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The roles of Bim-dependent apoptosis in controlling the responses of CD4+ T helper lymphocytes

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The roles of Bim-dependent apoptosis in controlling the responses of CD4+ T helper lymphocytes

by

Luke Barron

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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Biomedical Sciences

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GRADUATE DIVISION

of the

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I dedicate my dissertation to the UCSF scientists who became my friends and colleagues. Whether I was curious, frustrated, in need of advice or a break, or had forgotten to order an antibody, you always welcomed me.

To everyone who left a door open to me – thank you.
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Finally, thanks to the mice. Although, if they really are hyper-intelligent pan-dimensional beings [2], I’m in a lot of trouble.

The roles of Bim-dependent apoptosis in controlling the responses of
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ABSTRACT

Apoptosis can regulate CD4+ T helper lymphocyte responses by eliminating dangerous, superfluous, and non-functional cells. I investigated the roles of Bim-dependent apoptosis in controlling the responses of CD4+ T cells exposed to a systemic self antigen, deprived of a growth factor, or transitioning from the effector to memory stages of an immune response.

Ova-specific DO11 TCR transgenic CD4+ T cells divide, then cease responding and die when transferred into sOva transgenic mice that express Ova as a systemic self antigen. Bim-deficient DO11 cells were equally activated but protected from death in sOva recipients. Both mRNA and protein expression of Bcl-2 family members suggested that apoptosis was triggered by a post-translational mechanism. Despite surviving, Bim-deficient DO11 cells ceased dividing, failed to produce cytokines, and caused no signs of autoimmune disease. This anergic phenotype was not caused by differentiation into FoxP3+ regulatory T cells and did not require CTLA-4 or PD-1. Instead, CD69 induction and a failure to re-express S1P1 may contribute to tolerance by trapping self-reactive T cells in lymphoid organs where constant antigen exposure causes anergy.
FoxP3+ regulatory T cells require IL-2 to survive, and IL-2-deficient mice develop fatal autoimmune anemia. Bim deficiency enabled FoxP3+ T cells to survive without IL-2 and promoted the survival of IL-2Rα negative DO11 FoxP3+ cells. However, mice lacking IL-2 and Bim still died of anemia, despite maintaining a normal percentage of FoxP3+ cells, and regulatory T cells from these animals suppressed poorly in an in vitro co-culture assay. Thus, regulatory T cells require IL-2 both to survive and to maintain their proper suppressive function.

Bim-deficient DO11 cells also survived the effector to memory transition of the CD4+ T cell responses to VSV-Ova infection and Ova-pulsed dendritic cell immunization. However, besides increasing the number of DO11 cells, Bim deficiency did not alter the effector or memory phenotypes. This suggests that memory cells are chosen by a stochastic process.
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CHAPTER ONE

Introduction

OVERVIEW

The adaptive immune system arises from a combination of two processes. First, the processes that assemble antigen receptor genes randomize the antigen specificities of developing T and B lymphocytes. This step creates the possibility that any infection will be recognized and provoke an antigen-specific immune response, even if the cause of the infection has never been encountered before. However, the randomization of antigen receptors also produces non-functional and self-reactive lymphocytes. Therefore, the second step in the generation of the adaptive immune system is to select for cells that express functional antigen receptors but do not cause autoimmune disease [3]. Selection continues after development, within mature lymphocyte populations, as cells encounter new peripheral antigens and compete for limited resources.

The selection of both the developing and mature lymphocyte populations is ultimately enforced by apoptosis, a form of programmed cell death [4]. Apoptosis eliminates damaged, dangerous, and superfluous cells in an ancient and highly conserved process. First described by the orderly manner in which apoptotic cells disassemble themselves [5] this form of cell death facilitates the clearance of debris without, importantly, triggering inflammation.
My doctoral thesis research investigated the roles of apoptosis in controlling the responses of CD4+ helper T lymphocytes. CD4+ T cells coordinate immune responses, “helping” other cell types function properly. When activated by infections, CD4+ T cells help B cells produce antibodies [6], promote the ability of CD8+ T cells to attack infected host cells [7], prompt dendritic cells to activate more T cells [8], and trigger macrophages to destroy pathogens [9]. CD4+ T cells are also essential to maintain immune tolerance to self antigens. Regulatory T cells, a subset of the CD4+ population, are self-reactive but inhibit rather than promote the responses of other T cells and antigen-presenting cells [10].

In chapters two and three, I describe our studies on how apoptosis contributes to peripheral T cell tolerance. Autoimmunity can be prevented by multiple mechanisms, either because redundant pathways evolved to make tolerance more robust or because different tolerance pathways govern separate subsets of self-reactive lymphocytes [11]. T cells that recognize self antigens can be deleted by apoptosis, either during thymic development or after export to the peripheral lymphoid organs. Alternatively, self antigen recognition can lead to anergy and T cells cease responding. Both deletion and anergy are cell intrinsic mechanisms of tolerance; each pathologic self-reactive cell must die or lose responsiveness to prevent autoimmunity. Alternatively, some self-reactive cells differentiate to become regulatory T cells. Since regulatory T cells suppress other lymphocytes, this tolerance pathway is cell extrinsic and may safeguard against failures in deletion or anergy.
We previously developed an experimental system where mature naïve CD4+ T cells recognize a systemic self antigen [12]. The self-reactive T cells mounted an abortive response, undergoing apoptosis and becoming anergic, which resulted in tolerance. In the studies in chapter two, my goal was to assess the contribution of apoptosis to peripheral T cell tolerance in this system. I identified the apoptotic pathway responsible for deleting CD4+ T cells encountering a systemic self antigen, inactivated this pathway, and determined whether apoptosis was necessary for peripheral tolerance.

Chapter three addresses a different mechanism of tolerance, suppression by regulatory T cells. This CD4+ T cell subset is uniquely dependent on the cytokine IL-2 for survival, and IL-2-deficient mice develop autoimmunity [13]. My goal was to determine whether regulatory T cells also require IL-2 in order to function normally and suppress autoimmunity. By disabling apoptosis, I restored the regulatory T cell populations in mice lacking IL-2. This finding enabled me to assess the suppressive function of IL-2-deficient regulatory T cells in vitro and in vivo.

Adaptive immunity provides a tremendous advantage to defense against infections by creating immune memory. Lymphocytes specific for a particular pathogen may be rare at first encounter, but a larger pool of more capable antigen-specific cells survive to mount a superior response upon re-infection. However, most of the activated CD4+ T cells do not survive the transition from the effector to memory stage of the immune response and are eliminated by apoptosis [14].
The processes determining which activated T cells survive to differentiate into the memory population remain an open question. In the studies in chapter four, I sought to identify the apoptotic pathway that contracts a large pool of activated effector T cells into the smaller, long-lived memory subset. The ultimate goal of this study was to identify the causes of memory differentiation by comparing the CD4+ T cells that normally died to those that survived. If the differentiation of memory T cells is a selective process, then this comparison might reveal the causes of selection.

PATHWAYS OF APOPTOSIS

Apoptosis proceeds through two independent upstream pathways that converge to activate the proteases that begin dismantling dying cells [4]. One apoptotic pathway is linked to mitochondria and inhibited by the Bcl-2 family of anti-apoptotic proteins. The other is triggered by the ligation of “death receptors,” such as Fas, belonging to the Tumor Necrosis Factor family.

A critical step in both the mitochondrial and death receptor pathways is the activation of caspases [15]. These proteases, divided into initiators and effectors, are synthesized as inactive proenzymes. Initiator caspases associate with adaptor proteins that aggregate in response to death-inducing stimuli. This aggregation brings initiator procaspases close together and changes their conformation to enable enzymatic activity. Once activated, initiator caspases cleave themselves and other caspases, creating an amplifying cascade of proteolysis. Of the 9 mammalian initiator caspases, the mitochondrial pathway
usually relies on caspase-9 while death receptor signaling activates caspase-8. Initiator caspases next cleave and activate their effector counterparts, caspases-3, -6, and -7, which are primarily responsible for the cleavage of cellular substrates that in turn lead to irreversible changes, such as DNA fragmentation, that guarantee cell death. The mitochondrial and death receptor pathways converge at the level of effector caspase activation as both caspases-8 and –9 activate caspase-3.

Fas signaling is perhaps the best-characterized pathway of death receptor-induced apoptosis [16]. T cells constitutively express Fas as a transmembrane cell surface protein. The intracellular tail of Fas binds to the Fas-Associated Death Domain (FADD), the adaptor protein that recruits the caspase-8 proenzyme. Ligation of Fas oligomerizes the receptor complex, recruits pro-caspase-8 to FADD, activates this caspase, and initiates the proteolytic cascade that causes apoptosis. Naïve T cells resist Fas-mediated apoptosis because they express little Fas Ligand and high levels of FLIP, which competes with pro-caspase-8 for binding to FADD. T cell receptor plus IL-2 stimulation promotes Fas Ligand and inhibits FLIP transcription, as well as boosting Fas expression, rendering activated T cells vulnerable to Fas-induced apoptosis.

The mitochondrial apoptotic pathway is governed by the interactions between three types of proteins in the Bcl-2 family [4]. One group inhibits apoptosis and includes Bcl-2, Bcl-X, and Mcl-1. The second group, most importantly Bak and Bax, are very similar in structure to Bcl-2 but instead cause apoptosis. The third, and largest, group of Bcl-2 proteins shares only the short BH3 region of homology with the other family members. One of these “BH3-
only” family members, Bim, has proven essential for T cell apoptosis in response to thymic negative selection, cytokine withdrawal, and increased intracellular calcium.

The interactions between the three types of Bcl-2 proteins have proven difficult to define, and fundamental contradictions between experimental results and the models explaining them are not yet resolved [17]. Still, several key points about this apoptotic pathway are generally accepted [18, 19]. First, both death-preventing Bcl-2 and death-promoting BH3-only proteins lie upstream of Bak and Bax in this apoptotic pathway. Self-association of Bak or Bax on the surface of mitochondria is either an essential step towards cell death, necessary to permeabilize mitochondrial membranes and release cytochrome c, or at least correlates with and promotes apoptosis. Anti-apoptotic Bcl-2 proteins inhibit Bak and Bax, possibly by direct binding. Finally, the BH3-only proteins bind directly to anti-apoptotic Bcl-2 family members, but the affinity of this binding varies enormously between different pro- and anti-apoptotic protein pairs.

Given these conclusions, the simplest mechanism to explain the mitochondrial apoptotic pathway is a sequence of two inhibitory events [20-22]. First, Bcl-2 and other anti-apoptotic proteins bind to and inhibit Bak and Bax on mitochondria. Second, in response to death-inducing stimuli, BH3-only proteins displace Bak and Bax by binding to Bcl-2. The freed pro-apoptotic proteins self-associate, possibly oligomerizing into pores, and permeabilize the outer mitochondrial membrane to release cytochrome c. Cytoplasmic cytochrome c in turn triggers caspase-9’s adaptor protein, Apaf-1, to oligomerize, initiating a cascade of caspase activation and proteolysis. Thus, BH3-only proteins inhibit
anti-apoptotic Bcl-2 proteins, which in turn inhibit their pro-apoptotic counterparts.

A different model contends that at least some BH3-only proteins, particularly Bim, are capable of binding directly to Bak or Bax. Furthermore, without such binding, Bak and Bax remain inactive and do not trigger apoptosis [20, 23]. In this case, the anti-apoptotic Bcl-2 proteins function by sequestering BH3-only members.

A third possible explanation arises from the observation that Bcl-2 can still inhibit apoptosis in Apaf-1 and caspase-9-deficient cells, including thymocytes undergoing negative selection [24, 25]. This finding suggests that Bcl-2 may inhibit an unidentified adaptor of another initiator caspase, and that loss of mitochondrial integrity amplifies and enforces apoptosis rather than initiates the process [22].

Although the details of mitochondrial-linked apoptosis add complexity and nuance not described here, these models seem to explain the basic sequence of events that cause apoptosis. However, they do not address the more proximal question: how are the BH3-only proteins triggered to initiate apoptosis?

**BIM-DEPENDENT APOPTOSIS IN LYMPHOCYTES**

My thesis research investigated apoptosis triggered by three seemingly disparate stimuli: chronic stimulation by a self antigen, deprivation of a growth factor, and the clearance of a foreign antigen. In each case, Bim proved the essential BH3-only protein required to induce the apoptosis of the responding
CD4+ T cells. Diverse mechanisms have been shown to switch on the Bim pathway, including transcription, alternative splicing, mRNA stabilization, subcellular localization, altered binding to both Bcl-2 family and other molecules, and protein degradation. However, the mechanisms that actually control Bim, and so initiate apoptosis, are not fully conserved between different types of cells – even within the hematopoietic lineage. This introduction therefore emphasizes the phenotype of Bim-deficient T lymphocytes and some of the possible ways Bim may be controlled in this cell type.

The fourth BH3-only gene found, Bim (Bcl-2 Interacting Mediator of cell death) was identified by two independent screens of proteins interacting with Bcl-2 [26] or Mcl-1 [27]. Alternative splicing of Bim’s messenger RNA produces at least three isoforms. T cells express more of the extra-long than the long isoform and little, if any, of Bim’s short species. While many other isoforms can be created, they seem not to be expressed in primary lymphocytes [22]. Overexpression experiments demonstrated that the additional exons attenuated apoptosis, presumably because they subject Bim to additional inhibitory checks.

Bim-deficient mice are viable and grossly normal at birth, although born at non-Mendelian ratios due to uncharacterized embryonic lethality prior to day E10 [28, 29]. In newborn mice, both thymic architecture and thymocyte phenotypes appear normal. The number of Bim-deficient thymocytes does not exceed wild-type levels, even with age. However, adult Bim-deficient thymic populations contain more CD4 and CD8 single-positive and fewer double-positive cells than normal.
In contrast, Bim deficiency increases the numbers of all peripheral leukocytes, including CD4+ T cells [28]. This lymphoid hyperplasia progresses with age but, while a larger fraction of CD44 high – CD62L low cells are present, the majority of the CD4+ T cell population retains a naïve phenotype [29].

Both thymic and peripheral Bim-deficient T cells are markedly resistant to apoptosis when deprived of growth factors. Culture in serum-free media induces apoptosis via Bim, as does depriving in vitro activated T cells of IL-2 [28, 29]. Bim deficiency also rescued the survival of thymic and peripheral T cells in mice lacking IL-7 [30] or Bcl-2 [31].

Double-positive thymocytes have been assayed for Bim-dependent apoptosis against an array of in vitro stimuli. Cells lacking Bim robustly resist death induced by ionomycin but remain normally sensitive to, or at best weakly protected from, γ-irradiation, dexamethasone, PMA, etoposide, staurosporine, tunicamycin, and Fas Ligand [28, 29].

Strongly self-reactive thymocytes are deleted by calcium-dependent signaling, but can be protected by overexpression of Bcl-2 [3]. Thus, the initial observations of Bim-deficient mice suggested that this BH3-only protein might initiate the apoptosis of developing T cells that recognize self antigens. This role for Bim has been proven in a variety of experimental systems. Bim-deficient thymocytes survived αCD3 or SEB injection, and OT-II TCR transgenic thymocytes lacking Bim resisted deletion caused by both endogenous Mtv-9 superantigen and immunization with cognate ova peptide [25, 32]. In addition, the absence of Bim rescued HY antigen-specific TCR transgenic T cells from deletion in male mice. Indirect evidence for Bim’s contribution to thymocyte
negative selection has also been found. Central T cell tolerance is thought to be compromised in NOD mice, and independent screens to identify causes of this defect implicated weak Bim induction in thymocytes undergoing negative selection [33, 34].

Bim also eliminates peripheral CD4+ and CD8+ lymphocytes following TCR stimulation. This role for Bim was first demonstrated in response to superantigen (SEB)-mediated deletion [35]. Peripheral CD8+ T cells responding to a cross-presented self antigen were also found to die in a Bim-dependent manner [36]. Furthermore, the effector population of Bim-deficient CD8+ T cells failed to contract after Herpes Simplex Virus [37] or Lymphocytic Choriomeningitis Virus infections were cleared [38].

The results outlined above demonstrate that both thymic and peripheral T cells can require Bim to undergo antigen-induced apoptosis. Moreover, Bim contributes to the deletion of auto-reactive B cells [39]. However, and contrary to both expectations and the initial report, Bim-deficient mice seem generally protected from autoimmune disease. On a mixed 129SV X C57BL/6 background, mice lacking Bim initially suffered ~50% mortality by 1 year of age [28]. Death was suggested to result from kidney failure, as antibody deposits could be detected in this organ. But, when fully backcrossed onto the C57BL/6 background, Bim no longer altered the survival of deficient mice [31]. A subsequent study with 129SV X C57BL/6 mice also found that Bim deficiency did not increase mortality even though the deficient mice exhibited elevated levels of all antibody isotypes and produced antibodies reactive to double-stranded DNA [29]. This later study examined lung, liver, heart, and kidney histology and
noticed no inflammation. Thus, at the cellular level, Bim can be required to
delete self-reactive lymphocytes and contributes to immune homeostasis even
though Bim-deficient mice remain healthy. This apparent contradiction might be
resolved if another mechanism of peripheral tolerance, such as anergy or
suppression by regulatory T cells, is capable of compensating for impaired
apoptosis.

Multiple mechanisms have been described to control Bim’s ability to
induce apoptosis, but how Bim is regulated in T cells in vivo remains an
unresolved question [22, 40].

Thymocytes do contain more Bim when stimulated via the TCR to induce
apoptosis [32, 41]. While Bim levels typically only rise 2-5 fold, this magnitude of
change could be significant since Bim hemizygous cells show intermediate
sensitivity to various death-inducing stimuli [28]. However, Bim expression need
not increase in order to initiate apoptosis. Superantigen-stimulated peripheral T
cells die without inducing Bim protein because they instead lose Bcl-2 expression
[35]. These and similar findings have led to the concept that a cell’s “decision” to
initiate apoptosis depends on the net interactions between its full suite of BH3-
only and anti-apoptotic Bcl-2 proteins, thus integrating many survival and death-
inducing stimuli into a finely-tuned outcome [18].

Nevertheless, simply increasing Bim transcription could be sufficient to
induce apoptosis, especially if the levels of other anti- and pro-apoptotic proteins
are narrowly balanced. At least during in vitro stimulation, apoptosis of double-
positive thymocytes does depend on Bim transcription, which in turn requires
calcium signaling and PKC, but not calcineurin [41]. Conversely, IL-2 has been
proposed inhibit apoptosis by repressing Bim transcription [42]. When activated by IL-2, Akt can phosphorylate and thus inactivate FoxO3A / FKHRL1, preventing this transcription factor from driving Bim expression. Transcript stability may serve as an additional control point as cytokines have recently been shown to alter the association of Bim mRNA with heat shock proteins and other chaperones [43].

The level of Bim protein may be regulated by degradation as well as synthesis. Bim can be phosphorylated on multiple serine and threonine residues, and the phosphorylated forms of Bim are lost in thymocytes dying in culture [44]. Subsequent investigations demonstrated that Erk1/2 can phosphorylate Bim, and that the phosphorylated proteins were then ubiquitinated, marking them for proteasomal degradation [45]. Since growth factor signaling pathways often include Erk, depriving T cells of IL-7 or IL-2 might induce apoptosis by halting Bim’s degradation. Other kinases and phosphatases might similarly alter the level of Bim protein in response to different stimuli. For instance, TCR-stimulation of thymocytes caused a discordance between Bim mRNA and protein levels but not Erk-dependent phosphorylation of Bim [41].

One of the first described means of controlling Bim was to change the protein’s subcellular location and sequester it to the microtubule-binding complexes [46]. However, such sequestration has not been observed in T cells [47]. Instead, in T cells, Bim constitutively localizes to the outer mitochondrial membrane and portions of the endoplasmic reticulum where it would be in close proximity, if not already bound to, Bcl-2 proteins.
Rather than being separated by distance, Bim might instead be separated from Bcl-2’s inhibition by conformational changes. In addition to promoting degradation, Erk-dependent phosphorylation was also shown to dissociate Bim from Mcl-1 [48]. This finding raises the possibility that phosphorylation, and perhaps other post-translational modifications, could act as checkpoints by determining Bim’s affinity for its binding partners.

The expression of multiple isoforms of Bim complicates all these possibilities, as some forms of regulation may not apply to shorter species. For example, Bim’s long isoform seems not to be controlled by proteasomal degradation because this species is missing the phosphorylation sites that induce ubiquitination [45]. These complications do suggest that multiple signaling pathways, and hence diverse death-inducing stimuli, converge on Bim and that this BH3-only protein functions as a key initiator of T cell apoptosis.

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CHAPTER 2

*Interplay of apoptosis and anergy in systemic T cell tolerance*

SUMMARY

In an effort to better understand peripheral T cell tolerance, our investigations distinguished between apoptosis and anergy as potential mechanisms for preventing autoimmunity. We identified Bim as the essential apoptosis-inducing trigger of deletion when mature CD4+ T cells encountered a systemic self antigen. We then proved that apoptosis was not necessary for tolerance in this experimental system. The induction of FoxP3 also failed to explain why the self-reactive cells ceased responding. Instead, we found that cell-intrinsic anergy was sufficient to tolerize T cells, and anergy developed independently of the inhibitory receptors CTLA-4 or PD-1. We suspect that chronic antigen stimulation induced anergy by desensitizing or blocking proximal T cell receptor signaling. By maintaining high CD69 and low S1P1 expression, chronic antigen stimulation may also have trapped self-reactive T cells in lymph nodes. We propose that such entrapment may contribute to peripheral tolerance by giving no respite from antigen stimulation and hence no opportunity for T cells to escape anergy or regain their sensitivity.
BACKGROUND AND AIMS

Multiple mechanisms contribute to the induction and maintenance of peripheral T cell tolerance and it is unclear how these mechanisms interact with each other. Apoptosis deletes self-reactive T cells, and anergy renders them ineffective. But tolerogenic antigens usually trigger both apoptosis and anergy [49]. Whether deletion or anergy alone would be sufficient to induce tolerance is often not determined and could well depend on characteristics of the experimental system.

The goal of this series of studies was to separate apoptosis from anergy and define the contribution of each control mechanism to systemic T cell tolerance. We first sought to identify essential components of the apoptotic pathway(s) triggered by a systemic self antigen. We then anticipated we would be able to protect self-reactive T cells from deletion and determine whether apoptosis played a necessary role in peripheral T cell tolerance.

Our first aim was to identify which apoptotic pathway was activated in our experimental system and to protect T cells from this pathway. Two pathways of apoptosis exist in lymphocytes [4]. One, linked to mitochondria, is checked by Bcl-2 and related proteins and triggered by a family of sensors sharing only Bcl-2’s BH3 domain. The other pathway, independent of the first, is activated by the engagement of death receptors at the cell surface.

We hypothesized that Bim, a pro-apoptotic BH3-only sensor, would be essential to trigger apoptosis and that Bim-deficient T cells would not be deleted. Of all the BH3-only family members, only Bim plays a well established role in
eliminating activated T cells [50]. Bim also seemed a likely candidate because it is triggered by both TCR engagement and cytokine deprivation, both stimuli we expected T cells responding to a systemic self antigen to experience. Self-reactive thymocytes are deleted by Bim [32], and Bim deficiency rescues T cell development and survival in the absence of IL-7 [30]. Also, in vitro, culturing activated T cells with IL-2 reduces Bim-dependent death [29, 51].

We considered two alternative hypotheses in the event that Bim proved dispensable for deletion in our system. First, a different BH3-only protein might trigger apoptosis. To test this hypothesis, we used T cells overexpressing Bcl-X [52] which are thought to resist apoptosis induced by any BH3-only family member [50].

We also considered that the death receptor Fas might cause deletion because Fas deficient lpr T cells resisted apoptosis in other experimental systems of peripheral tolerance [53, 54]. Other death receptor ligands, TNF [55] and TRAIL (shown for CD8+ T cells; [56], have been shown to cause apoptosis in vivo. But, unlike the equivalent situation with BH3-only proteins, we had no practical tool for blocking signaling through all death receptors. All death receptors require FADD to initiate the apoptotic pathway, but a dominant negative construct of FADD also impaired T cell development, proliferation, and homeostasis [57].

If Bim proved essential for deletion in our system, our second aim was to determine whether changes in the expression of Bim, Bcl-2, or Bcl-X caused apoptosis. One of the most puzzling questions about this apoptotic pathway is what causes Bim to kill [22]. Bim, Bcl-2, and Bcl-X are already
expressed in naive T cells and form complexes associated with mitochondria [47]. This observation led to three general hypotheses explaining how Bim functions. First, the ratio of Bim to the anti-apoptotic regulatory proteins might increase. For example, injection of the superantigen SEB caused a drop in Bcl-2 in the responding Vβ8+ T cells [35]. Alternatively, cells can increase Bim expression via increased transcription or decreased degradation. Second, the key event in initiating the Bim pathway might be changes in the expression of other BH3-only sensors [18]. With more of another BH3-only protein present, fewer anti-apoptotic regulators would be available to inhibit apoptosis even if the levels of Bim and the regulators remain the same. Finally, the apoptotic potential of Bim might be determined post-transcriptionally. Bim can be spliced into at least three isoforms with different activities, and the longer isoforms can be phosphorylated in patterns that correlate with apoptosis.

By preventing deletion we could address our **third aim: to test whether apoptosis was necessary for peripheral T cell tolerance to a systemic self antigen.** We hypothesized that deletion would prove essential because, in our system, T cells should be unable to avoid stimulation. Without apoptosis even a rare failure to induce anergy could allow responsive T cells to accumulate. Then, driven by constant antigen stimulation, these few responsive cells could proliferate to form a population of non-tolerant, self-reactive T cells large enough to cause pathology.
RESULTS

The soluble Ova transgenic experimental system of tolerance

We devised a two part experimental system to study the CD4+ T cell response to a systemic self antigen. First, we chose the DO11.10 transgenic BALB/c mouse as a source of naïve antigen-specific CD4+ T cells. The DO11 mouse carries an αβ T cell receptor transgene specific for residues 323-339 of chicken ovalbumin peptide presented by the MHC class II molecule I-A(d). The ova-specific T cells that develop in these mice can be identified with KJ1.26, a monoclonal antibody specific for the DO11 TCR.

Second, we created the equivalent of a systemic self antigen for DO11 T cells. The soluble ovalbumin transgenic BALB/c mouse expresses ova from a transgene under the control of the metalliothionine promoter I [58]. Full-length ova protein is produced in the liver, secreted into the bloodstream, and circulates at a concentration of 10-20ng/mL throughout the sOva Tg mice.

Previous studies characterized the response of DO11 cells when exposed to systemic self antigen by adoptive transfer into sOva transgenic recipients, or by crossing the two strains of transgenic mice. These investigations focused primarily on the autoimmune reactions arising without endogenous lymphocytes and the generation and contribution of regulatory T cells to these reactions. The population of WT DO11 cells transferred into WT sOva transgenic recipients divided and expanded, then contracted, and the surviving cells were hyporesponse when restimulated [12]. Thus, apoptosis and anergy were
entangled in the sOva transgenic system. Tolerance depended on normal endogenous T and B cells [59] and was associated with an early burst of proliferation, the production of effector cytokine mRNA but not protein, and attenuated calcium flux in response to TCR stimulation [60]. We set out to untangle the processes of apoptosis and anergy in DO11 cells responding to systemic ova and to identify the causes of tolerance.

To expose CD4+ T cells to systemic self antigen, we adoptively transferred CFSE-labeled DO11 cells into sOva transgenic recipients. As a control, we transferred the same T cells into WT BALB/c recipients. To compare T cells that see self antigen with productively activated cells, we infected BALB/c recipients of naïve DO11 T cells with VSV engineered to produce Ova (see chapter 4). The Ova-specific DO11 cells were identified in lymph nodes and spleens by co-staining for CD4 and KJ1.26 by flow cytometry (Figure 1A).

Transfer into sOva Tg recipients activated the DO11 cells. Nearly all the DO11 cells in lymph nodes and spleen divided within 4 days, as shown by dilution of CFSE (Figure 1B). However, although the DO11 cells divided, they failed to produce cytokines. Encounter with self antigen did induce weak IL-2 production upon ex vivo restimulation 4 days after transfer. But this sOva transgenic IL-2 response was lost by day 7 and weaker than the BALB/c control if assayed
FIGURE 1
DO11 cells divided upon transfer into sOva Tg recipients but failed to produce cytokines.

CFSE-labeled DO11 cells were transferred into BALB or sOva Tg recipients. BALB recipients were left untreated (naive) or immunized with VSV-Ova (immunized). A) Detection of DO11 cells in unimmunized transfer recipients. BALB lymph nodes were stained with anti-CD4 and KJ1.26 7 days after transfer. Plots show the percentage of live cells in the DO11 gate. Excluding CFSE+ cells shows background staining. B) CFSE profiles of DO11 cells in lymph nodes and spleen of BALB and sOva Tg recipients 4 days after transfer. C) IL-2 and D) IFNγ cytokine responses after 4 and 7 days. Lymph nodes were restimulated 6 hours with ova peptide and analyzed by flow cytometry to determine the percentage of DO11 cells producing cytokine.
differently (Figure 1C and Figure 12A). More importantly, the DO11 cells never developed the ability to produce the effector cytokines IFNγ (Figure 1D) or, described later, IL-4 (Figure 12D) and IL-17 (Figure 16B). As noted, the characteristics of these self-reactive T cells implied that tolerance in the sOva transgenic system was induced by an abortive or incomplete activation [60].

**Mechanisms of apoptosis of self-reactive CD4+ T cells**

The number of DO11 cells increased to peak 4 days after transfer into sOva transgenic recipients [12]. This expansion was followed by a decline. By day 7 the number of DO11 cells fell back to the level of naive cells transferred into non-transgenic control BALB/c recipients. Within two weeks few, if any, DO11 cells were detected. Our next step was to examine the mechanism of the decline of the DO11 population.

We postulated that the decline in DO11 cell numbers was due to apoptotic death. To investigate this issue, we used DO11 cells with defects in different apoptotic pathways. Overexpression of Bcl-X retards all apoptotic pathways linked to mitochondria without, apparently, otherwise altering T cells. We obtained Bcl-X transgenic mice in which the Lck promoter drives constitutive overexpression of Bcl-X in T cells [52]. We then tested whether Bcl-X transgenic DO11 cells could survive in sOva transgenic recipients.
Donor DO11 Bcl-X transgenic cells exhibited the same naïve phenotype as their WT counterparts (data not shown). We co-transferred a 1 to 1 mixture of WT and Bcl-X transgenic DO11 cells into sOva Tg recipients. To distinguish the two DO11 populations, we used WT cells expressing the Thy1.1 allele and Bcl-X Tg cells expressing Thy1.2. We found equal numbers of WT and Bcl-X Tg DO11 cells 4 days later, at the peak of expansion (Figure 2A). But, unlike their WT counterparts, Bcl-X transgenic DO11 cells did not disappear within two weeks. And, over time, the ratio of the surviving populations increasingly favored cells overexpressing Bcl-X in sOva transgenic but not WT recipients (Figure 2B). This implied that WT DO11 cells were killed by a Bcl-X-repressible apoptotic pathway when exposed to systemic self antigen.

We chose to co-transfer WT and Bcl-X transgenic cells into the same recipient because it reduced the variability of our results. By comparing the WT and transgenic populations within the same animal, we controlled for variation in engraftment, kinetics, and the magnitude of expansion between mice. This experimental design did create the possibility that competition between WT and apoptosis-resistant cells biased the results. However, we observed a similar protection of Bcl-X transgenic to WT DO11 cells when the two populations were transferred separately into sOva transgenic recipients (data not shown).

We next checked that overexpression of Bcl-X had not altered the DO11 response. WT and Bcl-X transgenic DO11 cells displayed indistinguishable CFSE profiles, indicating equivalent proliferation (Figure 3A). Both populations also equally upregulated CD69, an early marker of T cell stimulation (Figure 3B).
FIGURE 2
WT DO11 cells were deleted after transfer into sOva Tg recipients, but Bcl-X Tg DO11 cells were protected.

WT DO11 Thy1.1 and Bcl-X Tg DO11 Thy1.2 cells were labeled with CFSE, mixed 1:1, and co-transferred into BALB or sOva Tg recipients. **A)** Number of DO11 cells in lymph nodes 4, 7, and 12 days after transfer. **B)** Ratio of Bcl-X Tg to WT DO11 cells.
FIGURE 3
WT and Bcl-X Tg DO11 cells were equally activated in sOva Tg recipients.

WT DO11 Thy1.1 and Bcl-X Tg DO11 Thy1.2 cells were labeled with CFSE, mixed 1:1, and co-transferred into BALB or sOva Tg recipients. A) CFSE profiles of WT and Bcl-X Tg DO11 cells in lymph nodes. Histograms are scaled to the absolute number of DO11 cells. Alternatively, WT and Bcl-X Tg DO11 were transferred separately. B) Expression of CD69 in lymph nodes 3 days after separate transfer. Endogenous levels of CD69 expression are shown for comparison.
Since WT and Bcl-X transgenic DO11 cells mounted an equal initial response to systemic ova, differences in activation could not explain the increase in Bcl-X overexpressing cells.

If improved survival explained the increase of Bcl-X transgenic relative to WT DO11 cells, then the transgenic cells should express higher levels of Bcl-X. We therefore confirmed that the transgenic DO11 cells expressed 2-3 fold more Bcl-X than their WT counterparts (Figure 4). Higher levels of Bcl-X were present in the transgenic cells both before (day 3) and after (day 6) the response peaked and showed no relationship to the number of times the DO11 cells divided (data not shown). We concluded that WT and Bcl-X transgenic DO11 cells were equivalently activated, but higher expression of Bcl-X in the transgenic cells prevented apoptosis.

In lymphocytes, Bcl-X does not affect sensitivity to apoptosis induced by death receptor signaling [61]. Since overexpression of Bcl-X protected DO11 cells, death receptors were not sufficient to delete these CD4+ T cells in the sOva transgenic system. Still, death receptors might explain the ~2 fold decline in Bcl-X transgenic DO11 cells between days 4 and 7 (Figure 2A). Fas seemed a likely candidate because death via the Fas pathway is associated with repeated T cell stimulation [62].

To test whether the Fas death receptor caused any deletion in the sOva transgenic system, we used DO11 cells from mice homozygous for the
**FIGURE 4**

WT DO11 expressed less Bcl-X than transgenic counterparts.

WT and Bcl-X Tg DO11 were labeled with CFSE and transferred into sOva Tg recipients. **A)** Bcl-X staining of WT and Bcl-X Tg DO11 cells in lymph nodes after 3 and 6 days. Isotype staining of transgenic cells is shown as a negative control. **B)** Mean fluorescence intensity of Bcl-X in DO11 cells.
FIGURE 5
Fas deficiency did not protect from deletion in sOva Tg recipients.

WT and Fas-deficient Ipr DO11 cells were labeled with CFSE and transferred into BALB or sOva Tg recipients. **A** Fas expression on DO11 cells before transfer. **B** CFSE profiles of WT and Ipr DO11 cells in lymph nodes 4 days after transfer. **C** Number of DO11 cells in lymph nodes 4 and 7 days after transfer.
lymphoproliferation (lpr) mutation. These mice express much reduced levels of Fas (Figure 5A;[63]). We compared the response of WT and lpr DO11 cells transferred into WT or sOva transgenic recipients. The absence of Fas did not change the proliferation or peak expansion of the DO11 cells encountering ova (Figure 5B, C). And, like WT DO11 cells, lpr cells were deleted between days 4 and 7. Thus, Fas was neither required for nor contributed to the apoptosis of self-reactive CD4+ T cells following recognition of systemic antigen.

Since Bcl-X proved protective, we next considered which death-inducing “sensor” might be essential in the mitochondria-linked apoptotic pathway engaged in our experimental system. We suspected Bim: TCR signaling and cytokine deprivation both trigger this sensor, and transfer into sOva transgenic mice leads to chronic antigen exposure with little and transient IL-2 production. We therefore obtained Bim-deficient knockout mice [28] to test the role of Bim-triggered apoptosis in this model of peripheral tolerance.

Bim KO donor DO11 cells displayed a naïve phenotype (data not shown). We co-transferred equal numbers of WT and Bim KO DO11 cells into WT or sOva transgenic recipients. Naïve WT and Bim KO cells engrafted equally and Bim did not change the peak expansion of DO11 cells encountering systemic ova, at day 4. But, unlike WT cells, the Bim-deficient cells survived encountering systemic self antigen (Figure 6). While the number of Bim-deficient DO11 cells declined ~2 fold between days 4 and 8, the drop in WT cells was ~10 fold greater.
FIGURE 6
Bim deficiency protected DO11 cells from deletion in sOva Tg recipients.

WT DO11 Thy1.1 and Bim KO DO11 Thy1.2 cells were labeled with CFSE, mixed 1:1, and co-transferred into BALB or sOva Tg recipients. A) Number of DO11 cells in lymph nodes 4, 8, and 13 days later. B) Ratio of Bim KO to WT DO11 cells.
Furthermore, the WT population disappeared entirely while the number of Bim deficient DO11 cells remained stable at least as long as 8 weeks after transfer (data not shown).

The absence of Bim did not alter the proliferation (Figure 7) or initial activation (Figure 8) of DO11 cells in sOva transgenic mice. Both WT and Bim KO cells expressed high levels of CD69 and low levels of IL-7Rα, as predicted of recently stimulated T cells. Both also induced OX-40, a secondary costimulatory receptor, and both expressed high levels of CD44 and low levels of CD62L. Thus, WT and Bim KO DO11 cells exhibited an equivalent phenotype characteristic of recently activated T cells.

How Bim initiates apoptosis remains unclear. One possibility is that the ratio of pro-apoptotic to anti-apoptotic proteins changes, freeing Bim from inhibition by Bcl-X and its family members. We therefore tracked the messenger RNA and protein levels of Bim and the likely antagonists of mitochondrial-associated apoptosis in DO11 cells following transfer into sOva transgenic recipients.

To measure expression at the mRNA level, DO11 cells were isolated by cell sorting 1 and 4 days after transfer into BALB or sOva transgenic recipients. RNA was extracted from the purified cells, reverse transcribed, and this cDNA was analyzed by quantitative PCR. Relative to the level in naïve cells, Bcl-2 mRNA
FIGURE 7
WT and Bim KO DO11 cells proliferated equivalently in sOva Tg recipients.

WT DO11 Thy1.1 and Bim KO DO11 Thy1.2 cells were labeled with CFSE, mixed 1:1, and co-transferred into BALB or sOva Tg recipients. CFSE profiles of lymph nodes are shown, scaled to the absolute number of DO11 cells.
FIGURE 8
WT and Bim KO DO11 cells displayed an equivalent, activated phenotype in sOva Tg recipients.

WT DO11 Thy1.1 and Bim KO DO11 Thy1.2 cells were co-transferred into BALB or sOva Tg recipients. Peripheral lymph nodes were analyzed on day 4 for expression of A) CD69, B) IL-7Rα, C) OX-40, D) CD44, and E) CD62L. Expression on endogenous CD4+ T cells is shown for comparison.
levels increased ~4 fold between 1 and 4 days after transfer into sOva transgenic recipients (Figure 9A). Bcl-X mRNA levels increased earlier, rising 30-40 fold within the first day and remaining ~15 fold above the naïve level after 4 days (Figure 9B). Mcl-1, another anti-apoptotic member of the Bcl-2 family, showed little change in its mRNA level (Figure 9C) but it is known to be regulated by altered ubiquitination and degradation rather than by varied transcription [64].

We found that transfer into sOva transgenic recipients also increased the level of anti-apoptotic proteins. DO11 cells analyzed by intracellular staining and flow cytometry showed an increase in Bcl-2 and Bcl-X protein expression 1 day after transfer (Figure 10). Both Bcl-2 and Bcl-X levels remained elevated through day 7, by which time most cells had been deleted. Thus, apoptosis in the sOva transgenic system seemed not to be caused by the loss of anti-apoptotic Bcl-2 or Bcl-X expression.

It was possible that any DO11 cell that reduced its anti-apoptotic proteins’ expression might die rapidly, and so not be detected. To rule out this possibility we also checked the level of Bcl-2 and Bcl-X proteins in Bim deficient DO11 cells 5 days after transfer. If apoptosis were associated with a decline in these molecules, then the protection conferred by Bim deficiency should also reveal a second population of DO11 cells expressing little Bcl-2 and Bcl-X that would otherwise have died. Instead, all Bim deficient DO11 cells expressed higher levels of Bcl-2 and Bcl-X in sOva transgenic than BALB recipients (Figure 11). The absence of Bim did lead to an abnormally low basal level of Bcl-2 protein, as
**FIGURE 9**
mRNA expression of genes in the Bim pathway did not correlate with apoptosis in sOva Tg recipients.

CFSE-labeled WT DO11 cells were transferred into BALB or sOva Tg recipients. DO11 cells were isolated 1 and 4 days later by cell sorting and processed to yield cDNA. Quantitative PCR was performed to measure mRNA levels of A) Bcl-2, B) Bcl-X, C) Mcl-1, and D) Bim. Results were normalized to HPRT and displayed as the ratio of expression in 2 sOva Tg recipients (white and gray bars) relative to the average expression in 2 BALB recipients. The day 4 sOva Tg group contained only divided cells.
FIGURE 10
Protein expression of genes in the Bim pathway did not correlate with apoptosis in sOva Tg recipients.

WT DO11 cells were transferred into BALB or sOva Tg recipients. Lymph nodes were analyzed by flow cytometry for A) Bcl-2 and B) Bcl-X expression in DO11 cells 1, 2, 4, and 7 days after transfer. C) Bim expression was similarly analyzed after co-transfer of WT DO11 Thy1.1 and Bim KO DO11 Thy1.2 cells. Bim-deficient cells served as a negative control for specific staining. D) Ratio of Bcl-2, Bcl-X, and Bim expression (mean fluorescence intensity) in DO11 cells transferred into sOva Tg relative to BALB recipients.
FIGURE 11

Apoptosis was not caused by loss of Bcl-2 or Bcl-X.

WT and Bim KO DO11 cells were transferred into BALB and sOva Tg recipients. Lymph nodes were analyzed on day 5 by flow cytometry to measure A) Bcl-2 and B) Bcl-X expression in DO11 cells. C) Results are presented as the average mean fluorescence intensity of Bcl-2 and Bcl-X staining in the four groups.
noted by others [35]. But, both Bcl-2 and Bcl-X expression rose close to WT levels in sOva transgenic recipients. We concluded that apoptosis following encounter with systemic antigen could not be caused by a loss of anti-apoptotic Bcl-2 or Bcl-X.

Instead, apoptosis might be triggered by an increase in Bim expression. We found that Bim mRNA levels increased 4 to 6 fold in DO11 cells transferred into sOva transgenic relative to BALB recipients (Figure 9D), but less than 2 fold at the protein level (Figure 10). Since the increase in Bim was at all times smaller than the increase in Bcl-2 plus Bcl-X (Figure 10D), a change in Bim expression also cannot explain what caused apoptosis in the sOva transgenic system.

The absence of a correlation between Bcl-2, Bcl-X, and Bim expression and survival suggested that a post-transcriptional mechanism of Bim regulated apoptosis to systemic self antigen. Possible explanations include changes in mRNA splicing to favor shorter Bim isoforms, Bim phosphorylation, and induction of BH3-only proteins besides Bim. But, setting aside these possibilities, the trigger causing Bim to delete DO11 cells in the sOva transgenic system is different from that previously described for SEB-induced T cell apoptosis [35]. Activation by SEB caused a drop in Bcl-2 and Bcl-X, while transfer into sOva mice boosted the levels of these anti-apoptotic proteins. Thus, an increase in the Bim to Bcl-2 ratio cannot generally explain what activates this pathway of T cell apoptosis.
Anergy of self-reactive CD4+ T cells

Transfer of WT DO11 cells into sOva transgenic recipients induced anergy as well as deletion. Within five days WT DO11 cells lost their responsiveness upon in vitro and in vivo restimulation [12]. Although protected from deletion, Bim KO DO11 cells still lost their responsiveness and became anergic, just as their WT counterparts (Figure 12). We transferred WT or Bim KO DO11 cells into BALB recipients, mice immunized with ova peptide-pulsed dendritic cells, or sOva transgenic recipients. Five days later we sorted peripheral lymph nodes and restimulated the purified DO11 cells with antigen presenting cells and graded doses of ova peptide. Both WT and Bim KO DO11 cells from sOva transgenic mice had lost the ability to produce IL-2, proliferated less than their naïve counterparts, and had not developed the potential to produce IFN\(\gamma\) or IL-4. Bim deficiency prevented apoptosis, but had not altered the induction of anergy.

Bim deficient DO11 cells also ceased dividing in vivo. We first noticed their cell cycle arrest when comparing CFSE profiles of the same population of DO11 cells over time. We observed little CFSE dilution beyond four days after transfer (Figure 7, compare day 4 to day 13). We confirmed that the surviving DO11 cells ceased to proliferate in vivo by measuring their uptake of BrdU (Figure 13). We treated BALB and sOva transgenic recipients with BrdU for the first or third week after transfer of Bim deficient DO11 cells. This assay proved less sensitive than CFSE (compare Figure 13 to Figure 1B), perhaps because BrdU levels in vivo decline within hours of injection [65]. Half the DO11 cells stained positive for BrdU – and so had entered the cell cycle – during the first week.
FIGURE 12
Bim deficiency did not prevent anergy.

WT and Bim KO DO11 were labeled with CFSE and transferred into BALB or sOva Tg recipients. BALB recipients were left untreated (naïve) or immunized with ova-pulsed DCs (immunized) one day later. On day 5, all (naïve) or divided (immunized, sOva Tg) DO11 cells were purified from lymph nodes by cell sorting. Sorted cells were restimulated in vitro with splenic APCs and varied concentrations of ova peptide. A) IL-2 production after 36 hours, measured by ELISA. B) Proliferation after 4 days, measured by 3H-thymidine incorporation. C) IFNγ and D) IL-4 production, measured after 3 days by ELISA.
FIGURE 13
Bim-deficient DO11 cells ceased dividing in vivo.

Bim KO DO11 cells were transferred into BALB or sOva Tg mice. Recipients were treated with BrdU for the first or third week after DO11 transfer, then analyzed by intracellular staining and flow cytometry. **A)** BrdU and isotype control stains, gated on DO11 cells in lymph nodes. **B)** Percentage of DO11 cells staining positive for BrdU. Equal labeling of endogenous CD4+ KJ- cells is shown as a technical control.
following transfer. But, when we measured BrdU uptake from the second to third week after transfer, the DO11 cells in sOva transgenic recipients proliferated even less than their naïve counterparts. The absence of BrdU+ DO11 cells was not due to unequal labeling as the uptake of BrdU by the endogenous CD4+ KJ1.26-population was the same in all groups. Bim deficiency permitted survival, but the surviving T cells still underwent cell cycle arrest.

We followed the recipient mice up to 8 weeks without observing any signs of autoimmune disease. DO11 donor cells cause a wasting disease and severe skin inflammation in RAG-deficient sOva transgenic recipients [59]. But Bim-deficient DO11 cells caused neither symptom in WT sOva transgenic mice (data not shown).

Without Bim, encounter with systemic self antigen resulted in a population of CD4+ T cells that did not proliferate or produce cytokines, but did have an activated phenotype. These characteristics are also true of regulatory T cells. We therefore determined whether DO11 cells induced FoxP3 and differentiated into regulatory T cells after transfer into sOva transgenic recipients.

We did find an early increase in the percentage of WT and Bim deficient DO11 cells expressing FoxP3 (Figure 14). After 4 days’ exposure to systemic ova, the percentage of FoxP3+ DO11 cells increased 5 to 10 fold versus cells transferred into BALB controls. Naïve DO11 populations were 2-3% FoxP3+ because of incomplete allelic exclusion by the ova-specific TCR transgene. RAG
FIGURE 14
Survival and anergy were not explained by differentiation into regulatory T cells.

Thy1.1 WT and Thy1.2 Bim KO DO11 cells were mixed 1:1 and co-transferred into BALB or sOva Tg recipients. Lymph nodes were analyzed 4 and 14 days later by intracellular staining and flow cytometry. A) FoxP3 expression in WT and Bim KO DO11 cells. The percentage of WT or KO DO11 cells staining FoxP3+ is shown. B) Number of FoxP3+ and FoxP3- DO11 cells in peripheral lymph nodes after 4 and 14 days. One-sided error bars are shown for clarity.
-deficient DO11 donor cells do not express FoxP3 [66] although some still became FoxP3+ when transferred into sOva transgenic recipients (data not shown). We could therefore not distinguish whether the increase in FoxP3+ cells was caused by new expression in previously FoxP3- cells, or whether the initial FoxP3+ cells divided preferentially.

However, all the WT DO11 cells died by day 14 regardless of their FoxP3 expression. And, in the Bim deficient population, the percentage of FoxP3+ DO11 cells returned close to the naïve fraction. This finding suggested that FoxP3 expression was only transient. WT FoxP3+ DO11 cells were not protected from apoptosis, and the survival of their Bim deficient counterparts was not explained by differentiation into regulatory T cells. The FoxP3+ fraction might initially have contributed to the inability of purified DO11 cells to proliferate and produce cytokines upon ex vivo restimulation (Figure 12). But the Bim deficient DO11 cells remained anergic in vivo (Figure 13) long after the FoxP3+ population returned to nearly naïve levels.

Contrary to our hypothesis, protecting self-reactive T cells from apoptosis failed to break tolerance to a systemic antigen. FoxP3 induction within the donor population did not explain this outcome. We expected that repeated antigen stimulation would drive the proliferation of at least a subset of T cells if these cells were protected from deletion. Instead we found a completely anergic population, perhaps because prolonged antigen exposure itself led to anergy.
Since peripheral tolerance seemed to depend on anergy, we hoped to identify molecules necessary for inducing anergy in our experimental system. T cell inhibitory receptors play essential if complex roles in anergy [67]. We noticed two such candidates while characterizing the phenotype of T cells responding to systemic self antigen. Transfer into sOva transgenic but not BALB recipients induced and sustained the expression of CTLA-4 and PD-1 (Figure 15).

To test whether either of these receptors contributed to apoptosis or induced anergy, we treated sOva transgenic recipients with antibodies against CTLA-4 or PD-1 during the first week following the transfer of WT DO11 cells. We began treatment the day prior to transfer so that the entire DO11 response occurred under the cover of the antibody blockade. Neither treatment increased the number of surviving WT cells or promoted their proliferation (Figure 16). Furthermore, both blockades failed to enable any IL-2, IFNγ, or IL-2 production after in vitro restimulation. CTLA-4 but not PD-1 blockade may have allowed <5% of DO11 cells to produce IL-17, but at little more than background levels. While possible that the blockades did not prevent all CTLA-4 or PD-1 engagement, a similar regimen completely reversed tolerance in a different experimental system [68]. Moreover, we also had found that CTLA-4 deficient DO11 cells were still deleted and tolerized in sOva transgenic mice [69]. We concluded that, although CTLA-4 and PD-1 are induced in our system, inhibition by these receptors was not responsible for apoptosis or anergy.
FIGURE 15
Transfer into sOva Tg recipients induced CTLA-4 and PD-1.

Thy1.1 WT and Thy1.2 Bim KO DO11 cells were mixed 1:1, co-transferred into BALB or sOva Tg recipients, and lymph nodes were analyzed 4 and 14 days later by flow cytometry. A) CTLA-4 (surface and intracellular) and B) PD-1 expression on WT and Bim KO DO11 cells.
FIGURE 16
Antibody blockade of CTLA-4 or PD-1 failed to prevent apoptosis or break tolerance.

WT DO11 cells were labeled with CFSE and transferred into sOva Tg recipients. As controls, CFSE-labeled Bim KO DO11 cells were transferred into sOva Tg mice and BALB mice and immunized with ova-pulsed DCs. All sOva Tg recipients were treated with rat IgG, αCTLA-4, or αPD-1 antibodies on days −1, 0, 1, 3, and 5. After 7 days lymph node and spleen cell suspensions were restimulated overnight with ova peptide and stained for analysis by flow cytometry. A) Number of DO11 cells present in lymph nodes of sOva Tg recipients. B) Cytokine versus CFSE profiles of WT DO11 cells from sOva Tg lymph nodes. DC-stimulated Bim KO DO11 cells from spleen are shown as a positive staining control. C) Percentages of DO11 cells producing cytokines.
Migration of T cells responding to a systemic self antigen

One potential complication of our studies was that the activated DO11 cells might migrate into non-lymphoid tissues. Since Ova was secreted into the serum, we expected DO11 cells to emigrate from lymph nodes and the spleen and home to other organs. For instance, intravenous injection of Ova peptide plus adjuvants or Ova-pulsed DCs enabled DO11 cells to migrate into the lungs. However, we were unable to detect DO11 cells in the lungs of sOva transgenic recipients 6 days after transfer even when the donor cells were protected from apoptosis by overexpression of Bcl-X (Figure 17A).

This finding suggested an additional implication of CD69’s high and sustained expression on chronically activated DO11 cells. T cell emigration from lymph nodes is regulated by reciprocal expression of CD69 and sphingosine-1 phosphate receptor (S1P1; [70]). Activation induces CD69 and represses S1P1 expression; cells must re-express S1P1 before migrating out of lymph nodes. As DO11 cells in sOva transgenic lymph nodes remain perpetually CD69+, we measured these cells’ S1P1 mRNA levels by quantitative PCR (Figure 17B). Activation by transfer into sOva transgenic recipients caused a greater than 10-fold drop in S1P1 versus naïve DO11 cells in BALB recipients. This decrease in S1P1 occurred within the first day and was not reversed by day 4. This raises the possibility that trapping T cells recognizing widespread self antigens in lymph nodes contributes to tolerance.
FIGURE 17
DO11 cells may have been unable to migrate into non-lymphoid tissues.

Bcl-X Tg DO11 cells were labeled with CFSE and transferred into sOva Tg or BALB mice immunized with intravenous ova plus LPS. Recipients were perfused 6 days after transfer, lungs were processed to extract lymphocytes, and lungs and lymph nodes were analyzed by flow cytometry. **A)** KJ1.26 versus CFSE plots of CD4+ cells from lymph nodes and lungs. The fractions of DO11 cells in the CD4+ gate are shown. **B)** S1P1 mRNA expression in DO11 cells 1 or 4 days after transfer into BALB or sOva Tg recipients. CFSE-labeled WT DO11 cells were purified by cell sorting, processed to yield cDNA, and analyzed by quantitative PCR. Only divided cells were isolated on day 4. Results were normalized to HPRT expression. Data from two recipients per group are shown separately.
DISCUSSION

The sequence of events leading to peripheral T cell tolerance

We found that T cell tolerance to a systemic self antigen followed a series of three events: expansion, anergy, and deletion. First, the self-reactive population of DO11 donor cells in sOva transgenic recipients proliferated and expanded in the lymph nodes and spleen. Nearly all these cells divided and upregulated activation markers. Next, the repeatedly stimulated CD4+ T cells lost their responsiveness and became anergic. They ceased proliferating and did not secrete cytokines. Finally, as anergy was induced, the DO11 cells were deleted by Bim-dependent apoptosis. We have focused on understanding the mechanism and significance of deletion in peripheral T cell tolerance.

Mechanisms of deletion

Our results demonstrated that apoptosis induced by a systemic self antigen could be blocked by overexpression of Bcl-X and required Bim. Bim plays a unique and major role in governing immune homeostasis. But, at least in T cells, it is not at all clear what changes trigger Bim to initiate apoptosis (see chapter 1 for possibilities). Loss of Bcl-2 rather than altered Bim has been advanced as the cause of deletion by superantigen [35]. In contrast, our results showed that persistent self antigen stimulation increased both mRNA and protein expression of Bcl-2 and Bcl-X. While Bim levels also rose, they increased to a lesser extent.
While our results showed no correlation between expression of Bim, Bcl-2, and Bcl-X and apoptosis, they remain open to two explanations. First, changes in other BH3-only proteins might have reduced the number of Bcl-2 and Bcl-X molecules available for binding Bim without altering their total expression. Alternatively, some qualitative change may have occurred to the Bim proteins we measured. Bim is predominantly expressed as its extra-long isoform, but can be spliced into long and short species with increasing apoptotic activities [26]. Since our reagents detected all isoforms, a change in Bim splicing might have triggered apoptosis. Changes in Bim’s phosphorylation have been correlated with the start of apoptosis, too, [44] although no evidence yet shows that adding or removing phosphate group acts as an apoptosis-inducing trigger.

Apoptosis caused by both antigen receptor signaling and growth factor withdrawal depends on Bim. The sOva transgenic system subjected DO11 T cells to both stimuli: chronic presentation of ova, coupled with the downregulation of IL-7 receptor and little IL-2 production. We favor that possibility that repetitive TCR stimulation engaged the Bim pathway because of two findings. First, DO11 cells that were activated in vitro before transfer were deleted faster than naïve donors (data not shown). These cells would have been exposed to their own IL-2 at relatively high concentrations, yet they were not protected. Second, we transduced the cultured DO11 cells with a constitutively active mutant of STAT5 to partially substitute for IL-2 and IL-7 signaling, and this also failed to protect against deletion (data not shown). These observations again contrast with superantigen-mediated deletion: here IL-2 inhibits death, at least in vitro, presumably by inducing Bcl-2 [71].
One way to resolve the contrasting results between the sOva transgenic and superantigen systems is to speculate that they reflect different pathways of Bim activation. In sOva transgenic mice, repetitive TCR stimulation could activate Bim by qualitatively changing the protein, perhaps by splicing or phosphorylation, to overcome increased expression of Bcl-2 and Bcl-X. Superantigen-mediated deletion could instead activate the Bim pathway in the same way as cytokine withdrawal, reducing the expression of Bcl-2 and relieving the inhibition of pre-existing and unmodified Bim.

We also showed that Fas did not contribute to apoptosis in the sOva transgenic system. While T cells constitutively express the Fas receptor, naïve cells express little Fas ligand and high levels of FLIP, which prevents death receptor signaling by competing with caspase 8. Activation can sensitize T cells to Fas-mediated apoptosis by upregulating Fas ligand and decreasing FLIP expression, but this sensitization to Fas depends on IL-2 [62]. Since the responding DO11 cells produced little IL-2 in sOva transgenic recipients they may have remained resistant to Fas-mediated killing.

**Mechanisms of anergy**

Besides triggering the Bim pathway, we believe that chronic antigen stimulation also induced anergy [72]. Repeatedly encountering antigen can desensitize T cells [73] and DO11 cells developed a defect in calcium mobilization upon αCD3 stimulation within three days of transfer [60]. While tolerant cells failed to respond to antigen presenting cells plus Ova peptide, bypassing the initial steps of T cell receptor signaling with PMA plus ionomycin treatment did
elicit a response (data not shown). These results implicate a block in signaling proximal to the T cell receptor as the cause of anergy.

Our evidence discounted three alternative mechanisms of anergy. The absence of costimulation did not cause anergy because tolerant DO11 cells responded poorly to immunization with mature ova-pulsed BMDCs, even though these antigen presenting cells expressed high levels of B7 and other costimulatory molecules [12]. T cell inhibitory receptors are also probably not responsible for anergy. Repeated encounter with self antigen did upregulate CTLA-4 and PD-1, but antibody blockade of these receptors failed to prevent anergy. Finally, the failure to proliferate and produce cytokines cannot be explained by differentiation into regulatory T cells. The DO11 cells expressing FoxP3 may themselves have seemed inert during in vitro restimulation. But these FoxP3+ cells could not have suppressed the response of the FoxP3- majority because of the culture conditions. More importantly, the increase in FoxP3+ DO11 cells was only transient, but the unresponsiveness of the Bim deficient DO11 population persisted for weeks.

The proposed biochemical explanations for anergy may differ from T cell desensitization caused, we believe, by persistently encountering self antigen. In vitro models point to the absence of costimulation or the combination of strong calcium with weak Ras/MAPK signals [74], but these are the opposite of our observations. A comparison of gene expression profiles in the DO11 X sOva doubly-transgenic mice showed that anergic cells expressed a distinct pattern of genes, but also presented no obvious candidate as a master regulator [60]. An open possibility is that E3 ubiquitin ligases, such as GRAIL or Itch, maintain
anergy by degrading key components of the TCR signaling pathway. It would also be interesting to test whether costimulation by OX40 could overcome the anergy and cell cycle arrest that we observed, as has been shown in two similar experimental systems [75, 76].

If chronic exposure to antigen does itself cause anergy, then trapping self-reactive T cells in lymph nodes may promote and maintain their desensitization. Such entrapment could maximize the frequency of antigen exposure. Furthermore, self-reactive T cells would be exposed to antigen presented by resting or immature dendritic cells which are known to have tolerizing effects on T cells [77].

A critical test of our desensitization hypothesis will be to isolate tolerant Bim-deficient DO11 cells from sOva transgenic recipients and re-transfer these cells into WT BALB/c hosts. If anergy is caused by an imprinted phenotype, then the previously tolerant DO11 cells should remain unresponsive when stimulated. If instead anergy results from desensitization to chronic antigen stimulation, then the DO11 cells should regain their ability to proliferate and produce cytokines. We anticipate that anergy will depend on continuous exposure to self antigen because of our results with mice expressing both the DO11 and sOva transgenes [60]. The CD25- DO11 cells from these mice are anergic. But, if isolated and transferred away from systemic autoantigen, these previously anergic cells cause autoimmunity in a diabetes model.
Contributions of apoptosis and anergy to tolerance

Apoptosis presents a teleologically attractive explanation for tolerance because it is rapid, cell intrinsic, and irreversible. But the available evidence has yet to prove that the apoptosis of peripheral T cells plays an essential role in maintaining immune tolerance. Both Bim and Fas deficiencies cause lupus-like autoimmune diseases, but only in certain strains of mice, in combination with impaired B cell deletion and, in the case of Bim, together with impaired thymic negative selection [4]. In contrast, deficiencies in anergy and regulatory T cells cause peripheral tolerance to fail. CTLA-4 [78], FoxP3 [79], and IL-2 [80] deficient mice all develop fulminant autoimmunity and die within 5 weeks of age without evidence of defective apoptosis.

When beginning this study, we considered what could mask the role of apoptosis. Anergy is one possibility: experiments inducing tolerance usually cause both apoptosis and anergy, and the interactions between these two T cell control mechanisms are not well defined. Much interest has focused on anergy because the T cells that survive tolerance induction lose their responsiveness. However, apoptosis could play two essential roles in this outcome. First, and most simply, apoptosis could reduce the number of self-reactive cells to a manageable scale. This role might be particularly important to compensate for inefficiencies in inducing anergy. Alternatively, apoptosis and anergy might control different subsets of T cells.

The mechanisms responsible for immune tolerance are thought to depend, in part, on differences between various autoantigens [69]. Apoptosis might be critical for tolerance to systemic and persistent self antigens but less important in
controlling T cell tolerance to transient or tissue-restricted antigens. In the superantigen and aqueous peptide models of tolerance the T cell stimulant is rapidly cleared; the responding cells don’t receive further T cell receptor stimulation until they are assayed to test their responsiveness. In consequence, these experimental systems may not allow a fraction of non-tolerant T cells to expand to a noticeable pool.

We hypothesized that apoptosis would play an essential role in our experimental system because the DO11 cells transferred into sOva transgenic recipients would be chronically stimulated. Without apoptosis, we expected that even slight inefficiencies in anergy would be revealed because any T cell that avoided tolerance would continue to proliferate. Instead, we found that anergy was sufficient to preserve tolerance even if defects in apoptosis prevented deletion. Tolerance did not require reducing the number of autoreactive cells and we found no evidence that apoptosis selectively eliminated subsets of T cells, such as those producing effector cytokines. One possible explanation for this result could be that apoptosis follows anergy rather than these two pathways being activated simultaneously. This possibility would explain why death of DO11 cells was not immediately evident, but rather occurred after the peak of the response and the onset of anergy.

Nevertheless, we cannot conclude that anergy is the key to peripheral tolerance and that apoptosis is dispensable. First, we were unable to perform complementary experiments that tested the importance of apoptosis when T cell anergy was defective. Also, if repetitive antigen stimulation induces anergy by
desensitizing T cells, then the sOva transgenic model may represent an extreme situation where anergy is extremely efficient and robust.

Our use of a single T cell receptor and peptide antigen also raises a potentially significant caveat. This approach enabled us to isolate an apoptotic defect in a cohort of monoclonal naïve T cells and so eliminate many confounding variables. But peripheral T cell populations have varying affinities for self antigen, ranging from a minimum recognition required for positive selection to the high self-reactivities of regulatory T cells [81]. The relative contributions of apoptosis and anergy to tolerance could well be related to T cell receptor affinity for self antigen, and our experimental system is not designed to address this possibility.

By preventing apoptosis, we believe the sOva transgenic system presents a promising opportunity to dissect the induction and maintenance of anergy. Besides no apparent contribution by CTLA-4, the phenotype of tolerant DO11 cells – initial proliferation followed by cell cycle arrest, loss of IL-2, failure to produce effector cytokines, and aberrant calcium response – are commonly shared characteristics of anergy in many systems [49]. By separating apoptosis from anergy, we hope that analyses traditionally limited to in vitro experiments can be applied to the mechanisms of in vivo tolerance.
REFERENCES


CHAPTER THREE

The roles of IL-2 and Bim in regulatory T cell survival and function

SUMMARY

The maintenance of FoxP3+ regulatory T cells requires signals provided by the growth factor IL-2. In this study, we showed that FoxP3+ T cells are restored in the absence of IL-2 if Bim-dependent apoptosis was disabled. However, despite restoring FoxP3+ cells, eliminating Bim failed to prevent the lymphoid hyperplasia and lethal autoimmune hemolytic anemia caused by IL-2 deficiency. Regulatory T cells from IL-2 – Bim doubly-deficient mice suppressed poorly in co-culture assays. We concluded that, without IL-2, regulatory T cells both died and ceased to suppress pathogenic self-reactive T cells, and that preventing their Bim-dependent apoptosis did not restore their ability to function properly. In addition, we found preliminary evidence with regulatory T cells specific for a defined antigen that supported this conclusion. Thus, IL-2 is required for both survival and proper suppressive function of FoxP3+ regulatory T cells.

BACKGROUND AND AIMS

The self-reactive regulatory T cell population suppresses the responses of other T cells, providing an essential mechanism for enforcing immune tolerance [13]. Regulatory T cells differentiate during thymic development following
recognition of self antigen and induction of FoxP3 [82]. Because suppression is a cell extrinsic mechanism of peripheral tolerance, regulatory T cells may compensate for any failure in cell intrinsic mechanisms, such as deletion and anergy.

Once in the periphery, regulatory T cells are critically dependent on IL-2 produced by other T cell populations [10, 83]. Without IL-2 or the alpha chain of its receptor, CD25, the percentage of CD4+ T cells expressing FoxP3 is reduced by half and mice develop lethal autoimmunity. Antibody blockade of IL2 causes similar effects, and CD25-deficient regulatory T cells are out competed by their WT counterparts. Such findings have led to the conclusion that IL-2 is required to maintain the survival of regulatory T cells in the periphery [13].

However, these results do not distinguish whether IL-2 also affects the ability of regulatory T cells to suppress, either by altering FoxP3 expression or by other means. Three observations indicate that IL-2 might play such a role. First, IL-2 enhances the level of FoxP3 expression [83, 84] and cells with low FoxP3 levels suppress weakly and incompletely [85]. Second, gene profiling by cDNA array suggests that, without IL-2, regulatory T cells become metabolically impaired [83]. Finally, IL-2-deficient mice die with even a partial reduction in peripheral FoxP3+ cells [83]. In contrast, treatment with antibodies to IL-2 or CD25 causes a similar drop in regulatory T cells but milder autoimmunity [86, 87]. This difference suggests that the effects of IL-2 on regulatory T cells are qualitative as well as quantitative.
Our goal for this study was to determine whether IL-2 is necessary for regulatory T cell function independent of these cells’ survival. It is difficult to address this question in germline IL-2 or CD25-knockout mice because these mice have few FoxP3+ cells. Therefore, our first step was to prevent regulatory T cells from dying despite the absence of IL-2 signals. We suspected that growth factor withdrawal would activate the same apoptotic pathway in different T cell populations. Bim is the principal sensor of growth factor deprivation in most cell types that have been examined, including lymphocytes [18].

Therefore, we hypothesized that IL-2 deprivation would activate the Bim pathway in regulatory T cells, and we could protect this population in IL-2-deficient mice by eliminating Bim. On the BALB/c background, IL-2 deficiency causes lethal autoimmune hemolytic anemia [80] faster than the inflammatory bowel disease that affects other strains. We bred IL-2 – Bim doubly-deficient mice and analyzed their regulatory T cell populations and autoimmune disease. Our plan faced a practical obstacle in identifying regulatory T cells because this population’s best marker, CD25, is only weakly expressed without IL-2. We solved this problem by using a reporter mouse strain with GFP knocked into the FoxP3 locus [88].

Our first aim was to determine whether Bim deficiency restored the FoxP3+ population in IL-2-deficient mice. Next, we aimed to test whether regulatory T cells could function normally without IL-2. We compared in vitro suppression by isolating FoxP3+ GFP+ cells from wild-type, Bim-deficient, IL-2-deficient, and mice lacking both IL-2 and Bim and comparing
their function in co-culture assays. We also assessed regulatory T cell function in vivo by monitoring the anemia and survival of the mice. If IL-2-deficient mice develop autoimmunity because too few regulatory T cells survive in the periphery, then we predicted that Bim deficiency would protect the animals. If, instead, regulatory T cells also required IL-2 to function properly, then we expected that the IL-2 – Bim doubly-deficient mice would still develop dysregulated T cell activation and anemia.

We also sought to confirm our findings with antigen-specific regulatory T cells. To do so, we crossed Ova-specific, DO11.10 T cell receptor transgenic mice with mice transgenically expressing Ova as a self antigen [60, 89]. The DO11 cells that develop in these animals undergo substantial deletion, but some differentiate into FoxP3+ regulatory cells with suppressive capabilities. We bred these DO11 X Ova transgenic mice with Bim-deficient animals and compared the phenotype and functions of CD25+ and CD25- DO11 populations.

RESULTS

Effects of Bim deficiency on regulatory T cell development and function

Since we planned to permit regulatory T cells to survive without IL-2 by eliminating Bim, we first established how Bim deficiency alone altered these cells. While Bim deficiency expands the number of all lymphocyte populations [28] its effect on the FoxP3+ subset has not yet been examined. In the thymus, we found that Bim deficiency expanded the fraction of cells staining positive for FoxP3
more than 6-fold, with the CD25- subset accounting for most of this expansion (Figure 1). Bim is known to alter thymic composition, and Bim-deficient thymi contained more CD4- CD8-, more CD4 and CD8 single positive, and fewer double positive cells than normal [32]. However, most of the FoxP3+ cells were still CD4+ CD8-. The total cellularity of the thymi changed little without Bim, so the gain in the number of FoxP3+ cells reflected the increased fraction of this population.

The increase in FoxP3+ cells lacking Bim suggested that many regulatory T cells are normally deleted during their development. Deletion may have occurred by negative selection, with self antigen recognition triggering apoptosis, a process known to involve Bim. But regulatory T cells may also require IL-2 to survive even before emigrating from the thymus [83]. Without Bim, the increase in CD25- FoxP3+ cells was greater than that of their CD25+ counterparts, supporting the possibility that the availability of IL-2 limits the regulatory T cell population even in the thymus. This finding also supported our hypothesis that regulatory T cells die via the Bim pathway when deprived of IL-2.

Bim deficiency caused a smaller difference in the FoxP3+ population in lymph nodes and spleen (Figure 2). The fraction of CD25+ FoxP3+ cells was the same with or without Bim and the percentage of CD25- FoxP3+ cells only doubled. While the total number of CD4+ cells did increase without Bim, a 2-fold change in the FoxP3+ CD25- fraction was the only disproportionate difference we observed. This suggested that some homeostatic mechanism, perhaps proliferation, compensated for the altered thymic output in Bim-deficient mice.
FIGURE 1
Bim deficiency expanded the thymic FoxP3+ population

Thymic cells from adult WT and Bim-deficient mice were counted and analyzed by flow cytometry. A) CD25 versus FoxP3 plots of total live thymocytes. B) CD8 versus CD4 plots of total and FoxP3+ thymocytes. C) Percentage of total thymocytes expressing FoxP3. D) Numbers of thymocyte subsets.
and returned the peripheral regulatory compartment to a normal fraction of CD25+ cells. FoxP3 levels varied with CD25 rather than with Bim expression (Figure 3). Whether wild-type or Bim-deficient, CD25+ cells stained nearly twice as brightly for FoxP3 than their CD25- counterparts.

We next tested whether Bim-deficient FoxP3+ cells suppressed normally in an in vitro co-culture assay. To do so, we combined Bim knockout mice with a GFP reporter knocked into the FoxP3 gene [88] and isolated CD4+ GFP+ cells by high speed cell sorting. Since the Bim-deficient mice contained twice as many CD25- regulatory T cells, expressing lower levels of FoxP3, we sorted wild-type and Bim-deficient cells to match their GFP fluorescence intensities (Figure 4A). The sorted cells, subdivided into high and low GFP expression, were titrated and co-cultured with wild-type CD4+ CD25- responder T cells, antigen presenting cells, and αCD3 antibody. We measured suppression by the decrease in proliferating responder T cells after 3 days in co-culture.

We found that Bim-deficient FoxP3+ regulatory T cells suppressed as well as their wild-type counterparts (Figure 4B, C). Both thymic and peripheral regulatory cells containing high GFP levels inhibited the proliferation of the responder cells but did not divide themselves. The peripheral cells with lower GFP expression also suppressed, but were 2-fold less potent. The thymic GFP low cells did not suppress but also did not proliferate. If this population were still in the process of differentiating it may not yet have acquired suppressive function. We concluded that eliminating Bim did alter the thymic development of regulatory T cells, but not their suppressive capabilities in vitro. More
FIGURE 2
Bim deficiency favored CD25- FoxP3+ cells in the periphery

Cells from peripheral lymph nodes and spleens of adult WT and Bim-deficient mice were counted and analyzed by flow cytometry.  
A) CD25 versus FoxP3 plots of CD4+ peripheral T cells.  B) Percentages of CD25+ FoxP3+ and CD25- FoxP3+ cells in the CD4+ population.  C) Number of peripheral T CD4+ T cell subsets.
**FIGURE 3**
Level of FoxP3 expression depended on CD25, not on Bim

Flow cytometry was used to measure the level of FoxP3 expression in peripheral regulatory T cells. **A)** CD25 versus FoxP3 plots of CD4+ WT and Bim KO lymph node cells. FoxP3+ cells are divided by CD25 expression with the indicated gates. FoxP3 expression is shown as overlaid histograms of CD25+ and CD25- gated FoxP3+ cells. **B)** Mean Fluorescence Intensity (MFI) of FoxP3.
FIGURE 4
Bim deficiency did not alter in vitro suppression by regulatory T cells

Thymic and peripheral regulatory T cells were purified by sorting cells from WT and Bim-deficient FoxP3-GFP reporter mice. Sort gates were set to divide CD4+ cells into GFP high and low populations and to match FoxP3 expression between WT and Bim KO cells. A) Levels of FoxP3, measured as GFP MFI, of sorted cells. Purified FoxP3-GFP+ cells were co-cultured in titration with splenic APCs, sorted WT CD4+ CD25- responder T cells, and αCD3 antibody. Proliferation of responder T cells was measured by 3H thymidine uptake after 3 days in culture to compare suppression by B) thymic and C) peripheral regulatory T cells. R: proliferation by responder cells cultured without regulatory T cells.
importantly, once in the periphery Bim-deficient regulatory T cells adjusted to a nearly normal fraction of the total CD4+ population and exhibited indistinguishable in vitro suppression from wild-type FoxP3+ cells.

**Bim deficiency restored FoxP3+ cells in IL-2 deficient mice**

IL-2 deficiency reduces the peripheral FoxP3+ population, presumably because these cells are short-lived. If so, we anticipated that the lack of IL-2 would trigger a Bim-dependent apoptotic pathway in regulatory T cells and we could rescue the peripheral FoxP3+ population by eliminating Bim. To test our hypothesis, we generated mice lacking IL-2, Bim, or both genes and compared them to wild-type littermates.

We found that, without IL-2, the percentage of FoxP3+ cells in the peripheral CD4+ population fell by half and few of the remaining cells expressed CD25 (Figure 5A, B). In Bim-deficient mice, by contrast, the elimination of IL-2 did not reduce the fraction of FoxP3+ cells although the regulatory T cell population still lost CD25 expression. However, changes in the percentages and numbers of FoxP3+ cells proved difficult to interpret because both IL-2 and Bim deficiencies increased the total number of peripheral T cells (Figure 7A). Regardless of Bim, loss of IL-2 decreased the number of CD25+ FoxP3+ cells, but this drop was more than offset by an increase in the size of the CD25- FoxP3+ population (Figure 5C). IL-2 deficiency actually raised the total number of FoxP3+ cells because of the increase in total T cell numbers, an effect exaggerated
FIGURE 5
Bim deficiency restored peripheral FoxP3+ cells in mice lacking IL-2

Peripheral lymph node cells from 3-4 week old WT, IL-2-deficient, Bim-deficient, and IL-2 – Bim doubly-deficient mice were counted and analyzed by flow cytometry. A) CD25 versus FoxP3 expression of CD4+ T cells with the percentage of CD25+ and CD25- FoxP3+ cells indicated. B) Percentage of FoxP3+ CD25+ and FoxP3+ CD25- cells in the CD4+ peripheral T cell populations. C) Numbers of CD4+ FoxP3+ peripheral T cells expressing or lacking CD25.
in mice also lacking Bim. Thus, IL-2 deficiency seemed to decrease the percentage of CD4+ cells expressing FoxP3 both via Bim-dependent apoptosis and by expanding the FoxP3- population.

**Proper regulatory T cell function required IL-2**

If a numerical shortage of FoxP3+ regulatory T cells was responsible for the autoimmune hemolytic anemia in BALB/c IL-2-deficient mice, then we might predict that mice lacking both IL-2 and Bim would be protected from this disease. We monitored the autoimmune pathology of wild-type or Bim-deficient mice also lacking either IL-2 or CD25, the alpha chain of the IL-2 receptor complex. We also checked whether the lymphoid hyperplasia and CD4+ T cell activation arising in IL-2-deficient animals still developed without Bim.

We found that mice lacking both IL-2 and Bim or CD25 and Bim still died of anemia (Figure 6). While onset of anemia varied, as expected [80], the hematocrit readings of 10 of 13 doubly-deficient mice were severely reduced. Analyses of red blood cell counts and hemoglobin concentrations matched the hematocrit readings (data not shown). This result showed no protection from the autoimmune pathology that developed in IL-2 or CD25-deficient mice expressing Bim, where 7 of 9 mice developed anemia. We detected no signs of anemia in mice lacking only Bim; all these animals registered hematocrit values within the normal range of 35-45. A larger sample size will be needed to determine whether Bim deficiency affected the onset of anemia.
FIGURE 6
Despite a restored FoxP3+ population, IL-2 – Bim and CD25 – Bim doubly-deficient mice still died of autoimmune hemolytic anemia.

IL2+/– Bim+/– or CD25+/– Bim+/– mice were interbred and their progeny monitored up to 6 weeks of age for anemia. Blood samples were analyzed to determine hematocrit (HCT, the fraction of blood volume occupied by erythrocytes) by age. The fraction of anemic (HCT < 30 versus 35-45 normally) mice is indicated. HCT readings below the threshold of detection were assigned a value of 1.
We also found that eliminating Bim exaggerated the lymphoid hyperplasia caused by IL-2 deficiency and failed to prevent widespread CD4+ T cell activation. Bim deficiency increased the number of cells in lymph nodes and spleens, but not as greatly as IL-2 deficiency (Figure 7A). The combination of IL2 and Bim deficiencies further promoted this lymphoid hyperplasia. Furthermore, removing Bim, and restoring the percentage of FoxP3+ cells, failed to reverse the increase in CD69 expression on CD4+ T cells or the loss of their CD44 low, CD62L high phenotype (Figure 7B, C).

Mice lacking both IL-2 and Bim died of anemia and lost control of CD4+ T cell activation even though Bim deficiency restored the percentage of peripheral FoxP3+ cells. These results suggested that, without IL-2, the FoxP3+ cells in these mice were unable to function normally and suppress pathogenic self-reactive T cells. To address the possibility, we tested the suppressive capabilities of FoxP3+ cells from IL-2 – Bim doubly-deficient mice in vitro. Because the FoxP3+ cells from IL-2 deficient animals expressed little CD25, we again used the FoxP3-GFP genetic reporter to isolate regulatory T cells. As for Bim-deficient animals, mice lacking both IL-2 and Bim contained a population of cells expressing less GFP that we separated from those expressing higher, wild-type levels (Figure 8A).

Suppression by FoxP3+ cells from IL-2 – Bim doubly-deficient mice was defective (Figure 8B). Even when co-cultured 1:1 with responders, the GFP high doubly-deficient cells decreased proliferation by only half, and an equivalent
FIGURE 7
Mice lacking both IL-2 and Bim developed exaggerated lymphoid hyperplasia and failed to control T cell activation

Lymph node and spleen cells of wild type mice or animals lacking IL-2, Bim, or both molecules were counted and analyzed by flow cytometry. A) Number of total live cells in peripheral lymph nodes and spleens. B) CD69 and C) CD62L versus CD44 expression on CD4+ lymph node T cells.
FIGURE 8
Without IL-2, Bim-deficient regulatory T cells suppressed poorly in vitro

Peripheral regulatory T cells were purified by sorting cells from WT, IL-2-deficient, and IL-2 – Bim doubly-deficient FoxP3-GFP reporter mice. A) Sort gates were set to divide doubly-deficient CD4+ cells into GFP high and low populations. Purified FoxP3-GFP+ cells were co-cultured in titration with splenic APCs, sorted CD4+ CD25- responder T cells, and αCD3 antibody. Proliferation of B) WT and C) IL-2-deficient responder T cells was measured by ³H thymidine uptake after 2 days in culture. R: proliferation of responder cells cultured without regulatory T cells.
suppression by wild-type or IL-2-deficient counterparts required 8 times fewer regulatory T cells. In a parallel experiment, to eliminate the possibility that IL-2 produced by the responder T cells affected suppression, we observed similarly weak suppression of IL-2-deficient responder T cells (Figure 8C). Furthermore, while the GFP high population exhibited at least weak suppressive activity, GFP low cells suppressed neither WT nor IL-2 deficient responders and proliferated themselves. We concluded that IL-2 was necessary for regulatory T cells to function properly. FoxP3+ cells in mice lacking both IL-2 and Bim could survive without their growth factor but suppressed weakly or not at all in vitro. In vivo, the restored FoxP3+ population failed to prevent a pathogenic T cell response leading to anemia and death.

**Development and survival of antigen-specific regulatory T cells**

Our findings with endogenous, polyclonal regulatory T cells could be confounded if Bim deficiency also changed the repertoire of these cells. One means of addressing this possibility was to fix the specificity of the regulatory T cells. Previous experiments demonstrated that Ovalbumin-specific, DO11.10 T cell receptor transgenic CD4+ T cells could differentiate into regulatory T cells when the DO11 transgene was crossed to mice expressing Ova as a self antigen [60, 89]. We extended these studies by breeding these DO11 X Ova transgenic mice to also lack Bim.

We examined regulatory DO11 T cells in two Ova-expressing transgenic strains. The RIP-Ova transgene expresses a membrane-bound form of Ovalbumin under the control of the Rat Insulin Promoter [90]. These mice
produce Ova in the pancreatic islets, and also in the thymus because this promoter can be activated by AIRE [91]. The DO11 cells developing in RIP-Ova transgenic mice would therefore encounter antigen in the thymus and pancreatic lymph nodes. The sOva transgenic mice produce Ova systemically and DO11 cells in this strain would recognize antigen both in the thymus and in all peripheral lymphoid organs (see chapter 2).

When Bim was present, both CD25+ and CD25- FoxP3+ DO11 cells developed in the RIP-Ova and sOva transgenic mice, accompanied by thymic negative selection of FoxP3- cells (Figure 9). Without Bim, thymic self antigen instead doubled the number of FoxP3- DO11 cells. Bim deficiency also dramatically altered the expression of CD25 by the FoxP3+ thymic population. Without Bim, the wild-type ratio of 3 CD25- to 2 CD25+ cells rose to 20:1 in the RIP-Ova and >100:1 in the sOva transgenic thymi (Figure 9A). As a result, the number of CD25+ FoxP3+ thymic DO11 cells was nearly the same in wild-type and Bim-deficient Ova transgenic mice while the size of the CD25- FoxP3+ thymic population increased >10 fold.

Bim deficiency caused similar changes in peripheral Ova-specific regulatory T cells. CD25-expressing DO11 FoxP3+ cells predominated in the lymph nodes (Figure 10) and spleens (data not shown) of both Ova transgenic strains. In the RIP-Ova transgenic mice, Bim deficiency changed the ratio of CD25- to CD25+ FoxP3+ cells from 1:12 to 1:1 (Figure 10A) equalizing the numbers of these two cell types (Figure 10C). In the Bim-deficient sOva transgenic animals, the wild-type ratio of 4 CD25+ per 1 CD25- FoxP3+ DO11 cell
FIGURE 9
Bim deficiency expanded Ova-specific FoxP3+ thymic T cell populations in RIP-Ova and sOva transgenic mice

WT and Bim-deficient DO11 TCR transgenic mice were bred to co-express the RIP-Ova or sOva transgenes. A) CD25 versus FoxP3 expression in thymic CD4+ KJ1.26+ DO11 cells. Percentages of CD25+ and CD25- FoxP3+ DO11 cells are shown. The average numbers of thymic DO11 cells of each subset are shown for B) wild-type (non Ova transgenic), C) RIP-Ova, and D) sOva transgenic mice.
FIGURE 10
Bim deficiency expanded Ova-specific FoxP3+ peripheral T cells populations lacking CD25 in RIP-Ova and sOva transgenic mice

Peripheral lymph node cells of mice from Figure 9 were counted and analyzed by flow cytometry. A) CD25 versus FoxP3 expression in DO11+ peripheral T cells. Percentages of DO11 cells expressing FoxP3 with and without co-expression of CD25 are shown. The average numbers of peripheral DO11 cells of each subset are shown for B) wild-type (non Ova transgenic), C) RIP-Ova, and D) sOva transgenic mice.
was reversed to 1 CD25+ per 5 CD25- cells (Figure 10A), making the FoxP3+ CD25- subset the largest population of DO11 cells (Figure 10D). Bim deficiency also increased the number of FoxP3- DO11 cells exposed to self antigen in the thymus (Figure 10C) or thymus and periphery (Figure 10D). As with the polyclonal regulatory T cells (Figure 3), FoxP3+ DO11 cells expressed higher levels of FoxP3 if CD25+ than if CD25- (Figure 11). This correlation held in the thymus, lymph nodes, and (data not shown) spleen. In contrast, Bim-deficiency did not generally correlate with lower levels of FoxP3.

These observations demonstrated that Bim normally deleted cells expressing low levels of FoxP3 and lacking CD25 expression. This deletion occurred naturally in polyclonal regulatory T cell populations and was exaggerated by fixing the T cell repertoire with a T cell receptor transgene. Bim deficiency favored the CD25- FoxP3+ cells in both Ova transgenic models, even though the DO11 cells would seldom have encountered self antigen in the periphery of RIP-Ova mice but been repetitively stimulated in sOva mice. This suggested that the CD25- FoxP3+ cells died independently of antigen stimulation and instead because they had been deprived of IL-2.

**CD25+, but not CD25-, antigen-specific regulatory T cells were suppressive**

If IL-2 stimulation contributed to the function ability of regulatory T cells, then CD25- FoxP3+ cells should prove less suppressive than their CD25+ counterparts. As an initial test of this prediction, we compared the in vitro suppression of CD25+ versus CD25- DO11 cells from sOva transgenic wild-type
FIGURE 11
Thymic and peripheral DO11 cells expressed higher levels of FoxP3 if co-expressing CD25

The levels of FoxP3 expression in WT and Bim-deficient DO11 cells from RIP-Ova and sOva transgenic mice were measured by flow cytometry. A) Thymic and B) lymph node FoxP3+ DO11+ cells were divided by CD25 expression, as shown in Figures 9A and 10A. FoxP3 expression in the CD25+ and CD25- subsets are graphed as the mean fluorescence intensity of the FoxP3 stains.
and Bim-deficient mice. We were limited to this indirect comparison because the FoxP3-GFP reporter was not yet available in combination with the DO11 and sOva transgenes, and because separation by other regulatory T cell markers, such as GITR and CD62L, failed to enrich the CD25- FoxP3+ population (data not shown). Nevertheless, since the Bim-deficient sOva transgenic CD25- DO11 population contained approximately 2 FoxP3+ cells for each FoxP3- cell, we could still infer suppressive capability.

We purified CD25+ and CD25- DO11 cells from sOva transgenic lymph nodes and co-cultured titrated numbers of these cells with CD25- naïve DO11 wild-type responder cells, antigen-presenting cells, and Ova peptide. We measured regulatory T cell function by the suppression of the responders’ IL-2 production (Figure 12A) and proliferation (Figure 12B). Both wild-type and Bim-deficient CD25+ DO11 cells, nearly all of which would have expressed FoxP3, suppressed IL-2 production and cell division. Although the Bim-deficient regulatory cells were slightly less efficient, the difference from wild-type suppression was only 2-fold. Regardless of Bim the CD25+ DO11 cells did not proliferate in culture.

However, neither wild-type nor Bim-deficient CD25- DO11 cells inhibited the IL-2 production or proliferation of the responder T cells. We expected the wild-type CD25- cells not to suppress because only 1 in 6 of these cells would have expressed FoxP3 (Figure 10A). In contrast, two-thirds of the Bim-deficient CD25- population should have been FoxP3+ yet we observed no suppression. While this experiment should be repeated with directly marked, GFP-expressing
FIGURE 12
In vitro suppression and proliferation of peripheral Ova-specific cells from DO11 X sOva doubly-transgenic mice

CD25+ and CD25- DO11 cells were separated by sorting the peripheral lymph nodes of WT and Bim-deficient DO11 X sOva doubly-transgenic mice. Sorted cells were co-cultured in titration with CD25- naïve DO11 WT responder T cells, splenic APCs, and Ova peptide for 4 days. A) Suppression of IL-2 secretion was measured by ELISA of supernatant samples after 40 hours. B) Suppression of proliferation was measured by 3H thymidine uptake between days 3 and 4. See Figure 10A for the estimated fraction of cells expressing FoxP3 in each tested population.
FoxP3+ cells, we could nonetheless infer that CD25- FoxP3+ T cells were functionally impaired. This preliminary result with antigen-specific regulatory T cells supports our conclusion that regulatory T cells required IL-2 to survive, but that IL-2 signaling also contributed to suppressive function of this population.

**DISCUSSION**

The proliferation, differentiation, and survival of effector T cells depends on IL-2 [92], and so it was at first surprising when IL-2-deficient mice were found to develop autoimmunity instead of immunodeficiency [93]. Extensive investigation has since established that peripheral T cell tolerance breaks down unless FoxP3+ regulatory T cells receive IL-2 signals [94]. However, why regulatory T cells require IL-2 has remained an open question. Direct investigation of the relationship between IL-2 and regulatory T cells has been limited because many of these cells died in the absence of the IL-2. Furthermore, the surviving cells could no longer be isolated on the basis of their high and constitutive CD25 expression, which was lost without IL-2 [10]. By combining a FoxP3-GFP reporter [88] with a genetic deficiency in apoptosis, we directly tested the contributions of IL-2 to the survival and suppressive function of regulatory T cells.

**IL-2 is a survival factor for regulatory T cells**

Mice lacking IL-2 develop a CD4+ T cell population containing half or less the percentage of FoxP3+ cells as in normal animals [83]. In contrast, the
FoxP3+ fraction did not decrease when IL-2 was deleted in Bim-deficient mice (Figure 5). Although Bim does affect T cell development [32], both thymic and peripheral Bim-deficient FoxP3+ cells suppressed normally in vitro (Figure 4). Furthermore, the absence of Bim favored the CD25- FoxP3+ populations of both polyclonal (Figures 1 and 2) and Ova-specific (Figures 9 and 10) CD4+ T cells. Thus, the subset of regulatory T cells which couldn’t have recognized IL-2 was selectively eliminated by Bim. Together, these findings demonstrate that when regulatory T cells were deprived of IL-2 they underwent apoptosis via the Bim pathway.

**Regulatory T cell homeostasis**

Since regulatory T cell survival depended on IL-2, it is surprising that the fraction of FoxP3+ cells was only halved by deletion of their survival factor. But, in addition to survival, homeostasis of the peripheral regulatory T cell population is determined by thymic output, de novo induction of FoxP3, and proliferation [10]. Regulatory T cells are also probably in constant competition for limited access to IL-2, which determines their proliferation and lifespan [84, 95-97]. Such competition would explain why Bim-deficiency enables 10-fold more CD25-FoxP3+ thymocytes to develop, but only 2-fold more of these cells to persist in the periphery (Figures 1 and 2). Similarly, minor changes in regulatory T cells with defective or partially defective IL-2 receptors are exaggerated when such cells are compared head-to-head with wild-type competitors in mixed bone marrow chimeras or adoptive transfer experiments [83, 84].
We suspect that IL-2 deficiency also affected how homeostatic mechanisms besides survival determined the size of the regulatory T cell population, and how regulatory T cells competed with each other. For instance, a shorter lifespan without IL-2 might cause little change in regulatory T cell numbers if new thymic emigrants replaced the dying cells. Further investigation will be needed to distinguish the relative contributions of survival, thymic export, and peripheral proliferation to regulatory T cell homeostasis.

**Possible roles of IL-2 for regulatory T cells: a requirement for proper suppressive function**

Three possibilities could explain why regulatory T cells fail to maintain peripheral tolerance without IL-2 [13]. The simplest explanation is purely quantitative: a narrow balance exists between suppression and autoimmunity and, without IL-2, too few FoxP3+ cells survive to suppress pathologic autoimmune T cell responses. Deletion of IL-2, IL-2Rα, IL-2Rβ, or treatment with antibodies to IL-2 all lead to autoimmunity and all reduce the percentage of FoxP3+ cells. But, contrary to this possibility, Bim-deficient mice showed no decrease in the fraction of peripheral CD4+ T cells expressing FoxP3 (Figure 5) and still developed lethal autoimmunity (Figure 6). While wild-type and Bim-deficient FoxP3+ populations may not be equivalent, and we are now comparing their phenotypes in detail, Bim did not alter suppression in vitro (Figure 4). The absence of Bim could increase the number and change the specificity of self-reactive T and B cells, as well as prolong survival of T, B, and other leukocytes contributing to the autoimmune disease, and it will be important to address both
these caveats. Nevertheless, this result indicates that IL-2 deficiency does not cause autoimmunity because of a decrease in the number of regulatory T cells.

An alternative explanation is that IL-2 plays an indispensable role in the development and differentiation of a regulatory T cell subset. FoxP3+ cells arise without IL-2, but his does not exclude the possibility that some T cells require IL-2 to induce or maintain FoxP3, or to activate pathways of suppression [82]. In support of this idea, mice engineered to express IL-2Rβ only in thymic T cells maintained a peripheral FoxP3+ population of cells, seemingly unable to respond to IL-2, and were protected from autoimmunity [84]. Nevertheless, no direct evidence has been shown that IL-2 alters the development of polyclonal [83] or monoclonal [98] regulatory T cells. The survival of Bim-deficient regulatory T cells without IL-2 presents an opportunity to revisit this unresolved question.

The final possibility is that regulatory T cells require IL-2 in order to function properly and suppress autoimmunity. Our findings supported this idea, demonstrating defective in vitro and in vivo suppression by regulatory T cells deprived of IL-2. FoxP3+ cells from IL-2 – Bim doubly-deficient mice, but not Bim-deficient mice, suppressed poorly when assayed by in vitro co-culture (Figures 4, 8). From our preliminary results, we could also infer that CD25-FoxP3+ TCR transgenic regulatory T cells were similarly defective (Figure 12). In vivo suppression also required IL-2 because deleting Bim in IL-2-deficient mice restored FoxP3+ cells but failed to prevent lymphoid hyperplasia, widespread T cell activation, and lethal autoimmune hemolytic anemia (Figures 6, 7).

Our conclusion that IL-2 is required for suppression is seemingly at odds with earlier results showing little or no difference in in vitro suppression between
WT and IL-2 or CD25-deficient regulatory T cells [83, 84, 98]. We propose that this contradiction arises because apoptosis disguised IL-2’s contribution to suppressive function by eliminating the least functional regulatory T cells. When deprived of IL-2, regulatory T cells both lose their ability to suppress and begin to die. Competition amongst FoxP3+ cells and between recent thymic emigrants and older peripheral cells could well accelerate and intensify the selection of robust and suppressive cells. But, in the IL-2 – Bim doubly-deficient mice, the functionally defective regulatory T cells accumulated instead of being eliminated. Bim deficiency negated IL-2’s role in regulatory T cell survival, revealing that IL-2 also plays an essential role in proper suppressive function.

**IL-2 and the mechanisms of suppression**

The cause of defective suppression in IL-2 deprived regulatory T cells seems likely to be associated with weaker FoxP3 expression [85]. However, we found that FoxP3 low peripheral regulatory T cells from IL-2 sufficient donors suppressed nearly as well as their FoxP3 high counterparts, whereas suppression by both FoxP3 low and high cells was severely compromised without IL-2 (compare Figures 4C and 8B). IL-2 probably boosts and maintains FoxP3 expression, but a lower level of FoxP3 was not in itself sufficient to compromise suppressive activity, at least in vitro.

IL-2 might instead be indirectly required for proper regulatory T cell function by its effects on cellular metabolism [88]. Lymphocytes require substantial energy input to support their responses to activation [99]. Perhaps IL-2 serves as an essential metabolic switch in regulatory T cells, such as by
triggering the Akt pathway. If so, then without IL-2 regulatory T cells may be unable to import the glucose or amino acids required for any cellular response, including suppression. This hypothesis could initially be tested by determining IL-2’s effects on the import channels, enzymes, and metabolites needed for energy production in regulatory T cells.

Alternatively, IL-2 might activate the suppressive mechanisms used by FoxP3+ regulatory T cells. The means by which regulatory T cells inhibit other T cells’ responses are controversial, in part because the phenotypes of FoxP3+ populations and their suppressive requirements vary between experimental systems [100-102]. One of the initial proposals is that regulatory T cells suppress effector T cells by direct T cell – T cell contact. In vitro testing of this model has shown suppression requires TCR stimulation, does not depend on TGFβ, IL-10, or IL-4, and can be overcome by providing excess IL-2 [101]. However, the molecular and biochemical requirements of this process have not been defined, and it is not clear whether this means of suppression is crucial to maintain tolerance in vivo.

Regulatory T cells have also been proposed to suppress effector responses by contacting antigen-presenting cells, either altering or eliminating them to prevent subsequent stimulation of effector T cells [100, 102, 103]. Another model of suppression is that cytokine production by FoxP3+ T cells prevents pathology. TGFβ or IL-10 derived from regulatory T cells has proven essential for suppression in some, though not all, experimental systems [100, 101, 104, 105]. Cytokine-mediated suppression could inhibit effector T cells directly and, also,
indirectly by locally affecting the antigen presenting cells, other cell types, and the cytokines, chemokines, and other aspects of the local environment.

If IL-2 directly affects suppressive function, then it might change the expression or activities of molecules involved in regulatory T cell – dendritic cell engagement as well as the cytokine profile of FoxP3+ cells. A cDNA microarray comparison showed few changes following IL-2 treatment of IL-2-deficient FoxP3+ cells [83]. TGFβ was induced ~5-10 fold by IL-2 while IL-10 did not change. Smaller increases were also noted in B7-H1, which could inhibit effector T cells via PD-1 ligation, perforin and granzyme B, which could enable regulatory T cells to lyse APCs, and hemoxygenase 1, which might inhibit T cell responses by producing carbon monoxide. However, this same study also reported no difference in suppression between WT and IL-2-deficient regulatory T cells in vitro (as discussed above). It would be interesting to repeat a similar screen with IL-2 – Bim doubly-deficient FoxP3+ cells, especially if IL-2 treatment restored their suppressive activity.

REFERENCES


CHAPTER FOUR

The role of Bim-mediated apoptosis in regulating the CD4+ T cell memory response

SUMMARY

In this study, we investigated the effector to memory stage transition of CD4+ T cells and the contribution of mitochondrial apoptosis to this process. We characterized the CD4+ T cell response to an acute viral infection, using Ova-specific DO11 cells activated by Vesicular Stomatitis Virus engineered to produce Ova. Immunization with VSV-Ova or dendritic cells pulsed with Ova created a large population of DO11 effector cells, most of which did not survive the transition to become memory cells. To better understand memory cell differentiation, we protected the effector population from apoptosis by deleting Bim. The Bim-deficient DO11 population displayed an unaltered effector phenotype but did not decline after antigen clearance. Besides the difference in cell numbers, the surviving Bim-deficient cells displayed a wild-type memory phenotype: re-expressing IL-7Rα at a high level, rapidly producing effector cytokines, and increased sensitivity to antigen. We concluded that Bim regulates the size of the CD4+ T cell memory pool mainly by promoting the contraction of antigen-activated lymphocytes following antigen elimination.
BACKGROUND AND AIMS

The transition from the effector to memory stage of an immune response changes a large population of active CD4+ T cells to a much smaller and quiescent but long-lived population capable of rapid re-activation [14]. Infections do not generate a homogeneous pool of responding T cells, and the total effector population contains subsets differing in their abilities to proliferate, migrate, and secrete cytokines [106]. While the memory population preserves such heterogeneity, memory cells are capable of converting from one phenotype to another [107]. This combination of heterogeneity and plasticity is probably vital to host defense, but also poses a challenge to understanding the survival and differentiation of memory T cells.

Several selective processes have been proposed to explain which CD4+ T cells give rise to the memory pool [108]. Memory cells have been hypothesized to arise from the best-stimulated cells [109], cells that have not fully differentiated, especially if committed to produce IFNγ [110], and cells first activated near the end of the immune response [111, 112]. Alternatively, memory cells could arise from stochastic survival of effector cells. In all these models, a small fraction of the total activated CD4+ T cells either gain a competitive advantage at the expense of the general population or switch their requirements for survival factors.

In order to better comprehend the CD4+ T cell transition from the effector to memory stage, we prevented the effector cells from dying after their antigen was cleared. Our goal was to define the factors determining memory cell fate by examining the phenotype of effector CD4+ T cells that
would normally have been eliminated. Bim caused the contraction of CD8+ effector cells after their response to Herpes Simplex Virus-1 [37]. Our first aim was to demonstrate that Bim caused the contraction of activated CD4+ T cells during the effector to memory transition. If so, then we could generate a population of previously activated Bim-deficient CD4+ T cells in vivo, most of which should have undergone apoptosis following the effector stage. We then aimed to characterize the phenotype and responsiveness of the surviving CD4+ T cells. If a selective process determined cell fate, then we expected the wild-type and Bim-deficient CD4+ memory T cells to exhibit differences, which could reveal the means of memory cell selection.

We anticipated that the re-expression of IL-7Rα would play a critical role in the selection of memory cells [92]. Both naïve and memory CD4+ T cells express IL-7Rα, but this receptor is downregulated following activation – in a reciprocal pattern to IL-2Rα, which in vivo is only transiently expressed by recently activated cells. Recent findings by our group demonstrated that signaling through IL-2Rα enhances CD4+ T cell memory development by promoting the re-expression of IL-7Rα [113]. Therefore, we expected to find only a subset of Bim-deficient cells to be IL-7Rα+ after activation, with those cells failing to re-express this receptor representing the population normally fated to die instead of differentiating into memory cells. By comparing the IL-7Rα positive and negative Bim-deficient CD4+ T cells, we hoped to identify the causes of memory cell differentiation.
RESULTS

Experimental systems for generating memory CD4+ T cells

We used the DO11.10 TCR transgenic adoptive transfer system to investigate memory CD4+ T cell differentiation. Previous studies relied on bone marrow-derived dendritic cells pulsed with Ovalbumin peptide and matured with LPS [113]. The responding DO11 cells required CD28 and IL-2 signaling for efficient differentiation to a memory phenotype [92]. Ova antigen was only encountered for the first 3 days following immunization, as DO11 cells transferred 3 days after immunization with Ova-pulsed DCs failed to respond, indicating that the antigen was functionally cleared [92].

We also established an experimental system to activate DO11 cells with an Ova-expressing recombinant virus. Vesicular Stomatitis Virus (VSV) is highly cytopathic Rhabdovirus related to rabies [114]. VSV rapidly replicates for the first 2 days after infection, but its single-stranded RNA genome provokes a strong type I interferon response that swiftly reduces viral replication [115]. VSV infection also triggers a strong and rapid IgM response, preventing virions from infecting new cells [116]. This combination of type I interferons and neutralizing antibodies is sufficient to protect mice from intravenous VSV infection.

We obtained a strain of VSV engineered to produce Ovalbumin protein [117]. Cells infected with VSV-Ova produce Ova in their cytoplasm, which is released when the host cells are lysed by the virus, but Ova protein is not included in the virion. VSV-Ova infection activates Ova-specific OT-I TCR transgenic CD8 cells, but OT-I cells fail to divide if transferred into recipients
infected 4 days previously, indicating that both virus and Ova antigen are cleared by this time [118].

Intravenous VSV-Ova infection provided three advantages to our study. First, DO11 cells were responding in an environment with concurrent innate and adaptive immune activation. Next, VSV-Ova’s abrupt eradication enabled us to clearly distinguish between the effector and memory stages of the DO11 response. Finally, since viral control and clearance did not depend on the DO11 response, any changes in the donor DO11 population would not create confounding changes in the course of the infection.

We first proved that DO11 cells could be specifically activated by VSV-Ova infection. Adoptively transferred DO11 cells proliferated after intravenous VSV-Ova infection, but not after immunization with the parental wild-type strain of VSV (Figure 1A). We identified dividing DO11 cells in the spleens, peripheral lymph nodes, mesenteric lymph nodes, and lungs of intravenously infected mice, whereas intraperitoneal or subcutaneous injection of VSV-Ova failed to activate DO11 cells (data not shown). While lower doses of virus caused fewer DO11 cells to divide, we observed no difference in the DO11 responses to $10^5$ to $10^7$ plaque-forming units of VSV-Ova (data not shown). As with Ova-pulsed DCs, VSV-Ova infection required CD28 costimulation in order to activate DO11 cells (Figure 1B).

DO11 cells divided rapidly between 2 and 3 days after VSV-Ova infection, increasing ~5 fold in numbers in the spleens and lymph nodes (Figure 1C). This burst of proliferation was delayed compared to the response to DCs+Ova as the virus had to infect host cells and replicate before Ova became available to activate DO11 cells. The number of activated effector DO11 cells stabilized from day 3
FIGURE 1
Response of DO11 CD4+ T cells to VSV-Ova infection

DO11 cells were labeled with CFSE and adoptively transferred into WT BALB/c recipients, then immunized i.v. the following day with PBS, the parental VSV strain, or VSV-Ova. The DO11 response was analyzed by flow cytometry. A) CFSE profiles of CD4+ KJ+ DO11 cells from the spleens of mice immunized 3 days previously. B) Proliferation of WT versus CD28 KO DO11 cells in the spleen 4 days after VSV-Ova infection. C) The number of DO11 cells in the spleens and peripheral lymph nodes was tracked after infection. DO11 cell numbers in two mice (circles) and their average (lines and bars) were determined at each time point.
until ~1 week following infection, then declined. More than 95% of the DO11 cells were lost during the transition of effector to memory cells, leaving on the order of $10^4$ divided DO11 cells persisting in the spleen and lymph nodes.

VSV is completely cleared from infected mice with intact immune systems. If not eradicated, infected mice develop lethal paralysis within 4 weeks [116] which we never observed following VSV-Ova infection (data not shown). However, although replicating virus was eliminated, Ova may have persisted and continued to stimulate DO11 cells.

We therefore determined how long after VSV-Ova infection the DO11 cells could detect Ova. We found that Ova was not persistently presented to T cells, and DO11 cells transferred only 3 days after VSV-Ova infection failed to divide (Figure 2A). To confirm this result we measured DO11 cell division in vivo by BrdU uptake at different times after VSV-Ova infection. While nearly all DO11 cells divided in the first 4 days after infection, proliferation returned to a naïve level between days 4 to 11 and 11 to 18 (Figure 2B, C). These results indicated that VSV-Ova infection provided a less than 3-day window of opportunity for DO11 cells to locate Ova on an APC. This short period of Ova presentation activated nearly all the DO11 cells, but these effector cells did not receive subsequent antigen stimulation.
**FIGURE 2**

Virus and Ova antigen were rapidly cleared

A) CFSE-labeled DO11 cells were transferred into recipients immediately following VSV-Ova infection (Day 0) or 3 days later. Spleens were analyzed by flow cytometry 3 days after DO11 transfer. CFSE profiles of CD4+ KJ+ cells are shown. B) DO11 RAG2 KO cells were transferred into BALB/c recipients and left uninfected or immunized with VSV-Ova the following day. Mice were treated with BrdU from 0 to 4, 4 to 11, or 11 to 18 days after infection. BrdU uptake was determined by flow cytometry at the end of each treatment period. Plots are gated on CD4+ KJ+ cells in spleens and the percentage of DO11 cells staining BrdU+ is shown in C). Isotype control antibody demonstrates specific staining.
**Bim deletion did not alter the effector stage of the CD4+ T cell response**

Immunization with either VSV-Ova or DCs+Ova induced a large population of effector DO11 cells that contracted to a smaller memory pool. We hypothesized that Bim-deficient DO11 cells would not die during the effector to memory transition. Before testing this hypothesis, we checked that Bim deficiency had not altered the effector phenotype.

Bim did not affect the number of effector DO11 cells responding to VSV-Ova (Figure 3A) or Ova-pulsed DCs (Figure 3B) on days 4-5, and CFSE profiles revealed equal initial proliferation of wild-type and Bim-deficient DO11 cells (Figure 3C). Bim also did not affect CD44 upregulation in response to either immunization (Figure 4A). VSV-Ova infection caused a transient loss of IL-7Rα expression, and an equal percentage of the wild-type and Bim-deficient DO11 population re-expressed this receptor after dividing (Figure 4B). Cytokine production was also unaffected by Bim deficiency. DO11 cells activated by VSV-Ova produced IL-2 and IFNγ, but not IL-4, with an equal response by wild-type and Bim-deficient DO11 cells (Figure 5A). Immunization with DCs+Ova induced IL-4 as well as IL-2 and a smaller percentage of IFNγ-producing DO11 cells with or without Bim (Figure 5B). Thus, Bim-deficient DO11 cells exhibited no change in their effector phenotype.
FIGURE 3
Bim deletion did not alter initial proliferation of DO11 cells

CFSE-labeled WT or Bim KO DO11 cells were transferred into BALB/c recipients. The following day recipients were immunized with VSV-Ova or Ova-pulsed DCs, or left unimmunized. Spleens and peripheral lymph node cells were counted and analyzed by flow cytometry to determine the number of DO11 cells **A)** 4 days after VSV-Ova infection and **B)** 5 days after DC immunization. **C)** Overlay of WT and Bim KO DO11 CFSE profiles from immunized mice.
FIGURE 4
Bim deletion did not alter the phenotype of effector DO11 cells

The DO11+ cells shown in Figure 3 were analyzed for CD44 upregulation and IL-7Rα re-expression. **A)** CD44 expression on CD4+ KJ+ cells in the spleens and lymph nodes 4 days after VSV-Ova infection or 5 days after DCs+Ova immunization. Expression on endogenous CD4+ populations are shown for comparison. **B)** IL-7Rα expression on the same DO11 cells responding to VSV-Ova, plotted versus CFSE dilution to show the average percentage of cells re-expressing this receptor after dividing.
FIGURE 5
Bim did not alter cytokine production by effector DO11 cells

Spleen cells from the mice shown in Figure 3 were restimulated overnight with Ova peptide and analyzed by flow cytometry for intracellular cytokines. IL-2, IFNγ, and IL-4 production by DO11 cells activated by A) VSV-Ova or B) DCs+Ova is shown as the percentage of DO11+ cells staining positive for each cytokine.
**Bim controlled the number but not differentiation of memory T cells**

After a peak response 5 days after VSV-Ova or DCs+Ova immunization, 90-95% of activated wild-type DO11 cells died during the effector to memory stage transition (Figure 6). In contrast, the size of the Bim-deficient DO11 population remained constant with no apparent loss of cells between the peak response and the number surviving weeks later. This implied that the contraction of the effector CD4+ T cell population was entirely dependent on Bim-mediated apoptosis.

If the development of CD4+ T memory cells occurred by a selective process, we expected that the surviving Bim-deficient DO11 population would be made of two subsets. One would be the bonafide memory cells, distinguished by high levels of IL-7Rα expression. The other would be derived from the effector cells that normally died instead of differentiating into a memory population.

Instead, we found the surviving wild-type and Bim-deficient DO11 populations exhibited the same phenotype. Regardless of Bim expression, VSV-Ova infection generated a population of DO11 cells that all re-expressed IL-7Rα and a majority of CD62L high cells (Figure 7A). When restimulated, wild-type and Bim-deficient DO11 cells displayed an equal ability to produce IL-2 and IFNγ (Figure 7B) and proliferated at lower concentrations of antigen than naïve counterparts (Figure 7C).

Immunization with DCs+Ova produced similar results to VSV-Ova infection. Both the surviving wild-type and Bim-deficient DO11 cells upregulated IL-7Rα and CD44 (Figure 8A). Bim expression did not affect IL-2 or IL-4
**FIGURE 6**
Bim caused the contraction of DO11 cells during the effector to memory transition

WT or Bim KO DO11 cells were labeled with CFSE, transferred into BALB/c recipients, and immunized with either **A)** VSV-Ova or parental VSV (Naïve), or **B)** DCs+Ova or left unimmunized (Naïve). The number of effector (day 5) and memory (week 4 or 7) DO11 cells in spleens, peripheral lymph nodes, and lungs was determined by flow cytometry.
FIGURE 7
Bim-deficient memory cells differentiated normally in response to VSV-Ova infection

A) DO11 cells from spleens of VSV or VSV-Ova infected mice shown in Figure 6 were analyzed by flow cytometry for IL-7Rα and CD62L expression 7 weeks after infection. B) These spleen cells were restimulated overnight with Ova peptide and DO11+ cells were assayed for intracellular IL-2 and IFNγ. C) Divided WT and Bim KO DO11 cells were purified by cell sorting of peripheral and mesenteric lymph nodes 11 weeks after VSV-Ova infection. Sorted cells were cultured with splenic APCs and titrated Ova peptide for 3 days. Proliferation was measured by thymidine uptake between days 2 and 3. As a control, naïve WT DO11 cells were sorted from a DO11 transgenic donor.
FIGURE 8
Bim-deficient memory cells differentiated normally in response to DCs+Ova immunization

A) DO11 cells from spleens of unimmunized or DCs+Ova immunized mice shown in Figure 6 were analyzed for IL-7Rα and CD44 expression after 4 weeks.  
B) Intracellular IL-2, IFNγ, and IL-4 staining of DO11 cells after these spleen cells were restimulated overnight with Ova peptide.  
C) Divided Bim KO DO11 cells were isolated from these mice by sorting the peripheral lymph nodes and assayed for proliferation as in Figure 7.
production upon restimulation, although IFN\(\gamma\) was reduced in some experiments (Figure 8B, but not others, data not shown), and the proliferation of the surviving Bim-deficient cells showed increased sensitivity to antigen (Figure 8C). Thus, the Bim-deficient DO11 cells differentiated into fully functional memory cells in response to either VSV-Ova infection or immunization with DCs+Ova.

**DISCUSSION**

Apoptosis during the transition from the effector to memory stages of the immune response normally eliminates >90% of the responding CD4+ T cells. Our study demonstrated that Bim caused this elimination and that, if Bim was eliminated, the size of the effector CD4+ T cell population did not decline. This result is similar to the previously described role of Bim-mediated cell death in reducing the number of CD8 effector T cells during the transition to the memory stage [37]. Our findings differed from those reported for Lymphocytic Choriomeningitis Virus (LCMV)-specific CD4+ T cells, where Bim-deficient mice ultimately maintained the same number of memory cells as wild-type controls [38]. However, we instead suspect that the eventual (after >100 days post infection) equilibration reported of wild-type and Bim-deficient LCMV-specific CD4+ T cells was at least partially caused by long-term proliferation. In this LCMV study, ~5 fold more antigen-specific cells were present 5 weeks post infection, after which the number of wild-type cells increased. Furthermore, in our study, only the DO11 cells lacked Bim whereas the LCMV memory response
was analyzed in Bim-deficient mice where changes in other cell populations could have affected the LCMV-specific CD4+ T cells.

Selective models of memory cell fate determination all imply that the effector cells that do not survive qualitatively differ from those giving rise to the memory pool. Bim deficiency protected activated DO11 cells from apoptosis but did not affect the phenotype of either their effector or memory phenotypes. Thus, our results suggest that memory cell fate is a stochastic process and not the result of selection in favor of a qualitatively different subset of CD4+ T cells.

Activated CD4+ T cells require IL-7 in order to transition to a long-lived memory population [119, 120]. Although IL-7 plays a complex role in T cell homeostasis, its effects are thought to influence the survival – but not function – of CD4+ memory T cells [108]. Bim has proven essential to eliminate T cells deprived of IL-7 [30].

In contrast to IL-7, IL-2 promotes the survival of CD4+ memory T cells but it also affects their differentiation [113]. Memory DO11 cells lacking IL-2Rα were both reduced in number and impaired in their ability to produce IFNγ and IL-4. However, the few surviving IL-2Rα-deficient memory cells expressed wild-type levels of IL-7Rα, CD44, and CD62L.

We expected that a subset of DO11 cells failed to re-express IL-7Rα after activation and died during the effector to memory transition, and that this population would survive without Bim. Instead, we observed no difference in IL-7Rα expression between wild-type and Bim-deficient effector or memory
populations (Figures 4B, 7A, 8A). Likewise, Bim did not alter IL-7Rα expression on CD4 T cells over the course or their response to LCMV [38].

We interpret these data to mean that CD4+ memory T cell differentiation and survival are controlled by distinct mechanisms. IL-2 and other signals, such as costimulatory receptors, are essential to program memory cell development and may secondarily promote survival, such as by hastening IL-7Rα re-expression or boosting this receptor’s level. However, the majority of effector CD4+ T cells, although they normally die, are capable of acquiring a fully functional memory phenotype. Our data indicate that the size, but not the characteristics, of the memory pool is set by competition for IL-7 and enforced by Bim-mediated apoptosis.

REFERENCES


CHAPTER FIVE

Materials and Methods

MICE

All mice were housed in the specific pathogen-free facility of the University of California, San Francisco in accordance with institutional guidelines. BALB/c mice were purchased from Charles River Laboratories (Wilmington, MA) and the Jackson Laboratory (Bar Harbor, ME). DO11.10 TCR transgenic BALB/c mice were obtained from Dr. K. Murphy (Washington University, St. Louis, MO) via the Jackson Laboratory. The BALB/c sOva transgenic mouse has been described previously [1, 2]. Bcl-X transgenic mice [3] were obtained from Dr. M. Boothby (Vanderbilt University, Nashville, TN) and back-crossed 5-10 generations onto the BALB/c strain. BALB/c Thy1.1 mice were provided by Dr. R. Locksley (University of California – San Francisco, San Francisco, CA). Fas-mutant lpr mice were purchased from the Jackson Laboratory and bred 10 generations onto the BALB/c background. Bim-deficient mice [4] were obtained from Dr. A. Strasser (University of Melbourne, Melbourne, Australia) and back-crossed 7-10 generations to the BALB/c strain. FoxP3-GFP reporter mice [5] were provided by Dr. A. Rudensky (University of Washington, Seattle, WA) on a mixed C57BL/6 X BALB/c background and bred 3 generations to BALB/c. IL-2 deficient mice [6] were obtained from the Jackson Laboratory and back-crossed >10 generations to BALB/c. The BALB/c RIP-Ova transgenic mice has been described previously [7,
8]. BALB/c CD28-deficient mice were provided by Dr. J. Bluestone (University of California – San Francisco, San Francisco, CA). Mice were genotyped by PCR, except when using flow cytometry to distinguish Thy1.1 from Thy1.2.

HEMATOLOGY

Blood samples were collected in EDTA-treated CapiJect tubes (Terumo Medical Corporation) and analyzed using a Hemavet 950 instrument (Drew Scientific).

ANTIBODIES

Unless otherwise noted, all antibodies were purchased from BD Biosciences and used in accordance with the manufacturers’ recommendations. The following antibodies were used: αB7.2 (GL1), αBcl-2 (3F11), αBcl-X (7B2.5, isotype control B10, Southern Biotech), αBim (10B12, Dr. L. O’Reilly), αBrdU (3D4, isotype control MOPC-21), αCD3ε (145-2C11, eBioscience), αCD4 (GK1.5, RM4-5), αCD8α (53-6.7), αCD19 (1D3), αCD25 (PC-61), αCD44 (IM7), αCD62L (MEL-14), αCD69 (H1.2F3), αCTLA-4 (UC10-4F10-11), αFas (J02), αFoxP3 (FJK-16s, eBioscience), αI-A^d^ (AMS-32.1), αIFNγ (XMG1.2), αIL-2 (JES6-5H4), αIL-4 (1B11), αIL-7Rα (AFS98, eBioscience), αIL-17 (TC11-18H10), KJ1.26 (Caltag Laboratories), OX-40, αPD-1 (J43), and αThy1.1 (OX-7). For flow
cytometry, antibodies were conjugated to FITC, PE, PerCP, PE-Cy7, or APC fluorescent dyes, or to biotin and detected with fluorescently labeled streptavidin.

CELL PREPARATIONS, PURIFICATIONS, AND ADOPTIVE TRANSFER

Cell suspensions

Single cell suspensions were generated from thymi, lymph nodes, and spleens by rubbing organs through a wire mesh followed by washing in media + 2% FCS and filtration. Erythrocytes in spleen samples were removed by hypotonic lysis by suspending cells in Tris-buffered 175mM NH₄ and incubating 5' at 37°C. Live cells were counted with a hemocytometer, using Trypan Blue staining, and by flow cytometry, using forward by side scatter gating and normalized with beads.

Purification of CD4+ cells

CD4+ cells were magnetically enriched by positive selection using the Dynabead (Dynal-Invitrogen) or either positive or negative selection using the EasySep (Stem Cell Technologies) reagents. Alternatively, CD4 cells were enriched by depleting CD8+ and CD19+ cells with magnetic αFITC beads (Qiagen). Magnetic purification protocols were based on the manufacturers’ recommendations.

Cells were also purified with a MoFlo high-speed cell sorter (DakoCytomation). Samples of sorted cells were analyzed on the same
instrument to confirm the purification. In some experiments, cells were magnetically enriched prior to cell sorting.

**CFSE labeling & adoptive transfer**

Donor cells were first checked by flow cytometry to verify purification and determine the fraction of DO11+ cells. Purified CD4+ cells were suspended at 10^7 cells/mL in PBS and labeled 10’ at room temperature with 2-5µM CFSE (Molecular Probes, Invitrogen). Excess dye was quenched by adding media + 10% FCS, incubating 10’ at room temperature, and washing 1-3 times with media. Labeled cells were filtered, re-counted, and washed and suspended in PBS. Each sex-matched recipient mouse received a tail vein injection of 2-5 million DO11+ cells in a 200-600µL volume.

**Isolation of lymphocytes from lungs**

Mice were treated with 150 units heparin (Sigma-Aldrich) in PBS by intra-peritoneal injection, then euthanized without cervical dislocation and perfused with PBS. Lungs were excised, washed in media, and mechanically dissociated. Samples were incubated in media plus 400 units/mL collagenase VIII (Sigma-Aldrich) for 30 min at 37°C, during which time the suspension was pipetted up and down 3 times for 1’. The resulting cell suspension was filtered and subjected to density gradient centrifugation using Lympholyte-M (Cedarlane Laboratories). Isolated lymphocytes were recovered from the interface, washed, and counted.
QUANTITATIVE RT-PCR

After purification by cell sorting, cells were transferred to RNase-free tubes, washed twice in PBS, snap-frozen as dry pellets in an ethanol – dry ice bath, and stored at -80°. Alternatively RNA was extracted from freshly sorted and washed, but unfrozen, cells. RNA was isolated with Trizol (Invitrogen), and mRNA was reverse-transcribed to cDNA using SuperScriptIII (Invitrogen), following the manufacturer’s suggestions. Complementary DNA was detected using SYBR Green fluorescence (Applied Biosystems) and an Opticon 2 instrument (Bio-Rad Laboratories) according to the manufacturer’s instructions. Relative gene expression was determined by normalization to HPRT. Both a melting curve analysis and gel electrophoresis confirmed that the primer sets amplified a single PCR product.

FLOW CYTOMETRY

Cell suspensions were washed with PBS + 1% FCS and incubated 5-20’ at 4° with αCD16/32 antibody. Fluorescently conjugated antibodies to surface antigens were added, cells were stained 10-30’ at 4°, washed 1-3 times in PBS + 1% FCS, and fixed 10’ at room temperature in PBS + 1-2% paraformaldehyde. Fixed cells were washed and suspended in PBS + 1% FCS and stored at 4° until samples were collected. Flow cytometry was performed on a FACSCaliber instrument using CellQuest software (BD Bioscience).

For intracellular analyses, fixed cells were washed, permeabilized for 10’ at room temperature, and stained for 30’ at 4° in PBS + 1% FCS + 0.5% saponin. Stained cells were washed 2-3 times in PBS + 1% FCS + 0.5% saponin and suspended in PBS + 1% FCS. In some experiments, Cytofix/Cytoperm and Perm/Wash (BD Biosciences) were substituted for paraformaldehyde and saponin.

Intracellular Bim staining

Cells were stained with KJ1.26-APC and αThy1.1-FITC, washed, fixed 20’ at 4° in Cytofix/Cytoperm, and washed with 1X Perm/Wash (BD Biosciences). All subsequent staining and washing was performed in Perm/Wash. Cells were stained with unconjugated αBim (10B12, reactive to all isoforms, provided by Dr. L. O'Reilly, University of Melbourne, Melbourne, Australia) or isotype control (Rat IgG2a, KLH/G2a-1-1, Southern Biotech), using 10ng antibody per million cells, for 30’ at 4°, then washed twice. Polyclonal αRat IgG F(ab')2-PE (Jackson
ImmunoResearch), using 1.25µg per million cells, was pre-absorbed in 2% normal mouse serum, then used to detect bound αBim by staining the cells for 30’ at 4°C, followed by two washes. Finally, cells were stained with αCD4-PerCP, pre-absorbed in 4% normal rat serum, for 20’ at 4°C. Cells were washed twice, then suspended in PBS + 1% FCS for analysis.

**BrdU labeling and intracellular staining**

For the experiments shown in Chapter 2, Figure 13, 5-Bromo-2’-deoxyuridine (Sigma-Aldrich) was dissolved in PBS and 1mg/mouse was administered by intra-peritoneal injection on days 0, 1, 2, 4, and 6 after transfer of DO11 cells, or the equivalent days beginning on day 14. Alternatively, as shown in Chapter 4, Figure 2, experimental groups were treated beginning on day 0, 4, or 11 after VSV-Ova infection. Mice were injected twice, ~4 hours apart, with 1mg BrdU, then provided with water supplemented with 1mg/mL BrdU and 5% glucose.

Cells were stained for surface antigens, then incubated for 30’ at room temperature in Cytofix/Cytoperm (BD Biosciences). Fixed cells were washed twice with Perm/Wash (BD Biosciences) and suspended in PBS + 1% FCS. To permeabilize the nucleus, cell suspensions were mixed an equal volume of 80% FCS + 20% DMSO and stored at -80°C until all time points of each experiment were collected.

Cells were thawed, washed with PBS + 1% FCS, fixed a second time with Cytofix/Cytoperm for 20’ at room temperature, and washed with Perm/Wash. To digest DNA, cells were incubated for 60’ at 37°C in PBS supplemented with 10µM
HCl, 150mM NaCl, 4.2mM MgCl$_2$ and 250 Kunitz units/mL freshly-dissolved DNase I (Sigma-Aldrich), then washed with Perm/Wash. BrdU was stained with 0.2 “tests” per million cells αBrdU-FITC (3D4, BD Bioscience) or isotype control (MOPC-21) in Perm/Wash for 30’ at room temperature, then washed twice with Perm/Wash and suspended in PBS + 1% FCS for analysis. In some experiments, Perm/Wash was replaced by PBS + 1% FCS + 0.5% saponin.

**Intracellular FoxP3 staining**

Cells were stained using the buffers and protocol provided by the manufacturer. Cells were fixed overnight and stained with αFoxP3 (FJK-16s, eBioscience) for 30’ at 4°C.

**CELL CULTURE AND IN VITRO ASSAYS**

T cells were cultured in RPMI 1640 media (Sigma-Aldrich) supplemented with 1mM each L-glutamine, non-essential amino acids, sodium pyruvate, HEPES, penicillin, streptomycin (all from Life Technologies), 50µM 2-ME, and 10% Fetal Calf Serum (FCS, Sigma-Aldrich, Omega Scientific). Cultures were incubated at 37°C in a 5% CO$_2$ atmosphere.

DO11 cells were activated with their cognate peptide antigen, chicken Ovalbumin 323-339 (Ova, Analytical Biotech Services). Concentrated Ova stocks were made by dissolving peptide in DMSO. Fresh peptide solutions were made for every experiment by diluting Ova stocks in media and filtration.
**Intracellular staining to assay cytokines**

Three to 7 million lymph node or spleen cells were cultured 6-24 hours with 1µg/mL Ova peptide in 24 well tissue culture treated plates. Brefeldin A (Epicentre Biotechnologies) was added at 10µg/mL for the final 2-3 hours of culture. Cells were washed, stained for surface antigens, fixed, permeabilized, and stained intracellularly for cytokines.

**Proliferation assay**

Cultures were set up in 96 well flat bottom tissue culture treated plates, varying only the concentration of Ova peptide. Each well contained: 10⁴ sort-purified DO11+ cells, 2.5 X 10⁵ antigen-presenting cells (APCs), 0-1µg/mL Ova peptide, and media to a 200µL volume. APCs were prepared from erythrocyte-depleted spleens by suspending cells at 25 million/mL in media plus 100µg/mL mitomycin C, incubating 45' at 37°, washing 5 times, and counting live cells. In some experiments, samples of supernatants were removed for analysis by ELISA and replaced by an equal volume of media.

Cultures were pulsed with 1µCi/well [H³]Thymidine 12-24 hours prior to endpoint and transferred to filtermats using a Harvester 96 Mach III (Tomtec). Proliferation was measured by scintillation counting by a Trilux 1450 Microbeta instrument (Wallac – Perkin Elmer).
ELISA to assay cytokines

Cytokines were measured by sandwich ELISA using antibodies from BD Biosciences. Culture supernatants were stored at –20°C until use. Ninety-six well plates were coated overnight at 4°C with purified “capture” antibodies to IL-2 (JES6-1A12, 1µg/mL), IFNγ (R4-6A2, 4µg/mL), or IL-4 (1B11, 1µg/mL) in PBS. Plates were washed 4 times using a Skan Washer 400 (Molecular Devices) with PBS + 0.01% Triton X-100, then blocked with PBS + 1% FCS for 1 hour at room temperature and washed twice. Cell culture supernatants were added to wells for 2 hours at room temperature or overnight at 4°C and plates were washed 4 times. Biotinylated αIL-2 (JES6-5H4), αIFNγ (XMG1.2), or αIL-4 (BVD6-24G2) were added in PBS + 1% FCS at the same concentration as the capture antibodies, and plates were incubated 45’ at room temperature then washed 6 times. Streptavidin-Alkaline Phosphatase (BD Bioscience) was diluted 1:400 in PBS + 1% FCS, added at 50µL/well, and plates were incubated 30’ at room temperature. Plates were washed 6 times and phosphatase substrate tablets (4-Nitrophenyl phosphate disodium salt hexahydrate, Sigma-Aldrich) were dissolved to 1mg/mL in DEA buffer. Substrate was added at 50µL/well and plates were read by a Spectramax Plus spectrophotometer (Molecular Devices) to measure absorbance at 405nm. Results were analyzed using SoftMax Pro software and data were converted from absorbance to concentration by comparison to a standard curve of recombinant cytokines.
Bone marrow-derived dendritic cells

Marrow was flushed from the femur, tibia, and humerus bones, mechanically disaggregated, and filtered. Samples were cultured at 10^6 cells/mL in 15cm suspension culture dishes. DCs were grown in IMDM media (Sigma-Aldrich) supplemented with 20ng/mL recombinant mouse GM-CSF (PeproTech), 1mM each L-glutamine, non-essential amino acids, sodium pyruvate, HEPES, penicillin, streptomycin (all from Life Technologies), 50µM 2-ME, and 10% Fetal Calf Serum (Sigma-Aldrich, Omega Scientific). Cultures were incubated at 37° in a 5% CO_2 atmosphere.

An equal volume of media was added after 3 days of culture. On day 6, non-adherent cells were collected by gently washing with PBS + 3mM EDTA, counted, and returned to culture as on day 0 but with 1ng/mL IL-4 added. An equal volume of media, 1-2µg/mL LPS (Escherichia coli O26:B6; Sigma-Aldrich), and 1µg/mL Ova peptide were added on day 7. Non-adherent, peptide-pulsed DCs were harvested on day 8, counted, and analyzed by flow cytometry.

“Mature” DCs were defined by bright staining of both αI-A^d and αB7.2. Cells were washed in PBS and 0.2-1 million mature DCs per mouse were injected intravenously.

Some DC cultures were adjusted as follows. Cells were passed on day 5, passed and treated with IL-4 on day 7, treated with LPS and Ova between days 8 and 10, and harvested the following day. In addition, surplus DCs were stored frozen. DCs were suspended at 1 million cells/mL in 50% FCS, 40% media, and
10% DMSO, then placed in a freezing chamber at -80°. After rapid thawing, frozen DCs were washed in media, counted, and injected as above.

**Co-culture suppression assay**

Cultures were set up in round-bottom 96 well plates, varying only the number of regulatory T cells per well. Regulatory T cells and responder T cells were purified by cell sorting, based on CD25 and/or FoxP3-GFP expression. Antigen-presenting cells were prepared from mitomycin C-treated splenocytes.

With polyclonal T cells, each well contained: 4 X 10⁴ APCs, 2 X 10⁴ responder T cells, 2.5 or 3µg/mL functional-grade αCD3 (145-2C11, eBioscience), media to a 250µL volume, and a titrated number of regulatory T cells. Cells were cultured 48 hours, then pulsed with [³H]Thymidine and assayed for proliferation as described.

With DO11 T cells, each well contained: 2.5 X 10⁴ APCs, 1.5 X 10⁴ naïve DO11 responders, 1µg/mL Ova peptide, media to a 200µL volume, and a titrated number of CD25+ or CD25- KJ1.26+ cells from DO11 X sOva Tg mice. After 40 hours of culture, supernatant samples were collected to measure IL-2 by ELISA and replaced by an equal volume of media. Cultures were pulsed with [³H]Thymidine after 72 hours to assay proliferation.
ANTIBODY BLOCKADE

Antibodies to CTLA-4 (4F10), PD-1 (RMP1-14), and Rat IgG negative control were produced as described by our collaborators [9]. Mice were given intra-peritoneal injections of 50µg antibody in PBS on days –1, 0, 1, 3, and 5 relative to DO11 cell adoptive transfer.

VIRUS PRODUCTION AND INFECTIONS

The Indiana serotype of Vesicular Stomatitis Virus (VSV) was previously engineered to express full-length chicken Ovalbumin [10]. Parental VSV and VSV-Ova stocks were provided by Dr. L. Lefrancois (University of Connecticut, Framington, CT). Viruses were produced and titered as described [10] and stored in aliquots at -80°. Mice were infected with 5-10 X 10^6 PFU VSV or VSV-Ova, diluted in PBS, by intravenous injection.

REFERENCES


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