key mechanistic clue as to how these proteins function was the network of physical associations among Bcl-2 family members [27]. This was initially described as the rheostat model to indicate how the physical association of Bcl-2 with Bax may explain either opposing functions (fig. 3) [80]. When Bcl-2 is in excess, Bcl-2 homodimers form and may promote survival, whereas when Bax is in excess, Bax homodimers form and may promote apoptosis. As additional Bcl-2 family members were identified, the network of physical associations between Bcl-2 family proteins

expanded [81], further strengthening the rheostat model with BH3-only proteins as key regulators to promote Bax homo-oligomers and cell death [27].

A key question of the rheostat model, however, was if the primary effect of the Bcl-2 family was to promote survival or promote death. Specifically, did Bcl-2 act directly to promote cell survival and Bax acted to inhibit Bcl-2, or did Bcl-2 act indirectly to promote survival by inhibiting an apoptotic function of Bax? The answer to this question has come from Bax−/−Bak−/− mice, where the mitochondrial forms of cell death that Bcl-2 family members can regulate, do not occur [45, 82–84]. In particular, mitochondrial death stimuli or transfection with pro-apoptotic BH3-only Bcl-2 family proteins were unable to cause apoptosis in the absence of Bak and Bax. If either Bax or Bak were present, even heterozygotically, apoptosis could occur [45]. Bak and Bax appear, therefore, to be functionally redundant and obligatory for mitochondrial apoptosis (fig. 3). In this model, signals that act to change the expression of anti- and pro-apoptotic Bcl-2 family members in B cells act to set a threshold for inhibition of Bax and Bak activity. If this apoptotic threshold is exceeded due to cell stress, downregulation of an anti-apoptotic family member, or upregulation of a pro-apoptotic family member, then Bax and Bak can induce mitochondrial damage by creating pores in the outer mitochondrial membrane [85] and cause apoptosis.

To induce mitochondrial damage, Bax and Bak must each first undergo a conformational change and oligomerization [86, 87]. Mechanisms that promote this conformation change remain uncertain, but interaction with specific BH3-only proteins may play a key role (fig. 3). Using BH3 peptides from various BH3-only Bcl-2 family members, the BH3-only Bcl-2 subgroup has been divided into sensitizes and death-inducers [33, 34]. BH3-only proteins, such as Bad and PUMA, are sensitizers and are unable to bind Bax. Instead they bind anti-apoptotic Bcl-2 family members and inhibit their function. This is not necessarily sufficient to cause Bax activation, but it relieves some inhibition on Bax and reduces the threshold for Bax-induced apoptosis. In constrast, the BH3-only protein Bid binds Bax and directly leads to Bax conformation change and mitochondrial damage after proteolytic cleavage to its active form, tBid. Bim has also been suggested to act as a death-inducing BH3-only protein in studies using purified BH3 peptide from Bim [33]. Intact Bim protein, however, has not been demonstrated to interact and cause Bax conformational change and activation [49, 88, 89]. Bim may, however, interact with Bax that has already undergone a conformation change to stabilize this conformation and enhance Bax activation [89]. Although interactions of BH3-only proteins with Bcl-2 family members are critically important in regulating cell death and can have significant impact on B cell autoimmunity, a great deal concerning the biochemistry of these complex protein interactions remains uncertain. It is clear

from the similarity of the phenotypes of Bax−/−Bak−/− and Bim−/− mice, however, that Bim is a critically important BH3-only protein in the hematopoietic system.

Metabolic Control of Bax Activation

With the discovery that Bcl-2 family proteins act to regulate mitochondrial integrity [20, 21], it became acknowledged that mitochondria act as central players in regulation of both cell death and cell metabolism. What remained unclear, however, was if these two critcal pathways in cell physiology and fate were intertwined and could affect each other. The most direct evidence that Bcl-2 family proteins and metabolic proteins may affect each other's function has come from the finding that the pro-apoptotic BH3-only protein Bad physically associates with glucokinase/hexokinase 4 in a multi-protein complex [90]. This interaction is regulated by Bad phosphorylation and is required for normal glucokinase activity and glucose homeostasis. Thus, regulation of cell metabolism does utilize Bcl-2 family proteins.

Conversely, it has also become apparent that activation of Bax can be regulated by changes in cell metabolism. A common feature of many B cell deaths that are important in the regulation of autoimmunity is that autoreactive B cells die as a consequence of failure to receive a survival signal (fig. 1). This appears to be the mechanism of cell death in follicular exclusion, where autoreactive B cells have an increased dependence on BAFF [61]. In addition, developing and mature B cells are dependent on IL-7 [77] and BAFF [67, 91], respectively, and dysregulation of this cell survival checkpoint can lead to lymphocytosis and/or autoimmunity [78, 92]. Analysis of how lymphocytes and lymphoid precursor cells die when deprived cytokine has shown that significant changes occur in cell metabolism in cytokine-deprivation that ultimately contribute to cell death. Prior to mitochondrial disruption and commitment to cell death, cytokine-deprived cells undergo cellular atrophy and do not sustain sufficient metabolic activity to maintain their viability [93]. In particular, lymphocyte expression of the glucose transporter, Glut1, and other glycolytic genes are decreased upon cytokine withdrawal and glycolytic rate sharply declines [94]. Cytokines such as IL-7 can prevent this decline and maintain glycolysis [74]. If glucose uptake is artificially maintained by constitutive expression of Glut1 and hexokinase 1, cells resist cytokine-withdrawal induced death (fig. 3) [94, 95]. Such nutrient-regulated resistance to cell death occurs through inhibition of Bax conformation change and is mediated by uptake of glucose and other nutrients [94–96]. These findings have led to the hypothesis that Bax is nutrient sensitive and access of lymphocytes to developmentally appropriate cytokines,

IL-7 or BAFF, for example, is required to maintain sufficient nutrient uptake to prevent Bax-activation and maintain cell survival.

The mechanism of how cytokines may mediate metabolic regulation and how changes in cell metabolism regulate Bax remain largely unknown. Clues, however, have come from studies of the oncogenic kinase, Akt/PKB. Akt is activated in B cells by a wide variety of signaling receptors, including cytokine and co-stimulation-induced signaling pathways and has potent anti-apoptotic functions [97, 98]. Akt activation can contribute to autoimmunity as constitutively active forms of Akt in transgenic mice with T cell specific Akt expression have shown increased T cell number and evidence of autoantibody production and autoimmunity with age [99, 100]. In addition conditional deletion of PTEN, a lipid phosphatase that inhibits Akt activation, in B cells significantly altered B cell homeostasis [101, 102].

Akt may promote disrupt lymphocyte homeostasis and lead to autoimmunity in part due to its affects on cell metabolism. Akt activation promotes multiple aspects of metabolism and leads to trafficking of glucose and other nutrient transporters to the cell surface [95, 103, 104], increased hexokinase activity and localization to mitochondrial membranes [95, 105, 106], and flux of glucose through glycolysis [95, 107] and the pentose phosphate pathway [95]. Unlike Bcl-xL, which can prevent cell death even when extracellular nutrients are limiting, Akt requires extracellular glucose and glucose uptake to prevent death [95, 105, 107]. It is unclear what aspect of cell metabolism is relevant for Akt-mediated survival, but regulation of hexokinase mitochondrial localization may be key [105, 106]. When associated with mitochondria, the hexokinase 1 and 2 isoforms associate with the voltage-dependent anion channel in a manner that can compete with Bax and prevent Bax toxicity [108]. Akt requires glucose to promote hexokinase targeting to mitochondria. If this targeting is disrupted and hexokinase dissociates from the outer mitochondrial membrane, Akt is no longer able to maintain cell viability [105, 106]. Akt did not require a fully hydrolyzable glucose, but could be replaced in these studies by the glucose analog, 2-deoxyglucose, suggesting that accumulation of phospho-2-deoxy-glucose was acting to promote hexokinase mitochondrial localization. Indeed intracellular phospho-glucose levels can affect hexokinase localization [109]. In this model, dissociation of hexokinase from mitochondria when intracellular glucose becomes limiting may allow recruitment of Bax to mitochondria to promote cell death [108]. Alternatively, rather than acting primarily as a signaling molecule, limited metabolism of glucose may be required for Akt-mediated cell survival. This is supported by observations that 2-deoxy-glucose is not completely non-hydrolyzable as is often reported, but can proceed down the pentose phosphate pathway to generate NADPH, which may by cytoprotective [110, 111]. In support of a glucose hydrolysis model are findings that while

2-deoxy-glucose can attenuate cell death, these effects are short-lived [95]. For Akt-mediated long-term cell survival, a fully hydrolyzable glucose source must be present to prevent Bax activation [95]. Because the Akt isoform, Akt1, is required to prevent the apoptosis of lymphocytes after a variety of cell death stimuli [112], it will be important in future studies to define the precise nature of Akt regulation of metabolism and metabolic pathways impact mitochondrial cell death. Cytokines may, therefore, signal via Akt to regulate B cell metabolism to prevent Bax activation.

Bax- and Bak-Independent Functions of Bcl-2

Bak−/−Bax−/− mice showed that pro-apoptotic Bcl-2 family members are required for mitochondrial apoptosis [45]. This left the role of anti-apoptotic Bcl-2 family members to be solely to prevent Bak and Bax conformation change and disruption of mitochondria and without any direct function of their own. Recently, however, it has become apparent that Bcl-2 and Bcl-xL can act to promote autophagy even in the absence of Bak and Bax (fig. 3) [113]. Autophagy is a process of self-digestion where intracellular contents are engulfed in vesicles called autophagosomes that fuse with lysosomes where vesicle contents are destroyed [114]. Autophagy can be either pro-survival or pro-death depending on the context and extent of autophagy. If Bcl-2 or Bcl-xL promote or allow autophagy to proceed too far, autophagic cell death can occur [113], thus making Bcl-2 or Bcl-xL appear to promote cell death. Autophagic cell death is morphologically distinct from apoptotic death and has been categorized as type II death, whereas apoptosis is type I death [114]. Autophagic cell death is blocked by caspase 8 [115] and generally occurs as a consequence of extreme nutrient deprivation or if cells are irreversibly damaged but fail to undergo apoptosis [113, 116]. Death occurs when digestion of intracellular contents exceeds the ability of the cell to maintain its integrity and the cell literally eats itself. This mechanism may play an important role in tumorigenesis because mice with defective autophagy due to targeted deletion of Beclin-1, a gene required for autophagy, develop a variety of cancers, including lymphomas [117, 118]. Autophagy is also required for cell survival in contexts of nutrient starvation, such as occurs in *C. elegans* dauer phenotype [119] or in post-natal mice [120]. The role of autophagy as a cell survival mechanism is not clear, but may be to provide substrates for mitochondrial metabolism. Autophagic Bak−/−Bax−/− cells undergo necrosis if autophagy is blocked, but necrosis can be prevented by providing cells with an alternate energy source [116]. A great deal remains to be understood about how autophagy is regulated, its role as a potential source of metabolites, and how autophagy can promote

cell survival in some contexts but cell death in others. An emerging model may be that in the absence of apoptosis, anti-apoptotic Bcl-2 family members promote autophagy to continue to provide mitochondria with substrates to prevent necrosis. If autophagy exceeds an undefined threshold, then cells undergo autophagic cell death. The role that this process may play in lymphocyte homeostasis and survival is unclear but due to the metabolic changes that occur in cytokine deprivation [93–95], autophagy may play an important role in survival of autoreactive B cells.

Regulation of Cell Death after Mitochondrial Damage and Release of Cytochrome *c*

In addition to regulation of mitochondrial integrity by members of the Bcl-2 family, numerous mechanisms exist that can regulate formation of the apoptosome even after mitochondrial disruption. The apoptosome consists of Apaf-1, caspase 9, and dATP and is critical to activate caspase 9 and initiate the caspase proteolytic cascade [121]. The role of the apoptosome in lymphocyte cell death is uncertain as some studies have shown that Apaf-1 and the apoptosome may function primarily to accelerate cell death rather than be required for death [122, 123]. In contrast, other studies have shown that defects in apoptosome formation or inhibition of caspase activity can impair thymic negative selection [124] or attenuate B cell death after BCR ligation and mitochondrial disruption [125, 126]. In addition, upregulation of endogenous caspase inhibitors correlates with autoimmunity [127]. A variety of mechanisms have been recently described that can inhibit apoptosome formation or caspase activation (fig. 4) and may contribute to survival of autoreactive lymphocytes and development of autoimmunity.

Inhibitor of Apoptosis Proteins

One of the first indications that caspase activity was regulated downstream of the mitochondria came with the discovery of the inhibitor of apoptosis proteins (IAPs) family of proteins. IAPs were identified in baculovirus infected cells as proteins that promote vial replication by inhibiting apoptosis, and cellular homologues have been cloned in many species including humans [128]. Addition of purified IAPs to cell extracts blocked cytochrome *c*-induced caspase activity [129, 130] and overexpression of IAPs inhibited apoptosis despite release of cytochrome *c* from the mitochondria [131, 132]. In addition, transgenic expression of XIAP in thymocytes protected cells from a variety of

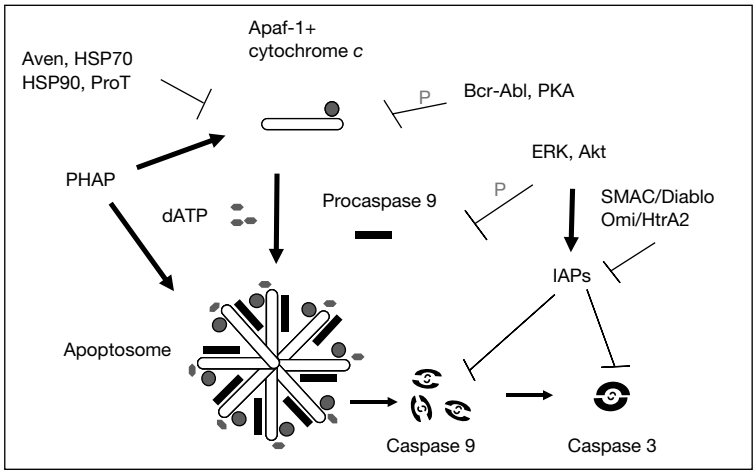

Fig. 4. Post-mitochondrial regulation of apoptosis. Following release of cytochrome *c* from the mitochondria both the formation and the activity of the apoptosome can be modulated. The inhibitory proteins Aven, Hsp70, Hsp90 and ProT have all been shown to bind to and inhibit Apaf-1 while PHAP can enhance the activity of the apoptosome through as yet undefined mechanisms. Caspase activity can be directly inhibited in the presence of IAPs and IAP activity is neutralized by SMAC/Diablo and Omi/HtrA2. Kinase signaling pathways also impinge on the function of the apoptosome where Akt and ERK target caspase 9 while Bcr-Abl and PKA appear to act at the level of Apaf-1.

apoptotic insults [133]. In mammalian cells, IAPs (XIAP, cIAP1, cIAP2 and ML-IAP) inhibit apoptosis by directly binding to and inhibiting the enzymatic activity of caspase 9, 3 and 7 [128]. XIAP, cIAP1 and cIAP2 contain a Ring finger domain that functions as an E3 ubiquitin ligase and IAPs have been reported to promote the ubiquitination and degradation caspases 3, 7 and 9 as well the pro-apoptotic protein, SMAC/Diablo (addressed below) [134–136]. This ubiquitin ligase activity appears to be important for the anti-apoptotic function of IAPs as mutation of the XIAP Ring domain resulted in a decreased ability of XIAP to inhibit apoptosis [137].

IAP-mediated inhibition of caspase activity is neutralized by the pro-apoptotic proteins SMAC/Diablo and Omi/HtrA2. SMAC/Diablo is synthesized as a precursor protein that is imported into the mitochondria where it is cleaved into the mature form. Following mitochondrial disruption, mature SMAC is released into the cytosol where it acts to relieve XIAP-mediated inhibition of caspases [138–141]. A motif at the amino terminus of SMAC, similar in sequence to the XIAP-binding region of cleaved caspase 9, confers binding of SMAC to XIAP [142]. SMAC then antagonizes both interaction of XIAP with

caspase 9 and XIAP ubiquitin ligase activity [134, 137]. The serine protease, Omi/HtrA2 is also released from the mitochondria in response to apoptotic stimuli, and can bind to and promote the inactivation of XIAP in part by cleaving XIAP [143–145]. The importance of IAP-mediated inhibition of caspase activity is exemplified in postmitotic sympathetic neurons which undergo apoptosis only if XIAP-mediated inhibition of caspase activation is relieved [146–149].

Decreased Levels of Apoptosome Constituents or Altered Cellular Compartmentalization

In several instances, resistance to cytochrome *c* has been shown to reflect a direct down-regulation of the core constituents of the apoptosome. For example, a strong correlation between chemoresistance and decreased Apaf-1 protein levels has been reported for some leukemic and other cancer cells [150–153]. Moreover, down regulation of caspase 3 protein correlated with inhibition of cell death in some breast cancer cell lines and tissues [151]. In addition to decreased levels of apoptosome components, altered sensitivity to cytochrome *c* may manifest through the mislocalization of Apaf-1. Sun et al. reported that Apaf-1 in cytochrome *c*-resistant Rajii cells (Burkett lymphoma cells) was localized to what appeared to be lipid rafts or some other 'non cytosolic' compartment. Intriguingly, forced redistribution of Apaf-1 to the cytosol restored cytochrome *c*-induced caspase activity in cell extracts and etoposide-induced apoptosis in intact Rajii cells [154].

HSP70, HSP90, Aven, Prothymosin-Alpha and PHAP

Several other proteins have been reported to associate with either Apaf-1 or the fully formed apoptosome to regulate its activation. Hsp70 and 90, two cytoprotective chaperone proteins, have been reported to bind to Apaf-1 and prevent the assembly of the apoptosome [155–157]. The CARD domain containing proteins Aven and APIP (apaf-1 interacting protein) inhibit the activation of caspase 9 by competing for binding to Apaf-1 [158, 159]. In a screen conducted to identify modulators of the apoptosome, Wang and colleagues discovered a novel pathway mediated by HLA-DR-associated proteins (PHAP) and oncoprotein prothymosin-alpha (ProT) that regulates mitochondria-initiated caspase activation. ProT appears to inhibit the apoptosome by preventing the recruitment of caspase 9 to Apaf-1, while PHAP was enhances the

activity of the apoptosome, possibly by promoting the formation of the apoptosome through enhanced binding of caspase 9 to Apaf-1 [160] or through a direct interaction with caspase 3 [161].

Protein Kinase-Mediated Regulation of Apoptosis Downstream of the Mitochondria

Kinase signaling pathways have also been shown to impinge upon the proper functioning of the apoptosome. Akt was shown to inhibit apoptosis downstream of the mitochondria by phosphorylating and stabilizing X-linked inhibitor of apoptosis protein XIAP [162]. Akt-mediated phosphorylation of XIAP prevented DNA damage-induced XIAP ubiquitination and degradation, and promoted cell survival after treatment of cisplatin. Moreover suppression of XIAP protein via siRNA or antisense induced apoptosis and inhibited Akt-mediated cell survival in ovarian cancer cells [162]. The Map kinase ERK can also modulate the function of the apoptosome by targeting caspase 9 activity. Activation of the B-Raf/Mek/ERK pathway was shown to inhibit caspase 3 activity after cytochrome *c* both in mammalian cells [163] and *Xenopus* egg extracts [164]. The ERK-mediated inhibition of caspase 3 activity appears to occur in part through phosphorylation of caspase 9 on threonine 125 to render the caspase catalytically inactive [165], although additional regulation may occur. Like Akt, ERK has also been suggested to maintain the protein levels of XIAP, which would then act to directly inhibit caspase 9, 7 and 3 [166].

Most recently, the proper formation and activation of the apoptosome itself was shown to be regulated by protein kinases [167, 168]. In the presence of the constitutively active oncogenic tyrosine kinase Bcr-Abl, cell extracts displayed a defect in the ability of caspase 9 to bind to Apaf-1, which in turn inhibited the activity of caspases 9 and 3 [167]. Unlike Akt and ERK, the Bcr-Abl-mediated inhibition of apoptosome function did not appear to occur *via* the regulation of caspase 9. Rather, in vitro evidence suggested that Bcr-Abl can indirectly mediate inhibitory phosphorylation of Apaf-1 [167]. Protein kinase A (PKA/cyclic AMP dependent kinase) was also reported to inhibit caspase 3 and 9 activity by modulating the apoptosome, via a mechanism similar to Bcr-Abl [168]. As was reported for Bcr-Abl, the ability of caspase 9 to bind to Apaf-1 was perturbed by PKA and PKA was shown to phosphorylate Apaf-1 in vitro. For both Bcr-Abl and PKA, whether or not the phosphorylation of Apaf-1 confers their inhibitory function on the apoptosome will await identification of de novo phosphorylation sites on endogenous Apaf-1. Nonetheless, these data provided the first evidence of apoptosomal inhibition by regulation of Apaf-1 through phosphorylation.

Summary and Future Prospects

Regulation of B cell death by mitochondrial cell death pathways is critical to maintain tolerance. It is now clear that a cell death can be regulated both pre- and post-mitochondrial disruption. Signals that regulate Bcl-2 family member expression or function are clearly of critical importance to affect mitochondrial stability as well as alternate pathways of cell death, such as autophagy. After mitochondrial damage, the combined affect of impaired mitochondrial function and activation of caspases then leads to cell death. If caspase activation is impaired by inhibition of caspase or apoptosome function, cells may survive and ultimately contribute to autoimmunity. A key challenge in future work is to define how these pathways are regulated and interact and to determine how the point of commitment to cell death – either at the point of mitochondrial disruption or afterwards – affects autoreactive lymphocytes and onset of autoimmunity.

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Role of Complement and Other Innate Immune Mechanisms in the Removal of Apoptotic Cells

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Abstract

The complement system is regarded as an ancient host defense mechanism that helps to promote phagocytosis and/or killing of foreign microorganisms. Less well known is the facilitatory role that complement and other closely related molecules of the innate immune system play in the removal of dying cells. In this chapter, we review the complement system and the mechanisms of complement activation that include natural antibodies and acute phase proteins. The effects of spontaneous and genetically engineered mutations on function of these proteins and their relationship to autoimmune diseases such as lupus are discussed. We also review the known function of non-complement receptors and their roles in recognition and removal of dying cells in normal cellular homeostasis and in inflammation.

Billions of cells undergo apoptosis daily as part of development, tissue remodeling, or senescence. It is vital that these cells be cleared rapidly before they progress to secondary necrosis, a process associated with loss of plasma membrane integrity and extravasation of potentially inflammatory contents into the extracellular space.

Since apoptotic cells need to be removed rapidly by phagocytosis, recognition and engulfment must occur either by neighboring cells (as occurs in embryogenesis) or elements of the innate immune system (as occurs in developing lymphoid tissue). Innate means to exist from origin, and represents the first or ‘original’ immune response. There is no memory response involved in the innate immune response to pathogen; therefore, the cells of the innate immune system must be able to recognize and respond to a broad spectrum of pathogens.

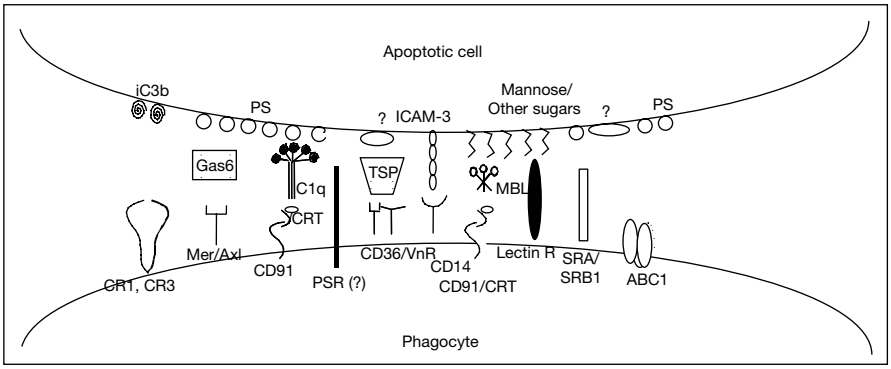

Fig. 1. Receptors, ligands, and bridging proteins reported to be involved in apoptotic cell clearance. These include (from left to right) complement receptors CR3 and 4 interacting with iC3b, CR1 that may bind to C1q and MBL. Mer and Axl are receptor tyrosine kinases that have been shown to bind to the bridging molecule Gas-6, which in turn adheres to phosphatidylserine (PS) on the apoptotic cell surface. Other proteins, including MFG-E8, β_2-microglobulin and annexin I also bind to PS on apoptotic cells. It is not clear whether a separate PS receptor exists. CD91/CRT bind C1q and the collectins as discussed in the text. The bridging protein thrombospondin (TSP) likely binds to both CD36 and the vitronectin receptor (VnR). CD14 has been shown to interact with ICAM-3 on the apoptotic cell surface. Lectins, such as the asialoglycoprotein receptor, have been implicated in the recognition of altered carbohydrates on the surface of apoptotic hepatocytes. Scavenger receptors, such as SR-A and SRB-1, recognize oxidized phospholipids and are thought to play a role in apoptotic cell removal. Finally, the ABC-1 transporter located in the membrane of the phagocyte has been implicated in extrusion of PS on the apoptotic cell plasma membrane.

Once a pathogen invades a tissue, it can be opsonized by components of the innate immune system. One example of this is the opsonization of invading pathogens in the lung by surfactant proteins, which leads to quick clearance of the particle by alveolar macrophages. At other sites, related proteins such as mannose-binding lectin (MBL), C1q and C3 can bind to pathogens, activate complement, resulting in opsonization and clearance by macrophages or the elicitation of an inflammatory response and destruction of the pathogen. Tissue macrophages may themselves recognize pathogens through Toll-like receptors (TLR) and initiate an inflammatory response. Other cells involved in the initial response to pathogens, such as neutrophils and monocytes, are called in as support by way of macrophage chemokine production.

Surprisingly, recent evidence suggests that mechanisms similar to those used by the innate immune system to recognize and dispose of pathogens are also used to recognize and dispose of apoptotic cells (fig. 1). Many of the proposed systems function as scavenger receptors or receptors for bacterial

components, reinforcing the idea that the innate immune system has evolved to scavenge both self as well as non-self material. What happens after clearance dictates how the innate immune system and, indeed, the adaptive immune system respond to the ingested particles.

The key components of innate immunity that facilitate removal of apoptotic cells are serum factors such as natural antibodies, complement and acute phase proteins that opsonize the apoptotic cell and the phagocytes such as monocytes, macrophages and dendritic cells that engulf the dying cells. In this chapter, we will discuss how components of the innate immune system play a pivotal role in recognition and clearance of apoptotic cells. We discuss how this process results in anti-inflammatory signals to the adaptive immune system thereby avoiding an immune response to self antigen (autoimmunity).

Appetizing Opsonins

Shortly after the cell dies, it expresses a number of 'eat me' signals to alert phagocytes that the cell must be removed. Although some phagocytic receptors recognize these eat me signals directly, an increasing number of examples of 'protein bridges' between the apoptotic cell and the phagocyte appear to be required in order to make the meal appetizing. These protein bridges act as opsonins [1] that amplify the number of potential receptors that recognize the same alteration of apoptotic cells.

Proteins that Bind to Phosphatidylserine

Apoptotic cells lose phospholipid asymmetry early in the apoptotic process. Aminophospholipid translocase activity decreases and the activity of another enzyme, a phospholipid scramblase, increases during apoptosis. This results in the 'flipping' of the anionic phospholipid phosphatidylserine (PS) to the outer leaflet of the plasma membrane of the apoptotic cell [2]. Human monocyte-derived macrophages stimulated with a digestible particle can be inhibited in the uptake of apoptotic cells by PS-containing liposomes in a stereo-specific fashion. This result indicates that the macrophages possess a receptor that can specifically interact with PS [3] or protein bound to PS. Whereas PS may be recognized directly by a putative phosphatidylserine receptor (PSR), proteins such as annexin I, Gas6, β-2-glycoprotein 1, and milk fat globule epidermal growth factor 8 (MFG-E8) also bind to PS, thereby allowing the protein bridge to interact with different receptors (fig. 1). Recent evidence suggests that these opsonins confer additional site selectivity or specialized

signals to phagocytes. For example, MFG-E8 facilitates apoptotic cell clearance in germinal centers (discussed elsewhere in this volume) whereas C1q deficiency leads to apoptotic cell accumulation in the kidney [4]. Complement components, such as C1q [5] and iC3b [6] also opsonize apoptotic cells for recognition and efficient clearance by macrophages as discussed below.

Complement

The complement system is composed of ~30 tightly regulated proteins that form a cascade of linked enzymatic reactions. Their role in host defense is either to coat foreign organisms for ingestion by phagoytes or to kill them by lysis. Much like the regulation of the caspase family of proteins, or the fibrinolytic system, serum complement components are, for the most part, synthesized as zymogens and become active after a triggering cleavage event. This regulation prevents the uncontrolled activation and thus protects the body from inflammation and destruction of healthy tissue, but allows for rapid activation of the system when it is needed.

There are three pathways of complement activation, the classical pathway, the alternative pathway, and the lectin-mediated pathway as illustrated in figure 2. The *classical pathway* of complement activation is typically activated following interaction of the Fab regions of the immunoglobulin with antigen resulting in an induced conformational change in the CH2 domain of the Fc portion of the molecule. C1q then undergoes a conformational change, rendering its 'neck' region open and available for binding C1r and C1s dimers, resulting in formation of the C1 complex. The resulting activation of C4 and C2 generates a C4bC2b convertase that cleaves C3. The lectin pathway is very similar to the classical cascade; it differs in the first component, MBL. MBL was found to activate complement in the absence of C1q [7]. MBL, after binding to pathogen surfaces, can bind to serine protease zymogens, MASP1 and MASP2, that are similar to C1r and C1s culminating in the generation of a convertase comprising MASP, C2 and C4. C1q and MBL share structural and functional homology not only with each other, but also with a family of proteins known as collectins. The *alternative pathway* is activated by certain bacterial surfaces and recruits serum Factors B and D to generate the alternative pathway C3 convertase, C3bBb. The C3 convertases formed by any of the pathways above cleave a 6 kDa portion off of the N-terminus of C3 generating C3b. C3b can also be generated by spontaneous hydrolysis of the sequestered thioester bond within C3 ('tickover'). If C3b binds to a surface, there are two possible fates: (a) it may be further cleaved and inactivated (C3bi) by factor I and the co-factor, factor H, as well as by membrane complement regulatory proteins or (b) it can generate the C5

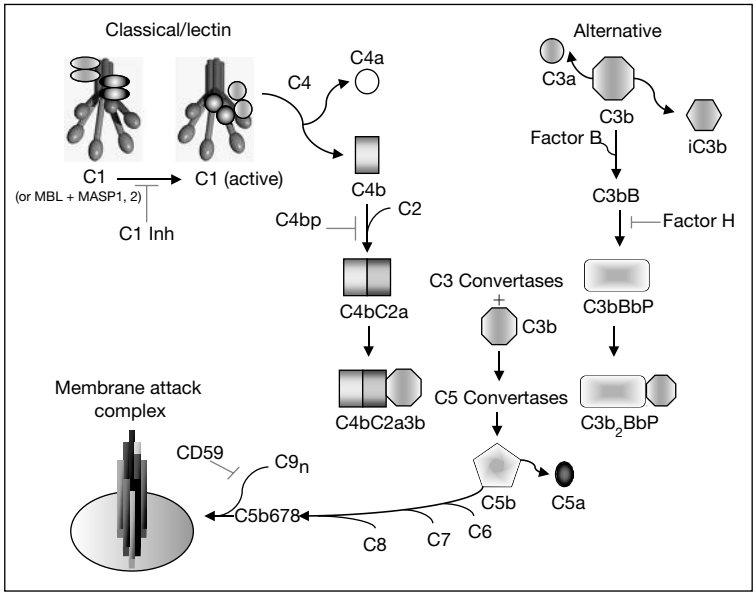

Fig. 2. The complement cascades. The classical pathway is activated by binding of C1q (coupled with C1r and C1s to form C1) to antigen-antibody complexes. Similarly, the lectin pathway is activated by MBL binding to carbohydrate residues on bacterial surfaces, and this activates MASP1 and MASP2. The lectin pathway shares the same downstream components as the classical pathway. The alternative pathway is activated by spontaneous 'tickover' of C3, by certain bacterial products and this pathway also functions as an amplification loop for C3 convertase formation. Activation of any of the three complement cascades, ultimately results in formation of the membrane attack complex (MAC) if not attenuated by regulatory proteins.

convertase (C4bC2aC3b) to initiate the assembly of the membrane attack complex (MAC). If C5b67 is not decayed, it binds the cell or pathogen surface and recruits C8, a three-chain glycoprotein that, when bound to C5b67, can penetrate the lipid bilayer of cells. C5b678 acts as an initiator for the polymerization of C9 in the cell membrane. Twelve to 15 copies of this single chain protein oligomerize in the cell membrane, forming a hole and resulting in cell lysis.

C1q – Key Initiator of the Classical Pathway

Deficiency in classical complement components are amongst the strongest known genetic risk factors for developing lupus and most individuals lacking functional C1q develop severe SLE [8]. C1q is a 460-kDa heterohexameric

protein comprised of structurally related, disulfide-linked A, B, and C chains. The complete protein forms a sertiform, or 'bouquet of tulips' configuration, with the collagenous tail forming the 'stalk' and the globular head region forming the 'flowers.' This structure is conserved among members of a related group of innate immune proteins called the collectins (discussed in detail elsewhere in this volume); C1q is not considered a collectin as it does not possess lectin activity in its globular head domain. C1q binds to anionic phospholipids as well as to the Fc portion of immunoglobulins.

C1q in humans is found in a plasma-soluble form and a macrophage membrane-bound form. Differences in the B chain of the protein cause the two distinct forms. Soluble C1q is present in the serum at a concentration of about 75 μg/ml. Membrane-bound C1q is known to exist on the surface of human macrophages and is a marker of maturity for these cells. It is thought that this membrane-bound variant may assist the macrophage with phagocytosis, and the polymerization of this C1q around the particle being engulfed may be the mode of operation [9].

C1q was initially detected on fixed, irradiated keratinocytes [10] but the ligand(s) for C1q binding was not elucidated. C1q may be activated directly by chemical alterations to the apoptotic cell surface or secondary to binding of natural antibodies or acute phase proteins. C1q binds to apoptotic cell surfaces in a punctate, clustered pattern. This binding may facilitate the clearance of apoptotic cells by professional phagocytes by forming a bridge between the calreticulin (CRT)/CD91 receptor complex and the apoptotic cell [5] and/or C1q activates the classical pathway of complement leading to deposition of C3b/bi and clearance through CR3 and CR4 [6]. C1q can also bind to constituents of necrotic cells, including phospholipid-bearing membrane fragments, DNA, chaperone proteins from the endoplasmic reticulum and likely aids in scavenging the debris from sites of tissue inflammation or breakdown [11].

The mechanism linking C1q deficiency to lupus remains to be fully elucidated. A requirement for C1q (or activation of the classical complement pathway) to facilitate clearance of apoptotic cells remains the strongest possibility, since it is supported by both in vivo [4] and in vitro [6] evidence. Failing clearance, the apoptotic cells progress to a state of necrosis and spill their potentially inflammatory contents, such as heat shock proteins, HMGB1 and nucleic acids, into the tissues, provoking inflammatory responses [12].

C3-Complement Clearinghouse

As mentioned above, C3b binds covalently to activating particles that include the outer surface of foreign (e.g. bacteria) and self (e.g. apoptotic cells)

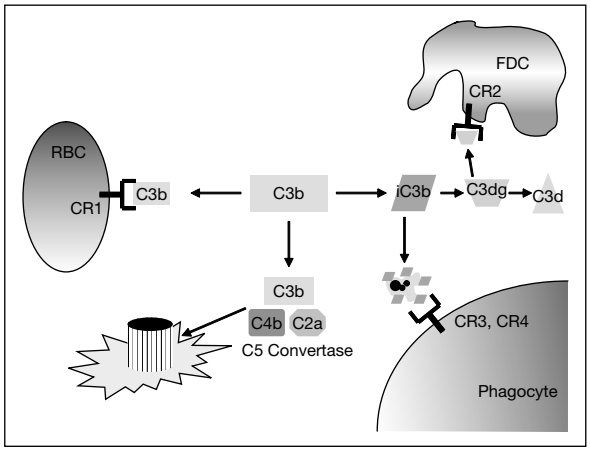

Fig. 3. Potential fates of C3. C3b attached to immune complexes may be bound to CR1 on red blood cells (RBCs), B cells, or phagocytes and rapidly cleared. Alternatively, C3b forms an integral part of the C5 convertase, resulting in release of the potent chemoattractant, C5a, and the initiation of the membrane attack complex (MAC). Finally, C3b may be sequentially cleaved to iC3b (a stable opsonin), C3dg (a ligand for CR2), and C3d.

particles. There are 3 potential fates for C3b (fig. 3): (1) it may immediately bind to its receptor, CR1 on red cells, phagocytes, B cells or FDC – this the common fate for immune complexes and results in disposal in the liver or spleen; (2) it may form part of the C5 convertase – this occurs when vast amounts of C3b are generated in the relative absence of complement regulatory proteins Factor I, DAF, MCP-1 and CD59 [13] – this is the common fate on bacteria and leads to the generation of the MAC (comprising C6–9) and lysis of the bacteria, and (3) it may be further cleaved to C3bi (inactive), C3dg and C3d. These cleavage products may result in different fates. C3bi is a ligand for the complement receptors, CR3 and CR4, that are expressed on phagocytes and help to promote phagocytosis of apoptotic cells. C3dg is the ligand for CR2 (CD21) that is expressed on FDC and B cells. FDC retain C3dg bound antigens for presentation to B cells in lymphoid tissue whereas C3d(g) lowers that threshold of activation of B cells to the bound antigen [14]. Thus impaired clearance of apoptotic cells may promote B cell reactivity to self antigens. That failure of apoptotic cell clearance could lead to the further cleavage of iC3b to C3dg on the apoptotic cell surface. Ligation of CR2 on autoreactive B cells may lower the threshold for autoantibody production by these cells (see below).

In view of two reports indicating that mice with deficiencies of serum IgM developed a lupus-like disease [15] we proposed that IgM might be the initiator of complement activation on the dying cells. Consistent with this idea, we recently observed that polyclonal IgM, but not IgG, readily binds to apoptotic cells. Tryptic digestion of IgM revealed that binding was an antigen/antibody reaction rather than non-specific binding through the Fcμ portion of the antibody.

Clues to the specificity of the IgM antibodies were provided by enzymatic hydrolysis of the cell membrane components and by antibody inhibition experiments. Exposing apoptotic cell membranes to phospholipase PLA_2 increased, whereas PLD reduced, IgM binding and complement activation. Studies using an $iPLA_2$ specific inhibitor, BEL, demonstrated that activation of endogenous $iPLA_2$ by caspase 3 contributed to the enhanced IgM cell surface binding during apoptosis (fig. 4). One of the membrane phospholipid substrates of $iPLA_2$ is phosphorylcholine (PC). Activated $iPLA_2$ hydrolizes the sn2 fatty acid in PC to produce lyso-PC (LPC). Absorption studies combined with direct plate binding assays revealed that the antigen recognized by serum IgM was the PC moiety on lysophosphatidylcholine. IgM antibodies failed to bind to phosphatidyl lipids such as PE and PS, but did recognize PC-Cl, PC-KLH, and lyso-PC [16].

This specificity of natural IgM for lyso-PC is strikingly similar to that described for CRP [17], an acute-phase protein that is also implicated in the clearance of self antigens during inflammation and tissue injury. Furthermore, Familian et al. [18] have reported that SAP also binds to apoptotic cells, most likely by specific interaction with phosphorylethanolamine. Together, these findings suggest that there are multiple proteins that recognize similar phospholipid(s) on the damaged cell membrane and promote the clearance of dead and dying cells. An intriguing question is whether IgM and the pentraxins compete for binding to the same membrane phospholipids. Regardless, these proteins must exert nonredundant functions since knockout of either sIgM [15] or SAP [19] leads to lupus-like diseases associated with impaired handling of apoptotic cells or their products.

We previously demonstrated that, when apoptotic cells were incubated in the presence of IgM-deficient serum in vitro, C3 deposition on the cells was substantially reduced, implying that natural IgM autoantibodies were largely responsible for complement deposition on dying cells [16]. We recently confirmed that IgM is required for optimal in vitro phagocytosis of apoptotic cells by bone marrow-derived macrophages and for efficient in vivo clearance of apoptotic cells. We observed that C3 was deposited on apoptotic cells in vivo and that both C3 deposition and rapid phagocytosis of apoptotic cells were dependent upon the presence of IgM antibodies (fig. 4). Since exposure of

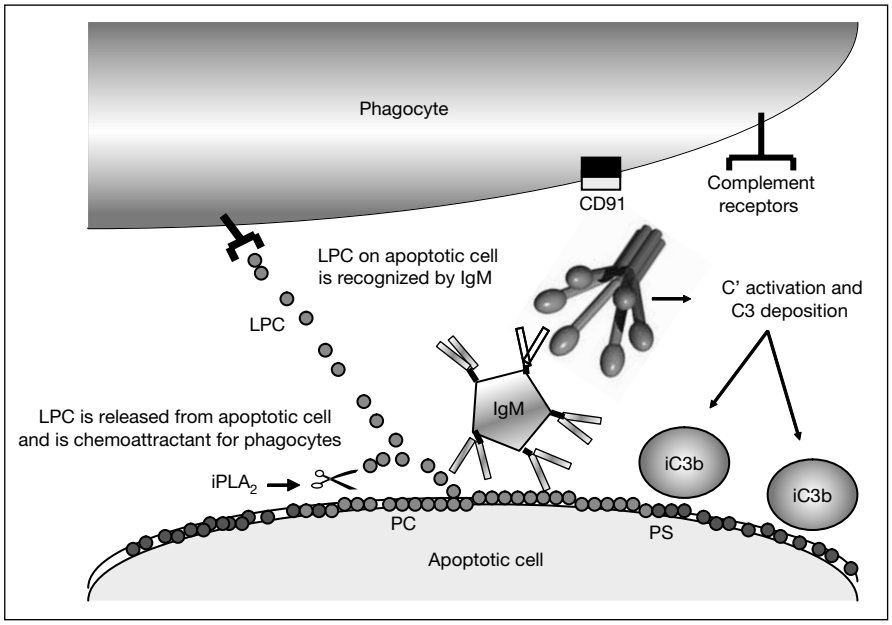

Fig. 4. Apoptotic cells activate complement. Apoptosis leads to activation of the calcium independent enzyme, iPLA2, which generates lysophosphatidylcholine (LPC) on the apoptotic cell surface. LPC is highly soluble and also acts as a macrophage chemoattractant. LPC exposed on the apoptotic cell surface is recognized by natural antibodies, leading to the activation of the classical complement pathway and covalent deposition of C3b molecules on the apoptotic cell surface. C3b is recognized by CR3 and/or CR4 on the phagocyte promoting clearance of the cell. C1q also binds to calreticulin (fig. 4, 5).

apoptotic cells to polyclonal IgM restored in vivo phagocytosis of apoptotic cells in sIgM deficient mice, but not in mice deficient in both C1q and sIgM, we concluded that IgM is required for classical pathway activation on apoptotic cells, at least under the well established conditions known to be aberrant in complement deficient mice [20] as well as in many spontaneous lupus models [21]. Although CRP, SAP, MBL and other potential activators of complement have been shown to deposit on dead or dying cells, rapid clearance required IgM [22].

Calreticulin

Calreticulin (CRT) was first identified in 1972 as a calcium-binding protein in the sarcoplasmic reticulum. CRT has been found to be associated with a

variety of proteins in the ER, and so, in addition to its role as a calcium storage protein, also acts as a chaperone in the ER [23]. CRT can act as a receptor, shuttling proteins such as protein kinase inhibitors, between the nucleus and the cytoplasm in HeLa cells. Focal adhesion disassembly mediated by thrombospondin is dependent on cell-surface CRT [24]. Here, CRT binds to the N-terminus of thrombospondin, resulting in activation of a signaling cascade resulting in actin rearrangement and focal adhesion reorganization.

CRT may be released from activated neutrophils, and, although there is some suggestion that it may bind to the globular heads of C1q, it also binds to C1q tails, acting as an inhibitor of complement activation by blocking C1r and C1s dimer binding and the formation of the C1 complex [25]. In addition, calreticulin, upon release from activated neutrophils, stressed cells (i.e. heat shock), or necrotic cells, can be a target for autoantigen production in diseases such as SLE. These circulating anti-calreticulin antibodies (found in 40% of SLE patients) [26] could theoretically enhance autoimmunity by inhibiting the collectin-mediated clearance of apoptotic cells.

The cC1qR and CRT are identical [27]. This discovery led to some controversy. While many groups adhere to the dogma that CRT is localized solely to the endosarcoplasmic reticulum, numerous investigators have shown that the protein is found in other cellular compartments, most especially on the cell surface [reviewed in 28]. How does CRT that is synthesized in the ER and has a KDEL sequence to ensure that it remains there, get to the cell membrane? CRT is a chaperone – it binds to many proteins as they are taken through the folding process in the ER. CRT has been shown to bind to one of these proteins and to remain associated with it until the protein, MHC I, is presented on the cell surface [29]. Inhibition of protein secretion with brefeldin A inhibits surface transport of CRT. Most recently, CRT has been demonstrated to interact with CD91 on the surface of macrophages. In a model system, CRT was released in a pool, along with related chaperones, the heat shock proteins, by necrotic cells and then became specifically associated with CD91 [30]. The possibility that calreticulin could be displayed on the surface of the apoptotic cell in either an altered state or configuration from how it is found on viable cells is currently under investigation.

Acute Phase Proteins

Although clearance of apoptotic cells has been described mainly in the contexts of development and homeostasis, removal of dead and dying cells as well as cellular debris is critical to resolution of inflammation [31]. Cytokines such as TNF-α and IL-1 not only promote inflammatory responses, they upregulate

acute phase proteins (APPs), many of which participate in the clean up operation (note that many complement components are also APP). Chief amongst the APP in humans is CRP, an acute phase protein produced by the liver that can increase more than 1,000-fold during inflammation.

C-reactive protein (CRP) was first characterized in 1930 as a protein that bound to the C polysaccharide of *Streptococcus pneumoniae* leading to the concept that CRP helped opsonize pathogens. More recently, CRP was noted to bind to self components such as chromatin and small nuclear ribonucleoprotein (SnRNP) particles [32]. We recently demonstrated that CRP can bind to cells prior to their rupture, most likely binding to cell surface LPC as discussed above. CRP markedly enhanced C3 binding to apoptotic cells but recruitment of the complement regulatory protein, Factor H, that acts as a co-factor for factor I, prevented activation of the MAC and lysis of the cells [33]. Furthermore, we demonstrated that CRP-facilitated apoptotic cell clearance promoted TGF-β production by macrophages, consistent with an immunosuppressive role for this scavenging.

Recent findings endorse the anti-inflammatory effects of CRP. When expressed as a transgene or injected as a purified protein into mice, CRP suppressed a lupus-like disease as well as experimental allergic encephalomyelitis [34, 35]. This result was not observed in IL-10 deficient mice, implying that CRP induced its anti-inflammatory effect by induction of this cytokine. Whether the anti-inflammatory effect was mediated by the scavenging function of CRP for dead and dying cells associated with TGF-β production or binding of CRP to Fc gamma receptors (a finding that is controversial), remains to be determined.

The homolog of CRP in mice is the pentraxin serum amyloid protein (SAP). This protein can also bind to chromatin and apoptotic cells, and deficiency in SAP predisposes to a lupus-like disease in certain strains of mice [19].

'Find Me' Signal

Supernatants from apoptotic cells have chemotactic properties for macrophages. Lauber et al. [36] observed that the key chemotactic component contained within apoptotic supernatants is the phospholipid LPC (fig. 4). Diffusible LPC in apoptotic cells was generated by the same pathway described above, namely caspase 3 cleavage of iPLA2. Thus, iPLA2 appears to be a pivotal enzyme facilitating both the opsonization of apoptotic cell as well as the directed recruitment of phagocytes to facilitate clearance of the dying cells. The question remains, which receptor is responsible for chemotaxis in response to LPC?

The Phagocytes

Phagocytes vary in their phenotype and their roles in the immune response. Monocytes patrol the systemic circulation. After entering the tissue, these cells differentiate into either macrophages or myeloid dendritic cells. This differentiation step may depend on the chemotactic factors that drew the cell to the tissue, the microenvironment of the tissue, or both. Although DCs are less efficient than macrophages at engulfing whole cells, their antigen processing machinery and potent antigen-presenting properties give them special status in terms of directing the immune response.

Macrophages

Macrophages are the cell type most intensively studied in the context of apoptotic cell clearance. However, macrophages are quite heterogeneous. They may be activated by inflammatory interferons (such as interferon-γ) and/or bacterial products (such as LPS) to an 'angry' or inflammatory state which results in increased expression of phagocytic receptors such as FcγR and complement receptors, production and secretion of pro-inflammatory cytokines such as TNF-α and IL-12, and cytotoxic chemicals such as reactive oxygen and nitrogen species. These have been dubbed 'M1' macrophages. The polar opposite of this phenotype is the 'M2' macrophage, also known as the 'alternatively activated' phenotype. These macrophages secrete primarily Th2 cytokines such as IL-10. These macrophages are unable to mount a pro-inflammatory response to stimuli such as LPS and have been likened to a tumor-associated macrophage (TAM) phenotype. These macrophages are unable to initiate an immune response to tumor and may even produce survival factors for the tumor cells [37].

M2 macrophages are more efficient in their ability to recognize and engulf apoptotic cells. This may be due to upregulation of certain cell-surface receptors such as CD14 for apoptotic cell engulfment. As clearance is thought to be a noninflammatory or anti-inflammatory event, this upregulation of apoptotic cell clearance may form a positive feedback loop, enabling a macrophage to quickly and efficiently clean up multiple cell corpses and aide healing by the promotion of an anti-inflammatory environment. Tumor cells may exploit this mechanism. For example, certain B cell lymphomas such as Burkitt's lymphoma maintain a constitutive subset of apoptotic cells, possibly as a result of the dysregulation of the c-myc proto-oncogene that is linked to tumorigenesis. Viable tumor cells produce the M2 cytokine IL-10, that inhibits M1 macrophage activation [37, 38] whereas the apoptotic tumor corpses induce the production of anti-inflammatory cytokines as well as growth factors, such as

BAFF [37]. BAFF, in turn, promotes the survival of the B cell tumor. In this way, macrophages can be subverted from acting as potent effector cells of the innate immune system to acting as support cells for tumor survival and growth.

Dendritic Cells

There are two principal subsets of dendritic cells (DCs), myeloid and plasmacytoid. Immature myeloid DCs (iDCs) are endowed with many of the same receptors as macrophages such as FcγR and complement receptors as well as subsets of TLR and C-type lectin receptors (CLRs) such as dectin-1 and DC-SIGN that are generally associated with recognition of foreign antigens. iDCS constantly sample their environment through a process of macropinocytosis. Ingestion of foreign antigens results in activation and maturation of DCs associated with antigen processing and presentation of peptides to T cells. In addition, maturation leads to downmodulation of many receptors and reduced engulfment of particles. iDCs also ingest apoptotic cells but, since the intact apoptotic cell does not mature the DCs, interaction with potentially self reactive T cells leads to anergy or tolerance of the T cell [39]. An important phenomenon that follows ingestion of self apoptotic cells and presentation to CD8+ cells is known as 'cross-tolerance'.

Plasmacytoid DCs (pDCs) act as 'professional interferon α/β cells' in the front-line response to viral infection. Although these cells have not been intensively studied in the context of apoptotic cell clearance it is of interest that exposure of lupus serum and apoptotic cells to peripheral blood mononuclear cells results in the production of high levels of α interferon [40], a cytokine that is upregulated in patients with SLE.

Amateur Phagocytes

Virtually every cell of the body is thought to be capable of ingesting apoptotic cells, at least at some stage of their maturation [41]. Receptors for amateur apoptotic cell clearance have not yet been fully elucidated, but the CD91/CRT complex (as well as a receptor for PS) is thought to be expressed on almost all cell types.

Receptors Implicated in Recognition of Apoptotic Cells

Metchnikoff first described the phenomenon of phagocytosis in 1892. However, the phagocytosis of apoptotic cells in the body was almost ignored for

decades likely due to the extremely efficient manner in which apoptotic cells are cleared. Since the early 1980s, improvements in microscopy and other simple tests for apoptosis brought this process to the forefront of research in normal physiology and in disease states. Despite the utilization of antibodies and other inhibitors in early studies, supplemented more recently by gene targeting approaches, no inhibitor or genetic deletion has completely abolished phagocytosis of apoptotic cells [42]. This fact is ascribed to the redundancy in the pathways for removal of dying cells, reflecting the multiplicity of receptor/ligand pairs as well as the heterogeneity of phagocytes and their states of activation.

Different phagocytes likely use different pathways, or perhaps combinations of mechanisms, for apoptotic cell clearance (fig. 1). These systems include scavenger receptors, complement receptors, CD14, lectin receptors, a putative phosphatidylserine receptor, the ABC-1 transporter and the mer/tyro/axl family of phosphotyrosine receptors as discussed below. Different receptor/ligand pairs also have different functions. According to the 'tether and tickle' model [43], some receptors, such as CD14 or CR3, serve as recognition structures and contribute to adhesion, others such as CD91 and the PS receptor convey signals for engulfment and yet others, such as SIRPα, preventing uptake.

The recent cloning of the scavenger receptor-like protein *ced-1* in *C. elegans* that appears to play a role in apoptotic cell engulfment suggests that these receptors are highly conserved not only to clear anionic debris, but also apoptotic cells. This receptor also contains NPXY and YXXL repeats in the cytoplasmic tail, sequences known to be involved in signaling by phosphorylation of tyrosine residues and for endocytosis [44].

Receptors Implicated in Interaction with Phosphatidylserine

Although a putative PSR was identified [3], this has now been shown to be a nuclear protein [45]. As discussed above, since a number of proteins including β-2-glycoprotein, annexin 1, MFG-E8 and Gas6 bind to PS, it is possible that these opsonins, rather than PS itself regulate binding to different receptors on phagocytes (fig. 1).

Complement Receptors

In vitro phagocytosis assays were initially performed in the absence of serum. However, Mevorach et al. [6] showed that phagocytosis of apoptotic cells was enhanced in the presence of serum and that this could be attributed to complement deposition on the apoptotic cell and recognition by CR3 and CR4

on the phagocytes (fig. 3–5). The β-2 integrin containing CR3 (CD11b/ CD18) and CR4 (CD11c/CD18) are known to recognize and help promote the phagocytosis of iC3b opsonized particles [46]. These receptors are also involved in adhesion and CR3 may signal anti-inflammatory cytokine release. The major manifestation of individuals with CD18 mutations is the disease, leukocyte adhesion deficiency and CD11b knockout mice have defective phagocytosis [47].

Complement receptor 1 (CR1, CD35) is expressed on a variety of other phagocytes as well as erythrocytes that transport immune complexes to the liver and spleen for disposal. Despite binding of C1q and MBL to CR1, this receptor has not been implicated in the clearance of apoptotic cells. CR2 is expressed on B cells and follicular dendritic cells. Since iC3b will be proteolytically degraded to C3dg if apoptotic cells are not removed, this may have important implications for promoting immune responses (fig. 5).

CD91 and Collectin Receptors

Collectins are known to act as opsonins and can facilitate phagocytosis of a variety of particles via what was called the common collectin receptor [48]. However, other C1q and collectin receptors were subsequently characterized and since these are multifunctional proteins, it is thought that they may use different receptors for their many tasks. Collectins and their receptors are dealt with in greater detail in the chapter by Stuart et al. [this vol.].

CD91 is a 600-kDa, type I transmembrane protein synthesized as a single polypeptide chain that is *N*-glycosylated and clipped in the late Golgi to form α and β chains. The α chain and the β chain are tightly, but noncovalently associated. The 515-kDa α chain exists on the extracellular side of the plasma membrane, while the 85-kDa β chain spans the membrane. The 100 amino acid cytoplasmic tail of the α chain has two NPXY endocytosis signal sequences and one YXXL tyrosine kinase activation motif. The β chain co-purifies with a 45-kDa protein known prosaically as the receptor-associated protein, or RAP. This protein is thought to act as a chaperone for CD91, assisting in transport of CD91 to the cell surface; however, RAP is thought to dissociate before CD91 reaches the cell surface.

CD91 is known as the α_2-macroglobulin (α2 m) receptor as well as the low-density lipoprotein-related receptor (LRP). α2 m is a large plasma glycoprotein of 200-kDa that binds proteinases and facilitates their clearance from the plasma. α2 m is found as a monomer, dimer or tetramer. Tetrameric α2 m/proteinase complexes bind preferentially to the receptor, and initiate endocytosis and clearance of redundant proteinases into macrophages. α2 m monomers bind to CD91

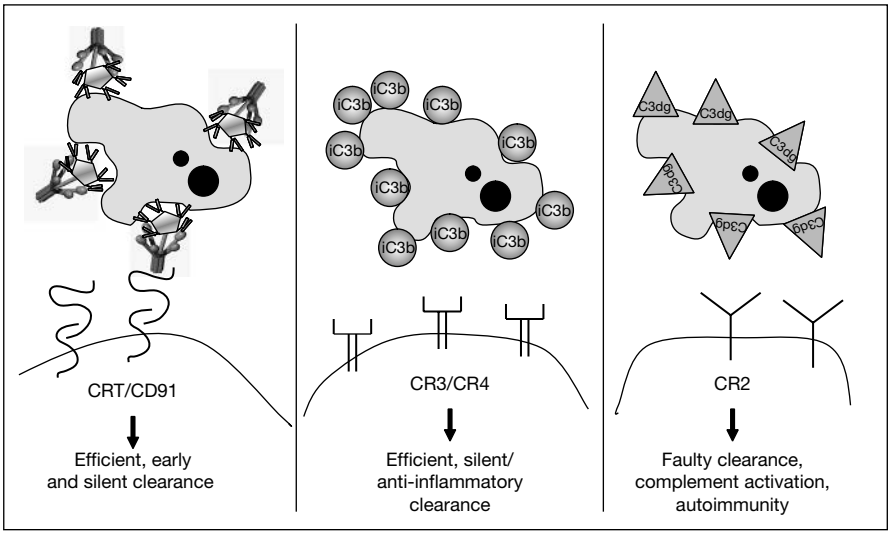

Fig. 5. Alternative fates of complement coated apoptotic cells. C1q binding to natural antibody may be coated by calreticulin (CRT) on the apoptotic cell surface followed by swift ingestion by phagocytes likely through the CD91 receptor. Complement activation by C1q may also activate the classical pathway leading to the generation of C3bi, which becomes covalently bound to the apoptotic cell surface as in figure 3. Delayed clearance of C3bi-opsonized cells leads to cleavage of C3b to C3dg, possibly resulting in binding to CR2-bearing antigen presenting cells such as B cells and follicular dendritic cells (FDC) promoting an autoimmune response.

with low affinity, whereas the tetrameric α2 m binds with high affinity, in what is called a 'bonus effect'. One CD91 protein has only one binding site for an α2 m/proteinase complex; therefore, clustering of the receptor results in optimal binding and clearance of α2 m/proteinase complexes.

The α chain of CD91 is composed of eight cysteine-rich complement-type repeats, two EGF-type repeats, and YWTD repeats. It is to a stretch of the former that ligand such as α2 m/proteinase complexes, RAP, and other ligands such as plasminogen activator/plasminogen activator inhibitor complexes, lipase-enriched $\alpha\alpha$VLDL, lactoferrin, and apolipoprotein E bind to the receptor. The α chain of CD91 also contains EGF-type repeats. Ligand binding appears to be calcium-dependent [49].

CD91 is found on hepatocytes, Kupfer cells, placenta, neurons, astrocytes, fibroblasts, smooth muscle cells, monocytes, and macrophages. Monocytes, however, have a low expression of CD91, whereas macrophages have a high level of expression, and so CD91 is classified as a differentiation antigen. CD91 knock-out mice are nonviable and die 13 day post-coitum [50].

Binding of the collagenous tails of the lung collectins to CD91 results in pro-inflammatory cytokine release by macrophages [51]. Whether or not this occurs in the presence of apoptotic cells has yet to be fully investigated.

Scavenger Receptors

Brown and Goldstein discovered scavenger receptors in 1979 [52]. This family of receptors is divided into class A (SR-A), class B (SRB-1, CD36), class D (CD68), and class E (Lox-1). The major site of expression of these scavenger receptors is on macrophage cell surfaces [53]. These receptors recognize anionic particles including acetylated and oxidized low-density lipoproteins. Monoclonal antibodies to oxidized low-density lipoproteins have been demonstrated to bind to apoptotic cells and to inhibit the uptake of these cells into macrophages [54]. Different types of scavenger receptors implicated in the clearance of apoptotic cells include CD36 (class B), CD68 (class D), the scavenger receptors B1, and scavenger receptors A.

Scavenger receptor (SR-A) was implicated in apoptotic cell uptake in the thymus but knockout mice do not have obvious alterations in the disposal of apoptotic cells [55]. This receptor also binds oxidized LDL and acetylated LDL (but not LDL or PS) and these lipoproteins compete for ingestion of apoptotic cells, presumably by blocking SR-A. Class A scavenger receptors, C1q, and collectins share polyanionic-ligand binding collagenous sequences of Gly-X-Y repeats [56] that likely contributes to their similar binding properties to apoptotic cells.

The CD36 receptor found on platelets, monocytes, and macrophages has long been thought to play a role in the uptake of apoptotic cells [31]. CD36 and the vitronectin receptor, integrin $\alpha v\beta 3$, bind to the multifunctional adhesive glycoprotein thrombospondin, which is bound to unknown ligands on the apoptotic cell. The cationic tetrapeptide RGDS is a known inhibitor of apoptotic cell clearance; it binds and blocks sequences on the vitronectin receptor that are recognized by the RGD sequences in thrombospondin [57].

Scavenger receptor B1, a newer member of the B family of scavenger receptors, may also play a role in the clearance of apoptotic cells. Overexpression of the scavenger receptors (SRB-1) receptor by transfected CHO cells enabled uptake of apoptotic cells [58]. The receptor mediates the uptake of apoptotic rat spermatogenic cells by Sertoli cells in the testis [59], apoptotic rat ovarian granulosa cells [60] and apoptotic thymocytes by nurse cells in the thymus [61].

Oxidation of exposed phospholipids on the external leaflet of the apoptotic cell may lead to recognition by scavenger receptors such as CD68, which is known to play a role in the clearance of oxidized LDL [62]; perhaps it recognizes similar ligand(s) on the apoptotic cell surface as natural antibodies have been shown to bind to oxidized phospholipids on LDL and apoptotic cells [63].

LOX-1 (lectin-like oxidized low-density lipoprotein receptor-1) is expressed primaril;y on endothelial cells and is proposed to play a major role in the clearance of apoptotic endothelial cells., possibly by recognizing and binding to exposed PS on the apoptic cell surface [64].

CD14

CD14 is a 55-kDa glycoprotein tethered to the cell membrane via a phosphatidylinositol glycan anchor. CD14 is expressed on the surface of monocytes and macrophages and was characterized as a receptor for complexes composed of lipopolysaccharide (LPS) and the LPS-binding protein (LBP). Ligation of CD14 with LPS/LBP complexes results in the internalization of these complexes and the release of TNF-α [65]. Since CD14 itself has no intracellular domain, it must complex with other transmembrane proteins, such as TLR4, on the cell surface [66]. CD14 has also been implicated in apoptotic cell uptake as evidenced by inhibition of apoptotic, but not necrotic, cell ingestion by the 61D3 monoclonal antibody [67]. CD14 likely works in tandem with another receptor to facilitate apoptotic cell clearance.

While CD14 can interact with various microbial proteins, such as LPS, peptidoglycan, uronic acid, as well as phospholipids such as PS [68], CD14 binds to ICAM-3 on the apoptotic cell surface. ICAM-3 is a member of the Ig-superfamily of proteins found on the surface of leukocytes, and is involved in cell-cell adhesion, primarily by binding to the leukointegrin, LFA-1. However, apoptotic cell ingestion by CD14/ICAM-3 is independent of LFA-1 or any other known ligand for ICAM-3 or CD14. This finding is consistent with the hypothesis that the binding domain of ICAM-3 (D1) may be altered on apoptotic cells. CD14 can facilitate clearance of ICAM-3 negative apoptotic cells, suggesting that CD14 may interact with other ligands on the apoptotic cell surface. CD14 knockout mice are viable, and while there is a significant apoptotic cell clearance defect in these mice, they do not develop autoimmunity, suggesting that the two events may be uncoupled in certain mouse strains [69].

Lectins and Their Receptors

Lectins have also been reported to facilitate apoptotic cell clearance. The asialoglycoprotein receptor on Kupfer cells in the liver enables these cells to recognize and remove apoptotic cells via recognition of N-acetyl-glucosamine residues. The uptake is partially abrogated by pre-incubation of macrophages with N-acetyl-glucosamine [70]. A related receptor, the mannose/fucose

receptor, has also been put forward as a potential clearance receptor, as preincubation of macrophages with free fucose and mannose can inhibit uptake of apoptotic cells [71]. Mannose receptor binds to the same ligands as the serum protein MBL, but is a type I transmembrane protein functioning primarily in the clearance of bacteria [72]. Recently, alterations to the polylactosamine-type carbohydrate chains of the multifunctional surface receptor CD43 were shown to facilitate very early binding of apoptotic cells to macrophages [73].

ABC-1 Transporter

The ATP-binding-cassette transporter 1 is a seven transmembrane-receptor located on the plasma cell membrane. Activation of this receptor results in activation of actin polymerization and localized phospholipid asymmetry, with both the apoptotic cell and the phagocyte displaying surface phosphatidylserine. Engulfment of apoptotic cells seems to rely, at least in some part, on functional ABC-1 transporter on the phagocyte surface [74].

Conclusions

There are three main circumstances where removal of apoptotic cells has been highlighted in invertebrate and mammalian physiology: (1) embryogenesis and tissue remodeling; (2) cell and tissue homeostasis; and (3) resolution of inflammation. As discussed in this brief review, the cells and receptors involved differ accordingly, although many details remain to be resolved.

The co-ordinate function of different components of the innate immune system in the removal of dead and dying cells has been emphasized. CRP is a good example in that CRP is induced up to 1,000-fold in the serum by inflammatory cytokines and functions to scavenge dying cells and cell debris leading to removal of cell debris and antagonism of inflammatory cytokine production. Many of the receptors implicated in the removal of apoptotic cells also function as scavenger receptors, likely interacting with modified self components.

There is considerable evidence that abnormalities in apoptosis or its clearance mechanisms predispose to lupus-like autoimmune disorders. It remains to be determined in humans with this disease as to whether the abnormalities are abnormal cell death, defective clearance of apoptotic cells [Gaipl et al., this vol.] or abnormal responses to dying cells and their cargo or some combination of these mechanisms.

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Collectins: Opsonins for Apoptotic Cells and Regulators of Inflammation

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Abstract

The collagenous C-type lectin family (collectins) members are humoral molecules found in the serum and on certain mucosal surfaces. In humans the family of collectins include the mannose-binding lectin, surfactant protein A and surfactant protein D. They demonstrate broad ligand specificity for both pathogenic bacteria and viruses. Over the past 5 years data have emerged indicating that these molecules are able to bind self-derived ligands in the form of apoptotic cells and regulate inflammatory responses. Furthermore, exciting new data from murine models have begun to define the in vivo importance of these molecules as regulators of inflammation and immunity. Here will discuss our current understanding of the process of collectin recognition of dying and damaged cells and its implications for autoimmune and inflammatory diseases.

The collagenous C-type (calcium-dependent) lectin family (collectins) and ficollins are humoral molecules found in the serum and on certain mucosal surfaces [as reviewed in 1–3]. In humans the family of collectins includes mannose-binding lectin (MBL) (known historically as mannose-binding protein), surfactant protein A (SP-A), surfactant protein D (SP-D) and two recently identified related molecules CL-L1 and CL-P1. In this chapter we will focus on

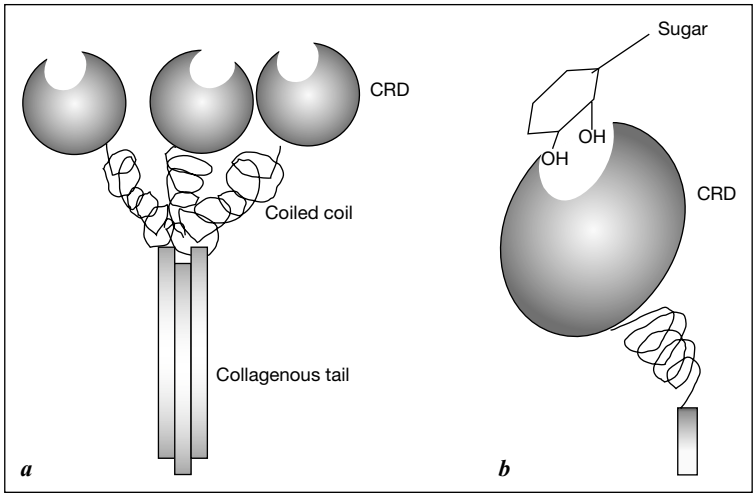

Fig. 1. Collectin structure. ***a*** Structure of the timer of collectin molecules (MBL) demonstrates the carbohydrate recognition domain (CRD), the α-helical coiled-coil domain and the 'collagenous' tail. ***b*** The carbohydrate recognition domain of MBL illustrating the recognition of the highly specific orientation of the OH groups on certain sugars.

MBL, SP-A and SP-D as they have all been implicated in clearance of apoptotic cells and in regulation of inflammation.

MBL and Surfactant Proteins: Structure Function

The collectins are encoded by a cluster of genes found on the long arm of chromosome 10 in humans and chromosome 14 in mouse. In mice the cluster contains SP-A, SP-D and MBLA but there is a second MBLC gene, thought to have arisen by a gene duplication event, and found on chromosome 19. The collectins are classified by very specific structural characteristics [4] (fig. 1) important for their function as innate immune opsonins and regulators of inflammation.

The structure of MBL is probably the best-defined [5] and highlights certain key points concerning the organization of these molecules that are generally true for all the collectins. MBL is defined as a collectin as it has a carbohydrate recognition domain (CRD) linked via a coiled-coil domain to a collagen 'tail'. The α-helical coiled-coil domain provides flexibility to the orientation of the CRD of MBL that recognizes hydroxy groups present in certain

sugars such as mannose and fucose. However, as a monomer, the CRD domains of MBL demonstrate only weak affinity for these sugars and the basis for high affinity binding is dependent on the ability of MBL to oligomerize through its collagenous region. Three monomers of MBL form a trimer in which the CRD is predicted to be separated by a minimum of 45 Angstrøms. This structure allows one trimer to recognize repeating patterns of sugars. In addition, the trimers further multimerize to form higher order structures such as dimers of trimers, trimers of trimers or heximers of trimers. This complex structure of the functional MBL multimer allows it to act as a true pattern recognition receptor, able to recognize polysaccharides that decorate the surface of pathogens with high affinity.

These oligomerization characteristics of MBL are shared with other collectins, SP-A and SP-D, and the related molecules ficollins [1, 3]. For SP-A, six homotrimers associate to form an octadecamer quaternary structure, resulting in a bouquet-like arrangement resembling MBL and C1q. In contrast, four SP-D homotrimers combine to form a SP-D dodecamer, resulting in a cruciform quaternary structure. The carbohydrate recognition domains of SP-A and SP-D demonstrate subtle differences from MBL and from each other, and these differences are responsible for the different array of sugars that they recognize. For example, both SP-A and SP-D bind avidly to mannose and glucose, but not to galactose. In contrast, SP-A and SP-D have distinctive binding characteristics in that SP-A binds to N-acetylmannosamine and *L*-fucose, and SP-D binds to inositol, maltose and glucose. These characteristics allow collectins to bind preferentially to non-host structures, like the glucose-containing oligosaccharide core of lipopolysaccharide and the sugars that decorate the bacterial outer wall. Interestingly, the CRD of SP-A and SP-D also plays a pivotal role in binding to phospholipids. In this context, SP-A binds to the surfactant phospholipids, dipalmitoylphosphatidylcholine, and to lipid A of gram-negative lipopolysaccharide. SP-D also binds to phosphatidylinositol and to glucosylceramide.

It is worth mentioning briefly some additional related molecules. The human collectins also contain two recently identified but poorly defined family members that are not encoded in the collectin cluster: CL-L1 is made in the liver and CL-P1 is a membrane bound receptor on vascular endothelia cells. The ficollins are structurally very similar to the collectins and, in humans, include L-ficollin, M-ficollin and H-ficollin. However, in ficollins, a fibrinogen-like domain replaces the C-type lectin domain of the CRD altering the specificity of binding. Finally, the overall organization of the hexamer of trimers of MBL and SP-A also resembles C1q, the first complement component, which however lacks the specific CRD domain and hence is not strictly included in the family of collectins. In this chapter we will focus on the

collectins (MBL, SP-A and SP-D) as they have all been specifically implicated in clearance of apoptotic cells.

Collectin Levels in Health and Disease

MBL is made predominantly in the liver but is also expressed in lung, kidney and GI tract suggesting that local production in certain tissues is likely. MBL is found as a circulating serum protein and is sequestered to sites of inflammation after tissue injury and endothelial damage. Circulating levels of MBL increase 2- to 3-fold upon infectious and inflammatory challenge but, unlike the archetypal acute-phase proteins such as serum amyloid P and CRP whose levels can increase by 10- to 1,000-fold, MBL levels are relatively constant in an individual. However, MBL levels are highly variant between individuals and determined by the inheritance of certain single nucleotide polymorphisms in the coding and promoter region that are associated with high, intermediate or low secreting haplotypes. Three common polymorphisms are found in codon 52, 54 and 57 which cause variations in the collagenous tail of MBL altering its ability to oligomerize. In addition numerous polymorphism in the upstream promoter region have also been identified that also determine serum levels. These differences result in variations in serum level that range 2–3 log orders of magnitude in healthy individuals and place MBL polymorphisms as one of the most dramatic examples of individual variations in an innate immune molecule. Recent work has emphasized that these different secretory haplotypes are associated with altered risks to certain infections, particularly in situations when the adaptive immune system is compromised such as in the early neonatal period or in patients rendered neutropenic from chemotherapy. Intriguingly, despite not being present in resting lung, patients with cystic fibrosis and low MBL secretory haplotypes have accelerated disease progression and early mortality (see below).

Surfactant proteins A and D are found predominantly in the lung where they are produced by alveolar type II cells that package the collectins into lamellar bodies along with surfactant lipids and other surfactant proteins, such as SP-B and SP-C, prior to secretion. Higher up in the airway, Clara cells also produce SP-A and SP-D, but secrete them without surfactant phospholipids. In addition SP-D (but not SP-A) are also found on other mucosal surfaces including the trachea, urogenital tract and lachrymal and salivary glands. Surfactant protein levels increase by up to 20-fold in the inflamed lung (as detected in bronchoalveolar lavage (BAL) fluid). However, unlike MBL polymorphisms, variations in SP-A and SP-D do not cause large variations between individuals. Five allelic variations of SP-A1, six of SP-A2, and four of SP-D have been identified. Although, the exact consequences of these differences have not been fully defined, there are reports of associations

with altered susceptibility to a number of diseases including TB, respiratory distress syndromes and chronic obstructive pulmonary disease.

Ligand Binding

The structure of MBL and the lung collectins results in broad ligand specificity, a feature they share with other multi-ligand receptors such as the scavenger receptors. MBL plays a vital role in host defense by virtue of its binding to a wide variety of known pathogens including, *Staphylococcus aureus*, certain rough mutants of *Escherichia coli*, *Candida albicans, Leishmania* and *Burkholderia cepacia*. It also recognizes numerous viruses including herpes simplex, influenza and gp120 of HIV. For all of these ligands MBL binding is inhibited by mannan and is cation dependent, indicating that they interact through the CRD.

Similar diversity in ligand binding has also been demonstrated for the lung collectins. Numerous studies have indicated that lung collectins bind both gram-positive and gram-negative bacteria, viruses and fungi. They have been shown to directly recognize lipopolysaccharides (LPS) and, in the case of SP-A, specifically recognize the lipid A moiety of the bacterial outer wall. These molecules also regulate responses to these organisms, possibly via their ability to bind the LPS binding molecule CD14 [6] and Toll-like receptors (TLR) [7]. However, the binding can either increase or decrease the inflammatory response (as discussed below).

In addition to their ability to recognize exogenous ligands, SP-A, SP-D and MBL also bind certain endogenous ligands and hence share with the scavenger receptors the ability to recognize 'modified-self'. Importantly, and pertinent to this chapter, they bind apoptotic cells [8–10] and nuclear material and thus play a role in the response to endogenous 'danger signals'. Recently, another important endogenous ligand for collectins has been identified as naked DNA [11] and it has been suggested that nuclear material released during cellular necrosis might provide a source of collectin ligands. However, this interaction is distinct from binding to other ligands in that collectin: DNA binding occurs, at least in part, through the collagenous region. Although it is possible that nuclear material localized on the surface of an apoptotic cells or in apoptotic blebs may be a ligand on dying cells that is recognized by the collectins, the exact nature of the ligand or ligands is unknown.

Collectin Receptors

The identity of the receptor or receptors for the collagenous tails of collectins remains to be fully defined [12]. The lung collectins may have specific

receptors: SP-A is thought to bind to SP-R210 (a molecule whose identity remains unknown), the CRD of SP-D binds to gp340 (also known as salivary agglutinin) and the CRD of both SP-A and SP-D binds SIRPα [13] (see below). Furthermore, data suggest that they may also bind to scavenger receptors and TLRs. In addition to these possible SP-A/SP-D receptors, numerous other molecules have been shown to increase the binding of collectins and C1q including CD93 [14], gC1qR, CD91 in a complex with calreticulin [8, 9], CR2 and CR3. However, definitive evidence of direct interaction with any of these receptors is lacking and we will provide two examples that illustrate the difficulty in formally identifying the receptor for these inherently 'sticky' molecules.

One putative collectin receptor, CD93, was proposed because it was shown to increase uptake of MBL and C1q opsonized particles [15]. However, more recent data has questioned its ability to directly bind these molecules as both MBL and C1q can still act as opsonins that upregulate phagocytosis in the absence of CD93 [16]. These data have led to the proposal that CD93 increases MBL and C1q-mediated clearance indirectly (possibly through regulation of expression of other undefined molecules such as integrins) or by modulating the activation state of the phagocyte that increase their phagocytic capacity. It is possible that similar scenarios may exist for other putative collectin receptors. As an example SP-A has been shown to increase bacterial uptake indirectly by regulating expression of the phagocytic receptors, SRA (scavenger receptor A) and mannose receptor [17, 18].

Another proposed receptor for collectins is calreticulin in a complex with CD91 [12, 19]. The initial observation that calreticulin bound collectins was surprising as calreticulin is known to be an ER protein that acts as a chaperone during protein assembly. However, recent data indicate that ER is recruited to the phagocytic cup and hence provides explanation for how this apparently intracellular molecule might participate in phagocytic recognition [20]. Furthermore, data from the model organism *Dictyostelium discoideum* indicate that GFP-calreticulin is found in the phagocytic cup and provide strong circumstantial evidence for its role in phagocytosis. These data are further supported by observations that antibodies to calreticulin and CD91 block collectin-mediated uptake of apoptotic cells by macrophages [8, 9].

In summary, current data on the identity of the receptor for the tail of collectins is ambiguous and the collectins may be promiscuous, using multiple receptors to trigger engulfment. Further data is needed to demonstrate direct interactions of molecules with the collagenous tail to confirm conclusively the identity of the true collectin receptor(s) and it is likely that additional information on the mechanism of recognition of collectins will emerge. However it is important to note that the unique ability of MBL to fix complement (see below)

suggests that in a physiological context it may act as an opsonin by mediating uptake not only via collectin receptors but also through the generation of iC3b that coats the targets and triggers uptake by CR3.

MBL, Complement Activation and Autoimmunity

MBL shares with C1q the ability to activate complement [21]. Upon binding to its ligand, MBL is thought to undergo conformational changes allowing it to associate with a family of MBL-associated serine proteases (MASPs) to activate the teleologically most ancient pathway of complement activation, the lectin pathway. MASPs co-opt the classical complement convertase, C4, leading ultimately to C3 cleavage. The ability to activate the lectin pathway of complement is also shared with the related molecules, the ficollins. In this regard MBL appears broadly similar to C1q suggesting that it may play a similar or complementary role in immune regulation.

The similarities between MBL and the first component of the classical complement pathway, C1q, are particularly pertinent when considered in the context of autoimmune disease. C1q deficiency is strongly linked to autoimmunity such that greater than 95% of patients with this deficiency develop an autoimmune syndrome with characteristics of systemic lupus erythematosus (SLE). This is further supported by the observation that C1q deficient animals develop auotantibodies and a nephritis characterized by accumulation of apoptotic cells. This has lead to the 'waste disposal' hypothesis that suggests that failure of prompt removal of effete cells is associated with autoimmunity. These uncleared apoptotic cells provide both the source of autoantigens that come to be targets in lupus and also generate danger signals when they undergo secondary necrosis that activate the immune response. The fact that MBL shares with C1q the ability to recognize apoptotic cells and that they are both circulating serum proteins has prompted the suggestion that MBL deficiency might also lead to autoimmunity. This idea is supported by the observed link between codon 54 mutations in MBL and SLE in certain populations [22–24]. Furthermore, MBL appears to alter the rate of disease progression and susceptibility to infection. However, although many studies suggest that low MBL levels may be detrimental in the context of SLE, other studies do not support a role for it in preventing autoimmunity. Indeed a recent study has indicated that SLE patients with low MBL develop lower titers of autoantibodies and later disease onset [25]. Thus, although MBL and C1q may have similar functions as systemic circulating opsonins for apoptotic cells and initiators of complement activation, they appear to have slightly different roles in autoimmunity.

Collectins and Apoptotic Cell Binding

The first identification of the role of collectins in apoptotic cell clearance was made in an in vitro context but has been confirmed and extended by in vivo studies. In these in vitro studies, the ability of MBL and C1q to bind apoptotic cells was compared. Both these molecules bound to apoptotic cells and increased phagocytic clearance. Specifically these experiments showed that the recognition of the collagenous tail was mediated by calreticulin in a complex with CD91 [8, 9] as discussed above. Subsequent work has addressed not only MBL but also SP-A and SP-D binding to dying cells and we will discuss these specifically.

MBL was shown to bind cells undergoing programmed cell death and it was specifically suggested to bind preferentially to cells in the late stages of apoptosis [26]. Initial work studying this phenomenon relied on serum purified MBL, known to associate in the circulation with immunoglobulin, specifically IgM, and binding was detected with anti-MBL antibodies. Natural antibodies are important opsonins that also recognize and mediate clearance of apoptotic cells and it is possible that serum derived MBL binding might be mediated, at least in part, through this contaminant found commonly in serum derived MBL preparations. We have recently undertaken a more exhaustive examination of the ability of MBL to bind dying cells using purified recombinant human MBL that was directly conjugated to the fluorophore Cy3 and allowed us to study the direct interaction of MBL with dying cells [27]. Using this approach we confirmed that MBL binds to apoptotic cells in a calcium-dependent, mannan-inhibitable manner implicating the CRD as the recognition domain for the ligands on apoptotic cells. Furthermore, it confirmed that MBL was able to bind dying cells directly and independently of other molecules.

Interestingly, using this strategy, we also found that MBL bound to freshly isolated thymocytes and a viable fibroblast cell line, also in a calcium-dependent, mannan-inhibited manner. These observations that MBL binds to certain live cells raise the question of why MBL binding to viable cells does not trigger engulfment. Two possible explanations exist: (1) MBL might be required to associate in a macro-molecular complex with another molecule or molecules (possibly IgM and C1q) to trigger activation and that these molecules do not assemble on viable cells or (2) the second possibility, and our preferred hypothesis, is that MBL on live cells is not in the correct configuration to recruit MASPs to trigger complement activation or engulfment. It has been shown previously that the distribution of C1q, SP-A and SP-D changes as cell death progresses [8, 9] and we also observed a similar redistribution of MBL, which became clustered on the cell surface and localized to apoptotic blebs. We would suggest that this alteration in ligand distribution is sufficient to cluster collectins and is necessary for them to act as opsonins to mediate engulfment (fig. 2). However, the exact nature of the

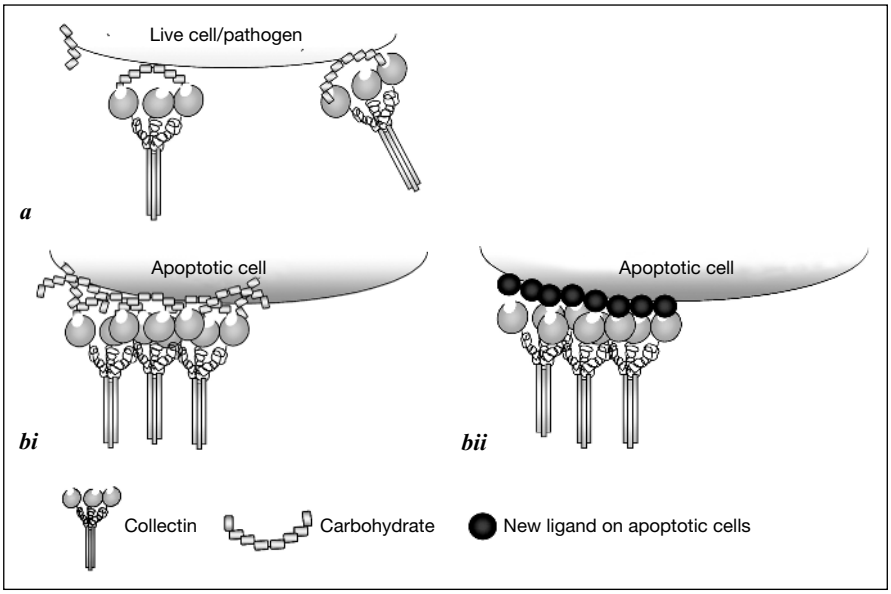

Fig. 2. Binding of collectins to live and dying cells. ***a*** Collectins recognize specific carbohydrates on pathogens and certain live cells. Redistribution of collectin binding as cells progress through apoptosis may represent either altered ligand distribution (***bi***) or exposure of neo-epitopes (***bii***) on the dying cell.

ligands for collectins is unknown and it remains to be determined if apoptotic cells expose a new ligand upon loss of membrane asymmetry or whether it is a redistribution of a ligand already present on live cells that results in clustering of collectins on the surface of apoptotic cells (fig. 2).

However, why MBL binding to apoptotic cells does not induce complement activation is unclear. One possibility is that there is an inhibitor of the lectin pathway that is present on the surface of live cells that prevents full complement activation and that such an inhibitor is lost as cells progress through apoptosis into necrosis. However, unlike the classical and alternative pathway, no inhibitor of the lectin pathway has been identified. In this regard, recent proposals that the alternative pathway acts as an amplification-loop for both the classical and lectin pathways may be pertinent. This raises the interesting possibility that the lectin-pathway is regulated indirectly via the classical or alternative complement pathways. Failure to activate the alternative pathway on live or early apoptotic cells may be the vital step that prevents amplification of the lectin pathway, controlling full complement activation. It is evident that many questions remain concerning

collectin recognition of dying cells such as, whether MBL co-operates with IgM or C1q to bind apoptotic cells to trigger complement and internalization in vivo. Essential steps in further elucidating these facts will require generation of compound knockout animals deficient in all of these factors.

Regulation of Apoptosis

Interestingly, not only do collectins bind apoptotic cells but may also regulate their generation [28]. The lung collectins transduce important survival signals in the lung that provide natural protection from apoptosis. Two studies by White et al. demonstrated that SP-A protected rat type II epithelial cells from undergoing apoptosis by ultraviolet light irradiation or etopopside, and protected a Clara cell tumor cell line from staurosporin-induced apoptosis [29, 30]. This SP-A-mediated effect appeared to depend upon activation of the PI3K/AKT pathway and dephosphorylation of the forkhead transcription factor, FKHR. Recent work has expanded these findings by showing that both SP-A and SP-D inhibit spontaneous and oxidant-induced neutrophil apoptosis through a PI3K/AKT dependent pathway [Gardai SJ, et al., unpubl. data]. Interestingly, for the neutrophil, this effect is mediated through collectin interaction with signal inhibitory regulatory protein α (SIRPα) suggesting a novel consequence of SIRPα ligation.

Collectins as an Opsonin for Dying Cells in vivo

Cells undergoing programmed cell death are rapidly cleared from tissues such that free apoptotic cells are rarely seen in vivo. Interestingly, a number of molecules that have been implicated in apoptotic cell recognition in vitro do not appear to have a role when studied in knockout animals. As discussed previously, MBL, SP-A and SP-D all bind dying cells and could be shown to increase clearance in vitro. However, it was important to address their physiological relevance in vivo. The first in vivo confirmation for the role of these molecules necessitated the use of an in vivo phagocytosis assay to study SP-A and SP-D in their physiological context, the lung [31]. Instillation of apoptotic cells directly into the lung of mice deficient in SP-A and SP-D demonstrated an absolute requirement for SP-D (but not SP-A) to efficiently remove apoptotic cells [9]. The failure to demonstrate a role for SP-A in these assays is likely to reflect that other molecules such as SP-D or MBL (see below) compensate in its absence. Interestingly, C1q (which had been shown to have a role in the

peritoneal clearance) was not required for apoptotic cell clearance within the lung indicating for the first time that molecules act as opsonins only in specific tissues. These data were the first to demonstrate that in vivo studies of molecules involved in apoptotic cell recognition were best interpreted in the relevant physiological context.

MBL is also an important opsonin for apoptotic cells, able to increase phagocytosis of apoptotic cells by both macrophages and dendritic cells in vitro [32]. The role for serum MBL and C1q-mediated clearance of apoptotic cells has been further confirmed by blocking antibodies studies [8]. However, it was intriguing that in these in vitro experiments, MBL provided a greater proportion of serum mediated opsonization of apoptotic cells than C1q and it was of interest to define the relative importance of these molecules in vivo. Murine MBL is encoded for by two genes MBLA and MBLC and mice deficient in both forms of MBL have recently been generated, providing the required tool to study the role of MBL in vivo [33]. Using the same assay that had established the in vivo importance for C1q (in which apoptotic cells are instilled into the peritoneum and phagocytosis by recovered macrophages determined [34]) we have recently addressed the role of MBL in clearance in vivo. These mice demonstrate impaired clearance of apoptotic cells in the peritoneum, with a defect comparable to the defect reported in the C1q deficient animals. These observations are intriguing and suggest that both MBL and C1q, either alone or in co-operation, mediate engulfment of dying cells in the peritoneum. Furthermore, we have also found a surprising role for MBL in clearance of dying cells from the alveolar space [Stuart LM, Vandivier RW and Ezekowitz A, unpubl. data]. These data are particularly intriguing, as MBL is not detected in the resting lung, but nonetheless suggest that MBL and SP-D may co-operate in vivo to regulate cell numbers and remove cell debris from the airway (as discussed below).

Collectin Knockout – Insights into in vivo Functions

The peritoneal and lung assays described above have only a limited ability to define the physiological role of molecules involved in apoptotic cell clearance. Specifically, they do not address the context in which a mode of clearance is dominant and, by inference, most relevant. We would suggest that a more informative approach is to study the tissue homeostasis in animals in which clearance mechanisms have been perturbed. These sorts of models occasionally result in accumulation of apoptotic material in a certain tissue site and provide conclusive data for a non-redundant role for a specific molecule in certain circumstances or in certain organs. As an example, C1q deficient mice demonstrate uncleared inflammatory cells in the glomeruli. These observations are

likely to reflect two aspects of C1q deficiency; firstly that the glomeruli are a particular target for inflammatory damage in the systemic autoimmune syndrome associated with C1q deficiency, and secondly that C1q plays a significant and non-redundant role in clearance of dying cells in the kidney which cannot be compensated for by other mechanisms.

Similarly, the examination of SP-A and SP-D null animals has also been informative. SP-A-deficient mice have a normal lung phenotype, and despite the involvement of SP-A in surfactant processing, surfactant lipids are unchanged. SP-A deficient mice are defective in their ability to clear a variety of microbes, including group B *Streptococcus*, *Hemophilus influenzae*, *Pseudomonas aeruginosa*, *Mycoplasma pulmonis*, and respiratory syncytial virus [35]. Regulation of the inflammatory response is also impaired in SP-A deficient mice, in that pro-inflammatory mediators are high and anti-inflammatory mediators are low [3]. In contrast to SP-A, SP-D-deficient mice develop a complex pulmonary phenotype [36] characterized by emphysema and sub-pleural fibrosis, as early as three weeks of age; phospholipid pools (8-fold), lipid-laden macrophages (10-fold), matrix metalloproteinases, inflammatory mediators (e.g. IL-1β), and hydrogen peroxide (10-fold) are also increased in these mice. Pertinent to the subject of apoptotic cells clearance, SP-D null animals develop profound accumulation of inflammatory cells with in the alveolar spaces and increased numbers of dying cells are recovered in the BAL fluid [28]. These data support the observation that SP-D plays an important role in clearance of dying cells and regulation of inflammation in the lung and suggest that this is the context in which SP-D-mediated clearance is most relevant.

In this regard it is pertinent that we have observed that tissue homeostasis in the lung is abnormal in the absence of MBL [Stuart LM, Vandivier RW and Ezekowitz A, unpubl. data]. These observations provide important new insight into the role of MBL in vivo: they suggest that MBL is important in regulating inflammation in the lung and that the lung is a major site in which MBL mediated clearance may be important. Importantly, MBL appears to share this role with the lung collectins, SP-A and SP-D. Surprisingly, our studies of MBL null animals did not support a role for MBL in preventing autoimmunity as MBL null mice neither developed ANA or dsDNA at titers higher than WT controls, nor was there evidence of end-organ damage consistent with an autoimmune-like syndrome [27]. However these mice did accumulate B1 cells in the peritoneum suggesting that MBL is important for B cell homeostasis and it remains possible that these mice may develop overt autoimmunity on different genetic backgrounds (as seen with the C1q-deficient animals). Taken together, these data suggest that the in vivo role of MBL is more similar to that of SP-D than to C1q.

Collectins as Modulators of Inflammation

Regulation of inflammation in the lung is vital because of the massive surface area (the size of a tennis court) that is exposed to 10,000 liters of air each day that contain an array of potential inflammatory stimuli, including micro-organisms, particulate matter, oxidative stress and endotoxin. However, the exact role of lung collectins in the regulation of inflammation and lung homeostasis has been difficult to define. Some of this confusion arises from conflicting in vitro studies where SP-A and SP-D have been shown to have both anti-inflammatory and pro-inflammatory effects. However, in vivo, SP-A and SP-D deficient and overexpressor mice suggest that the lung collections are important mediators of lung quiescence and suppressors of lung inflammation.

A recent study has addressed one possible molecular mechanism that might account for these divergent results. Gardai et al. [13] have proposed a novel mechanism whereby the lung collectins exert either anti-inflammatory or pro-inflammatory effects, depending upon the orientation of the lung collectin and its particular binding partner (fig. 3). They found that SP-A and SP-D CRDs preferentially bind to SIRPα on alveolar macrophages and epithelial cells in the naïve lung. Engagement of SIRPα activated the tyrosine phosphatase, SHP-1, and blocked pro-inflammatory signaling through the *src*-family kinase, Hck, and p38 MAP-kinase. Interestingly, these anti-inflammatory effects were not seen with C1q or MBL. Another intriguing outcome of the interaction of lung collectins with SIRPα may be to inhibit the phagocytic capacity of naïve, quiescent, alveolar macrophages [Dickinson MG, et al., unpubl. data]. This possibility may explain the relative inefficiency with which alveolar macrophages phagocytose apoptotic cells when compared to macrophages from other tissues. However, when collectins were allowed to opsonize apoptotic cells or cell debris via their CRD, their collagenous tails are then recognized by calreticulin/CD91 on alveolar macrophages, to increase uptake with pro-inflammatory consequences.

Further work addressing the role of the collectin in regulating inflammation have extended these observations and have specifically focused on the role of MBL in experimental models of ischemia-reperfusion injury in vivo. Ischemia-reperfusion injury is characterized by cell damage resulting in exposure of neoepitopes on damaged cells and/or induction of apoptosis or cell death. The role of complement in mediating this damage has been inferred from the deposition of C3 in injured tissues and the protection of mice that lack distal complement components from organ damage. However, the mechanism of complement activation had not been defined. Recently, two independent models of ischemia-reperfusion injury studying the kidney and GI tract and myocardium have addressed this question [37, 38]. In all these models the presence of MBL

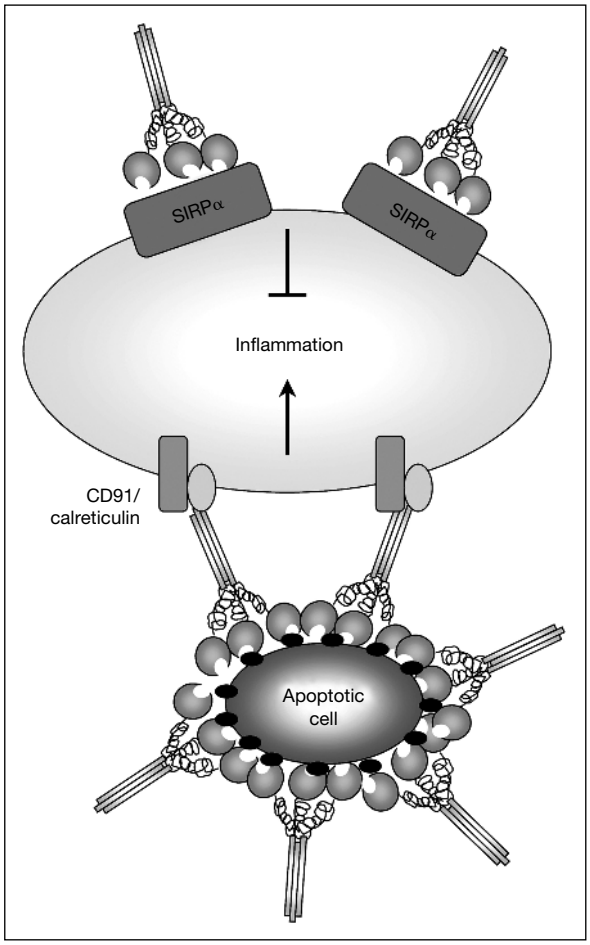

Fig. 3. Proposed molecular mechanism of divergent response to collectins. Collectins can bind to phagocytes via their globular heads that interact with SIRPα and inhibit inflammatory response. However, if the collectins are opsonizing a particle the collagenous tails are exposed and interact with a calreticulin/CD91 complex to increase proinflammatory responses.

greatly worsened the extent of tissue inflammation and MBL null animals were protected from damage indicating an important role for this collectin in regulating sterile inflammation. Intriguingly, mice deficient in soluble IgM (but not C1q) were also protected in the GI model, implicating MBL and natural antibody in triggering complement activation and local tissue damage in this site. These data suggest that these molecules may co-operate to recognize neo-epitopes exposed on the damaged tissue and/or recognize the dying cells

associated with this hypoxic stress. However, unlike the recognition that is mediated by C1q that appears to protect from autoimmune inflammatory damage, MBL/sIgM recognition appears detrimental for the animal. Thus, in the context of reperfusion injury, MBL and natural antibodies appears to contribute to pro-inflammatory response and detrimental clearance of injured tissue. Importantly these observations are consistent with the in vitro studies by Gardai et al. [13]. Taken together these data support suggestions from in vitro data that the collectins may have either pro-inflammatory or anti-inflammatory roles, as seen in ischemia-reperfusion injury and the lung, respectively. It is likely that the outcome is dependent on tissue site and the context of the injury that occurs. Further work in this area will be required to define the relative contributions of the collectins in other models of sterile injury and in different organs in vivo.

Experiments of Nature

The evidence presented indicating an important but complex role for the collectins in regulating inflammation and disposing of dying cells and this is supported by patient studies. As an example SP-D is increased in several acute and chronic lung diseases, including asthma, allergic bronchopulmonary aspergillosis, hypersensitivity pneumonitis, idiopathic pulmonary fibrosis, radiation-, amiodarone- and methotrexate-induced lung injury [39]. In contrast, SP-A and SP-D are decreased in the lungs of smokers and decreased SP-D is associated with the development of neonatal chronic lung disease [40]. SP-A and SP-D alleles have also been associated with a variety of lung diseases, including chronic obstructive lung disease (COPD), respiratory distress syndrome, tuberculosis and the propensity to develop severe RSV infections [3]. We will discuss briefly one specific example of how failure of apoptotic cell clearance can be related to defects in collectin function and disease, by addressing their role in cystic fibrosis (CF).

CF is a common inheritable disease arising from a recessive defect in the cystic fribrosis transmembrane regulator (CFTR). These patients accumulate thick mucus secretions and their lungs become colonized at an early age with *Pseudomonas aeruginosa* and *Burkholderia cepacia*. One study we have performed has examined sputum of patients with bronchiectasis and CF, which contained large numbers of uncleared apoptotic cells and cellular debris [41]. Specifically we addressed the mechanism of failed clearance and demonstrated that neutrophil elastase cleaves one of the receptors for apoptotic cells (the phosphatidyl-serine receptor) thus leading to defective apoptotic cell removal [42]. Furthermore, SP-A and SP-D are reduced in CF patients, likely to further compound this defect in apoptotic clearance.

In another extensive study, low MBL secretory haplotypes were studied in the context of CF [43]. Low MBL levels were linked with increased rate of decline of lung-function such that these patients died on average eight years before those with normal or high MBL levels. This attrition could not be explained solely on the rate of bacterial colonization of the patients as MBL levels did not correlate with age of colonization by *Pseudomonas* but rather suggested that MBL may play a role in this disease for other reasons. Our in vivo data from MBL null animals suggest that, although MBL is not detected in resting lungs, its lack is associated with increased inflammation in the alveolar spaces and accumulation of uncleared apoptotic cells. It is possible that MBL mediated clearance of dying cells is of particular relevance in the context of CF and provides an explanation of why MBL levels correlates with poor disease phenotype in these patients. Taken together these data indicate that the collectins appear to make an important contribution to resolving lung injury through their ability to act as opsonins for apoptotic cells in addition to their other diverse immunoregulatory roles.

Conclusions

The collectins are a unique family of humoral molecules, present both in serum and on many mucosal surfaces. They have broad ligand specificity including the ability to recognize many pathogens, apoptotic cells and cellular debris. They function not only to mediate opsonic uptake of dying cells but also to regulate the cytokine responses associated with them and thus play an important role in tissue injury and in the resolution of inflammation. Future work will need to focus on how collectins act in their physiological context and in particular tissue sites to regulate disease.

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MFG-E8-Dependent Clearance of Apoptotic Cells, and Autoimmunity Caused by Its Failure

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Abstract

Apoptotic cells are swiftly engulfed by macrophages and immature dendritic cells. Inefficient clearance of apoptotic cells has been implicated as a cause of inflammation and autoimmune diseases. Milk fat globule-EGF factor 8 (MFG-E8) and developmental endothelial locus-1 (Del-1) are glycoproteins secreted from macrophages that pass apoptotic cells to phagocytes. MFG-E8, but not Del-1, is expressed in the tingible-body macrophages at the germinal centers of the second lymphoid tissues. MFG-E8-deficient mice carry many unengulfed apoptotic cells in the germinal centers of the spleen, and develop a lupus-like autoimmune disease. In this review, we discuss the importance of the MFG-E8-mediated clearance of apoptotic cells in the prevention of autoimmune diseases.

Factors Involved in the Engulfment of Apoptotic Cells

Billions of cells undergo apoptosis in our bodies every day, and must be rapidly removed to maintain the integrity and functions of their surrounding tissues [1, 2]. The clearance of apoptotic cells is mainly mediated by professional phagocytes (macrophages and immature dendritic cells), which recognize ‘eat me’ signals exposed on the apoptotic cells [3, 4]. Among many proposed molecules, the best characterized candidate for ‘eat me’ signals is phosphatidylserine [5]. Phosphatidylserine is a component of the cell plasma membrane, and is

kept exclusively on the inner leaflet of the lipid bilayer in healthy cells by a putative ATP-dependent aminophospholipid translocase [6]. When cells undergo apoptosis, phosphatidylserine is quickly exposed on the outer leaflet of the dying cells via a mechanism that has not been well characterized [6]. When the cell surface is replete with phosphatidylserine, the cells can be recognized by phagocytes as targets to be engulfed [7]; thus, phosphatidylserine fulfills the criteria for an 'eat me' signal.

A number of molecules have been proposed as the receptor for phosphatidylserine on apoptotic cells. These are the scavenger receptor and LDL receptor [8, 9], which are expressed on phagocytes, and several soluble proteins [β_2-glycoprotein, growth-arrest specific protein 6 (Gas 6) and milk fat globule EGF factor 8 (MFG-E8)] that bridge apoptotic cells and phagocytes [10–12]. A molecule called 'phosphatidylserine receptor (PSR)' was identified by Fadok et al. [13]. PSR-deficient mice die as embryos, and two groups claim that these mice have a defect in the engulfment of apoptotic cells [14, 15]. On the other hand, Bose et al. [16] also established PSR-deficient mice, and reported with more convincing data that the PSR-deficient macrophages have no defect in the engulfment of apoptotic cells. PSR has no clear transmembrane domain and a very low affinity for phosphatidylserine. It has significant homology to the 'Jumonji' chromatin remodeling factor [17], which regulates mouse development. It is likely that the lethality of the PSR-deficient mice is due to defects in embryogenesis, and not to failure to engulf the apoptotic cells per se.

MFG-E8 and Developmental Endothelial Locus-1

The engulfment of apoptotic cells co-cultured with macrophages has been assayed by counting the number of labeled or unlabeled apoptotic cells inside the macrophages. However, we noticed that apoptotic dying cells often stick to the macrophages, and it is not easy to distinguish the cells inside macrophages from those attached to them.

Caspase-activated DNase (CAD) is essential for apoptotic DNA degradation in dying cells [18]. CAD-deficient cells do not undergo DNA fragmentation, but their DNA is cleaved when the dying cells are phagocytosed [19]. Using this knowledge, we established an assay system for the engulfment of apoptotic cells by macrophages [11]. Thymocytes from CAD-deficient mice were induced to undergo apoptosis and used as prey for macrophages. The generation of TUNEL-positive DNA in macrophages was then quantified by flow cytometry. We used this assay to screen a library of monoclonal antibodies (mAb) that reacted with thioglycollate-elicited peritoneal macrophages for the mAbs' ability to affect the engulfment. One mAb enhanced the engulfment of

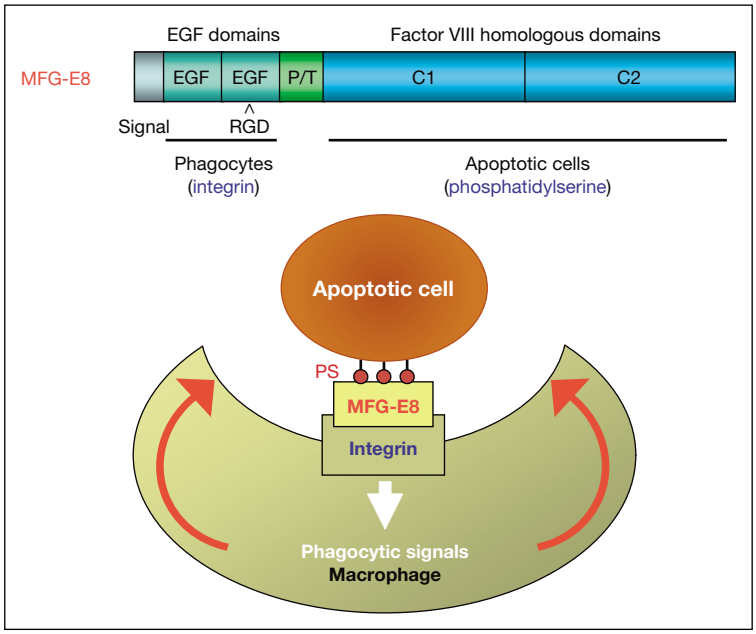

Fig. 1. Linking of apoptotic cells to phagocytes by MFG-E8. The structure of MFG-E8 is shown schematically. MFG-E8 is a secreted glycoprotein with two EGF domains (containing an RGD motif) and two factor VIII homologous domains (C1 and C2). It binds to phosphatidylserine on apoptotic cells via the C1 and C2 domains, while it binds to integrins on phagocytes via the EGF domains. Thus, it works as a bridge between apoptotic cells and the macrophages, stimulating engulfment of apoptotic cells.

apoptotic cells by peritoneal macrophages, and the purification of its antigen showed it to be a protein called MFG-E8 [11].

MFG-E8 was originally identified as a surface protein of mammary epithelial cells [20]. It is a secreted glycoprotein that consists of a signal sequence, two epidermal growth factor (EGF) domains, a proline/threonine (PT)-rich domain, and two factor-VIII-homologous domains (C1 and C2) (fig. 1). An RGD (arginine-glycine-aspartate) motif that can be recognized by some members of the integrin family [21] is present in the second EGF domain. Mice have two MFG-E8 variants (MFG-E8L and MFG-E8S); the PT-rich domain is missing in MFG-E8S [22]. It seems that human has only one variant, which corresponds to mouse MFG-E8S [23; Miyasaka, unpubl. results]. In database searches for molecules homologous to MFG-E8, a protein called ‘developmental endothelial locus-1 (Del-1)’ [24] was identified [25]. It has an

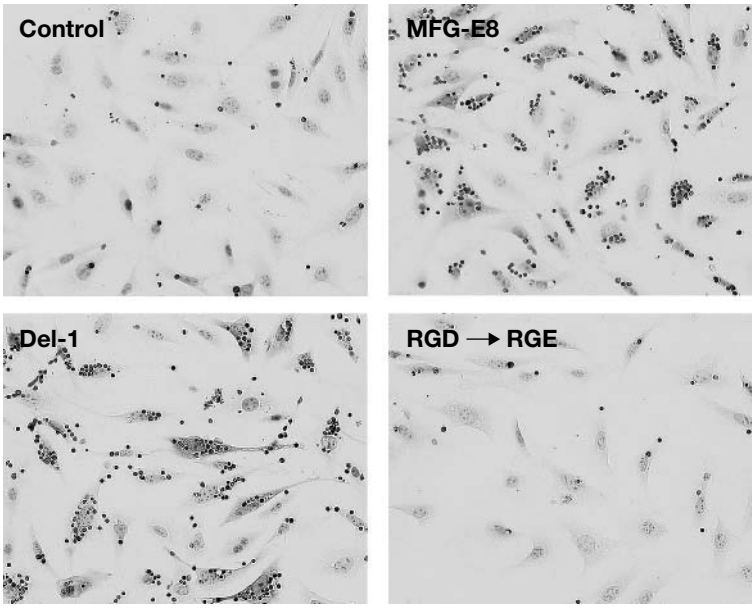

Fig. 2. MFG-E8-dependent engulfment of apoptotic cells. Thymocytes from CAD-deficient mice were induced to undergo apoptosis by treating for 4 h with dexamethasone. Mouse NIH3T3 cell transformants expressing $\alpha_v\beta_3$ integrins were co-cultured with the apoptotic thymocytes in the absence (control) or presence of 0.1 μg/ml of MFG-E8, Del-1 or an MFG-E8 mutant carrying the Asp to Glu replacement at amino acid 89.

identity of about 50% with MFG-E8 at the amino acid level, and is expressed in the endothelial cells of the fetus, particularly in the heart.

Recombinant MFG-E8 and Del-1 bind apoptotic cells but not healthy ones. Like other members of the discoidin family, to which MFG-E8 and Del-1 belong, MFG-E8 and Del-1 bind phosphatidylserine through their C1 and C2 domains. MFG-E8 and Del-1 have a high affinity for phosphatidylserine with a dissociation constant (K_d) of 2 n*M*, and have no affinity for other phosphoglycerolipids, including phosphatidylinositol, phosphatidylcholine, and phosphatidylethanolamine. When MFG-E8 and Del-1 are bound to phosphatidylserine, they also bind $\alpha_v\beta_3$ or $\alpha_v\beta_5$ integrins on phagocytes through their RGD motif in the second EGF domain. Thus, MFG-E8 and Del-1 work as a bridge between apoptotic cells and phagocytes. In fact, when mouse fibroblast NIH3T3, which do not phagocytose apoptotic cells, are engineered to express $\alpha_v\beta_3$ integrins, they efficiently engulf apoptotic cells in the presence of MFG-E8 or Del-1 (fig. 2). Integrins are known to activate small G proteins such as Rac and Rho [26]. We and others recently found that the MFG-E8-stimulated

phagocytosis of apoptotic cells is mediated by a signal cascade involving CrkII/Dock180/Rac1 [27; Nakaya, unpubl. results].

Expression of MFG-E8 in a Distinct Set of Macrophages

In addition to mammary epithelial cells, MFG-E8 is abundantly expressed in thioglycollate-elicited peritoneal macrophages [11]. As expected, the thioglycollate-elicited macrophages from MFG-E8-deficient mice have a reduced ability to engulf apoptotic cells. Immature dendritic cells are another kind of professional phagocytes that engulf apoptotic cells. Bone marrow-derived immature dendritic cells, generated by culturing bone-marrow cells in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF), expressed at least 30 times more MFG-E8 than bone marrow-derived macrophages [28], and their ability to engulf apoptotic cells was severely reduced by a lack of MFG-E8. Langerhans cells, immature dendritic cells of the skin, also expressed MFG-E8 abundantly, although the physiological role of MFG-E8 in the Langerhans cells is not clear.

The secondary lymphoid organs, such as the spleens and lymph nodes, contain many different subsets of macrophages, as regards their expression of CD68, F4/80 and MOMA-1 [29]. MFG-E8 is specifically expressed in 'tingible-body macrophages' [macrophages carrying stainable bodies (nuclei)], which are CD68-positive macrophages present in the germinal centers of the spleen and lymph nodes. In response to antigens, B lymphocytes are activated at the germinal centers of the spleen and lymph nodes, proliferate, and mature into plasma cells that produce antibody [30]. In this process, B lymphocytes that have low affinity for the antigen are also activated, and they undergo apoptosis. Tingible-body macrophages have been thought to be responsible for engulfing these apoptotic B cells [31]. However, the molecular mechanism underlying how the tingible-body macrophages recognize and engulf apoptotic cells has been elusive.

MFG-E8-Mediated Clearance of Apoptotic Cells in Germinal Centers

MFG-E8-deficient mice are normal at birth. However, as they grow older, MFG-E8-deficient mice develop splenomegaly with enlarged white pulp and numerous germinal centers [32]. Many IgG-producing plasma cells are found at the marginal zone of the enlarged germinal centers. The tingible-body macrophages in the germinal centers are associated with TUNEL-positive apoptotic

cells, which is in contrast to few or no TUNEL-positive cells found in the tingible-body macrophages in wild-type mice. An electron microscopy analysis indicated that the wild-type tingible-body macrophages carry many apoptotic condensed nuclei inside themselves. In contrast, most of the apoptotic cells associated with MFG-E8-deficient macrophages lie outside of the cells. It seems that tingible-body macrophages without MFG-E8 can recognize apoptotic cells, but cannot engulf them.

Hoffmann et al. [33] proposed a 'tethering and tickling' two-step model for the phagocytosis of apoptotic cells. In this model, apoptotic cells are first tethered to macrophages via a specific ligand-receptor pair, followed by the tickling step, which causes the engulfment of the apoptotic cells. Accumulation of unengulfed apoptotic cells on the MFG-E8-deficient tingible-body macrophages suggests that MFG-E8 is involved in the tickling step of the engulfment. Some unidentified molecules seem to be involved in the tethering step for MFG-E8-mediated engulfment of apoptotic cells. We recently showed with one mouse macrophage cell line that the interaction of CD47 expressed on apoptotic target cells with SHPS-1 expressed on macrophages is essential for the phosphatidylserine-mediated engulfment of apoptotic cells [34]. However, CD47 is exposed on the cell surface not only of apoptotic cells but also of living cells, and it is not clear how CD47 is involved in the tethering step of apoptotic cells to macrophages.

Development of Autoimmune Disease in MFG-E8-Deficient Mice

Engulfment of apoptotic cells has been regarded as a process that prevents the release of noxious and antigenic materials from dying cells, thus preventing the development of autoimmune diseases [35]. In fact, MFG-E8-deficient mice, which have defects in the engulfment of apoptotic cells, develop autoimmune diseases of the systemic lupus erythematosus (SLE) type. At the age of 40 weeks, MFG-E8-deficient mice produce a large quantity of anti-double stranded DNA and anti-nuclear antibodies (fig. 3). They develop glomerulonephritis from a massive deposition of immunoglobulins in the glomeruli, and suffer from proteinuria. As has been seen in other animal models of autoimmune diseases and human SLE patients [36], the female mice have more severe phenotypes than the male mice. This phenotype does not depend on the mouse background genes. That is, severe glomerulonephritis can be found in MFG-E8-deficient mice with a mixed background from the 129 and C57BL/6 strains, and in mice with the C57BL/6 background [37], indicating that the development of autoimmune diseases is an intrinsic phenotype caused by the lack of

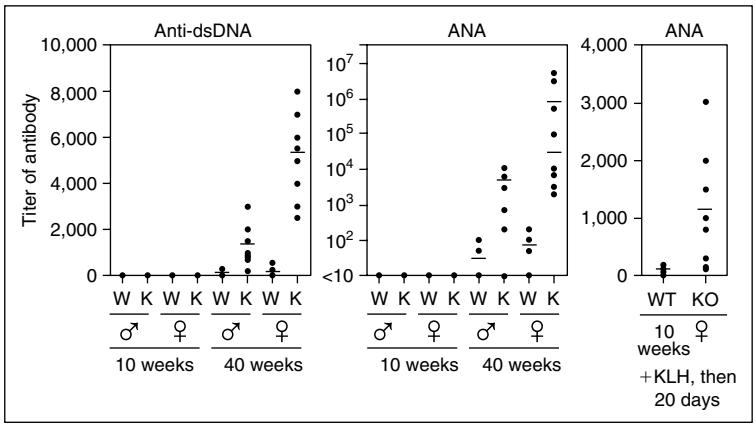

Fig. 3. Production of autoantibody in MFG-E8-deficient mice. The titers of the double-stranded DNA-specific antibody (anti-dsDNA, left) and antinuclear antibody (ANA, middle) in the serum of male and female mice at 10 or 40 weeks are plotted. W = Wild-type mice; K= MFG-E8-deficient mice. Right panel: 10-week-old MFG-E8+/+ (WT) and MFG-E8−/− (KO) mice were immunized twice with keyhole limpet hemocyanin (KLH), and the ANA titer was determined at day 20.

MFG-E8. Furthermore, when B lymphocytes are activated by immunizing the mice with keyhole limpet hemocyanin (KLH), MFG-E8-deficient female mice, even young ones, quickly generate anti-nuclear antibody. These results confirm that if apoptotic cells, in this case B lymphocytes activated in the germinal centers, are not efficiently engulfed by macrophages, the animals will develop autoimmune diseases.

Conclusion and Perspective

Although many cells undergo apoptotic cell death in our bodies, it is not easy to detect the dying cells in situ. This is because apoptotic cells are swiftly engulfed by phagocytes. It seems that immediately after cells are triggered to undergo apoptosis, they are recognized by phagocytes and engulfed. Thus, most of the morphological and biochemical changes of apoptotic cells seem to occur in the phagocytes, which may block the access of the immune system to the intracellular materials that can be exposed on apoptotic cell surfaces [38]. The administration of apoptotic cells to non-autoimmune mice induces the transient production of anti-phospholipid autoantibodies [39]. A mutant of MFG-E8

in which the RGD-integrin binding motif is changed to RGE binds to phosphatidylserine but not to integrins on macrophages, and can mask the phosphatidylserine exposed on the surface of apoptotic cells [11]. Thus, when apoptotic cells are administered to mice after being treated with the RGE mutant of MFG-E8, the production of autoantibodies (anti-cardiolipin and anti-phosphatidylserine) and the development of glomerulonephritis are greatly enhanced [40], confirming that the phosphatidylserine-dependent engulfment of apoptotic cells plays a role in preventing the development of autoimmune diseases.

Several mouse model systems for lupus-like autoimmune diseases have been developed using gene-knockout mice [35]. Among them, mice deficient in C1q show an autoimmune phenotype similar to that of the MFG-E8-deficient mice [41]. C1q, a member of the collectin family, is the first component of complement, and binds to the immune complex to clear it. It seems that the lack of C1q causes accumulation of the immune complex, which is deposited on the glomeruli causing glomerulonephritis. Involvement of C1q in the engulfment of apoptotic cells has been suggested [42]. However, C1q binds to apoptotic cells late in apoptosis, in an IgM-dependent manner, suggesting that the Clq-mediated engulfment of apoptotic cells is a backup system [43, 44]. Thus, we think that the MFG-E8-deficient mice are the best model for the autoimmune disease caused by the failure of macrophages to engulf apoptotic cells. Monocyte-derived macrophages or tingible-body macrophages at the germinal centers from SLE patients often have an impaired ability to engulf apoptotic cells [45, 46]. It will be interesting to examine whether MFG-E8 or its related molecules are properly expressed in these SLE patients.

How does the inefficient engulfment of apoptotic cells lead to autoimmune responses? When apoptotic cells are left unengulfed, they undergo secondary necrosis, and their cellular contents are released. These cellular components are ingested by macrophages or dendritic cells, and their antigens may be presented, triggering the immune reaction. In another scenario, autoreactive B cells recognizing DNA or chromatin are known to be present in normal mice as inferred from the Fas-deficient *lpr* mice [47]. These B cells have a low affinity for the antigens, and remain quiescent. However, when DNA or chromatin is released from dying cells, it may activate these B cells to produce a large amount of autoantibodies (fig. 4) [48]. The release of cellular contents often activates the inflammatory responses, which may further enhance the development of autoimmune diseases. In any case, the development of autoimmune diseases in MFG-E8-deficient mice provides strong evidence that apoptotic cells must be cleared rapidly to prevent these diseases. We hope that the characterization of MFG-E8-deficient mice will contribute to the understanding of the molecular mechanism of human autoimmune diseases.

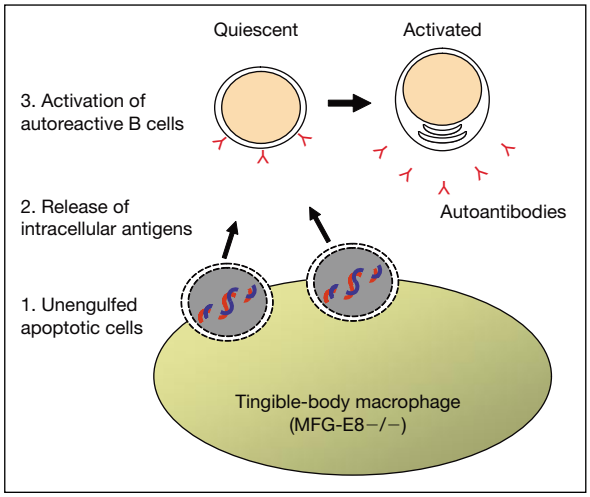

Fig. 4. A model for the autoimmune responses caused by inefficient engulfment of apoptotic cells. In germinal centers, apoptotic cells are swiftly engulfed by tingible-body macrophages in an MFG-E8-dependent manner. These apoptotic cells are left unengulfed in MFG-E8-deficient mice, and undergo secondary necrosis, which lead to the release of intracellular antigens (such as DNA or chromatin). These antigens then activate autoreactive B cells that normally remain quiescent, to produce a large amount of autoantibodies.

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Clearance of Apoptotic Cells in Human SLE

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Abstract

Systemic lupus erythematosus (SLE) is characterized by a diverse array of autoantibodies, particularly against nuclear antigens, thought to derive from apoptotic and necrotic cells. Impaired clearance functions for dying cells may explain accumulation of apoptotic cells in SLE tissues, and secondary necrosis of these cells may contribute to the chronic inflammation in this disease. The exposure of phosphatidylserine (PS) and altered carbohydrates on dying cells are important recognition signals for macrophages. Furthermore, serum factors such as complement, DNase I, pentraxins (e.g. C-reactive protein) and IgM contribute to efficient opsonization and uptake of apoptotic and necrotic cells. Defects in these factors may impact the development of SLE in humans and mice in a variety of ways. We observed impaired clearance of apoptotic cells in lymph nodes and skin biopsies of humans with lupus, as well as intrinsic defects of macrophages differentiated in vitro from SLE patients' CD34+ stem cells, demonstrating that apoptotic cells are not properly cleared in a subgroup of patients with SLE. This altered mechanism for the clearance of dying cells may represent a central pathogenic process in the development and acceleration of this autoimmune disease.

The immune system is a crucial element in all biologic systems. It invokes an effective defense against invading pathogens or tumor cells and at the same time discriminates self from non-self to avoid autoimmunity. Genes and environmental factors play important roles in the pathogenesis of autoimmune diseases, which can affect almost any organ or body system.

In this article, we will focus on the autoimmune disease, systemic lupus erythematosus (SLE). What are the self antigens in this disease and how are

they rendered accessible to the immune system? Chromatin and nuclear proteins are the primary targets for autoantibodies in SLE [1]. Therefore, protection from these autoantigens requires a fast and efficient removal of dying cells and their remnants.

Dying Cells Modulate the Immune Response

Cells can die via two main pathways, i.e. apoptosis and necrosis. Because apoptotic cells can maintain their membrane integrity for a limited time, their immediate clearance by neighboring cells ensures that no inflammation occurs. The recognition and uptake by macrophages of apoptotic cells leads to the release of anti-inflammatory signals such as IL-10 and TGF-β, and to the suppression of inflammatory mediators such as IL-12 or TNF-α [2, 3]. Nevertheless, some cells dying via programmed cell death become accessible to dendritic cells and tolerance is induced, since the antigenic peptides are presented without co-stimulation. In contrast, primary necrotic cells induce inflammation and immune reactions, and are characterized by enlargement of cells and their organelles causing cell membrane disintegration. Apoptotic cells also lose membrane integrity upon entry into the late stages of apoptosis, and are often referred to as secondary necrotic cells. It has been shown that the high mobility group B1 (HMGB1) protein, which is immobilized on the chromatin of apoptotic cells, remains so even under conditions of secondary necrosis. In contrast, in the case of primary necrosis, HMGB1 is released and acts as an inflammatory cytokine [4, 5]. Therefore, primary and secondary necrotic cells display different inflammatory signals with a stronger proinflammatory response of the former.

Clearance of Dying Cells

Several receptors and adaptor molecules are involved in the recognition and uptake of dying cells by phagocytes [reviewed in 6]: Collectin receptors, calreticulin/CD91, Fcγ receptors, c-Mer, the β_2-glycoprotein-1 receptor, integrins such as $\alpha_v\beta_3$, lectins, CD14, ABC transporters, scavenger receptors including CD36, and an inconclusively identified phosphatidylserine (PS) receptor recognized and blocked by the monoclonal antibody mAb 217G8E9. The vast redundancy of ligands, receptors, and bridging molecules between dying cells and phagocytes points out the importance of an efficient recognition and clearance system, exemplified by the fact that 8 of 14 genes regulating apoptosis in *Caenorhabditis elegans* are necessary for the engulfment process [7].

The receptors and ligands responsible for clearance of apoptotic cells are discussed elsewhere in this volume so that only a few key points will be mentioned here.

Phosphatidylserine as a Recognition Signal for Phagocytes

The exposure of PS on the outer leaflet of apoptotic and necrotic cells is an important primary signal recognized by phagocytes [8]. However, there are also viable PS-exposing cells (e.g. activated B cells, monocytes) that are not phagocytosed. To illuminate this controversial feature of PS exposure, we analyzed the binding of annexin V (AxV), a protein that specifically binds to PS, to viable and dying monocytes, and demonstrated a cooperative AxV interaction with dying (apoptotic and necrotic) monocytes. Conversely, AxV binds to viable monocytes without co-operation [9], suggesting that cell membranes of dying cells have a higher lateral mobility of PS, and that AxV requires a critical density or clustering of PS molecules. It is also possible that AxV requires an undefined co-factor only present on dying cells.

The recognition of exposed PS triggers the release of immunosuppressive cytokines, which quench inflammation and prevent the maturation of antigen presenting DC. Because the immunosuppression induced by PS-dependent clearance might represent a major obstacle to immunization against cancer cells, we analyzed whether apoptotic cell immunogenicity could be restored by blocking their PS-dependent clearance, and demonstrated that this was the case [10]. Moreover, treatment with AxV decreased apoptotic cell uptake by peritoneal macrophages and concomitantly increased apoptotic cell uptake by CD8+/11c+ DC. Vaccination with AxV-treated irradiated tumor cells augmented tumor rejection [11]. We suggest that the impaired clearance of the dying tumor cells enhances the immune response.

Altered Carbohydrates as a Recognition Signal for Phagocytes

Recognition of PS is a major factor in the phagocytosis of apoptotic cells, but there are many other membrane alterations during apoptotic cell death that lead to the recognition of apoptotic cells by additional adaptor molecules. Examples of those molecules are: the complement component C1q, surfactant protein A (SPA) and D (SPD), the long pentraxin PTX3, and CRP as discussed elsewhere in this volume. Carbohydrate-binding proteins (lectins) also play a role in the innate immune system. Two decades ago, it was suggested that lectin-like molecules on the surface of phagocytes recognize changes in the

cell-surface carbohydrates of cells undergoing apoptosis [12]. Galactose- and mannose-specific receptors are considered to be important to the recognition of dying cells [13]. Certain lectins, such as ficolin and the mannose-binding lectin, have been described to bind and opsonize apoptotic cells and enhance their uptake [14, 15]. However, the exact role of altered carbohydrates in the apoptotic clearance process remains elusive. We observed an increased binding of lectins specifically recognizing mannose-, N-acetylglucosamine- and fucose-containing epitopes to apoptotic and necrotic cells [16]. The expression of these modified sugar moieties displayed delayed kinetics compared to PS and represents a major membrane alteration preceding secondary necrosis. Therefore, the exposure of modified sugars may represent a further back-up mechanism for clearance for apoptotic cells that had escaped the early PS-dependent phagocytosis by macrophages.

Serum Factors Involved in the Clearance Process

Complement and DNase I

It has long been known that deficiencies in early complement proteins, especially C1q and C4, often lead to the development of human SLE [17, 18]. We and others have shown that complement is important for the removal of apoptotic cells in vitro and in vivo [19–23]. Furthermore, we observed a dominant co-operation of DNase I and C1q in the clearance of necrotic cell-derived chromatin in human serum [24]. C1q did not increase the DNase activity for the digestion of free DNA, indicating the action of an indirect mechanism during digestion of necrotic cell-derived chromatin. Two alternative possibilities for the acceleration of the DNase activity by C1q can be hypothesized: (1) Analogous to SAP [25], C1q increases the access of DNase to the DNA of the nucleoprotein complex in the chromatin of necrotic cells. (2) C1q interferes with the immobilization of DNase by components of the actin cytoskeleton [26, 27]. We have examined sera obtained from autoimmune patients with regard to their capability to degrade necrotic cell-derived chromatin. A significant reduction of DNase I activity in sera of patients with SLE and with rheumatoid arthritis (RA) in comparison to normal healthy donors (NHD) was observed. Most interestingly, SLE sera showed a strongly reduced degradation capacity of necrotic cell-derived chromatin in comparison to RA sera and NHD sera [own unpubl. data]. This might be due to the reduced complement activity detected in the sera of SLE patients. We conclude that an additional protection from chromatin implicated in the development of autoimmune disorders such as SLE can be achieved by the C1q and DNase I-dependent degradation of chromatin and the C1q-dependent uptake of the degraded chromatin [24].

Pentraxins

Pentraxins are highly conserved cyclic pentamers that also play a role in several clearance processes. PTX3 is the prototypic long pentraxin, structurally related to the short pentraxins, C-reactive protein (CRP) and serum amyloid P component (SAP) [28]. CRP is the prototypical acute-phase reactant in humans and a component of the innate immune system. Once bound, CRP induces complement activation via the classical pathway, which, in turn, triggers the influx of neutrophils, decorates the surfaces of several ligands with opsonizing complement fragments and enhances phagocytosis of material that has bound CRP and complement. CRP also interacts with Fcγ receptors on phagocytic cells and acts as an opsonin. CRP binds to nuclear constituents of apoptotic and necrotic cells that are targeted by autoantibodies of SLE patients [29]. Recently, it has been shown that human-derived CRP suppressed kidney inflammation in NZB/W F1 mice [30], suggesting an immune modulatory effect of CRP in addition to its ability to act as an opsonin for apoptotic and necrotic cells [reviewed in 31].

IgM

Natural IgM antibodies have also been shown to bind to dying cells and to play an important role in their opsonization by complement [32]. IgM antibodies are necessary for the C1q-dependent uptake of apoptotic thymocytes by murine bone-marrow-derived macrophages [33].

Deficiencies in molecules implicated in the disposal of effete cells may promote the development of lupus. Molecules such as DNase I, CRP, C1q, MBL, IgM and others digest, mask, and opsonize autoantigens and cellular debris and promote their clearance.

Systemic Autoimmunity in Humans and Mice – Similarities and Differences

SAP is the major acute-phase reactant in mice, whereas CRP plays this role in humans. Mice with targeted deletion of the SAP gene spontaneously develop autoimmunity, show enhanced anti-DNA responses to immunization with extrinsic chromatin, and develop a severe SLE-like syndrome [34]. However, this strong effect may also depend on background genes located on the chromosome 1 [35, 36]. Very severe SLE is caused by C1q deficiency in humans [37], but it induces only a mild form of autoimmunity in mice. Deficiency of the Fas pathway invariably causes mild autoimmunity (anti-lymphoproliferative syndrome; ALPS) in humans [38] but a severe form of SLE-like disease in mice on the MRL background. As mentioned above, DNase I co-operates with C1q for the degradation of extracellular chromatin in humans, similar to the murine system

Table 1. Anti-nuclear autoimmunity in humans and mice

Humans
C1q deficiency causes severe SLE
CRP is the major acute phase reactant
Deficiency of the Fas pathway causes a mild autoimmunity (ALPS)
DNase-I co-operates with C1q for the degradation of extracellular chromatin
Penetrance is low
Mostly polygenic
Mice
C1q deficiency induces mild autoimmunity
SAP is the major acute phase reactant
Deficiency of the Fas pathway causes a SLE-like disease in MRL mice
DNase-I co-operates with plasminogen for the degradation of extracellular chromatin
Penetrance is high (inbred)
Models often monogenic depending on background genes

wherein plasmin and DNase I have been shown to co-operatively serve this function [39]. In contrast to gene-targeted mice, the development of autoimmunity in humans and spontaneous mouse lupus models is mostly polygenic and of low penetrance. However, the genetic background also strongly influences the development of autoimmunity in mice. The accumulation of late apoptotic cells, debris, or chromatin seems to be associated with anti-nuclear autoimmunity in humans and mice, but the pathways involved may differ (table 1).

Impaired Clearance of Apoptotic Cells in Germinal Centers of some SLE Patients

Impaired clearance of dying cells may explain the accumulation of apoptotic, and subsequently of secondary necrotic, cells in tissues of SLE patients [40, 41]. We analyzed lymph node biopsies from such patients to determine whether a defect in engulfment of apoptotic cell material can also be observed in germinal centers (GC). A characteristic feature of the GC is the presence of specialized phagocytes, usually referred to as tingible-body macrophages (TBM). Under normal conditions, TBM efficiently remove apoptotic cells in the early phases of apoptosis. In a subgroup of SLE patients, apoptotic cells were found to accumulate in the GC of the lymph nodes, and the numbers of TBM usually containing engulfed apoptotic nuclei were significantly reduced. In contrast to controls, apoptotic material was observed associated with the surfaces of follicular dendritic cells (FDC) [42] (fig. 1). In the case of the GC, it is of major

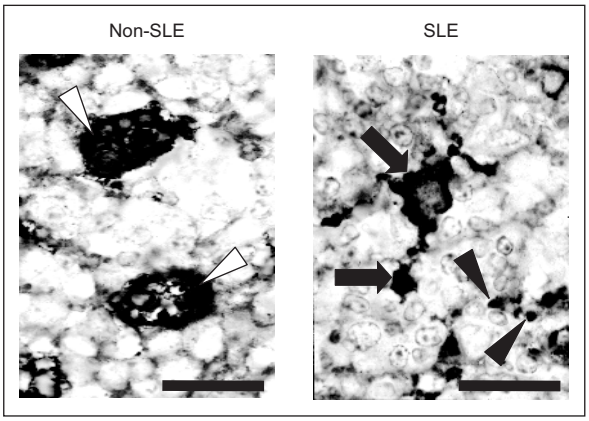

Fig. 1. Apoptotic nuclei are associated with follicular dendritic cells (FDC) in lymph nodes from some systemic lupus erythematosus (SLE) patients. Apoptotic nuclei ingested by tingible-body macrophages are marked with a white arrowhead. Un-ingested apoptotic nuclear material decorating the surfaces of FDC is marked with black arrows. Free apoptotic material is marked with black arrowheads. Scale bar = 50 μm.

importance to remove potential autoantigens that could otherwise serve as antigen for affinity maturation of autoreactive B cells [43]. FDC may serve as autoantigen repositories in the case of clearance failure. Accumulated nuclear debris may consecutively activate complement, resulting in opsonization and binding to the complement receptors of FDC. In accordance with the extremely low phagocytic activity of FDC [44], the nuclear material on the surfaces of FDC remains accessible to autoreactive B cells. Therefore, we propose that ANA induction, at least in one subgroup of SLE patients, results from inadequate clearance of GC apoptotic centrocytes in the early phase of apoptosis, either due to impaired phagocytic activity or the absence of TBM. Apoptosis can progress and the cells can enter the late stages of apoptotic cell death, including secondary necrosis. In this state, activation of the classical complement cascade may result in deposition of C3b on cell surfaces. Via C3b and its fragments, the apoptotic cells and nuclear debris bind to CR2/CD21 on FDC. These complement receptors are necessary for the generation of an antigen-specific IgG response [45]. The disintegrated membranes of apoptotic cells permit access to potential intracellular autoantigens, and the latter can consequently provide a short-term survival signal for those B cells that have accidentally gained a B cell receptor that is reactive with nuclear antigens during the random process of somatic mutation. Taken together, nuclear autoantigens bound to FDC may provide a survival signal for autoreactive B cells, thereby overriding an important initial control mechanism of B cell tolerance (fig. 2).

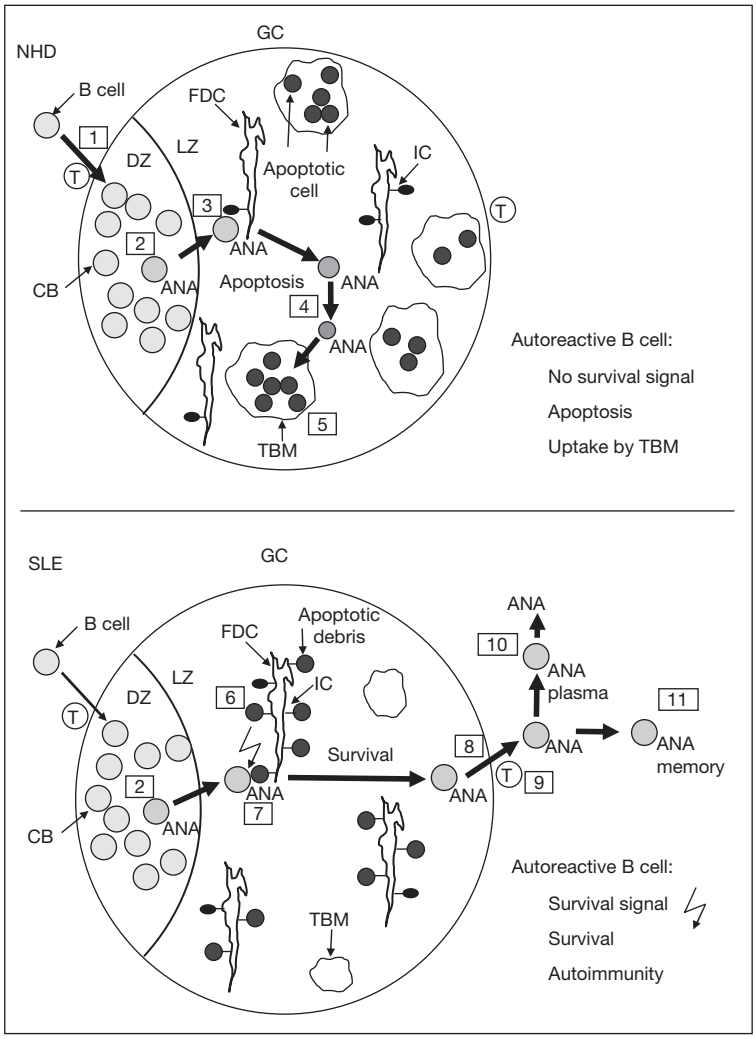

Fig. 2. Fate of B cells that have acquired autoreactivity against nuclear material during somatic hypermutation in germinal centers (GC) of normal healthy donors (NHD) and patients with systemic lupus erythematosus (SLE). Affinity maturation of B cells is dependent on T cell help (1) and takes place in the GC of secondary lymphoid organs. In the dark zone (DZ) of GC, surface immunoglobulin-negative B cells proliferate as large centroblasts (CB). After a few divisions, centroblasts stop cycling and express surface immunoglobulin. Somatic hypermutation of the B cells occurs and autoreactive B cells against nuclear material can be generated randomly (2). The B cells enter the light zone (LZ) of the GC and contact antigen bound to follicular dendritic cells (FDC) (3). Usually, antigen is bound to the surface of FDC as an opsonized immune complex (IC). A lack of antigen or somatic mutations of the B cell receptor lead to reduced affinity for the antigen, and the B cells do not receive the necessary

Impaired Clearance of Apoptotic Cells in the Skin of Patients with Cutaneous Forms of Lupus erythematosus

Cutaneous lupus erythematosus (CLE) defines a wide spectrum of clinical manifestations with variable evolutions and is increasingly being recognized as more common disease than previously thought [46]. The skin has not received the experimental attention of other organ systems, and the pathogenesis of skin lesion formation in CLE remains poorly understood. The preferential involvement of sun-exposed areas and the induction of skin lesions after solar or artificial UV irradiation suggest that photoexposure is an important element in the pathogenesis of CLE [47, 48]. It has been proposed that UV exposure might cause exacerbation of local and systemic autoimmunity by inducing changes in the expression and binding of keratinocyte autoantigens [49–51]. Furthermore, Casciola-Rosen et al. [1] have demonstrated autoantigen clustering at the cell surface of cultured keratinocytes with UV-irradiation-induced apoptotic changes. Translocation of autoantigens to the cell surface of apoptotic blebs may allow circulating autoantibodies to gain access to these autoantigens, which are usually sequestered inside the cells. Antibody binding to the exposed antigens is proposed to result in tissue injury by complement or inflammatory cells [52], which may be especially important if the anti-inflammatory clearance of apoptotic cells is impaired or delayed and the apoptotic cells consecutively undergo secondary necrosis [2, 53].

Using in situ labeling methods for the detection of DNA strand breaks, an increased number of apoptotic keratinocytes in primary lesions of CLE patients compared with controls has been described [54–56]. Our group demonstrated that a significant increase of apoptotic nuclei was also found in lesional skin biopsy specimens after provocative phototesting of patients with CLE [57]. Tissue sections taken 1 day after a single UV exposure showed an increased number of epidermal apoptotic nuclei in controls compared with tissue from CLE patients taken under the same conditions before lesion formation. In sections collected 3 days after irradiation, a significant decrease of the apoptotic nuclei count was observed in controls consistent with proper clearance of apoptotic cells 1–3 days after UV

survival signals and rapidly undergo apoptosis (4). Apoptotic B cells are normally removed immediately (5) by specialized phagocytes referred to as tingible-body macrophages (TBM). In the case of SLE, the number of TBM containing engulfed apoptotic nuclei is significantly reduced, and uningested apoptotic debris, including potential autoantigens, becomes coated with the complement component C3d. Similar to IC, opsonized apoptotic debris binds to complement receptor2/CD21 on FDC (6). Survival signals for autoreactive B cells (ANA) are consecutively provided (7). Positively selected B cells survive, leave the GC (8), and migrate into the mantle zone, where they must interact with CD4+ T lymphocytes in a cognate manner to receive further co-stimulation (9). The cells can now differentiate into either anti-nuclear autoantibodies (ANA)-secreting plasma cells (10) or memory cells (11), respectively.

exposure. In striking contrast, the majority of CLE patients showed a significant increase in the number of apoptotic nuclei in this period, suggesting that late apoptotic cells accumulate in the skin of a subgroup of CLE patients. Analogous to the clearance defects in apoptotic cells and the consecutive accumulation of apoptotic nuclei reported for SLE patients [40–42, 58], we propose that impaired or delayed clearance of apoptotic cells occurs in the skin of most CLE patients. Consequently, this condition may result in the accumulation of high numbers of apoptotic cells that undergo secondary necrosis and cause local tissue inflammation [59, 60].

Intrinsic Clearance Defects in SLE

Impaired clearance of dying cells is thought to play an essential role in the pathogenesis of autoimmune diseases. As discussed above, factors extrinsic to phagocytic cells may play a role in the reduced uptake of dead and dying cells in SLE. For example, the hereditary C1q deficiency shows many typical characteristics of human SLE disease and provides the strongest proof of the importance of this complement protein in the pathogenesis of autoimmunity against nuclear structures [61]. However, many findings support the hypothesis that monocytes and macrophages from SLE patients have intrinsic defects. We found impaired uptake of apoptotic cells and cellular debris by monocyte-derived macrophages from SLE patients independent of serum factors [40], and others have reported that monocytes from SLE patients display decreased macrophage density in vitro, are altered in phenotype and function, show an abnormal differentiation and altered expression of cell surface molecules [62, 63].

We analyzed the phagocytic potency of macrophages differentiated from CD34+ stem cells derived from the peripheral blood of SLE patients or controls, respectively, and found similar proliferation of the stem cells in vitro. However, differentiation into macrophages was reduced in some SLE stem cell cultures. Fewer macrophages differentiated from CD34+ stem cells. Some SLE stem cell-derived macrophages were smaller, showed a strongly reduced adherence, died earlier and had a reduced phagocytic capacity [64].

In patients with SLE phagocytic defects were observed in multiple cell types: monocytes, macrophages, granulocytes, and phagocytes derived from in vitro cultivated CD34+ stem cells. We further examined in vitro the uptake of various particles by macrophages and granulocytes derived from SLE patients and NHD. Macrophages and/or granulocytes of some SLE patients showed a strongly reduced uptake of albumin-coated beads, immunoglobulin-coated beads, apoptotic and necrotic cells as well as degraded chromatin. Very importantly, phagocytes from different SLE patients showed in part different defects. The phagocytic defects observed were therefore heterogeneous.

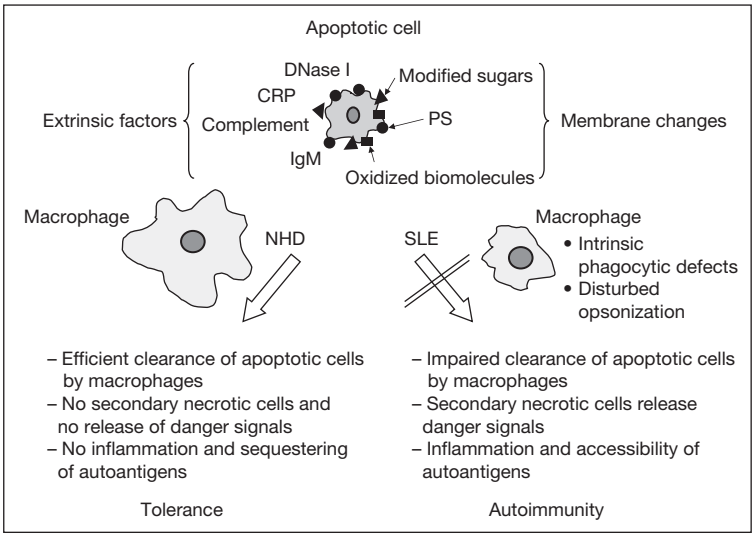

Fig. 3. Impaired clearance of apoptotic cells in SLE patients. Efficient recognition and uptake of apoptotic cells normally leads to tolerance. In SLE, impaired uptake of apoptotic cells by macrophages that have in part intrinsic functional defects, leads to loss of tolerance and fosters autoimmunity (see text for details). CRP = C-reactive protein; DNase I = deoxyribonuclease I; NHD = normal healthy donor; PS = phosphatidylserine; SLE = patients with systemic lupus erythematosus.

These results further support the notion that intrinsic defects of phagocytes from SLE patients, combined with extrinsic defects, contribute to the impaired clearance of dying cells frequently observed in such patients.

Conclusion

The early recognition and engulfment of apoptotic cells is necessary to avoid cells entering into the late stages of apoptosis. Characteristic membrane changes of apoptotic cells such as exposure of oxidized molecules, PS, and modified sugar structures, serve as 'eat-me' signals of apoptotic cells prior to apoptotic cell disintegration. Several receptors on macrophages, as well as bridging molecules and opsonizing proteins such as complement, CRP, and IgM, normally contribute to efficient removal of dying cells by phagocytes. Clearance defects that allow dying cells to accumulate and release danger signals and autoantigens becoming accessible to generate a pro-inflammatory milieu, ultimately lead to a chronic autoimmune response (fig. 3). Altered clearance mechanisms for dying

cells, therefore, represent a central pathogenic process in the development and acceleration of autoimmune diseases like SLE and CLE.

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Apoptosis and Glomerulonephritis

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Abstract

Glomerulonephritis (GN) is a form of autoimmunity in which apoptosis may be a double-edged sword. Resolution of GN can be promoted by apoptosis of infiltrating leucocytes and excess resident glomerular cells, leading to efficient anti-inflammatory clearance by macrophages and mesangial cells. However, unscheduled apoptosis in glomerular cells, especially epithelial cells ('podocytes') may drive progression of GN to hypocellular, non-functional scar. Defects in clearance of apoptotic cells may also have deleterious local effects, in addition to promoting autoimmunity itself. Nevertheless, there is strong promise for novel therapies based on new knowledge of apoptosis in GN, especially in regulation of leucocyte clearance from the inflamed glomerulus.

Introduction: The Inflamed Glomerulus and Apoptosis

Autoimmunity is an important cause of many histologically defined patterns of glomerular injury and has been extensively reviewed elsewhere [1]. In turn, glomerulonephritis (GN) is an important cause of chronic renal failure requiring renal replacement therapy [1, 2]. Although glomerular inflammation can resolve completely, as exemplified by post-streptococcal GN, it frequently persists leading to scarring and loss of organ function. While glomerulosclerosis (glomerular scarring) is prominent in failing kidneys affected by GN, tubulointerstitial inflammation and fibrosis are also prominent and may determine outcome of the glomerular lesion.

Apoptosis was first described in glomerulonephritis by Harrison [3]. However, studies of programmed cell death in the glomerulus emphasise the histologically inconspicuous nature of apoptosis, which is due to rapid phagocytic clearance and degradation of dying cells. Various strands of evidence demonstrate that in most tissues little more than an hour is required for a dying

cell to become recognisably apoptotic, taken up by a phagocyte and degraded beyond histological recognition [4]. In the rat, in keeping with normally low rates of cell birth by mitosis (~1 in 10,000 in a classical study [5]), we observed similarly low rates of histologically-demonstrated apoptosis in normal glomeruli, with only 1 in 10,000 cells appearing apoptotic at any given time [6].

Nevertheless, as explained below, the detection of very high rates of apoptosis in a mouse model of autoimmune GN provided a key insight into the links between failed clearance of apoptotic cells and autoimmunity [7]. Furthermore, there is now compelling evidence that autoimmunity affecting the glomerular vasculature can also be associated with disruption of safe cell clearance by apoptosis and subsequent phagocytosis [8]. These discoveries could have important implications for treatment, as discussed below.

Death of Cells Implicated in Glomerulonephritis

The glomerulus exhibits a stereotyped response to inflammatory injury, with all histological types of GN exhibiting subtly different degrees of three key abnormalities [1]. All have links to apoptosis.

First, increased infiltration with leucocytes is now recognised in virtually all forms of glomerular injury, although subtle increases in glomerular macrophages in disorders such as diabetic glomerulopathy can be contrasted with classical acute inflammatory leucocyte infiltration in glomerular vasculitides, for example, with prominent recruitment of neutrophils, monocyte/macrophages and lymphocytes. In keeping with inflamed sites elsewhere, apoptosis plays an important role in deletion of leucocytes attracted to inflamed glomeruli [9], as discussed further below.

Second, increases in intrinsic glomerular cell number are common and variably prominent. The glomerular mesangial cell can rapidly adopt a myofibroblastic phenotype, increased numbers in GN being analagous to the 'healing' response in skin wounds, in which a wave of myofibroblast proliferation precedes wound closure. Glomerular endothelial cells can also proliferate in disorders such as post-streptococcal GN. However, although the highly specialised glomerular epithelial cells or podocytes appear able to engage some elements of the mitotic machinery there is little evidence that adult podocytes can proliferate. Nevertheless, all three intrinsic glomerular cell types have been reported to undergo apoptosis [9, 10].

Third, a truly universal feature of glomerular inflammation is a change in the amount and composition of extracellular matrix (ECM) in the injured structure. For example, over-expression of (plasma type) fibronectin and type I collagen are prominent in the mesangial matrix of injured glomeruli. This may

increase the susceptibility of glomerular cells to apoptosis, since in vitro studies demonstrate that fibronectin and type I collagen fail to provide mesangial cells with β1 integrin-mediated survival signals, by contrast with normal constituents of glomerular ECM such as laminin and type IV collagen [11].

Apoptosis in Glomerular Leucocytes

Neutrophil polymorphonuclear granulocytes are the classical 'rapid response' force of the acute inflammatory response, being summoned to injured tissue by a range of chemoattractants. Indeed, their presence in most tissues is a reliable marker of inflammation since they are normally confined to their birthplace in the bone marrow, the vasculature in which they circulate for ~6 h and 'graveyards' in liver, spleen and bone marrow. Neutrophils are terminally differentiated cells incapable of division. They are prominent in various severe forms of GN. Furthermore, there is no evidence that they emigrate from inflamed tissues by return to the blood or via lymphatics (at least not in significant numbers). Instead, there is now compelling evidence that neutrophils and their toxic granule contents are usually deleted from inflamed sites by undergoing apoptosis leading to swift, non-phlogistic phagocytosis by 'professional' macrophages and 'semi-professional' phagocytes such as glomerular mesangial cells [12]. Inflamed glomeruli exhibit apoptotic neutrophils [9]. Furthermore, although the authors are not aware of reports of eosinophil granulocyte apoptosis in less common glomerular conditions characterized by local eosinophilia (such as Churg-Strauss vasculitis), there is also compelling in vitro and in vivo evidence that eosinophil granulocytes are also deleted by apoptosis [12].

Some important principles of regulation of leucocyte apoptosis are illustrated by controls on neutrophil programmed cell death. Although isolated blood neutrophils engage constitutive apoptosis, dying in vitro with a half-life around 18 h, a wide range of inflammatory mediators normally delay such death [12]. One common 'pro-survival' pathway in granulocytes involves activation of NF-κB transcription factor, which impinges on multiple downstream survival pathways. Clearly, therefore, if granulocyte apoptosis is to occur at inflamed sites in vivo there must be powerful systems to overcome the pro-survival 'tone' of inflammatory mediators. One such 'pro-death' influence is phagocytosis by granulocytes of bacteria opsonised by endogenous complement components. This event triggers the 'respiratory burst' leading to production of oxygen radicals within neutrophils which then drive death by mechanisms including caspase activation and changes in the balance of proteins in the Bcl-2 family [13]. Similarly, local release of 'pro-death' cytokines of the TNF-α family, particularly Fas ligand (FasL) is likely to overcome pro-survival

influences on granulocytes. Nevertheless, there remains some debate about the cellular source of FasL in such circumstances. Some studies indicate that neutrophils do not express FasL, a relevant source being neighbouring monocyte/macrophages that release the cytokine in response to phagocytosis, including uptake of apoptotic cells [14]. Other reports suggest that under certain circumstances neutrophils can express functionally significant levels of FasL and that this is normally kept in check by members of the forkhead transcription factor family such as Foxo3a [15]. However this debate is resolved, it is clear that regulation of FasL expression at inflamed sites represents a new therapeutic target in inflammatory disorders (see below).

Monocyte recruitment occurs soon after neutrophil infiltration in classical acute inflammatory responses, infiltrating monocytes differentiating into inflammatory macrophages. The macrophage complement of the inflamed tissue can also be increased by local proliferation of resident macrophages. In severe forms of GN such as crescentic nephritis, in which there is leakage of blood cells from glomerular capillaries into Bowman's space, there is good evidence of both monocyte recruitment and local monocyte/macrophage proliferation [16]. Furthermore, in these special circumstances there may be local deletion of monocyte/macrophages by apoptosis [16], which is likely directed because of the normal propensity of inflammatory macrophages to resist pro-apoptotic stimuli [12]. Nevertheless, rather than local death by apoptosis, it appears that the usual fate of inflammatory macrophages is integrin-mediated emigration from the inflamed site via lymphatics [17]. Indeed, there are compelling reports of macrophage trafficking from inflamed glomeruli to draining lymph nodes [see 16 for details]. Once emigrating macrophages reach draining lymph nodes their fate is currently obscure, although it can be speculated that such macrophages undergo apoptosis in the node. A further intriguing issue for future study will be to define the influences that cause macrophages to emigrate the inflamed site. Lessons may be learnt from a closely related phagocyte, the dendritic cell, which when laden with apoptotic cells can traffic to lymph nodes from tissues. Preliminary data strongly indicate that binding of apoptotic cells may be a potent stimulus to macrophages for emigration [Watson et al., unpubl. data].

Lymphocytes are also prominent in many types of GN, although mononuclear leucocyte (monocyte/macrophages and lymphocytes) infiltration of the tubulointerstitial compartment of the kidney is often more prominent than that observed in glomeruli. Furthermore, lymphocytes are in many ways characterized by their propensity to circulate through healthy and diseased tissues, coursing from blood to lymph. Nevertheless, in keeping with other lineages of inflammatory leucocytes, lymphocytes can undergo apoptosis at inflamed sites [18].

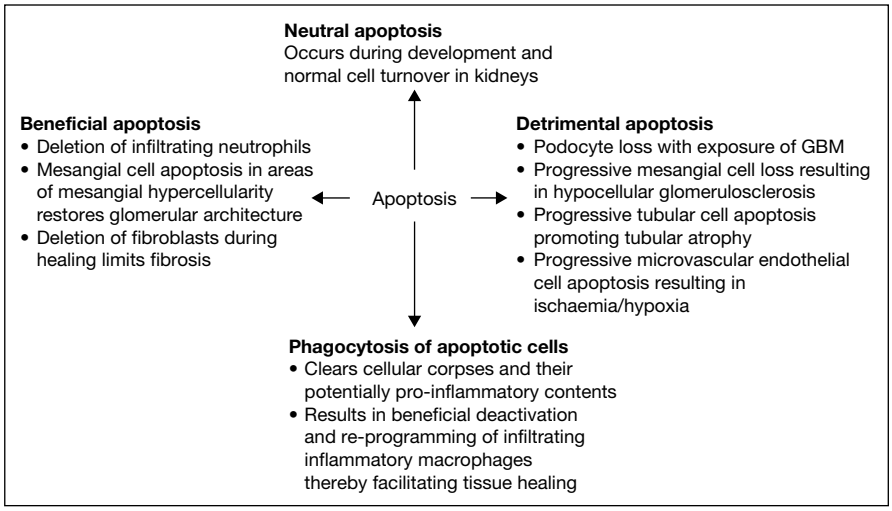

Fig. 1. Apoptosis in glomerulonephritis. Apoptosis occurs within the kidney as part of normal tissue homeostasis. Apoptosis occurring during renal inflammation may have detrimental or beneficial effects depending upon the biological context in which it takes place.

Apoptosis in Resident Glomerular Cells

Apoptotic deletion of infiltrating leucocytes can be readily appreciated as a 'force for good', subject to appropriate clearance of such cells (as discussed below). However, apoptosis in glomerular cells appears to be a 'double-edged sword', in that glomerular cell death can promote resolution of inflammatory glomerular injury but, as discussed in the next section, if excessive or unscheduled can result in unwanted progression to non-functional, hypocellular scar (fig. 1). Consequently, it is important first to rehearse key concepts of programmed death in the three main cell types of the glomerulus.

Apoptosis of Mesangial Cells

Many types of GN exhibit increased glomerular mesangial cell number and, with appropriate or no treatment, are potentially reversible indicating that excess mesangial cells may be beneficially deleted by apoptosis. The complete resolution of mesangial hypercellularity in the rat associated with self-limited mesangial proliferation induced by anti-Thy1.1 antibody is a dramatic example of this reparative capacity [6]. Although undoubtedly complex, evidence suggests

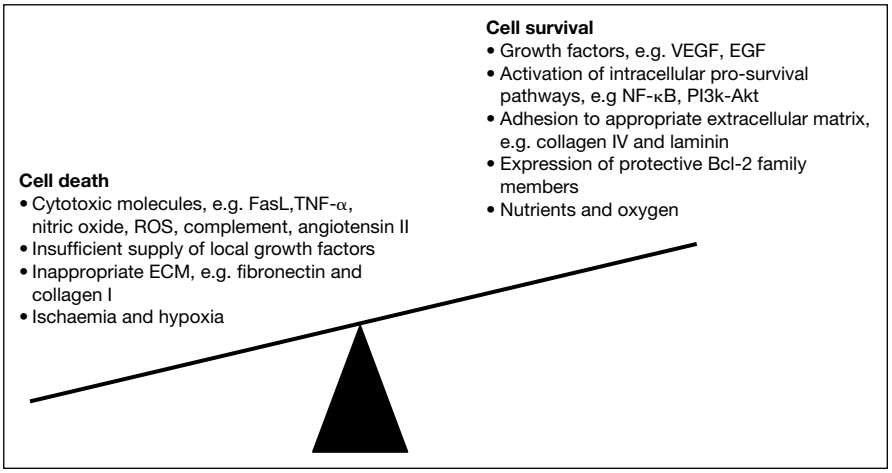

Fig. 2. The balance between life and death in glomerulonephritis. All cells are susceptible to undergoing apoptosis and the occurrence of apoptosis during renal inflammation may be regulated at a cellular level by the balance that exists between the pro-apoptotic factors and survival signals to which individual cells are exposed. These factors will vary according to the lineage and location of the cell.

that the balance between survival factors and pro-apoptotic death factors may be key in regulating mesangial cell apoptosis in disease (fig. 2).

Cytokine Survival Factors

The peak of mesangial cell proliferation and apoptosis coincide in the Thy1.1 model of mesangial proliferative glomerulonephritis [6]. This suggests a potential regulatory role for survival factors as proliferating cells are significantly more vulnerable to apoptosis if survival factors are in a limited supply. The resultant competition for survival factors at the peak of mesangial cell hypercellularity is, therefore, a candidate mechanism for apoptotic deletion of surplus mesangial cells. Mesangial cell apoptosis induced by serum starvation is inhibited by insulin-like growth factor-1 (IGF-1), IGF-2 and basic fibroblast growth factor (bFGF) whilst transforming growth factor-β_1 (TGF-β_1), epidermal growth factor (EGF) or platelet-derived growth factor (PDGF) are not protective [19]. These observations emphasize that survival controls are cell lineage-specific such that different sets of factors would be required to promote the survival of glomerular endothelial or epithelial cells. Furthermore, survival factors may not protect cells from all pro-apoptotic stimuli e.g. IGF-1, IGF-2 or bFGF do not block mesangial cell apoptosis induced by Fas ligation [19]. Activation of the transcription factor nuclear factor-kappa B (NF-κB) promotes mesangial cell survival and inhibition of NF-κB activity sensitises mesangial

cells to tumour necrosis factor-α (TNF-α)-mediated apoptosis [20]; a death effector utilized by inflammatory macrophages [21]. Interestingly, methylprednisolone inhibits mesangial cell NF-κB activity and ameliorates hypercellularity in Thy 1.1 glomerulonephritis [22]. It is unknown whether this action plays a major role in the beneficial effect of corticosteroids in patients with mesangial proliferative glomerulonephritis.

Recombinant erythropoietin (EPO) is a survival factor for tubular cells exposed to pro-apoptotic stimuli such as hypoxia and can protect tissues from diverse insults including ischaemia-reperfusion injury. It is therefore of interest that EPO also protects mesangial cells from pro-apoptotic stimuli in vitro [reviewed in 23]. Currently, there is no data available regarding whether EPO ameliorates experimental glomerular inflammation. Although still somewhat controversial, the administration of recombinant EPO may slow the rate of progression of chronic renal failure [23]. Beneficial effects may be secondary to improved renal oxygenation but an inhibitory effect upon chronic renal inflammation is also a possibility – further work is needed in this area. Lastly, chemokines may exert effects upon mesangial cell proliferation and apoptosis [23] but there is no data as to whether these effects are relevant in vivo.

Extracellular Matrix

Cells in all tissues, glomeruli included, are surrounded by an extracellular matrix (ECM) rich in proteoglycans. ECM is dynamic and modulates the behaviour and phenotype of cells with 'appropriate' ECM imparting survival signals. As mentioned above, collagen IV and laminin, components of normal mesangial ECM, provide β1 integrin-mediated protection of rat mesangial cells exposed to pro-apoptotic stimuli [11]. In contrast, collagen I and fibronectin, typically found in diseased or scarring glomeruli, do not support mesangial cell survival [11]. Thus, glomerular accumulation of 'survival-neutral' ECM may increase the vulnerability of mesangial cells to pro-apoptotic stimuli and potentially facilitate further mesangial cell loss. Indeed, mesangial cells cultured upon damaged oxidised ECM exhibit increased apoptosis associated with reduced NF-κB activation, emphasising the potential pathogenic importance of disrupted survival signalling [reviewed in 23].

Pro-Apoptotic Stimuli

A range of factors appear capable of inducing mesangial cell apoptosis including complement, immune complexes, reactive oxygen species, cytokines, Fas ligation, etc. Mesangial cell Fas expression has been noted in cases of diffuse proliferative lupus nephritis. Indeed, Fas ligand (FasL) is upregulated in human lupus nephritis with recent work indicating that mesangial cells comprise the majority of FasL positive cells [reviewed in 23]. The function of mesangial cell

FasL expression is unclear but potential roles may include limiting further leucocyte infiltration, inducing apoptosis of neighbouring Fas bearing mesangial cells (so-called 'fratricide') or the release of soluble FasL into the glomerular microenvironment. Despite these experimental and observational insights, the exact role of the Fas/FasL system in human glomerulonephritis requires further investigation. Similarly, TNF-α is undoubtedly important in the pathogenesis and progression of glomerulonephritis and can induce mesangial cell apoptosis in vitro [20, 21]. However, studies specifically examining the exact role of TNF-α in mesangial cell apoptosis in vivo are currently lacking.

Inflammatory macrophages play an important role in glomerulonephritis [reviewed in 23] and may induce mesangial cell apoptosis [21, 24]. In vitro co-culture experiments in a model rodent system indicate a key role in mesangial cell killing for macrophage-derived nitric oxide (NO), a lesser role for TNF-α and no demonstrable involvement of FasL [21, 24]. In addition to inducing mesangial cell death, macrophage-derived NO inhibited mesangial cell proliferation; an ideal combination of effects to combat mesangial proliferative nephritis. Although the role of NO in human disease is controversial, a significant correlation was found between the numbers of cells expressing inducible nitric oxide synthase (iNOS) and glomerular cell apoptosis in lupus nephritis renal biopsies. However, experiments in mice targeted for the deletion of the iNOS gene have not produced a clear picture of the exact role of iNOS-generated NO in glomerulonephritis [reviewed in 25]. Similar in vitro co-culture studies of human monocyte-derived macrophage-mediated apoptosis of vascular smooth muscle cells (that share some features with myofibroblastic mesangial cells) indicate the involvement of FasL, TNF-α and NO with NO acting to enhance FasL-mediated death. Also, human macrophages induced FasL-dependent apoptosis of antibody-coated human mesangial cells (antibody-dependent cellular cytotoxicity, ADCC) [reviewed in 23]. Thus, mesangial cells coated with antibody or immune complexes may become vulnerable to injury from macrophages. Recent reports employing an experimental system for inducible macrophage deletion emphasise the importance of macrophages in governing progression and resolution of glomerular disease [26].

Apoptosis of Glomerular Endothelial Cells

This is important in both renal inflammation and healing. Patients with SLE may have detectable anti-endothelial cell antibodies and such antibodies are implicated in the microvascular injury found in patients with scleroderma. The presence of active endothelial injury in human disease was recently demonstrated in patients with active ANCA-associated small vessel vasculitis. Patients with active disease exhibited significantly increased numbers of

circulating necrotic endothelial cells with numbers falling following initiation of immunosuppressive treatment [27]. The importance of disordered apoptosis in GN is considered below.

Important endothelial cell survival factors include VEGF, angiopoietin-1, integrin-mediated adhesion, eNOS-derived NO and shear stress. Endothelial cell apoptosis may result from inflammatory pro-apoptotic stimuli, reduced survival signals or both. Although, macrophages actively induce endothelial cell apoptosis during development [28], there is no direct evidence that inflammatory macrophages actively induce microvascular endothelial cell apoptosis during renal inflammation. However, macrophages may modulate endothelial cell survival indirectly. For example, macrophage infiltration colocalises with both reduced podocyte immunostaining for VEGF and loss of glomerular capillaries in the remnant kidney model in rats suggesting that macrophages may lower the 'threshold' at which endothelial cells may undergo apoptosis. Administration of exogenous VEGF is protective in this model as well as a mild model of Thy 1.1 glomerulonephritis thereby reinforcing its key role [reviewed in 23]. Prevention of endothelial injury/apoptosis during acute disease would be predicted to reduce the detrimental microvascular rarefaction implicated in the progression of renal disease.

Apoptosis of Glomerular Epithelial Cells

Podocyte apoptosis and loss is a key factor in the development of glomerulosclerosis following injury. Podocytes adhere to the external surface of the glomerular tuft and interactions with extracellular matrix play a major role in the regulation of podocyte survival [29]. Low numbers of detached podocytes may be found in normal urine but patients with glomerular disease exhibit markedly elevated numbers of urinary podocytes (388 podocytes/mg creatinine versus <0.5 podocytes/ mg creatinine; disease versus control). Apoptosis may result in cell detachment whilst cell detachment may result in apoptosis and consequently it is unclear what exact contribution apoptosis plays to such podocyte detachment. Numerous factors may induce podocyte death including reactive oxygen species, fibroblast growth factor, complement, angiotensin II, mechanical strain, endothelin and transforming growth factor-β [reviewed in 23]. Recent work indicates that CD2-associated protein acts to inhibit transforming growth factor-β induced podocyte apoptosis [29]. In addition, although VEGF is primarily regarded as an endothelial cell mitogen/ survival factor, VEGF also inhibits podocyte apoptosis and acts, at least in part, by promoting phosphorylation of nephrin [30]. Podocyte apoptosis may be amenable to modulation as hepatocyte growth factor is protective in vitro whilst retinoids reduce podocyte injury and apoptosis induced by puromycin both in vivo and in vitro. In addition, the

administration of endothelin 1 antagonists inhibited spontaneous age-dependent glomerulosclerosis in rats and protected podocytes from puromycin-induced apoptosis in vitro [reviewed in 23]. The downregulation of endogenous survival factors would be predicted to predispose podocytes to undergoing apoptosis and it is therefore of interest that the downregulation of podocyte Bcl-2 expression is associated with a worse outcome in patients with chronic IgA nephropathy [31].

Disordered Apoptosis and Glomerulonephritis

Disordered Leucocyte Apoptosis

Autoimmune vasculitis associated with anti-neutrophil cytoplasmic antibodies (ANCA) commonly causes severe glomerular inflammation with necrotizing capillaritis and crescentic change. It is now clear that ANCA can also disrupt the normal progress of apoptosis in neutrophils that have been exposed to stimuli that 'prime' the respiratory burst and superoxide production, such as low concentrations of LPS or TNF-α, or culture in the presence of serum. This is because primed neutrophils relocate small amounts of certain granule contents to their cell surface, including myeloperoxidase (MPO) and proteinase 3 (Pr3), the principal autoantigens recognised by ANCA. Consequently, immune complexes form at the neutrophil cell surface, activating neutrophils and triggering the respiratory burst and oxygen radical production [32].

The consequences for neutrophil apoptosis of such effects of ANCA are most intriguing [32]. While there is acceleration of the 'nuclear program' of apoptosis, with more rapid nuclear condensation and progression to eventual secondary necrosis, there is no change in the rate of appearance on the neutrophil surface of 'eat me' signals such as phosphatidylserine (PS) exposure, reducing the time window for safe phagocytic clearance. The speculation that such disordered apoptosis promotes necrosis of neutrophils prior to safe clearance needs testing but would be consistent with the key histological feature of ANCA-associated vasculitis – leukocytoclasis, the falling apart of neutrophils in affected tissues [32].

Since monocytes also express ANCA antigens it will be of interest to examine whether monocyte survival or death is also affected by ANCA.

Disordered Glomerular Cell Apoptosis

Since there are reports of autoantibodies reactive with all three glomerular cell types it can be speculated that autoantibody-mediated description of the intrinsic death program, rendering this 'less safe' in some way, might be a more general mechanism promoting persistent inflammation in some types of GN.

However, whether the death program itself is normal or abnormal, it is already clear that glomerular cell apoptosis can be disordered in GN in the sense that there is too much apoptosis occurring at the wrong time and in the wrong place. The first reports of mesangial cell apoptosis emphasized a role in beneficial remodelling of an expanded glomerular cell complement as this returned to normal [6]. Subsequent data have emphasized that increased glomerular cell apoptosis can also be a final common pathway by which persistently inflamed glomeruli progress to hypocellular, non-functional scar [reviewed in 23].

Thus glomerular cell apoptosis is truly a 'double-edged sword'. Undoubtedly beneficial in resolution of glomerular hypocellularity, apoptosis becomes a bad thing when it occurs too frequently, at the wrong time or in the wrong cell type. In particular, loss of podocytes appears to be a crucial threat to the mechanical stability and viability of the glomerular tuft. As described above there are multiple pro-death stimuli that will need ordering for importance where glomerular cell death becomes deleterious.

Disordered Clearance of Apoptotic Cells and Glomerulonephritis

Haslett should be credited with the discovery that apoptosis was crucially involved in resolution of inflammation [33]. His early writings predicted that defects in safe clearance of apoptotic cells would be associated with persistent inflammation and autoimmunity [33]. As will be apparent from a number of earlier contributions in this volume, there is now very strong evidence that autoimmune glomerulonephritis can be associated with specific disorders of apoptotic cell clearance, due to deficiency of soluble 'bridging' factors such as C1q and MFGE8, and with generalised autoimmune conditions with defects in clearance of apoptotic cells that require further characterization such as lupus [see chapters by Henson, Elkon, Nagata, Hermann and coworkers, this vol.]. Although we have an incomplete understanding of the complex molecular mechanisms involved in phagocyte recognition of cells undergoing apoptosis, available data indicate roles for various 'eat me' signals on the apoptotic cell surface, a range of soluble 'bridging' molecules and several classes of phagocyte receptors [reviewed in 34, and chapters by Henson, Elkon, Nagata, Hermann and coworkers, this vol.].

Local Effects of Disordered Clearance

In patients and animal models of systemic lupus erythematosis and related conditions such as C1q deficiency [7] free apoptotic cells can be observed in inflamed glomeruli. Furthermore, probable progression to secondary necrosis

without efficient phagocytic clearance is likely to account for the presence of cell fragments in severe lupus GN, sometimes described by histopathologists as 'nuclear dust'. Nevertheless, while we might expect inappropriately liberated cell contents to have direct local pro-inflammatory effects [12, 33], more work is required to examine this important issue, given some similarities between necrotic and apoptotic cells [34].

A second key facet of locally disordered clearance of apoptotic cells concerns the regulation of macrophage function. For example, apoptotic neutrophils opsonised with ANCA can stimulate rather than suppress macrophage secretion of pro-inflammatory mediators [32]. Indeed, we need to understand more of the subtleties of apoptotic cell regulation of macrophage function, especially in the presence of infection. Thus, not only may macrophages exposed to bacterial ligands for Toll-like receptors demonstrate an initial pro-inflammatory response to apoptotic cells [35] but also we need to understand how neutrophils driven into apoptosis by pathogens may elicit heat shock protein-mediated activation of macrophages [36]. Furthermore, we must characterize which of the many molecules involved in clearance of dying cells mediate anti-inflammatory signalling in the inflamed glomerulus. Thus, although $\alpha_v\beta_3$ integrin, CD36 and thrombospondin 1 have all been implicated in anti-inflammatory triggering of macrophage TGFβ1 secretion by apoptotic cells [34], preliminary work on knockout mice deficient in these molecules reveals no defect in anti-inflammatory signalling [Lacy-Hulbert et al., unpubl. data]. Furthermore, the PS receptors involved in triggering TGFβ1 release appear to require further characterization [37]. Roles for other macrophage molecules, such as those of the TREM (triggering receptor expressed on myeloid cells) family, will need to be sought [38]. Defects in signalling of anti-inflammatory effects of apoptotic cells may be particularly important in progressive glomerular injury, since there might be failure to inhibit macrophage-driven apoptosis of glomerular cells [21, 24, 26] with increased risk of deleterious cell loss and scarring. Furthermore, there might be a lack of beneficial secretion of glomerular cell growth/survival factors [39] (fig. 3).

Systemic Effects of Disordered Clearance

A key issue for future work, already addressed in this volume [chapters by Henson, Elkon, Nagata, Hermann and coworkers, this vol.] is to understand how defects in clearance of apoptotic cells are linked to promotion of the autoimmune state which may then affect the glomerulus and induce or exacerbate GN. In particular, we need to understand why defective apoptotic cell clearance can also be clearly dissociated from defective cell clearance, as exemplified by knockout mice deficient in mannose-binding lectin (MBL) [40].

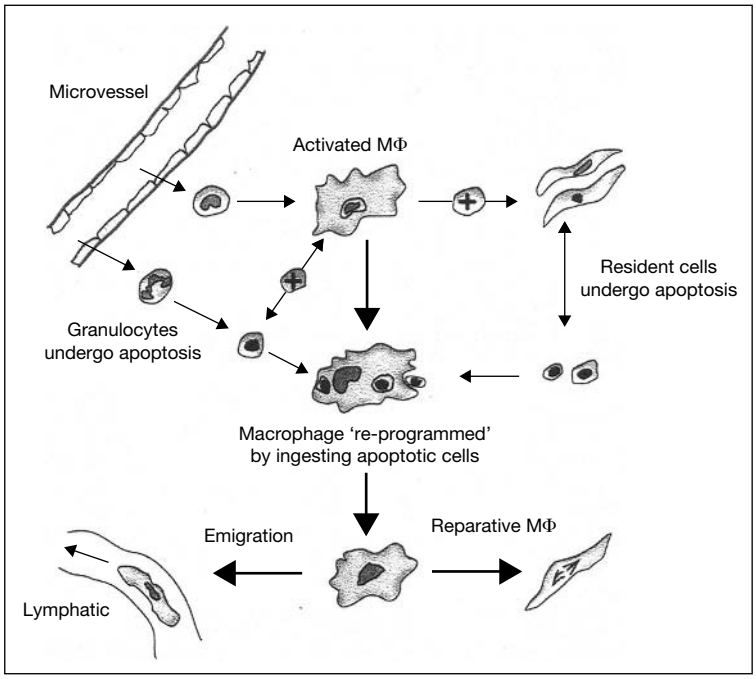

Fig. 3. Hypothesis for regulation of macrophage activation by interaction with apoptotic cells. Activated macrophages (MΦ) can accelerate leucocyte apoptosis and trigger resident cell apoptosis. Subsequent phagocytosis of the apoptotic progeny deactivates or 'reprograms' the macrophage, which then receives signals to promote, repair and/or emigrate.

Furthermore, possible systemic inflammatory effects of defective apoptotic cell clearance also need to be understood because inflammation at a distant site is well known to exacerbate autoimmune GN [1, 2]. Thus, even though *MBL−/−* mice do not display primary autoimmune GN, they can develop lung inflammation which might exacerbate GN developing for other reasons – further work is required to test these ideas. Nevertheless, mice deficient in CD14 fail to display either systemic inflammation or autoimmunity [41].

Prospects for Therapy

New insights into the diverse roles in GN of apoptosis and clearance of apoptotic cells seem likely to be the basis of novel therapeutic approaches

designed to promote resolution of glomerular inflammation and/or arrest progression to hypocellular scar.

Manipulation of Leucocyte Clearance

Serhan and colleagues [reviewed in 42] have discovered that during the course of self-limited inflammation there is a switch in local synthesis of lipid mediators from pro-inflammatory agents, to species that arrest inflammatory responses (such as prostaglandin D_2 and lipoxins) or have potential to promote resolution (omega−3 polyunsaturated fatty acid derivatives of the 'resolvin' family). Excitingly, prostaglandin D_2 can override NF-κB-mediated granulocyte survival and induce apoptosis [43], while lipoxins can promote beneficial phagocytosis of apoptotic cells at perturbed sites in vivo [44]. These are new therapeutic leads.

Further work will also be required to investigate the 'pro-resolution' effects of glucocorticoids, familiar anti-inflammatory agents bedevilled by systemic toxicity. Intriguingly, glucocorticoids can directly promote acquisition of capacity for phagocytosis of apoptotic cells [45] as well as trigger apoptosis in some leucocyte types such as eosinophils and lymphocytes [12]. A realistic prospect of targeting such glucocorticoid effects is raised by the recent discovery that phagocytic capacity for apoptotic cells can be controlled by macrophage expression of 11β-hydroxysteroid dehydrogenase-1 [46], an enzyme that reactivates inactive circulating glucocorticoids such as cortisone. Furthermore, the well known anti-inflammatory effects of the glucocorticoid-inducible protein annexin-1 and annexin-1-derived peptides may include the capacity to promote phagocytosis of apoptotic cells [47].

Manipulation of Glomerular Cell Apoptosis

Although one might theoretically promote resolution of hypercellular GN by inducing beneficial apoptosis of glomerular mesangial or endothelial cells, or prevent progression by inhibiting inappropriate apoptosis of glomerular cells (especially podocytes), these therapeutic objectives seem relatively distant given potential pitfalls. Thus, in glomerular hypercellularity one would need to target a pro-apoptotic stimulus (such as TNFα combined with a NF-κB inhibitor [20]) to just the right number of cells at just the right time, or else risk deleterious glomerular cell loss. Conversely, theoretical risks of inhibiting inappropriate apoptosis in progressive GN with cytokines such as VEGF include the development of tumours. Nevertheless there is enthusiasm for and experience in the use of pro-survival cytokines in renal disease [reviewed in 23].

Conclusions

Apoptosis is now established as a key feature of glomerular inflammation, regulating both the local complement of intrinsic and inflammatory cells and systemic aspects of inflammatory and immune responses. Defects in the frequency and execution of the apoptotic program are being uncovered in glomerular diseases, as are perturbations of safe clearance of apoptotic cells by phagocytes. Candidate therapies that target these control points have been identified and are being investigated.

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