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Apoptosis and Autophagy in the Regulation of T Lymphocyte Function

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Abstract

During the development and normal function of T lymphocytes, the cells are subject to several checkpoints at which they must "decide" to live or die. At these critical times and during homeostasis, the molecules that regulate the classical apoptotic pathways and survival pathways such as autophagy have critical roles in controlling this decision. Our laboratory has focused on the roles of apoptotic and autophagic proteins in T lymphocyte development and function. Using genetic models in mice and in vitro analyses of T cell functions, we have outlined critical roles for the Bcl-2 family (regulators of the intrinsic pathway of apoptosis), c-FLIP (an anti-apoptotic protein in the extrinsic pathway of apoptosis), and autophagy in T lymphocytes.

Keywords

apoptosis; autophagy; T lymphocytes; Bcl-2; Mcl-1; c-FLIP

Introduction

Throughout their lifespan, T lymphocytes undergo several developmental checkpoints as well as periods of expansion and contraction as they mature and perform their immunological functions. During development in the thymus, hematopoietic progenitors progress through a series of developmental stages, each of which is carefully regulated not only to promote the development of a pool of mature cells that is able to respond to foreign antigens but also to eliminate self-reactive cells. Once thymocytes have exited the thymus into the peripheral lymphocyte compartment, they require a low level of stimulation from self peptide presented on the major histocompatibility complex (MHC) and cytokines, particularly interleukin-7 (IL-7), to survive in the periphery and to be ready to respond to their cognate antigens (1, 2). Once a specific antigen has been encountered under the appropriate co-stimulatory conditions, responding T cells undergo robust expansion and differentiation into effector populations, including cytotoxic T lymphocytes (CTLs) and T helper cells. Subsequently, the effector cell population undergoes a period of contraction in which a majority of cells die by apoptosis. While it is estimated that the size of the responding T cell population increases by up to 1,000-fold, most of the responding cells die during contraction and only a subset of responding cells are retained to form a memory T cell pool that is capable of rapidly responding to a repeat encounter with the same antigen (3, 4).

In each of the stages described above (development, naive cell homeostasis, the effector phase, contraction, and memory), T cells utilize classic processes to regulate cell death and

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cell survival. One such process is apoptosis, a form of programmed cell death that is important in a variety of developmental and other contexts in most metazoan organisms (5). During apoptosis, cells undergo nuclear contraction as cytoplasmic contents are compartmentalized and bleb off of the cell. Apoptotic cells and debris are cleared by phagocytic cells such as macrophages, typically without inducing inflammation or an immune response.

At the molecular level, apoptosis is carried out by a complex called the apoptosome, which consists of APAF-1, caspase 9, and cytochrome c, and can be regulated upstream by one of two major pathways: the intrinsic pathway, and the extrinsic pathway (5, 6). In the intrinsic pathway, cells are induced to undergo apoptosis by signals of cellular damage or stress, such as DNA damage and endoplasmic reticulum stress. The subsequent cascade, regulated by the Bcl-2 family, leads to mitochondrial depolarization, release of cytochrome c, and activation of downstream caspases. In the extrinsic pathway, the initiating signal comes from the ligation of death receptors on the cell surface, for example the tumor necrosis factor receptor (TNFR) or Fas. Ligation of these receptors leads to the activation of initiator caspases in the cytoplasm and ultimately, converging with the intrinsic pathway, activation of downstream caspases and the apoptosome. Both the intrinsic and the extrinsic pathways have been shown to be important for T cell apoptosis and have distinct roles in a variety of situations (6, 7). These roles, particularly the contribution of the anti-apoptotic proteins, have been a major focus of our research and will be discussed in more detail in this review.

Recently, there has been a growing interest in the role of metabolic and catabolic processes in the immune system. The cells of the immune response must be able to respond to growth factors and other stimuli with an activation and/or proliferative response. T cells in particular undergo periods of relative quiescence interspersed with periods of rapid growth, each phase having different metabolic needs. The metabolic regulators Akt and mammalian target of rapamycin (mTOR) have been shown to have important roles in T cell function and survival (8, 9). The catabolic process of autophagy is classically considered to be a mechanism for the recycling of cytoplasmic contents, particularly in times of starvation (10). However, autophagy has been reported to have several distinct roles in the immune system, including clearance of intracellular pathogens, antigen presentation, and lymphocyte homeostasis (11, 12). In addition, autophagy had been proposed to be a mechanism of programmed cell death, and autophagy proteins have been shown to interact with molecules in both the intrinsic and extrinsic apoptotic pathways (13–16). Given these connections, examining the roles of autophagy in T cells has become a major focus of our lab, and our laboratory was the first to extensively characterize autophagy in T lymphocytes.

To study autophagy and apoptosis in T lymphocytes, our approach has been to use genetic models in mice in combination with in vitro analyses to examine the roles of several of the critical proteins in these important processes. In this review, we will discuss the current understanding of the roles of apoptosis and autophagy in T lymphocyte survival and function as it relates to the research in our laboratory.

Bcl-2 and the intrinsic pathway of apoptosis

The Bcl-2 family of proteins, the members of which are defined by sharing at least one of four Bcl-2 homology (BH) domains with the protein Bcl-2, is well known to control the intrinsic pathway of apoptosis. Members of this family are either pro-apoptotic or anti-apoptotic. The pro-apoptotic members can be divided into two groups: the multidomain pro-apoptotic proteins, and the BH3-only proteins. The multidomain Bcl-2 family members Bak and Bax homo-oligomerize to form pores in the mitochondrial membrane and are required for apoptosis through the mitochondrial pathway (17, 18). Interestingly, in most loss-of-

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function studies, Bak and Bax are able to compensate for one another, and only when both proteins are lost is there an observable phenotype (18–21). However, subtle differences in the way these proteins are regulated are still possible and may have important effects in certain contexts. The other group of pro-apoptotic proteins is the BH3-only proteins. The BH3-only proteins, such as Bim, Bad, Bid, Noxa, and Puma, share only one BH domain with Bcl-2 and are the initiators of the apoptotic pathway. These proteins respond to different intracellular stimuli and are able to initiate apoptosis through their interactions with other Bcl-2 family members. However, the mechanism by which BH3-only proteins activate downstream pro-apoptotic molecules (whether it involves direct activation of Bak and/or Bax or indirect activation by the neutralization of the anti-apoptotic proteins) has been debated (22–28).

The anti-apoptotic proteins of the Bcl-2 family are Bcl-2 itself, Bcl-x_L (a splice product of the *Bcl-x* gene), Mcl-1, A1, and Bcl-w (a viral protein). The anti-apoptotic proteins share multiple BH domains with Bcl-2 and have been shown to bind Bak and/or Bax and BH3-only proteins. Although the interactions between the anti-apoptotic proteins and both the BH3-only proteins and Bak/Bax have been described in several cell-free and cellular systems, it was still uncertain whether one of these functions (binding BH3-only proteins or binding Bak/Bax) is dominant in vivo (22, 23, 26, 27).

Because of their importance in preventing apoptosis, it is perhaps no surprise that the anti-apoptotic members of the Bcl-2 family have been shown to be important during developmental processes. In fact, the Bcl-2 homolog CED-9 was originally described by its role in *C. elegans* development in some of the first studies to delineate the apoptotic pathways, and the Bcl-2 family has been shown to be involved in developmental processes in several other model organisms (5). In mice, the anti-apoptotic proteins have been shown to be critical for survival at different developmental stages of the organism and in the hematopoietic system. Although the *Bcl-2* gene knockout mice (Bcl-$2^{-/-}$) are born, they develop a lethal polycystic kidney pathology and hematopoietic failure within the first few weeks of life (29, 30). Bcl-$x^{-/-}$ mice die at embryonic day 13, and the embryos exhibit extensive cell death in the developing brain and nervous system and increased apoptosis in the fetal liver, the major site for embryonic hematopoiesis (31). Knockout of the *Mcl-1* gene causes peri-implantation lethality in embryos (32), but conditional knockout systems have shown that Mcl-1 is important for many hematopoietic lineages, including hematopoietic stem cells (HSCs), T cells (discussed further below), B cells, and neutrophils (33–36). The phenotypes of knockout models of the BH3-only proteins are less severe but have illustrated specific roles of the different proteins in different tissues (37). Bak/Bax double knockout mice develop physical and neurological abnormalities as well as splenomegaly and lymphadenopathy (20, 21). Thymocyte development is perturbed in these mice, and $Bax^{-/-}Bak^{-/-}$ T cells are resistant to death by a variety of stimuli (20, 21).

Several studies have shown that the expression of the anti-apoptotic Bcl-2 family proteins is actively regulated in T cells. Mcl-1 is expressed throughout thymic development and in peripheral T cells and can be further induced by the cytokines IL-7 and IL-15 and activation through the TCR (34, 35). Bcl-2 and Bcl-x_L are both expressed in thymocytes, but their levels are regulated in a converse pattern: Bcl-2 is downregulated during the $CD4^+CD8^+$ double positive (DP) stage, while Bcl-x_L is more highly expressed at this stage (38–42). In peripheral T cells, Bcl-2 is expressed in naïve cells, downregulated to some extent in the effector phase, and highly expressed in memory cells; meanwhile, Bcl-x_L is most highly expressed in activated/effector T cells (43–45).

The work of our laboratory and others has demonstrated the importance of the Bcl-2 family in T lymphocytes. Studies using Bcl-$2^{-/-}$ or chimeric mice showed that although Bcl-2 is

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not required for early neonatal T cell development, it is required for the development and maintenance of the T cell compartment after this early developmental period (29, 30, 46, 47). After approximately three weeks of age, the Bcl-$2^{-/-}$ thymus undergoes a drop in cell number, and the profile is skewed toward the $CD4^{-}CD8^{-}$ double negative (DN) compartment, indicating that after three weeks of age, Bcl-2 is required for progression to the DP stage (29, 47). Additionally, the number of Bcl-$2^{-/-}$ peripheral T cells was also severely decreased after a few weeks of age, indicating that Bcl-2 is required for the maintenance of the mature T cell compartment (29, 47). The reason for this shift in phenotype after a few weeks of age could be attributed to a difference between fetal liver-derived and bone marrow-derived cells in their dependence on Bcl-2 (46).

In contrast to Bcl-2, studies using Bcl-$x^{-/-}$Rag$2^{-/-}$ chimeric animals showed that Bcl-x_L is not required for the development of T cells (31, 42). However, Bcl-$x^{-/-}$ DP thymocytes displayed reduced survival in vitro in medium alone, when cultured on anti-CD3-coated wells, and upon treatment with apoptotic stimuli such as γ-irradiation or the steroid dexamethasone (31, 42). No increase in sensitivity was observed in Bcl-$x^{-/-}$ single positive (SP) or resting peripheral T cells (42). Thus, while not critical for developmental progression or resting T cell survival, Bcl-x_L still functions to promote DP thymocyte survival.

In order to better study the role of Bcl-x_L specifically in T cells and to eliminate the possibility that defects in other cell types could interfere with the results and interpretations of T cell functional studies, we created a conditional knockout “floxed” allele of *Bcl-x* (Bcl-$x^{f/f}$) and crossed the mice expressing this allele to mice that express the Cre recombinase under the control of the *Lck* gene regulatory elements (LckCre) (48). The LckCre gene induces deletion of the floxed allele specifically in thymocytes at the DN2/DN3 stages (49). The Bcl-$x^{f/f}$LckCre mice had reduced thymocyte and peripheral T cell numbers, but similar to the chimeric mouse studies, the percentages of cells within the different developmental compartments were similar to controls, indicating that Bcl-x_L is not required for thymocyte development (48). In addition, we likewise found that the DP cells from the Bcl-$x^{f/f}$LckCre mice were more sensitive to cell death in vitro, but mature T cells were not (48). Interestingly, in spite of the fact that Bcl-x_L is upregulated in response to activation through the T cell receptor (TCR) and the coreceptor CD28 (45), both the primary and the memory responses of Bcl-x-deficient T cells toward the pathogen *Listeria monocytogenes* were normal (48). Thus, while Bcl-x_L promotes DP thymocyte survival, it is not required for normal T cell function.

Because of the early lethality of Mcl-$1^{-/-}$ mice, examining the role of Mcl-1 in T cells also required the use of a conditional knockout system. Using a floxed allele of *Mcl-1* (Mcl-$1^{f/f}$) crossed to the LckCre mice, the Korsmeyer group showed that loss of Mcl-1 in thymocytes caused a profound loss of cells and a block in development at the DN stage (34). Using Mcl-$1^{f/f}$ mice generated in our own lab (36) crossed to the LckCre line, we similarly observed that Mcl-1 is required for thymocyte survival and progression past the DN stage (35). To examine the requirement for Mcl-1 at the later stages of thymic development, we bred Mcl-$1^{f/f}$CD4Cre mice. Although thymocytes from these mice delete Mcl-1 late in the DN stage (49), we found that Mcl-$1^{f/f}$CD4Cre thymocytes progressed normally through the DP stage but could not reach maturity in the SP compartment (35). This loss of mature SP cells led to a severe reduction in T cell numbers in the spleen, and the T cells in the spleen appeared to have escaped thymic deletion of Mcl-1 based on Mcl-1 protein levels. Thus, the SP stage contains a second “checkpoint” at which Mcl-1 is required. Interestingly, while neither Mcl-1 nor Bcl-x_L appeared to be singly required for DP cell survival in vivo, deletion of both of these genes caused a severe loss of DP cells as demonstrated by the

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severely reduced DP cell number in Mcl-$1^{f/f}$Bcl-$x^{f/f}$CD4Cre mice (35). The functional reason for this redundancy at the DP stage is not yet clear.

There is a clear requirement for Mcl-1 at multiple stages of thymocyte development. Additional evidence suggests that Mcl-1 is also required for the survival of mature peripheral T lymphocytes. Using the interferon-inducible MxCre knockout system, it was shown that deletion of Mcl-1 from mature T cells caused a decrease in cell viability, even in the presence of IL-7 (34). The reduced survival of T cells following loss of Mcl-1 was also shown in vivo by the adoptive transfer of Mcl-$1^{f/null}$MxCre cells to $Rag2^{-/-}$ mice. Upon treatment with polyinocinic-polycytidylic acid (pI-pC) to induce deletion, a significant decrease in the number of T cells in the spleen was observed within two days of pI-pC treatment (34). To avoid any immune cell phenotype caused by the use of interferons, we examined the impact of deletion of Mcl-1 in mature T cells using a mouse model in which Cre is expressed as a fusion protein with the estrogen receptor (ERCre mice) so Cre activity could be induced (and Mcl-1 deleted) by treatment with the drug tamoxifen or its active metabolite 4-hydroxy-tamoxifen (4-OHT) (50). Mcl-$1^{f/f}$ERCre T cells exhibited reduced survival in culture in medium alone, in medium supplemented with IL-7, and in the presence of the activating anti-CD3 and anti-CD28 antibodies (35).

A fourth mammalian anti-apoptotic Bcl-2 family protein, A1, is also expressed in thymocytes. Because multiple conserved isoforms of A1 exist at separate locations on the chromosome, gene targeting of all of the A1 isoforms has not been feasible (51). However, it has been suggested that A1 may be upregulated to promote survival in response to pre-TCR signals (52).

While the studies above outlined the specific requirements for the anti-apoptotic Bcl-2 family members in T cells, other studies have focused on how the pro-apoptotic family members affect T cell development and function. One of the most interesting cases is that of the BH3-only protein Bim. Bim-knockout mice have increased numbers of hematopoietic cells, including T cells and thymocytes, and develop autoimmune kidney disease (53). Thymic development is disrupted in $Bim^{-/-}$ mice as evidenced by an expansion of the SP compartment, particularly $CD4^{+}$ SP cells (53). The autoimmunity displayed in $Bim^{-/-}$ mice can be explained by a defect in negative selection because $Bim^{-/-}$ SP thymocytes are resistant to TCR-induced death, and autoreactive TCR-transgenic cells are not deleted efficiently when they are crossed onto a Bim-deficient background (54, 55). Furthermore, $Bim^{-/-}$ T cells are resistant to a number of apoptotic stimuli in vitro (53), and Bim has shown to also be responsible for T cell apoptosis following activation in vivo (56, 57).

Because each of the Bcl-2 family members appears to have a distinct role in T cells, the models described above provided an opportunity to define the specific interactions between the pro-apoptotic and the anti-apoptotic Bcl-2 family members using genetic models. A number of molecular interactions have been shown in cell lines and in cell-free systems, but our understanding of the in vivo relevance of these interactions in specific contexts was still limited. Crossing Bcl-2-deficient mice onto a Bim-knockout background rescued the survival of Bcl-2-deficient thymocytes (58, 59). Thus, in the thymus, the major role of Bcl-2 is to counteract the activity of Bim.

In examining the expression patterns and the requirements for the different anti-apoptotic proteins, it was striking that Bcl-2 and Mcl-1 are often co-expressed, for example at the DN stage, and yet both Bcl-2 and Mcl-1 are required for progression past these stages. This suggested two likely explanations: 1) that the combined level of Bcl-2 and/or Mcl-1 must be above a certain threshold and that the single knockouts were unable to reach this threshold, or 2) that Mcl-1 and Bcl-2 have molecularly distinct roles in developing thymocytes. To

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distinguish between these possibilities, we crossed the Mcl-$1^{f/f}$Cre lines with mice expressing human Bcl-2 on a transgene under the MHC class I promoter (60). As previously reported, the Bcl-2 transgene product increased the number of thymocytes and peripheral T cells on the wild type Mcl-1 background (60), but the presence of excess Bcl-2 was unable to rescue the developmental defects of the Mcl-1-deficient thymus (61). These data suggested that Bcl-2 and Mcl-1 have distinct roles in thymocytes. To further define these roles, the Mcl-$1^{f/f}$ mouse strains were crossed to mice with knockout alleles of the pro-apoptotic genes *Bim*, *Bax*, and *Bak*. Interestingly, only the gene knockout that provided a significant rescue of the Mcl-1-deficient thymocytes at both the DN and the SP stages was that of Bak (61). This suggests that the dominant role of Mcl-1 in thymocytes is to antagonize Bak. These data provided in vivo, genetic evidence for the differential binding of the anti-apoptotic proteins to Bak, a specific interaction that had previously been shown biochemically (62).

The importance of many of the members of the Bcl-2 family in the development and function of T cells is now clear. Our laboratory and other laboratories have demonstrated that the regulation of the Bcl-2 family members and the context-specific roles of the anti-apoptotic Bcl-2 family proteins are critical in regulating T cell survival. In addition to this, the regulation of the different pro-apoptotic proteins in different circumstances is also critical in determining cell fate. Current research efforts on the Bcl-2 family of proteins should continue to examine the regulation of both the pro-apoptotic and the anti-apoptotic Bcl-2 family members and the interactions between these two opposing groups.

c-FLIP and the extrinsic pathway of apoptosis

While the intrinsic pathway of apoptosis is a response to intracellular damage signals, the extrinsic pathway of apoptosis is a mechanism by which cells are signaled to die by soluble ligands and/or other cells. Some of the canonical factors that trigger this pathway are tumor necrosis factor (TNF), TRAIL, and Fas ligand (FasL). These ligands can bind receptors such as TNFR and Fas on the cell surface to trigger an apoptotic cascade that is summarized below (for further review of this pathway, see references (5–7)). Upon binding of the death receptors, death domains on the intracellular portion of the receptor recruit the death domain-containing adaptors, such as Fas-associated death domain protein (FADD) and TNF-receptor-associated death domain protein (TRADD). FADD then binds to pro-caspase 8 via interactions between the two proteins' death effector domains (DEDs). Pro-caspase 8 then undergoes homodimerization and self-cleavage to form the active caspase. Active caspase 8 then initiates the activation of downstream caspases, leading to apoptosis.

Cellular FLICE-like inhibitory protein (c-FLIP), also known as CASH, MRIT, I-FLICE, CLARP, usurpin, Casper, and FLAME-1, is a caspase homolog that is an important regulator of the extrinsic pathway of apoptosis (63–70). Differential spicing of the c-FLIP transcript results in the expression of three isoforms in human (c-$FLIP_L$, c-$FLIP_S$, and c-$FLIP_R$) and two isoforms in mouse (c-$FLIP_L$ and c-$FLIP_S$/c-$FLIP_R$) (71–74). All of the isoforms of c-FLIP share similar structural homology to caspase 8 in that they contain two DED domains. The long isoform also contains a caspase 8-like region containing amino acid substitutions that render it catalytically inactive. The c-$FLIP_R$ and c-$FLIP_S$ isoforms lack the caspase 8-like region entirely and differ from each other by the presence or absence of only a few terminal amino acids. Although the mouse protein is more similar to human c-$FLIP_R$, it has also been referred to as c-$FLIP_S$ because the human c-$FLIP_R$ isoform was identified more recently than the long and short isoforms (mouse "c-$FLIP_{S/R}$" is used in this review) (71, 72). Whether or not there is a functional difference between c-$FLIP_S$ and c-$FLIP_R$ is not yet known. All isoforms are able to bind pro-caspase 8 through their DED domains, in essence acting as dominant negative inhibitors of caspase 8 (73, 74). Because of its ability to

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allow at least partial cleavage of caspase 8, there is some conflicting evidence that c-FLIP$_L$ may in some cases activate caspase 8, and c-FLIP$_L$ has been shown to have a role in T cell signaling pathways (74–79). However, because these studies generally used overexpression of c-FLIP, loss-of-function models were necessary to assess the role of endogenous c-FLIP.

Several studies have shown roles for the death receptors and their ligands in T cell biology. Naturally occurring mutations in the death receptor Fas (the *lpr* allele) or its ligand FasL (*gld*) have been shown to lead to lymphoproliferation and autoimmunity in mice (80, 81). The Fas/FasL interaction was shown to cause activation-induced cell death (AICD) in T cells, particularly using an in vitro model of activation in IL-2, resting, and restimulation (82, 83). However, in some contexts, it has been demonstrated that Fas and TNFR are not required for the deletion of activated T cells in vivo (56, 57, 84, 85). Interestingly, the involvement of Fas in T cell death in vivo may depend on the nature of the infection/stimulus because while Bim has been shown to be the critical mediator of T cell death using staphylococcal enterotoxin B (SEB) and herpes simplex virus (HSV), it has been shown that Bim and Fas cooperate to induce T cell death and prevent immune pathology in a chronic infection model using mouse γ-herpesvirus and for the clearance of effector cells during acute infection with LCMV (56, 57, 86, 87).

Because the death receptor pathways are clearly important in T cell function, we examined the role of c-FLIP, an inhibitor of the death receptor-mediated cell death pathways, in T cells. c-FLIP$^{-/-}$ mice die during embryogenesis around day 10.5 (88), precluding the study of T cells in conventional knockout mice. To examine the role of c-FLIP in T cells, we created a floxed *c-FLIP* allele (c-FLIP$^{f/f}$) that could be deleted specifically in T cells by crossing the mice expressing this allele to the LckCre line of mice (89). The total number of thymocytes in the c-FLIP$^{f/f}$LckCre mice was comparable to the cell number in control mice. However, while the DN and DP populations appeared normal, the c-FLIP-deficient thymocytes had reduced percentages of both CD4$^+$ and CD8$^+$ SP cells. Additionally, the SP cells that were present exhibited a more immature phenotype based on a reduced level of downregulation of the opposing CD8 or CD4 coreceptor and the marker CD24 (also known as heat-stable antigen/HSA). The spleens and lymph nodes of c-FLIP$^{f/f}$LckCre mice contained severely reduced numbers of both CD4$^+$ and CD8$^+$ T cells, and those cells that were present appeared to have escaped deletion of c-FLIP (89).

The data above suggested that c-FLIP is essential for the development, survival, and/or expansion of mature T cells. Consistent with this, c-FLIP-deficient SP thymocytes displayed reduced amounts of proliferation and reduced expression of IL-2 and CD25 after in vitro culture in the presence of the activating antibodies anti-CD3 and anti-CD28 (89). Under these conditions, a significant decrease in cell survival could be detected in c-FLIP-deficient cells within a number of hours after TCR stimulation, indicating that it is likely increased apoptosis that is responsible for the observed defects (89). c-FLIP-deficient DP, CD4$^+$ SP, and CD8$^+$ SP cells all showed increased rates of apoptosis in response to treatment with anti-Fas, which stimulates the Fas pathway (89). Consistent with these observations, freshly isolated DP and SP thymocyte populations from c-FLIP$^{f/f}$LckCre mice contained a higher percentage of cells that were annexin V-positive (89). Finally, although c-FLIP has been shown to have a role in the Erk and NF-κB pathways (90), Erk activation and the degradation of the NF-κB inhibitor IκBα were normal in TCR-stimulated c-FLIP-deficient SP cells (89). These data indicate that the major role of c-FLIP in SP thymocytes is likely anti-apoptotic but do not rule out that c-FLIP may also have a role in the proliferation and activation of thymocytes and mature T cells.

Because the c-FLIP$^{f/f}$LckCre mice largely lacked peripheral T cells, in order to determine the role of c-FLIP in mature T cells, we used the tamoxifen-inducible ERCre deletion

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model. When c-FLIP$^{f/f}$ERCre cells were cultured with 4-OHT then activated with anti-CD3 treatment, the c-FLIP-deficient T cells exhibited a greatly increased level of cell death compared to control cells from c-FLIP$^{f/f}$ (ERCre-negative) mice (91). This increase in cell death was inhibited by the caspase inhibitor zVAD, indicating that the death was occurring by apoptosis and not by any caspase-independent mechanism. Because the c-FLIP-deleted cells were also more sensitive to TNFα- and Fas-induced apoptosis, we crossed the c-FLIP$^{f/f}$ERCre mice onto the Fas mutant (*lpr/lpr*), the TNFα$^{-/-}$, and the double *lpr/lpr*TNFα$^{-/-}$ background to determine whether signaling through the Fas or TNF death receptors were responsible for the TCR-induced cell death of c-FLIP-deficient T cells (91). While the *lpr* mutation completely rescued the sensitivity of c-FLIP-deficient cells to anti-Fas treatment, it only partially inhibited the increased cell death of c-FLIP-deficient cells in response to TCR engagement (91). Meanwhile, loss of TNFα had no effect in this context. These data indicate that c-FLIP promotes the survival of TCR-activated T cells in part by inhibiting Fas-mediated death signals and in part through a yet unknown mechanism.

In order to determine whether one specific isoform of c-FLIP was responsible for the specific roles of c-FLIP observed in the knockout, we crossed the c-FLIP conditional knockout mice to mice in which cDNA for either c-FLIP$_L$ or c-FLIP$_{S/R}$ had been inserted into the *c-FLIP* locus on a BAC transgene (91, 92). Upon deletion of the endogenous *c-FLIP* alleles, the transgenic mice expressed only c-FLIP$_L$ or c-FLIP$_{S/R}$ under the control of the normal *c-FLIP* regulatory elements on the BAC transgene, thus functioning as a c-FLIP$_{S/R}{}^{-/-}$ or c-FLIP$_L{}^{-/-}$, respectively. When expressed in this manner, both the c-FLIP$_L$ and the c-FLIP$_{S/R}$ isoforms could rescue the survival of c-FLIP$^{f/f}$ERCre T cells after TCR stimulation in vitro, indicating that both isoforms are sufficient to protect T cells after activation through the TCR (91). In vivo, we showed that c-FLIP$_L{}^{-/-}$ (c-FLIP$^{f/f}$LckCre-c-FLIP$_{S/R}{}^{Tg}$) thymocyte development is normal and that the mice have similar numbers and percentages of T cells in the periphery (92). However, c-FLIP$_L{}^{-/-}$ T cells showed an impaired T cell response to in vivo infection with *Listeria monocytogenes* (92). They also exhibited a defect in proliferation and IL-2 production after TCR stimulation in vitro (92). In concordance with our data in c-FLIP-deficient thymocytes, c-FLIP$_L{}^{-/-}$ T cells showed normal levels of Erk, Jnk, and NF-κB activation, so these pathways are likely not regulated by c-FLIP$_L$ in T cells (92). These data suggest that while both the long and short isoforms can protect T cells from death under certain conditions, there is a clear requirement for c-FLIP$_L$ in the T cell response in vivo.

Autophagy in T lymphocytes

Autophagy, meaning “self-eating,” is a conserved process by which intracellular contents are degraded (93). It was originally described in yeast as a process that occurs during periods of starvation, but it also appears to be a critical process for mammalian cells (93, 94). Different forms of autophagy have been described, including microautophagy, chaperone-mediated autophagy, and macroautophagy. For the purpose of this review, we will largely focus on what would be considered macroautophagy. Autophagy is initiated by the formation of an isolation membrane in the cytoplasm of the cell (93, 94). This membrane is elongated and fused to encapsulate cytoplasmic contents including cytoplasmic proteins and whole organelles within a double-membrane structure called the autophagosome. The autophagosome then fuses with the lysosome for the degradation of the autolysosomal contents.

Several molecules are known regulators of the process of autophagy. A number of autophagy-related genes, including the Atg genes by the current nomenclature, were originally identified in yeast, and many of these genes are involved at the level of the initiation and the elongation of the isolation membrane (95–97). Two separate pathways,

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which have been described as “ubiquitin-like” in that they involve a protein being sequentially processed upon covalent interactions with mediator proteins, appear to be critical for this process (93, 94). In one, the protein Atg12 is first bound to Atg7 then passed through a series of covalent bonds to Atg10 and finally to Atg5. In the other pathway, the protein LC3 is enzymatically processed from its LC3-I form to its LC3-II form through interactions with Atg7 and Atg3 (93, 94). Upstream of these pathways, the Bcl-2-interacting protein Beclin1 (yeast Atg6) and the PI3-kinase Vps34 have been shown to be important in the initiation of autophagy in yeast and some cell lines, but the roles of these proteins in T cells is not yet clear (15, 94). Because autophagy is induced under starvation conditions, proteins involved in cell metabolic pathways have been shown to be important for crosstalk with autophagy pathways (10, 93). The activity of the metabolic regulator mTOR has been shown to inhibit autophagy, and treatment with rapamycin, which targets mTOR, can induce autophagy in T cells (10, 98).

In addition to being important under starvation conditions, other roles for autophagy have been shown. Some studies have suggested that autophagy is responsible for the clearance of organelles, for example mitochondria in a process known as “mitophagy” (99–105). In addition, while autophagy is largely considered to be pro-survival, some studies have shown a pro-death role for autophagy, and autophagic cell death has been termed “type II programmed cell death” (13, 14). In one study, $Bax^{-/-}Bak^{-/-}$ double-knockout embryonic fibroblasts could be induced with etoposide and staurosporine (typical inducers of the intrinsic pathway of apoptosis) to die what appeared to be an autophagic cell death because it could be inhibited by the PI3-kinase inhibitor 3-methyladenine (3-MA) or silencing of Atg5 or Beclin1 (106). It has also been shown that the caspase inhibitor zVAD could induce autophagic cell death, and the cell death induced by this method could be inhibited by RNAi-mediated suppression of Atg7 or Beclin1 (107). However, because both of these studies required the manipulation of classical apoptosis to observe autophagic cell death, the relevance of this process in vivo is unclear.

In mice, several important roles of autophagy have been shown. Consistent with a role in survival during starvation conditions, knockout mice lacking the genes for Atg5 or Atg7 are born, but are unable to survive the first approximately 24-hour period before they begin to nurse (108, 109). Interestingly, when the Atg5-deficient neonates were hand-fed to prevent this starvation period, the neonates survived longer (108). Autophagy has also been shown to be important for neural development (110, 111). In the immune system, autophagy has been shown to be important in the clearance of intracellular pathogens such as group A Streptococcus and *Mycobacterium tuberculosis* (112, 113). Importantly, it was shown that autophagy could be induced in macrophages by interferon-γ (IFN-γ), indicating that the clearance of intracellular bacteria by autophagy is a regulated process rather than a process of circumstance (113).

Several autophagy-related proteins have been shown to cross-talk with apoptotic pathways. Notably, Bcl-2 and Bcl-X_L bind to and inhibit Beclin1, and Atg5 has been shown to interact with FADD (15, 16, 114). Because of these interactions, our lab hypothesized that autophagy may also be an important process in regulating the survival of T lymphocytes. Previous studies identified autophagosomes in human T lymphocytes using transmission electron microscopy (TEM) and fluorescence microscopy to observe clustering of LC3-GFP (115, 116). Using semiquantitative RT-PCR, we found that the autophagy-related proteins Atg5, LC3, and Beclin-1 are expressed in primary mouse thymocytes and in both freshly isolated and activated T cells (117). Additionally, in freshly isolated and activated T cells, we observed double-membrane structures with the characteristic appearance of autophagosomes by transmission electron microscopy (117). We also observed the processing of LC3-I to LC3-II by Western blotting, and the relative level of LC3-II was

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increased in response to both amino acid starvation and activation using an anti-CD3 antibody (117). In another study, Li et al. observed the formation of autophagosomes in activated $CD4^+$ T cells by TEM and LC3-GFP puncta formation (98). These measures indicated that autophagy does indeed occur in T cells. What's more, in addition to starvation conditions, autophagy could be observed in T cells in response to activation through the TCR. Whether this is related to metabolic pathways and the metabolic requirements of T cell activation (thus mimicking starvation conditions) or whether a different signaling pathway induces autophagy in response to T cell activation is still unclear.

The transfer of $Atg5^{-/-}$ fetal liver cells into lethally irradiated adult mice allowed the further study of the role of autophagy in the immune system. After reconstitution, mice that received $Atg5^{-/-}$ hematopoietic cells showed a decreased number of thymocytes compared to those reconstituted with wild type fetal liver cells. However, the profile of the thymic subsets in the $Atg5^{-/-}$ chimeras showed that thymic development was normal, indicating that autophagy may be dispensable for thymocyte development (117). In contrast, the numbers of $CD4^+$ and $CD8^+$ T cells were severely reduced in the peripheral compartments of $Atg5^{-/-}$ chimeric mice (117). These T cells, most strikingly the $CD8^+$ T cells, showed an increased rate of apoptosis, indicating that autophagy likely promotes the survival of mature T cells (117). Further, $Atg5^{-/-}$ T cells proliferated less than wild type cells in response to both signaling through the TCR and after stimulation with phorbol 12-myristate 13-acetate (PMA) and ionomycin, which bypasses membrane signaling (117). The T cell phenotypes observed in the $Atg5^{-/-}$ chimeric mice were also observed in T cells in which autophagy was interrupted by using a floxed allele of *Atg7* (109) that was deleted specifically in T cells using LckCre, indicating that the defects observed in the $Atg5^{-/-}$ chimeras were likely due to the interruption of autophagy and not a separate role of Atg5 (118).

The data above suggested that autophagy is critical for the survival of mature T lymphocytes. However, the mechanism behind this requirement was not yet clear. One potential reason that autophagy could be required is for the maintenance of metabolism in mature T lymphocytes. However, the addition of the metabolic substrate methylpyruvate could not rescue the defect in the proliferation of $Atg5^{-/-}$ chimeric T cells (unpublished data from our laboratory), indicating that a non-metabolic role for autophagy may be important in this context. Autophagy has been shown to have a role in the clearance of mitochondria under several conditions (99–102). By examining the mitochondrial content of Atg7-and Atg5-deficient T cells using the dyes MitoTracker Green and TMRE and by measuring mitochondrial DNA and protein levels, we observed that the mitochondrial compartment of autophagy-deficient T cells was expanded (118). An increase in mitochondrial levels could also be detected in SP thymocytes in the Atg7-deficient but not the Atg5-deficient model (118). Interestingly, in wild type thymocytes and T cells, the mitochondrial levels as detected by MitoTracker Green dramatically decreased in correlation with the transition of the thymocyte subsets into the peripheral T cell subsets (118). This decrease suggests that T cell mitochondrial levels may be developmentally regulated, but the reason for the clearance of mitochondria upon maturation is not yet clear. Because the autophagy-deficient DN and DP cells showed similar levels of mitochondrial staining to control cells, the data suggested that autophagy-deficient T cells have a defect in the developmentally regulated clearance of mitochondria (118). Consistent with the increased mitochondrial content, we observed that Atg7-deficient T cells contained increased levels of reactive oxygen species (ROS) (118). Because ROS production has been shown promote the death of T cells (119), it is possible that the increased levels of ROS could contribute to the reduced survival of autophagy-deficient T cells. Additionally, we observed differences in the expression levels of mitochondrial proteins that have roles in apoptotic pathways, such as cytochrome c and apoptosis-inducing factor (AIF), between autophagy-deficient and control T cells (118). The levels of certain Bcl-2 family members were also changed: although Bcl-2 expression was

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higher in Atg7-deficient T cells, the level of pro-apoptotic Bak was also increased, and Mcl-1 expression was unchanged (118).

Collectively, the data from our laboratory and others suggest that autophagy is critical for the survival and proliferation of T lymphocytes (12, 117, 118). We have shown that autophagy has a role in the developmentally regulated clearance of mitochondria from T cells, and that the disruption of this process leads to increased ROS production and an imbalance in apoptotic proteins. In addition to the role of autophagy in mitochondrial clearance and T cell survival in a developmental context, the observations that autophagy is also induced by TCR activation and appears to have a critical role in T cell proliferation are also quite interesting. Current research efforts in our laboratory are to examine the role of autophagy in activated T cells and the mechanism behind the defect in proliferation.

Concluding remarks

Our laboratory has focused on using genetic models to delineate the in vivo functions of several key molecules in T cell apoptotic and autophagic pathways. In combination with the work of other groups, our data give us a clear understanding of the roles of the different anti-apoptotic Bcl-2 family members in developing thymocytes. We have shown that Mcl-1 is critical for T cell survival at multiple stages due to its ability to neutralize Bak (35, 61). Bcl-x_L appears to have a largely supporting role: it is not required for development or function but does promote survival of DP thymocytes (35, 48). Other groups have shown that Bcl-2 is also critical for T cell survival, and one of its roles is to counteract the BH3-only protein Bim (29, 30, 46, 47, 58, 59). These data confirm that the intrinsic pathway of apoptosis is regulated at multiple levels in T lymphocytes. Future studies should continue to define the signals that alter the balance in the Bcl-2 family members and the interactions between the different proteins in different in vivo T cell environments.

Our studies have also shown that the extrinsic apoptotic pathway is critical for T cell survival. Loss of the apoptotic regulator c-FLIP in thymocytes and T cells has shown that c-FLIP is important for the survival of developing thymocytes and peripheral T cells, particularly after activation through the TCR (89, 91). Although overexpression studies have shown a role for c-FLIP in signaling, we have not observed any defect in signaling pathways in c-FLIP-deficient T cells, indicating that the dominant role for c-FLIP in T cells is one of survival (89, 91). Interestingly, c-$FLIP_L^{-/-}$ T cells, which express c-$FLIP_{S/R}$, have defective responses to infection, indicating that isoform-specific roles may exist in T cells (92). The mechanism behind the specific roles of c-$FLIP_L$ and c-$FLIP_{S/R}$ remains to be determined.

Finally, our interest in cell survival and intriguing evidence of a molecular connection with apoptosis brought us to the study of the catabolic process of autophagy. Autophagy is a critical process in T cells that appears to be developmentally regulated (12, 117). It can be induced not only by classical starvation conditions, but also by TCR stimulation (117). T cells that are deficient in autophagy have increased levels of mitochondria and ROS as well as decreased survival (118). Further, they cannot proliferate as well as wild type cells in response to TCR stimulation (117). The mechanism by which autophagy controls TCR-induced proliferation is currently of great interest.

Our studies to date have dissected the roles of individual proteins in T cell survival pathways. Beyond this, the next step will be to examine the levels at which these pathways interact. While the intrinsic pathway and the extrinsic pathway converge at the level of apoptosome formation, they are also known to crosstalk at the level of the BH3-only protein Bid, which can be cleaved by caspase 8 and feed into the intrinsic pathway (120). Autophagy has been shown to couple with cell death in Bax/Bak-deficient cells (106). Beclin1, which is part of the autophagic pathway in some cell types, has been shown to

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interact with Bcl-2 and Bcl-x_L, and overexpression of Bcl-2 inhibits Beclin1-mediated autophagy in both yeast and mammalian cell lines (15, 114). Some evidence also suggests that Atg5 interacts with FADD, an important regulator of the extrinsic pathway of apoptosis (16). In addition to these direct interactions, autophagy and the Bcl-2 family can both be regulated by the Akt and mTOR pathways in T lymphocytes (98, 121). The balance of these types of intracellular signals will likely determine whether a cell survives or dies and through which mechanism.

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