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CONTRACTING THE ‘MUS CELLS’ – DOES DOWN-SIZING SUIT US FOR DIVING INTO THE MEMORY POOL ?

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Summary

Maintenance of T-cell homeostasis is critical for normal functioning of the immune system. After thymocyte selection, T cells enter the peripheral lymphoid organs, where they are maintained as naive cells. Transient disruption of homeostasis occurs when naive T cells undergo antigen-driven expansion and acquire effector functions. Effector T cells then either undergo apoptosis (i.e. contraction at the population level) or survive to become memory cells. This apoptotic process is crucial: it resets T-cell homeostasis, promotes protective immunity, and limits autoimmunity. Although initial studies using *in vitro* models supported a role for death receptor signaling, more recent *in vivo* studies have implicated Bcl-2 family members as being critical for the culling of T-cell responses. While several Bcl-2 family members likely contribute to T-cell contraction, the pro-apoptotic molecule Bim and its anti-apoptotic antagonist Bcl-2 are essential regulators of the process. This review discusses the progress made in our understanding of the mechanisms underlying contraction of T-cell responses and how some cells avoid this cell death and become memory T cells.

Keywords

T cell; apoptosis; memory

Introduction

During an acute infection, activated antigen-presenting cells (APCs) drive a dramatic antigen-specific T-cell clonal expansion. In general, following clearance of the antigen, the immune response wanes, and most of the expanded T cells die off while some remain and become memory cells. This T-cell attrition restores homeostasis, avoiding increased metabolic costs and autoimmunity. If the expanded T cells are not culled, lymphadenopathy would develop after a few infections and the persisting activated T cells could also be a predisposing factor for autoimmunity. Further, when T cells are not appropriately eliminated, a lethal hyperinflammatory reaction can develop if the pathogen is re-encountered (1). If too many expanded T cells are eliminated after the clearance of infection, the establishment of protective T-cell memory is compromised. Thus, mechanisms that control the apoptosis of effector T cells are central to our understanding of both immunity and autoimmunity.

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The tracking of T-cell clonal expansion and contraction has been aided greatly by developments in T-cell receptor transgenic (TCR Tg) mice and major histocompatibility complex (MHC) 'tetramers' that have allowed the precise quantitation of antigen-specific T-cell responses *in vivo* (2-5) (Fig. 1). Analysis of endogenous T-cell responses using MHC tetramers have revealed that previous studies vastly underestimated the magnitude of T-cell responses (6-9). Further, these analyses showed that the numbers of antigen-specific T cells declined massively (~10-20 fold decrease) in the week following the peak of the response to acute viral infection (2,5). These results have confirmed earlier studies in a superantigen model in which the robust programmed cell death of the clonally expanded T-cell population was observed (10). Thus, many of the effector T cells generated during immune responses are destined to die. Here, we review the progress in our understanding of the molecular mechanisms underlying the death of effector T cells after the peak of the response.

Death receptor signaling and contraction of T-cell responses

In general, apoptosis is controlled by two major pathways, the 'intrinsic' or mitochondrial pathway and the 'death-receptor' pathway. The death receptor pathway is activated through cell surface receptors that are linked directly to caspase proteases. For example, trimerization of Fas by Fas ligand (FasL) results in the formation of a 'death-inducing signaling complex' (DISC) into which the Fas-associated death domain containing protein (FADD) and caspase-8 are recruited (11). Efficient activation of caspase-8 by Fas requires the downregulation of the Fas, FADD-like IL1 β converting enzyme inhibitory protein (FLIP), an enzymatically inactive homologue of caspase-8 (12). Once activated, caspase-8 initiates the apoptotic cascade by cleaving and activating executioner caspases, such as caspase-3, which in turn cleave proteins involved in cell structure and integrity.

Mutations in either Fas or FasL result in generalized lymphadenopathy and accumulation of B and T lymphocytes, but the involvement of the Fas/FasL pathway in the contraction of T-cell responses remains controversial. Initial studies on the death of activated T cells observed that T-cell hybridomas and primary T cells died, instead of proliferating when stimulated through their TCR, leading to the use of the term 'activation-induced cell death' (AICD) (13,14). Around the same time, it was discovered that spontaneous mutations in either Fas or FasL were responsible for lymphadenopathy and autoimmunity that developed in *lpr* and *gld* mice, respectively (15,16). So, when three publications (17-19) clearly showed that Fas/FasL interactions were essential for T-cell death in the *in vitro* AICD model, it was assumed that the death of activated T cells *in vivo* was driven largely by Fas/FasL interactions. However, extrapolation of these *in vitro* data to *in vivo* models has resulted in some controversy. Several papers have clearly shown that apoptotic contraction of T-cell responses *in vivo* does not require Fas/FasL interactions (20-22), while others have shown a role for Fas/FasL in seemingly similar model systems (23-25). One potential reason for the discrepancy between these studies is the nature of the antigenic stimulation. Most of the *in vivo* studies implicating a critical role for Fas/FasL signaling involve repeated antigenic stimuli, while the studies suggesting a Fas/FasL independent cell death entail a single round of antigenic stimulation. Thus, similar to the *in vitro* models, repeated antigenic stimulation *in vivo* appears to render T cells susceptible to the death receptor pathway.

Although these early experiments involved repeated injections of non-replicating superantigens, more recent studies have utilized infectious disease models to test the hypothesis. In two such viral infection models, the death receptor pathway and the mitochondrial pathway appeared to synergize to promote survival of activated viral-specific T cells (26,27). In these reports, both Fas-dependent and Fas-independent mechanisms contribute to the death of activated CD8⁺ T cells. One system employed a model of chronic viral infection, where prolonged and repeated antigen stimulation was likely, both Fas-

dependent and Fas-independent death contributed to the loss of viral-specific T cells (26). In contrast, during acute viral infection, where repeated antigen stimulation is less likely, Fas-driven death played only a minor role (27). Furthermore, in both of these studies, the mice were globally deficient in Fas, making it unclear whether or not Fas signaling on T cells themselves is critical for driving the death of activated T cells. Specific deletion of Fas in germinal center B cells resulted in increased numbers of activated T cells due to prolonged antigenic stimulatory capacity of B cells in germinal centers (28). Such prolonged T-cell responses were mitigated when the germinal center Fas-deficient mice were bred onto a CD28^{-/-} background. Thus, one possibility is that the 'repeated antigenic stimulation' model is not due to T-cell specific signaling effects of Fas but rather to prolonged antigenic stimulation of T cells in germinal centers (28). Indeed, another recent paper clearly showed that conditional loss of Fas in granzyme A-expressing cells (presumably mostly T cells), did not prevent the death of T cells following immunization (29). Although these mice did develop spontaneous autoimmunity and this was ascribed to failure of autoreactive T cells to undergo apoptosis, this was never formally demonstrated. Therefore, whether or not the spontaneous autoimmunity was due to the failure of autoreactive T cells to undergo apoptosis or to another role of Fas is unknown. It remains unclear to what degree the synergistic effects of death receptor-dependent and -independent pathways are due to cell-intrinsic or cell autonomous mechanisms. Further, during acute immune responses evidence indicates that the death receptor pathway does not appear to be the dominant pathway by which activated T cells are killed.

Bcl-2 family members and contraction of T-cell responses

In contrast to the death-receptor apoptotic pathway, the mitochondrial pathway is largely controlled by members of the Bcl-2 family. Bcl-2 family members can be divided into three major groups. The first group, BH3-only proteins, are pro-apoptotic and act as 'messengers'. T cells express several BH3-only proteins including Bim, Bad, Puma, Noxa, Bid, NIX, and BNIP3 (30). Of the BH3-only proteins, Bim has the most profound effect on T-cell homeostasis. BH3-only proteins appear to transmit apoptotic signals to the Bax/Bak-like molecules (members include Bax, Bak, and Bok). The Bax/Bak-like family members are genetically downstream of the BH3-only molecules, as mice lacking both Bax and Bak are completely resistant to overexpression of the BH3-only messengers (31). Although Bax and Bak are thought to be completely redundant, it is intriguing that mice deficient in Bim and Bax do not have the same phenotype as mice deficient in Bim and Bak (32). This may be because different mechanism(s) are involved in restraining Bax and Bak (i.e. Bax is cytosolic and in a folded conformation, while Bak is constitutively localized to the mitochondria), to tissue-specific expression of either molecule, or to differential activation of either Bax or Bak by different BH3-only molecules. Even if their functions are not 100% redundant, mice deficient in both Bax and Bak have profound lymphadenopathy, which includes massive accumulation of T cells. The T-cell accumulation in Bax/Bak double-deficient mice is more severe than that observed in Bim-deficient mice, suggesting that other BH3-only proteins contribute to T-cell homeostasis. Puma is another BH3-only protein that appears to cooperate with Bim in modulating T-cell survival (33). Nonetheless, Bax/Bak-like molecules, once activated by the messengers, are believed to form large pores in the outer membrane of the mitochondria promoting the release of cytochrome *c*. These events trigger subsequent caspase activation, and ultimately proteolytic destruction that is characteristic of apoptosis.

The third group of Bcl-2 family members includes the anti-apoptotic molecules, of which Bcl-2, Mcl-1, Bcl-xL, and A1 are all expressed in T cells (30). Bcl-2-deficient mice appear to develop normally but between 4-8 weeks of age start to die, exhibiting a profound loss of naive T cells (34,35). Mcl-1 is also an essential anti-apoptotic regulator required for the

survival of naive T cells *in vivo* (36), but neither A1 nor Bcl-xL appears to be strictly required for naive T-cell survival (37,38). Anti-apoptotic Bcl-2 family members function largely by their physical interaction with pro-apoptotic molecules. The BH1, BH2, and BH3 domains of the anti-apoptotic molecules form a hydrophobic pocket that binds to the BH3 domains of the pro-apoptotic molecules (39,40). Although the binding of anti-apoptotic molecules to pro-apoptotic molecules is generally thought to inhibit apoptosis, how apoptotic signals are propagated in cells that possess multiple types of anti-apoptotic molecules is unclear.

Two models have been proposed to explain the propagation of apoptotic signaling. One model proposes that BH3-only proteins sequester anti-apoptotic molecules away from Bax/Bak-like molecules causing their self-oligomerization and activation (41). A second model proposes that BH-3 only molecules interact directly with Bax/Bak-like molecules and drive their activation (42). In this model, certain 'sensitizer' BH3-only molecules (Bad and Noxa) titrate anti-apoptotic molecules away from other potential 'activator' BH3-only molecules (e.g. Bim, Bid, and Puma), allowing these activator molecules to bind to Bax/Bak and transmit their apoptotic signal. This model has been referred to as a 'hit and run' model because physiologic interactions between activator proteins (other than tBID) and Bax or Bak have been notoriously difficult to detect due to the low affinity and/or transient nature of these interactions. One study provided support for the first model as it was shown that that Bax and Bak were still able to cause apoptosis when the 'activators' were absent (41). Another recent study in which a novel interaction between a stabilized Bim-BH3 peptide and the α helical regions 1 and 6 of Bax was identified supports the second model (43). Further, mutagenesis of these α helices on Bax inhibited their ability to interact with the Bim-BH3 peptide and also partially inhibited their ability to transmit apoptotic signals in cells (43). We note that these two different models are not necessarily mutually exclusive as BH3-only proteins might drive cell death via both routes. Depending upon the cell type, the levels and ratios of BH3-only proteins and anti-apoptotic molecules, and cell death stimuli, one mechanism may predominate. Indeed, a recent tour-deforce study in which the BH3 domains of various pro-apoptotic molecules were genetically swapped in various strains of knockin mice provided evidence that both mechanisms probably contribute to apoptosis initiation (44). Either way, it is clear that Bcl-2 family members play critical roles in controlling T-cell homeostasis.

Specific interactions between pro- and anti-apoptotic Bcl-2 family members

As stated previously T cells (actually most cells) express multiple pro- (BH3-only) and anti-apoptotic Bcl-2 family members. Thus, recent work suggest that cell death and survival is controlled not simply by overall ratios of anti- to pro-apoptotic molecules but rather by specific interactions between BH3-only molecules and anti-apoptotic Bcl-2 family members. For example, using peptides corresponding to the BH3 domains of pro-apoptotic family members, the affinity of various pro-apoptotic molecules for their anti-apoptotic counterparts was assessed (45). This study demonstrated that individual anti-apoptotic antagonists have different specificities for the different BH3-only molecules. For example, both Bim and Puma peptides bound with similar affinity to all anti-apoptotic molecules, while Bad bound selectively to BclxL, Bcl-2, and Bcl-w, and Noxa bound selectively to Mcl-1 and A1 (45). These isolated biochemical experiments studying individual binding interactions appear to be holding true in real cells where multiple Bcl-2 family members are expressed. Indeed, Bcl-2 is the major anti-apoptotic molecule bound to Bim in T cells (46) and the lethal disease that ensues in Bcl-2-deficient mice is alleviated by additional genetic ablation of Bim (47,48). Although Mcl-1 can also interact with Bim (36), a recent study suggests that Mcl-1 may target Bak, as deficiency in Bak partially restores the survival and blocks in T-cell development found in Mcl-1-deficient animals (49). However, as the

expression levels of Bcl-2 family members is dynamic across T-cell development and activation status, the interactions between individual pro- and anti-apoptotic Bcl-2 family members and are likely to vary. Thus, the temporal regulation of expression of Bcl-2 family members adds another layer of complexity to the control of apoptotic signaling in T cells.

Bim is critical for contraction of T-cell responses

Despite early reports showing that overexpression of Bcl-2 in the T-cell compartment was sufficient to protect activated T cells from death *in vivo* following a single superantigen injection (50), little progress was made in understanding the role of the mitochondrial pathway in activated T-cell death for several years. This was largely because of the assumed dominant role of the Fas pathway in the process. We re-examined the role of death receptor versus mitochondrial pathways *in vivo* using a superantigen model system. Superantigens drive expansion of T cells bearing particular V β regions as part of their TCR followed by massive contraction of these expanded cells (10). As Strasser and Cory had found years earlier, we also showed that Bcl-2 overexpression was sufficient to block superantigen-induced T-cell death *in vivo* (20). While these Bcl-2 overexpression studies implicated the mitochondrial pathway in activated T-cell apoptosis, they did not identify particular pro-apoptotic molecules directly involved in the process. Also, these studies did not directly implicate Bcl-2 itself, as Bcl-2 overexpression may promote interactions between Bcl-2 and other molecules that are not relevant under physiologic conditions. However, following activation *in vivo* by either superantigens or viral infection, we and others (51,52) showed that the levels of endogenous Bcl-2 significantly decreased in antigen-specific T cells at the peak of the response, just before most of these cells will go on to die. These data suggested that the decline in Bcl-2 levels rendered activated T cells susceptible to death. The development of tools to assess antigen-specific T cells *ex vivo* (i.e. MHC tetramers, TCR Tg mice, etc.) made these observations possible, because *in vitro* models did not reveal striking differences in Bcl-2 expression. One potential explanation for this is that in tissue culture systems, common γ chain cytokines [interleukin-2 (IL-2), IL-4, etc.] are often added or produced in excess and significantly elevate levels of Bcl-2 within T cells (52).

Reasoning that the downregulation of Bcl-2 was critical for rendering activated T cells susceptible to apoptosis, we began screening pro-apoptotic Bcl-2 family members for their ability to accelerate T-cell apoptosis using a retroviral overexpression system (20). We found that a previously described Bcl-2 antagonist, Bim (53,54), was critical for the apoptotic deletion of superantigen-reactive T cells *in vivo* (20). Similarly, deficiency in both Bax and Bak also prevented apoptotic deletion of superantigen-reactive T cells further implicating the mitochondrial pathway (55). The critical role for Bim has since been validated in several different mouse viral infection models, including herpes simplex virus (HSV), lymphocyte choriomeningitis virus (LCMV), and influenza, (27,56-58) and has been implicated in the death of activated macaque T cells responding to simian immunodeficiency virus (SIV) (59) and in human T cells responding to hepatitis B infection (60). Further, we recently showed that Bim is critical for controlling mouse T-cell responses to the parasite *Leishmania major* (61). Combined, these studies suggest a critical and largely non-redundant role for Bim in the death of activated T cells *in vivo*.

Although the Bim-deficient mice have profound phenotypes regarding *in vivo* T-cell responses, all of the aforementioned studies have used mice in which Bim is missing from all tissues. Bim-deficient mice have multiple immunological impairments (62), raising the possibility that the lack of Bim in other cell types such as dendritic cells or B cells could be contributing to the enhanced T-cell responses in Bim-deficient mice. Although one study showed that prolongation of dendritic cell survival through Bcl-2 overexpression could enhance *in vivo* T-cell responses (63), the effect was largely restricted to expansion of T-cell

responses, and contraction of T-cell responses occurred normally in these mice. We found that when activated T cells from Bim-deficient mice were transferred to congenic recipients or were cultured *in vitro*, they survived better than their wildtype counterparts (58). However, even in these studies, it remained possible that altered stimulation in the Bim-deficient environment may have imparted a 'long-lived' phenotype to the T cells. This issue was addressed by Bevan's group (64) when they adoptively transferred naive TCR Tg T cells from Bim-deficient mice and found, after infection with recombinant *L. monocytogenes* infection, that the contraction of these Bim-deficient TCR Tg T cells was impaired. These data strongly suggest that the effects of Bim on contraction of T-cell responses are largely T-cell intrinsic.

Control of Bim activity

Bcl-2 as the gatekeeper for Bim

If Bim has a cell-intrinsic effect on T-cell apoptosis, how is its function controlled in T cells? One simple explanation could be that Bim is not expressed in naive T cells but is upregulated in activated T cells. However, naive T cells contain significant amounts of Bim protein that are only slightly increased in T cells activated *in vivo* (20,46). Interestingly, Bim mRNA is increased substantially in activated compared to naive T cells (30). It is unclear if the increased Bim mRNA in activated T cells is due to increased transcription or to increased stabilization of Bim mRNA. Further, it is unclear if the discordance between Bim protein versus mRNA is due to the death of cells having increased levels of Bim or if post-translational mechanisms keep Bim protein levels from increasing commensurate with the increases in mRNA. Nonetheless, naive T cells contain significant amounts of Bim (20,46), and most of the Bim within naive T cells is bound to Bcl-2 at the mitochondria (46). While we and others (20,50,65) showed that Bcl-2 overexpression could block activated T-cell apoptosis driven by certain stimuli, others reported no effect of Bcl-2 overexpression on contraction of T-cell responses to viral infection (66,67). Why Bcl-2 might be sufficient to prevent death under only certain circumstances is unclear. It is possible that, during infections, stronger TCR signals are generated which actively inhibit Bcl-2. Indeed, we have seen that during infection levels of the Bcl-2 transgene are decreased substantially in antigen-specific T cells (authors' unpublished data). Thus, levels of Bcl-2 in certain transgenic animals may not have been sustained sufficiently to block Bim in these previous studies.

Such interactions between Bcl-2 and Bim are critical to maintain naive T-cell viability as we and others showed that T-cell attrition in Bcl-2-deficient mice was alleviated by concomitant loss of Bim (47,48). As mentioned previously, after T-cell activation, Bcl-2 levels significantly decrease increasing the amount of uncomplexed or 'free' Bim. This free Bim would then be predicted to initiate apoptotic signaling by either directly and/or indirectly activating Bax/Bak. Thus, one critical mechanism underlying the Bim activation is the modulation of Bcl-2 expression. Despite the critical role for Bcl-2 in controlling T-cell viability, the mechanism(s) controlling Bcl-2 expression in T cells are just beginning to be unraveled.

While Bcl-2 levels are decreased following T-cell activation, expression of Bcl-xL, A1, and Mcl-1 are all increased in activated T cells (30,49). The degree to which these anti-apoptotic molecules contribute to the survival of activated T cells is just beginning to be understood. No role for A1 has been described, and Bcl-xL appears to be dispensable for the survival of activated T cells after bacterial infection (38). Conversely, Mcl-1 is critical for activated T-cell survival *in vitro* (49) and *in vivo* (our unpublished results). However, the degree to which Mcl-1 antagonizes Bim remains unclear. Further work is required to determine the roles of individual anti-apoptotic molecules in promoting survival of activated T cells.

Controlling the gatekeeper

Several mechanisms have been implicated in decreasing Bcl-2 expression in activated T cells (Fig. 2). We and others (68,69) had previously shown that the production of reactive oxygen species (ROS) controls apoptosis of activated T cells *in vitro*. Further, we found that a synthetic catalytic antioxidant, manganese III tetrakis (5, 10, 15, 20-benzoic acid) porphyrin (MnTBAP), increased expression of Bcl-2 within T cells (70). The anti-apoptotic effects of MnTBAP could be recapitulated by catalase overexpression and required Bcl-2 expression in T cells *in vitro* (70). In hippocampal neurons, ROS antagonized Bcl-2 expression by inhibiting the transcriptional activity of the cyclic-adenosine monophosphate (cAMP) response element binding protein (CREB) (71), although it is unclear if this mechanism is relevant in T cells. *In vivo* administration of MnTBAP lessened the magnitude of T-cell contraction during acute LCMV infection, but it also limited expansion of the T-cell response (72,73). Furthermore, it is unclear whether the effects of MnTBAP *in vivo* are due to effects in T cells or other cells, as MnTBAP was not targeted specifically to T cells. Thus, the role of ROS in controlling Bcl-2 expression in activated T cells *in vivo* remains uncertain.

Another mechanism for regulation of Bcl-2 expression is cytokine signaling. Several common γ chain cytokines can increase the levels of Bcl-2 within T cells both *in vitro* and *in vivo* (52,74-76). Further, overexpression of Bcl-2 prevented the loss of T cells in IL-7- or IL-7R-deficient mice (77,78). IL-7 may contribute to maintenance of Bcl-2 in effector CD8⁺ T cells, as the cells expressing high levels of IL-7R (CD127) were shown to also express higher levels of Bcl-2 compared to their CD127^{lo} counterparts (79). We also found that IL-7 given *in vivo* prevented the contraction of the CD4⁺ T-cell response and that this effect required Bcl-2 (74). However, we found that IL-7 blockade, using antibody-mediated neutralization, failed to exacerbate contraction of the CD4⁺ T-cell response, suggesting that IL-7 is not limiting for most effector T cells to survive (74). In addition, transgenic CD127 expression in T cells failed to prevent contraction of CD4⁺ or CD8⁺ T-cell responses (80,81). Importantly, Bcl-2 was not increased in CD127 Tg T cells, which may be at least part of the reason the T cells were not rescued from death.

IL-15 also contributes to maintaining Bcl-2 expression within effector CD8⁺ T cells. Contraction of CD8⁺ T-cell responses was enhanced in IL-15-deficient mice, and effector CD8⁺ T cells in IL-15-deficient mice have significantly less Bcl-2 when compared to their wildtype counterparts (82-84). We have found that neutralization of IL-7 in IL-15-deficient mice during contraction of the response significantly decreases the numbers of antigen-specific CD8⁺ T cells (Tripathi *et al.*, manuscript submitted). Moreover, we also found that signal transducer and activator of transcription 5 (Stat5), a signaling molecule shared by IL-7 and IL-15, is essential for maintaining effector CD8⁺ T cells and their expression of Bcl-2 *in vivo* (Tripathi *et al.*, manuscript submitted). As both cytokines may become limiting for expanded populations of effector T cells, this form of 'cytokine withdrawal' may contribute to their decreased expression of Bcl-2 by failing to maintain adequate signaling through Stat5.

Another potential mechanism controlling Bcl-2 expression is via transforming growth factor- β (TGF- β). In a recent study, overexpression of a dominant negative TGF- β receptor (dnTGF- β R) in T cells resulted in a significantly increased expansion of adoptively transferred TCR Tg T cells (85). Inhibition of TGF- β R signaling in T cells resulted in a significantly greater expansion of effector CD8⁺ T cells. This effect correlated with increased expression of Bcl-2 within dnTGF- β R-expressing T cells. Also, TGF- β R signaling may negatively impinge on IL-15 signaling, as transfer of dnTGF- β R TCR Tg T cells into IL-15 knockout mice partially relieved the loss of Bcl-2 observed in IL-15-deficient animals. However, the increases in Bcl-2 expression in dnTGF- β R TCR Tg T cells was transient, as

Bcl-2 levels declined later after infection. In addition, despite the significant increase in expansion afforded by the inability to respond to TGF- β , the activated T cells underwent a similar, if not enhanced, contraction. Although TGF- β has suppressive effects on cytokine induced T-cell survival in splenocytes from mice whose T cells cannot respond to TGF- β , suggesting that at least some of the inhibitory effects of TGF- β are non-T cell intrinsic (85). This idea is further supported by the observation that TGF- β R expression decreases dramatically in activated CD8⁺ T cells, making it unlikely that they are able to respond to TGF- β (85). Thus, while TGF- β signaling likely contributes to the diminished expression of Bcl-2 and may contribute to the death of some activated T cells, whether its effects on T cells are direct or indirect remain unclear.

The Yin-Yang of Bcl-2-Bim

Recent data have highlighted the complex relationship between Bim and Bcl-2. We first found that the levels of Bcl-2 were decreased by about twofold in Bim-deficient T cells (20). One possibility is that the absence of Bim allows Bcl-2^{lo} cells the opportunity to survive, since a major function of Bcl-2 is to restrain Bim (47,48). Mechanistically, this might be due to the effects of IL-7 on maintaining Bcl-2 in peripheral T cells at a level sufficient to counteract Bim. The increased numbers of T cells in Bim-deficient mice may effectively dilute out the IL-7, thereby lowering Bcl-2 levels. Normally, this would be a mechanism for controlling peripheral T cell numbers; when a cell does not receive sufficient IL-7 signaling Bcl-2 levels are not maintained sufficiently to counteract Bim. Indeed, Bcl-2 overexpression as well as Bim deficiency can independently rescue peripheral T-cell homeostasis in CD127-deficient mice (77,78,86). Marrack's group (87) recently showed that transient, *in vitro* overexpression of Bcl-2 also resulted in increased levels of Bim. Following up on these observations, we have found that effector CD8⁺ T cells with the highest level of Bcl-2 also maintain the highest level of Bim (authors' unpublished data). As stated above, one possible explanation for these data is that Bcl-2 levels must be maintained at a certain level to oppose Bim triggered apoptosis. We have started to explore this possibility, and our preliminary data suggest that when Bcl-2 levels were limited in activated T cells, Bim levels plummeted, and cell numbers were significantly reduced (authors' unpublished data). These data suggest that the reciprocal control of Bim and Bcl-2 expression may be a reflection of apoptotic selection of cells based on their levels of Bcl-2 and Bim. However, we cannot rule out the possibility that other mechanisms such as protein turnover and degradation might also contribute to modulating Bim and Bcl-2 levels in the absence of apoptotic selection.

Control of Bim expression

The Bim gene produces at least three distinct isoforms (BimEL, BimL, and BimS) due to alternative splicing (53,54). Of the three isoforms, nearly all of the Bim expressed in T cells is of the BimEL variety, with a little amount of BimL and undetectable levels of BimS (20). This ratio stays virtually constant following T-cell activation *in vivo* (20). As stated previously, Bim protein levels within T cells are only marginally increased following activation *in vivo* (20,58). At first glance, these data suggest that increased expression of Bim is not a major contributor to activated T-cell death. However, as stated before, increased expression of Bim would lead to rapid induction of apoptosis, and therefore, increased levels of Bim protein may be difficult to detect. This is also consistent with the aforementioned discordance between Bim protein and mRNA levels in activated T cells. Thus, we cannot rule out the possibility that increased expression of Bim contributes to contraction of T-cell responses.

Expression of Bim is controlled at the transcriptional, post-transcriptional, and post-translational levels. At the transcriptional level, AKT signaling has been shown to restrict the ability of the forkhead box subclass O transcription factor 3a (FOXO3a) to promote Bim

expression (88). FOXO transcription factors are evolutionarily conserved targets for cytokine-induced AKT signaling (89-91). AKT phosphorylation of FOXO family members inhibits their transcriptional activity by causing their cytosolic retention. In T cells, IL-2/IL-7 phosphorylation of FOXO3a AKT activation correlated with repression of Bim expression and increased memory T-cell survival, although effector T-cell survival was not examined (92). Also, overexpression of a constitutively activated AKT (caAKT) was sufficient to promote survival of superantigen-activated T cells *in vivo* (93), which was likely due to increased expression of Bcl-xL (94). Unfortunately, the effects of caAKT on Bim or on FOXO3a were not determined in these studies. Another transcription factor, E2F1, has also been shown to be an important transcriptional regulator of Bim (95). E2F family members are important downstream effectors of the retinoblastoma (Rb) protein (96). However, contraction of T-cell responses is normal in E2F1-deficient mice, suggesting that E2F1-mediated regulation of Bim is likely not a major contributor to the process (97). More work clearly needs to be done to understand the transcriptional regulation of Bim in activated T cells. Nonetheless, it is possible that lack of cytokine signaling could be a double-edged sword by failing to drive AKT activation sufficient to restrain FOXO3a transcriptional control of Bim and failing to drive Stat5 activation sufficient to maintain adequate levels of pro-survival Bcl-2 family members.

Post-transcriptional control of Bim

Bim is also regulated at the post-transcriptional and post-translational levels. Post-transcriptionally, Bim levels are regulated by microRNAs of miR-17-92 family. Genetic deletion of the miR-17-92 cluster in mice causes defects in B-cell development due to increased expression of Bim at the pro-B to pre-B transition (98). Conversely, mice overexpressing a miR-17-92 cluster transgene exhibit lymphadenopathy and accrual of B and T cells and have decreased expression of Bim (99). In these studies however, it was unclear whether these effects were due to decreased expression of Bim or the decreased expression of PTEN, as both are MiR-17-92 target genes (98). Alternatively, it is possible that other miR-17-92 target genes in addition to Bim and/or PTEN contribute to promote lymphadenopathy. Further, although Bim may be regulated by microRNAs during lymphoid and T-cell development, their role in controlling Bim levels in activated T cells remains unclear.

Post-translational control of Bim

Bim levels are also regulated by protein phosphorylation and turnover. Bim has been reported to be phosphorylated on serines (e.g. S55, S65, S73, and S100) and on threonine 112 (100,101). Before the phosphorylation sites on Bim were mapped, one group showed that phosphorylation of Bim correlated with T-cell survival (102). Bim phosphorylation appears to be regulated predominantly by mitogen-activated protein kinase signaling, including extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 molecules (100,101). In both BaF3 and PC12 cells, ERK-1/2 signaling was shown to be critical for phosphorylation of Bim at S65 (101,103,104). Phosphorylation of Bim at S65 facilitated phosphorylation of Bim at S55, S73, and S100 and also promoted ubiquitinylation and proteasomal degradation of Bim (101). Indeed, S69A mutants of Bim accumulate in cells, have a longer half-life, and cause significant toxicity (105,106). These data suggest a role for an E3-ubiquitin ligase in targeting phospho-Bim for ubiquitination. c-Cbl is an interesting candidate, given the altered T-cell phenotypes in c-Cbl-deficient mice (107,108); however, recent data suggest that Bim degradation proceeds normally in c-Cbl-deficient fibroblasts (109). It is possible that c-Cbl may be important for Bim degradation in T cells and that redundant mechanisms may exist to degrade Bim in fibroblasts. Indeed, β -TrCp has been identified as an E3 ubiquitin ligase important for modulating Bim protein turnover in fibroblasts (110). In this study (110), ERK signaling was critical for Bim phosphorylation

and subsequent association of Bim with β -TrCP. The role of β -TrCP alone or in conjunction with c-Cbl in controlling Bim turnover in T cells is unknown. Consistent with a role for ERK in Bim phosphorylation and turnover, we have found that Bim turnover increases following T-cell activation *in vivo* (authors' unpublished data). In addition to ERK, JNK has also been described to phosphorylate Bim at S69; however, these data are derived largely from the use of partially specific inhibitors as well as constitutively active kinases, both of which can have non-specific effects (101). Instead, it appears that JNK is critical for threonine phosphorylation of Bim at T112 (100). In general, both ERK and JNK likely contribute to Bim phosphorylation depending on the cell type and the cellular context, with ERK promoting serine phosphorylation and JNK promoting threonine phosphorylation.

To examine Bim phosphorylation more closely *in vivo*, Davis' group (100) recently generated knockin mice in which serines 55, 65, and 73 were all mutated to alanines (Bim^{3SA} mice) and another strain of knockin mice in which threonine 112 was mutated to alanine (Bim^{T112A} mice). Surprisingly, *in vivo* analysis of thymocytes showed no role for the three serines in double positive thymocyte apoptosis driven by either dexamethasone or negative selection (100). In contrast, Bim^{T112A} mice were partially resistant to double positive thymocyte apoptosis in response to either stimulus (100). Interestingly, phosphorylation of T112 was required for Bim to interact with Bcl-2, although this was studied in cell lines and not primary T cells. Nonetheless, it raises the possibility that in addition to decreased Bcl-2 transcription, dephosphorylation of T112 of Bim may be another mechanism to prevent Bim from interacting with Bcl-2 and thereby promote downstream apoptotic signaling. As Bim interacts with multiple anti-apoptotic Bcl-2 family members, it will be interesting to determine if T112 phosphorylation is critical for these interactions as well. For example, T112 phosphorylation may underlie differential binding of Bim to Bcl-2 versus Mcl-1. This differential binding may facilitate apoptosis as predicted by the indirect signaling model whereby Bim promotes Bax/Bak oligomerization by sequestering anti-apoptotic molecules like Bax and Bak from their cognate anti-apoptotic molecules. While substantial progress has been made, more work is clearly needed to determine the role of Bim phosphorylation in apoptosis in activated T cells.

Effector T cells: all created equal?

Of the effector T cells that exist at the peak of the response, only a minority become memory T cells. Recent data have defined subsets of effector T cells that may have differential propensities to generate memory CD8⁺ T cells. During viral infection, it was first found that effector CD8⁺ T cells having increased expression of CD127 were enriched as the response contracted (79). Since then, effector CD8⁺ T cells have been further subdivided based on their expression of killer cell lectin-like receptor subfamily G, member 1 (KLRG-1). Effector CD8⁺ T cells having increased expression of CD127 but lacking expression of KLRG-1 survived more during contraction of the response (111). These cells have been termed 'memory precursor effector cells' (MPECs) (111). Conversely, effector CD8⁺ T cells having decreased expression of CD127 but increased expression of KLRG-1 have been called 'short-lived effector cells' or (SLECs) (111). Adoptive transfer studies demonstrated that MPECs contained a population of cells that had increased potential to become memory T cells, while SLECs had a decreased potential to generate memory T cells (111).

The aforementioned studies led to a whirl of speculation that contraction of T-cell responses was controlled by limiting amounts of IL-7. However, the idea that IL-7R is somehow selective for effector CD8⁺ T-cell survival is somewhat myopic, given that the receptor for IL-15 (CD122) is upregulated on nearly all effector CD8⁺ and CD4⁺ T cells (112). Data suggest a limited role for IL-7 in the selection of surviving effector T cells. First, weeks after

contraction of the response, only ~ 50-60% of the cells express CD127, demonstrating that although many CD127^{lo} cells died during the response, some also survived (58). Second, transgenic overexpression of CD127 failed to prevent or even alter the contraction of the CD8⁺ T-cell response (80,81). Third, we showed that neutralization of IL-7 *in vivo* did not enhance contraction of virus-specific CD4⁺ T-cell responses (74). Fourth, immunization strategies can elicit copious numbers of IL-7R^{hi} effector CD8⁺ T cells at the peak of the response, but these cells do not survive well afterwards (113). Thus, while cells having increased potential for memory may reside within a subpopulation of effector CD8⁺ T cells bearing low expression of KLRG-1 and high expression of CD127, these markers are clearly not all encompassing. The use of the SLEC/MPEC nomenclature is thus somewhat misleading, as it implies that all of the SLECs die during contraction of the response, which is clearly not the case.

Given that most effector CD8⁺ T cells increase expression of CD122 and some of these cells also have increased expression of CD127, it is possible that IL-7 and IL-15 play partially redundant roles in effector CD8⁺ T-cell survival. Indeed, using an adoptive transfer of OT-1 TCR Tg T cells into IL-15-deficient recipients followed by infection with recombinant vesicular stomatitis virus expressing OVA (VSV-OVA), Rubinstein *et al.* (83) found that, while almost all OT-I effector T cells were lost in the absence of IL-15, the loss was greater in the KLRG1^{hi} subset. In this study, the authors also attempted to show that IL-7 contributed to survival of KLRG1^{lo} CD127^{hi} effector CD8⁺ T cells. However, cell surface CD127 levels were not detectable, because an anti-IL-7R α neutralizing antibody was used to prevent IL-7R signaling (83). In addition, OT-1 cell numbers were not decreased by anti-IL-7R α antibody or by transfer of OT-I cells into IL-7-deficient mice (83). We have recently found that neutralization of IL-7 or loss of IL-15 resulted in a significant loss of KLRG-1^{lo}CD127^{hi} CD8⁺ T cells and that this was additive when both cytokines were blocked (Tripathi *et al.*, manuscript submitted). Thus, IL-15 appears to play a more critical role than IL-7 in promoting effector T-cell survival, although there may be redundancy in a small sub-population of effector T cells.

It remains unclear whether KLRG-1^{hi}CD127^{lo} and KLRG-1^{lo}CD127^{hi} cells represent two separate lineages of cells that are committed at the effector stage or whether there is some plasticity in expression of these markers. One problem with using markers to identify cell populations is that the stability of expression of particular cell surface markers is often unclear. This is especially true for expression of cytokine receptors, whose regulation is complex and dynamic and often influenced in an autoregulatory fashion (114). Indeed, expression of CD127 is extremely dynamic in the immune system. During thymocyte development, CD127 is repressed at the DP stage and then upregulated in single positive cells (115). Further, IL-7 can repress expression of IL-7R, at least in an *in vitro* setting (114). However, a recent paper suggested that in effector CD8⁺ T cells, expression of CD127 is controlled by opposing actions of the transcriptional repressor growth factor independence-1 (gfi-1) and transcriptional activator GA-binding protein- α (GABP- α) (116). Although the initial repression of CD127 in activated CD8⁺ T cells was independent of gfi-1, the re-expression of CD127 was associated with histone H3K9 deacetylation likely via HDAC1 (116). Further, cell transfer experiments have showed that viral-specific KLRG-1^{hi}CD127^{lo} effector CD8⁺ T cells did not readily convert to a CD127^{hi} phenotype unless they were deficient in gfi-1 (116). In addition, cell sorting and gene microarray experiments have identified different 'gene expression signatures' of these two cell populations, and both of these were different from those of memory T cells (79,117,118). Thus, it is likely that further education is required for development of effector T cells into full-fledged memory T cells.

If indeed the KLRG-1^{hi}CD127^{lo} cells represent terminally differentiated effector cells that will not enter the memory compartment, then one would expect that promoting their survival would not affect memory development, because the numbers of memory precursor cells are already determined prior to contraction. However, our and others' recent data suggest a more complex picture. In a mouse model of *L. major* infection, we found that the absence of Bim significantly increased effector T-cell responses (61). Further, when Bim-deficient mice were re-challenged with *L. major*, they had significantly improved memory compared to wildtype mice (61). In other models where antigen-specific T cells are more easily tracked, the loss of Bim affords survival of both CD127^{lo} and CD127^{hi} effector T cells (58). Another group recently showed that adoptively transferred Bim-deficient CD8⁺ T cells survived better than their wildtype counterparts and that they were enriched in KLRG-1^{hi}CD127^{lo} cells at later times after infection (64). Regardless of the cell surface markers, both studies came to the same conclusion that in the absence of Bim, functional memory CD8⁺ T cells were significantly increased, indicating that Bim is essential for limiting the number of effector T cells available to enter the memory pool.

Why make so many effector T cells if most of them are destined to die, especially given that they die so quickly after the peak of the response? It is possible that excessive amounts of effector T cells are produced to clear infection as quickly as possible. The way to overcome their short half-life is to produce them *en masse*. Their short half-life, in turn, ensures that they will not overtake the lymphoid compartment or stay around to cause prolonged immunopathology. Thus, the amount of memory induced is simply a byproduct of effector T cells that escaped cell death. The lack of complete elimination of these effector cells allowed them to persist and protect from re-infection. If this is the case, what would the selection pressure be for establishing a memory compartment? If an animal has survived an infection once, why would they need memory cells to protect them from a secondary infection again? It is likely that those animals better able to rid themselves of a secondary infection were more fit and able to pass on these traits. Indeed, the intermittent re-activation of these cells by secondary infection may have selected for animals that had enough response to protect from a second infection but not too much to cause fatal hyper-responsiveness. This sort of pressure may have selected for animals in which some effector T cells are programmed early during the effector response with attributes that facilitate their development into memory T cells. Others may hang around long enough to get with the program.

Future directions

Over the last 10-15 years, significant progress has been made in our understanding of kinetics of T-cell responses to infectious pathogens. In general, the peak of the response is very short and most effector T cells die during the response. While early *in vitro* studies suggested a role for death receptors, our and others' data suggest that Bim is critical for the culling of T-cell responses *in vivo*. Further, examining the cell death signaling by Bcl-2 family members has revealed a highly regulated process driven by specific interactions between family members, rather than overall levels of pro- versus anti-apoptotic molecules. Despite these advances, several questions remain, especially regarding mechanism(s) that control Bim function in T cells. Although Bcl-2 is one mechanism that restrains Bim, it is likely that several other mechanisms contribute. For example, the contribution of transcriptional and post-transcriptional regulation of Bim as well as the potential for other anti-apoptotic molecules to combat Bim in activated T cells remains unclear. In addition, how this regulation is controlled in subpopulations of activated T cells is also currently unknown. Finally, while the balance of Bim and Bcl-2 appears critical for culling CD4⁺ T-cell responses, it is unclear if the paradigms developed for survival and memory development of CD8⁺ T cells will be the same for CD4⁺ T cells. Understanding these interactions is not just an academic exercise, as further elucidation of these mechanisms

could also be relevant to potential therapeutic interventions. Indeed, small molecule BH3-mimetics are being developed for cancer therapeutics and one could envision their use for ablating populations of activated T cells in autoimmune and/or hypersensitivity conditions. Conversely, antagonists of Bim are also being developed for potential use as vaccine adjuvants. Together the ability to manipulate Bim function and/or expression could prove to be a potent immunomodulator with broad therapeutic implications.

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Fig. 1. Kinetics of a typical T-cell response to acute infection

Following their development in the thymus, newly generated T cells enter the secondary lymphoid organs and are maintained there as naive T cells. After encountering antigen-bearing, activated antigen-presenting cells, clonal pools of T cells undergo massive expansion. The majority of these expanded cells die, while some remain and become memory T cells. These memory cells are maintained in the tissues as well as the secondary lymphoid organs.

Fig. 2. Mechanisms controlling Bcl-2 expression and function in T cells

IL-7 and IL-15 signal through Stat5 to drive Bcl-2 expression in T cells. Bcl-2 is negatively regulated in T cells by at least three distinct mechanisms. First, cessation of cytokine signaling could reduce Bcl-2 by failing to maintain sufficient levels of active Stat5. Second, TGF- β R signaling could impair Bcl-2 expression, although the underlying mechanisms remain unclear. Third, ROS can also negative regulate Bcl-2 expression, potentially by negatively affecting CREB driven transcription of Bcl-2.