UvA-DARE (Digital Academic Repository)

Understanding human immunology through the study of primary immune deficiency disorders

de Oliveira Filho, J.B.

Link to publication

Citation for published version (APA):
de Oliveira Filho, J. B. (2011). Understanding human immunology through the study of primary immune deficiency disorders.

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: https://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.

UvA-DARE is a service provided by the library of the University of Amsterdam (http://dare.uva.nl)

Download date: 14 Jun 2020
CRITICAL ROLE OF BIM IN T CELL RECEPTOR
RESTIMULATION-INDUCED DEATH

Snow AL,1* Oliveira JB,2* Zheng L,1 Dale D,3 Straus SE,3 Fleisher TA, Lenardo MJ.1
*co-first authors;

1Molecular Development Section, Laboratory of Immunology, National Institute of
Allergy and Infectious Diseases; 2Department of Laboratory Medicine, Clinical Center; 3
Laboratory of Clinical Infectious Diseases, National Institute of Allergy and Infectious
Diseases, National Institutes of Health, Bethesda, MD 20892-1508, USA

Biology Direct. 2008; v.3, p.34-44, 2008.
ABSTRACT
Background: Upon repeated or chronic antigen stimulation, activated T cells undergo a T cell receptor (TCR)-triggered propriocidal cell death important for governing the intensity of immune responses. This is thought to be chiefly mediated by an extrinsic signal through the Fas-FasL pathway. However, we observed that TCR restimulation still potently induced apoptosis when this interaction was blocked, or genetically impaired in T cells derived from autoimmune lymphoproliferative syndrome (ALPS) patients, prompting us to examine Fas-independent, intrinsic signals. Results: Upon TCR restimulation, we specifically noted a marked increase in the expression of BIM, a pro-apoptotic Bcl-2 family protein known to mediate lymphocyte apoptosis induced by cytokine withdrawal. In fact, T cells from an ALPS type IV patient in which BIM expression is suppressed were more resistant to restimulation-induced death. Strikingly, knockdown of BIM expression rescued normal T cells from TCR-induced death to as great an extent as Fas disruption. Conclusion: Our data implicates BIM as a critical mediator of apoptosis induced by restimulation as well as growth cytokine withdrawal. These findings suggest an important role for BIM in eliminating activated T cells even when IL-2 is abundant, working in conjunction with Fas to eliminate chronically stimulated T cells and maintain immune homeostasis.

INTRODUCTION
Proper homeostasis is achieved during an immune response by controlling the appropriate size and activity of the effector T cell pool to maximize immunity and minimize immunopathology. After an immune response, homeostasis depends on the efficient contraction of the expanded T effector pool. Both processes require the selective death of effector T cells1-5. When resting T cells become activated and proliferate under the influence of growth cytokines, they display heightened sensitivity to apoptosis2,4,6. The mechanisms by which apoptosis is provoked have been thought to differ depending on the level of antigen in the T cell milieu. In one simple schema, T cell apoptosis proceeds through either an "intrinsic" (Bcl-2 superfamily/mitochondrial-dependent)
Role of BIM in RAICD

program when antigen levels are low, or an "extrinsic" (Fas/CD95/APO-1/death receptor-mediated) pathway under conditions of high or repeated antigen stimulation. In the first case, antigen clearance at the conclusion of an immune response results in diminished growth and survival cytokines (such as interleukin-2 (IL-2)), thus activating the mitochondrial death program. Cytokine withdrawal apoptosis (CWA) dramatically reduces the expanded T effector population to re-establish homeostasis, but permits a small population to persist as memory T cells. CWA is principally regulated by the pro- and anti-apoptotic members of the Bcl-2 family. In particular, the pro-apoptotic "BH3-only" proteins Bim and Puma have been implicated in CWA, as revealed by expanded memory T cells in knockout mice. These "BH3 only" members of the Bcl-2 superfamily cause caspase activation and apoptosis by binding pro-survival congeners and releasing the proapoptotic proteins Bax and Bak. Moreover, we recently discovered that a gain-of-function mutation in N-RAS, which suppresses Bim expression via constitutive extracellular signal-related kinase (ERK) activation, could cause a novel form of ALPS in humans. Indeed, Bim expression is tightly controlled by several transcriptional and post-translational mechanisms that underscore its role in central and peripheral T cell tolerance.

On the other hand, the extrinsic apoptosis pathway involves restimulation of activated T cells with high doses of antigen during the immune response; a pathway often referred to as "activation-induced cell death (AICD)". However, it is important not to obfuscate the critical functional distinction between "activation" – the process entrained to the antigen receptor that causes resting cells to cycle, expand, and acquire effector function – and the death mechanism induced by TCR restimulation of those effector T cells that counterpose their expansion. So the term "TCR restimulation" or "TCR-induced" apoptosis will be used herein. The key immunoregulatory consideration is why restimulation by same antigen that produced the immune response, can kill the participating T cells in a highly specific way. At first glance, this event would seem to debilitate the immune response since the antigen, and presumably its pathogenic source, are still present. However, it is best understood as a negative feedback mechanism that constrains effector T cell proliferation to avoid immunopathology, previously termed "propriocidal" regulation. Propriocidal or TCR-induced death increases
proportionately with high or persistent levels of antigen in IL-2. TCR-induced death has hitherto been primarily equated with the Fas death receptor. Indeed, the upregulation of Fas ligand (FasL) on the surface of restimulated T cells engages Fas on effector T cells in cis ("suicide") or in trans ("fratricide") leading to apoptosis\textsuperscript{15-18}. Moreover, debilitating mutations in Fas or FasL result in defective lymphocyte homeostasis and autoimmunity first characterized in mice (lpr and gld, respectively) and later in humans with ALPS type Ia or Ib\textsuperscript{19,20}.

The Bim vs. Fas paradigm recently restated for intrinsic vs. extrinsic T cell apoptosis is appealing in its simplicity but illusory. For instance, other BH3-only proteins such as PUMA are likely instrumental in CWA\textsuperscript{9,10}. Also, the evidence suggests that Fas may not be the sole mediator of TCR-induced death and that TNF or nonapoptotic pathways may be involved\textsuperscript{21,22}. Data from conditional knockout mice in which Fas is ablated or blocked in distinct hematopoietic compartments indicate that Fas-mediated apoptosis may also counter autoimmunity by ensuring the removal of antigen presenting cells, including B cells and dendritic cells rather than T cells\textsuperscript{23,24}. Although autoreactive T cells accumulate in T cell-specific Fas knockout mice, surprisingly, loss of Fas confers no selective survival advantage for T cells exposed to repeated antigen challenge\textsuperscript{24}. Also, Fas engagement can intersect with the intrinsic pathway through a caspase-8 activating cleavage of Bid – a Bcl-2 superfamily member that can trigger mitochondrial apoptosis. Based on these insights, we asked whether death effector pathways other than Fas, including intrinsic signals routed through mitochondrial activation, were important for TCR-induced death of human T cells.

In re-examining human T cells in which FAS signaling is blocked or genetically impaired, we found that TCR-induced apoptosis can proceed through rapid induction of BIM expression in the absence of FAS signals, which contributes to mitochondrial permeabilization and cell death in the presence of IL-2. Knockdown of BIM expression partially rescued cells from TCR-induced death, particularly for CD8\textsuperscript{+} human T cells. Moreover, we show that TCR-induced apoptosis is normal for ALPS Ia patients displaying elevated BIM expression, but impaired in an ALPS type IV patient in which BIM expression is repressed. Collectively, these data indicate that FAS and BIM can cooperate as independent effector molecules in TCR-induced apoptosis. Our results show
BIM plays a key role in T cell contraction even when cytokines are abundant, indicating that FAS- and BIM-mediated T cell apoptosis are not mutually exclusive pathways as recently reinforced in the literature.

**METHODS**

*Cells and treatments*

Patients were enrolled and blood samples were obtained with informed consent under protocols approved by the National Institutes of Health (NIH). Peripheral blood lymphocytes (PBL) from normal donors were isolated by Ficoll density gradient centrifugation, and T cells were activated by either 5 µg/ml ConA or 1 µg/ml OKT3 mAb (Ortho Biotech, Raritan, NJ) plus 25 U/ml rhIL-2 (Peprotech, Rocky Hill, NJ), washed 3× in PBS, then cultured in 100 U/ml rhIL-2 for at least 7 days before apoptosis assays were performed. Activated T cell subsets were separated using CD4 or CD8 Microbeads and MACS magnetic bead cell separation (Miltenyi Biotec, Auburn, CA). In some experiments, inhibitors to caspase 8 (IETD-fmk) or caspase 9 (LEHD) (BioVision, Palo Alto, CA) were added at 20 µM. Caspase 9 enzymatic activity was measured using a Caspase 9 Colorimetric Assay Kit (BioVision) according to the manufacturer's instructions.

*Flow Cytometry*

Apoptosis assays were performed as previously described [12]. Briefly, activated T cells were resuspended in fresh media + IL-2 and stimulated for 24 h with soluble OKT3 mAb, agonistic anti-Fas mAb APO1.3 (Alexis, San Diego, CA) plus 200 ng/ml Protein A, or 2 µM staurosporine. In some experiments, 1 µg/ml of an antagonistic Fas blocking Ab (clone SM1/23, Alexis) was added to cells 30 minutes prior to OKT3 restimulation. The level of apoptosis was determined by staining with 1 µg/ml propidium iodide and flow cytometry analysis using constant time acquisition as previously described. Mitochondrial permeability was measured by staining with 40 nM 3,3'-dihexyloxacarbocyanine iodide (DiOC6) (EMD Biosciences, San Diego, CA) for 15 min at 37°C before flow cytometry analysis. For surface staining, cells were stained with 5 µg
anti-CD4-fluorescin isothiocyanate (FITC), anti-CD8-phycoerythrin (PE), or anti-CD95-PE (BD Biosciences).

**Electron Microscopy**
Treated cells (5 \times 10^6) were pelleted and overlaid with 2% glutaraldehyde in 0.1 M cacodylate buffer fixative for 2 h at room temperature (RT). Sample preparation and electron microscopy was performed at the Image Analysis Laboratory of the National Cancer Institute (Frederick, MD).

**Microarray Analysis**
RNA was isolated from two normal donor activated T cells at 0 or 6 h after OKT3 restimulation using Trizol (Invitrogen) and RNeasy mini-columns (Qiagen, Valencia, CA). Purified RNA was amplified using the Ovation Aminoallyl Amplification System (NuGEN, San Carlos, CA), labeled with Cy5 using the Cy5 Reactive Dye Pack (GE Healthcare, Piscataway, NJ), and cleaned up using Vivaspin columns (VivaScience AG, Hanover, Germany). Amplified RNA (2 µg) was hybridized to Hsbb 23K human spotted arrays (NIAID Microarray Research Facility) versus Cy3-labeled reference RNA pooled from six normal donor cycling T cells. Data was analyzed using GenePix and mAdb software.

**Immunoblotting**
Cells were lysed in 1% NP-40 lysis buffer for 15 min on ice, then cleared by centrifugation. Protein concentration was determined by BCA assay (Pierce, Rockford, IL), and 20–30 µg total protein was separated by SDS-PAGE. Blots were probed with the following antibodies (Abs): anti-BIM (Stressgen, Ann Arbor, MI); anti-BAX, anti-cytochrome c (clone 7H8.2C12), anti-BCL-xL, anti-BCL-2, anti-MCL-1 (BD Pharmingen); anti-PUMA (Alexis); anti-β-actin (clone AC-15, Sigma). Bound Abs were detected using appropriate horseradish peroxidase-conjugated secondary Abs (Southern Biotech, Birmingham, AL) and ECL (Pierce).
siRNA Transfections

Activated human PBL were transfected with 200 pmol of either specific small interfering RNA oligoribonucleotides (siRNA) or a non-specific (NS) control oligo (Invitrogen, Carlsbad, CA) using the Amaxa Nucleofection system (Amaxa, Koln, Germany). Assessment of knockdown efficiency and all subsequent assays were performed 4 days (human) post-transfection. siRNA sequences are available from Invitrogen (Stealth Select).

RESULTS AND DISCUSSION

TCR restimulation induces apoptosis signals independent of FAS

To examine TCR-induced death in human T cells, activated peripheral blood lymphocytes (PBL) from normal donors were restimulated with the anti-CD3 mAb OKT3 after cycling in IL-2 for 7–14 days. The majority of these cells are CD4⁺ and CD8⁺ T cells, with the latter generally more abundant in culture. Data was obtained for numerous human donors. We found that apoptosis was readily induced in restimulated T cells, marked by chromatin condensation and shrinkage (Fig. 1A). This was followed by loss of membrane integrity due to secondary necrosis. Apoptosis was verified by PI exclusion; however, we noted that blocking FAS with an antagonistic Ab (SM1/23) provided only partial protection against TCR-induced (Fig. 1B) Flow cytometric analysis of restimulated T cells also confirmed cell shrinkage and loss of mitochondrial membrane potential, as indicated by decreased DiOC₆ staining following 12 h of OKT3 treatment, signifying apoptosis (Fig. 1C) Again, blocking Fas with an antagonistic Ab (SM1/23) only partially rescued this drop in mitochondrial membrane potential and cell viability. Remarkably, T cells from an ALPS Ia patient with a FAS death domain mutation also showed only a modest loss of mitochondrial membrane potential and viability (Fig. 1C), suggesting a mitochondria-dependent apoptotic signal could proceed despite compromised FAS function. Similarly, cytochrome c released from mitochondria in response to OKT3 restimulation was only modestly decreased by FAS blockade (Fig. 1D). We also tested caspase 9 activation, which occurs downstream of cytochrome c release and "apoptosome" formation. As expected, caspase 9 activation was only partially reduced in restimulated cells in the presence of FAS blocking Ab, but completely
abrogated in the presence of the caspase 9 specific inhibitor LEHD-fmk (Fig. 1E). In contrast, the SM1/23 Ab effectively blocked APO1.3 anti-Fas induced apoptosis, indicating that the cells were competent for FAS-mediated death (Fig. 2). Taken together, our data confirms that TCR-induced death relies in part on intrinsic mitochondrial signals triggered independently of FAS-FASL interactions.

**Role for BIM induction in TCR-induced death**

Initial studies of AICD indicated that *de novo* transcription was required for the execution of apoptosis in response to T cell restimulation. Since our data pointed toward a mitochondrial component, we surveyed expression of several pro- and anti-apoptotic BCL-2 family members using microarrays following TCR restimulation of activated human PBL for 6 h. As a positive control, we detected significant induction of FASL expression. Notably, we detected an even greater increase (> 5 fold) in BIM transcription in response to OKT3 stimulation (Fig. 3A) Only BCL-xL was also increased with restimulation, whereas other BCL-2 family members remained largely unchanged or slightly decreased. The expression of all three BIM protein isoforms (extra long (EL), long (L), and short (S)) also increased substantially over time with OKT3 restimulation, whether Fas blockade was applied or not (Fig. 3B) Although BCL-xL protein levels also increased, the ratio of BIM:BCL-xL expression rose substantially over time, suggesting heightened Bim expression represents a "tipping point" for overcoming the anti-apoptotic function of BCL-xL and related proteins in driving mitochondrial depolarization. PUMAβ levels also showed a minor increase (Fig. 3B). Remarkably, the quick induction of BIM upon restimulation occurred in the presence of IL-2, which is required for TCR-induced death. IL-2 signaling alone can destabilize BIM mRNA or promotes BIM protein degradation via Raf/ERK or phosphoinositide kinase 3 (PI-3K) signaling pathways. However, our results suggest the TCR restimulation overrides this signal to allow for rapid BIM upregulation. These data are consistent with previous observations indicating BIM expression can be induced upon TCR triggering in human CTL clones, depending on the agonistic peptide used. However, these studies did not establish whether loss of BIM expression had functional consequences for TCR-induced apoptosis sensitivity, or how this related to FAS-FASL signaling.
Figure 1. TCR re-stimulation signals mitochondrial-dependent apoptosis independent of FAS. (A) Electron micrographs (upper panels, 2500× magnification or lower panels, 10000×) of activated human PBL either not restimulated (NRS) or restimulated with OKT3 mAb for 18 h. Arrows indicate apoptotic cells. (B) Activated human T cells were untreated (NRS) or restimulated with OKT3 for 24 h in the presence of FAS blocking Ab (SM1/23) or isotype control Ab. Cells were stained with PI and analyzed by flow cytometry; gates indicate % viable cells. (C) Activated human T cells from a normal donor or ALPS 1a patient were untreated (NRS) or restimulated with OKT3 for 12 h in the presence of FAS blocking Ab (SM1/23) or isotype control Ab. Cells were stained with DiOC6 and analyzed by flow cytometry (right panels). Viable gates are shown at left, and the percentage of DiOC6 low cells are indicated in the histograms on the right. (D) Cytosolic extracts from activated human PBL were immunoblotted for the presence of cytochrome c following stimulation with OKT3 or staurosporine (STS) for the indicated timepoints, in the presence or absence of SM1/23 Ab. (E) Lysates prepared as described in (D) were incubated with the caspase 9 specific substrate LEHD-pNA for 2 h, and caspase 9 enzymatic activity was quantitated as OD at 405 nm minus background (OD405 at 5 min).
To definitively test whether BIM contributes to the TCR-induced apoptosis signal, we silenced BIM expression by RNA interference (RNAi) in activated PBL and restimulated them with OKT3 with or without FAS blockade. Knockdown of BIM expression significantly reduced the sensitivity of activated PBL to TCR-induced death (Fig. 3C). Control immunoblots showed that BIM expression was silenced effectively in cells that received BIM-specific siRNA both before and after restimulation (Figure 3D, Figure 4). As noted above, FAS blockade also partially rescued cells from death in these experiments, and had an additive protective effect when BIM expression was reduced (Fig. 3C). The protective effects of BIM suppression and Fas blockade were noted in multiple human donors (Fig. 3E). Knockdown of FAS associated death domain (FADD) rescued cells from TCR-induced death to a similar extent, further illustrating that death receptor signaling is only part of the apoptotic signal triggered by TCR restimulation (Fig. 5). In addition, knockdown of PUMA also provided some protection from TCR-induced death (Fig. 6) although this effect was variable in different donors tested. Collectively, our data definitively shows that intrinsic apoptosis mediators, particularly BIM, are required for optimal apoptosis triggered by TCR re-engagement separate from extrinsic FAS-induced apoptotic signals.
Role of BIM in RAICD

Figure 3. Induction of BIM expression contributes to TCR-induced apoptosis. (A) Microarray analysis of designated Bcl-2 family members was performed using RNA purified from activated human PBL either untreated (0 h) or stimulated with OKT3 for 6 h. Relative expression values normalized to reference RNA from normal human PBL are shown at left; fold change following TCR restimulation is quantitated at right. (B) Activated human PBL were stimulated with OKT3 for the indicated times, and whole cell lysates were prepared and immunoblotted for the proteins indicated on the right. All three isoforms of BIM (extra-long (EL), long (L), short (S)) were detected. Spot densitometry analysis of the ratio of BIM-EL to BCL-xL (normalized to β-actin loading control) is plotted below. (C) Activated human PBL were transfected with nonspecific (NS) or Bim-specific siRNA, rested 4 days, and then restimulated for 24 h with increasing doses of OKT3 in the presence or absence of SM1/23. Percent cell loss was calculated in triplicate by PI exclusion. Differences in apoptosis sensitivity (relative to NS alone) were statistically significant for each dose of OKT3 (p < 0.04), except for SM1/23 treated NS cells at 1 µg/ml. (D) Lysates from cells transfected in (C) were immunoblotted for BIM as in (B). β-actin serves as a loading control. (E) Average extent of TCR-induced apoptosis inhibition (relative to NS siRNA alone) is shown for each condition described in (D) for 6 different normal donor PBL tested.
Chapter 6

Figure 4. Bim siRNA effectively suppresses restimulation-induced BIM expression in activated T cells. Activated human PBL, purified CD4+ and CD8+ T cells were transfected with nonspecific (NS) or Bim-specific siRNA and rested for 4 days. Lysates were made from cells left untreated (0 h) or restimulated for 8 h with 100 ng/ml OKT3. Knockdown of protein expression was confirmed by immunoblotting.

Figure 5. Knockdown of FADD or Bim expression results in partial resistance to TCR-induced death. Activated human PBL were transfected with nonspecific (NS), FADD-specific, or Bim-specific siRNA, rested for 4 days, and then restimulated for 24 h with increasing doses of OKT3 in the presence or absence of Fas blocking Ab (SM1/23). Percent cell loss was calculated in triplicate by PI exclusion (left panel). Knockdown of protein expression was confirmed by immunoblotting in whole lysates 4 days post-transfection (right panel).
Relative contribution of BIM in CD4$^+$ versus CD8$^+$ TCR-induced death

We next tested whether BIM induction played a role in TCR-induced death of both CD4$^+$ and CD8$^+$ T cells. Purified CD4$^+$ and CD8$^+$ T cells sorted from activated PBL were transfected with NS or BIM-specific siRNA and tested for sensitivity to OKT3-induced death. Whereas Fas blockade alone substantially rescued the apoptosis of purified CD4$^+$ T cells, knockdown of Bim expression had little effect (Fig. 7A). Conversely, CD8$^+$ T cells relied on both FAS and BIM for TCR-induced apoptosis signaling. Although BIM expression was consistently higher in CD4$^+$ T cells compared to CD8$^+$ T cells from multiple donors (Fig. 7B) BIM induction from steady state levels was as good or better in CD8$^+$ T cells upon restimulation (Fig. 4 & data not shown). We cannot rule out that residual BIM expression in CD4$^+$ T cells following BIM siRNA transfection contributed to the Fas-independent of apoptosis observed. However, other experiments revealed that BIM knockdown using the same siRNA provided greater protection from IL-2 withdrawal apoptosis in CD4$^+$ T cells (Fig. 8) suggesting BIM levels could be sufficiently depleted to hinder BIM-dependent death. Collectively, the data suggests that human CD8$^+$ T cells rely on BIM more extensively for TCR-induced deletion than CD4$^+$ T cells,
which are largely dependent on FAS signaling. This idea agrees with landmark studies that implicated FAS in TCR-induced apoptosis, which focused primarily on CD4\(^+\) T cell lines or clones from humans or mice\(^{15-18}\). Moreover, our data potentially explain new studies suggesting BIM drives Ag-specific CD8\(^+\) T cell deletion in establishing peripheral tolerance in both mice and humans\(^{30,31}\).

**Figure 7. Bim is important for TCR-induced apoptosis of CD8\(^+\) T cells.** (A) CD4\(^+\) or CD8\(^+\) T cells purified from activated human PBL were transfected with NS or Bim-specific siRNA, rested 4 days, then restimulated with increasing doses of OKT3 in the presence or absence of SM1/23. Percent cell loss was calculated in triplicate by PI exclusion. Differences in apoptosis sensitivity were statistically significant for SM1/23 treated CD4\(^+\) cells (NS and Bim) compared to NS cells alone (p < 0.007), except for SM1/23 treated NS cells at 1 \(\mu g/ml\) OKT3 (p < 0.07). Differences in apoptosis sensitivity for CD8\(^+\) T cells (relative to NS alone) were all statistically significant (p < 0.05). (B) Lysates from cells transfected in (A) were immunoblotted for BIM. \(\beta\)-actin serves as a loading control.

**Bim and Fas cooperate in TCR-induced apoptosis of murine T cells**

In light of our findings in human T cells, we re-examined TCR-induced death in murine T cells. Surprisingly, we observed that activated splenic T cells from Fas-deficient lpr mice showed only minor resistance to anti-CD3-induced death induced by restimulation, whereas bim knockout mice showed no difference in sensitivity compared to WT cells (Fig. 9A). We also tested for Bim induction in restimulated WT and lpr T cells in the presence of IL-2. Consistent with data in human T cells, activated mouse T cells (WT or lpr) showed a clear increase in BimEL expression after 6 hours of restimulation (Fig. 9B). We also detected a change in the migration of BimEL and BimL
isoforms, suggesting post-translational modifications may affect of bim function in mice, perhaps via phosphorylation.

**Figure 8. BIM siRNA impairs IL-2 withdrawal apoptosis in both CD4+ and CD8+ T cells.** Purified CD4+ and CD8+ T cells were transfected with nonspecific (NS) or Bim-specific siRNA and rested 24 hrs in IL-2. IL-2 was removed by thorough washing, and percent cell loss was calculated 72 and 96 hrs later by PI exclusion (left panel). The percent of apoptosis inhibition afforded by BIM siRNA (relative to NS) is graphed in the right panel.

Next, we reasoned that differences in apoptosis sensitivity caused by loss of Fas or Bim may differ in CD4+ and CD8+ T cell cultures, as noted for human T cells. Therefore, we assayed TCR-induced apoptosis sensitivity in purified CD4+ and CD8+ T cells from WT, lpr, and bim−/− mice. As expected from previous reports, CD4+ lpr cells showed a profound defect in restimulation-induced death (Fig. 9C). This concurred with our results in human CD4+ T cells using Fas blocking Ab (Fig. 7A) indicating Fas is necessary for CD4+ T cell restimulation apoptosis. In contrast, there were no differences in CD8+ T cell death between restimulated WT and lpr cells, explaining the cumulatively minor rescue of TCR-induced death in total splenic T cells when Fas is absent. Furthermore, genetic ablation of bim had little protective effect for activated CD4+ T cells upon TCR restimulation, and no discernible effect on apoptosis in CD8+ T cells (Fig. 9D).
Figure 9. Fas and Bim cooperate in driving TCR-induced apoptosis of murine T cells. (A) Activated splenic T cells from wild-type (WT), lpr, or bim<sup>−/−</sup> mice were restimulated with platebound anti-CD3 for 24 h. Percent cell loss was calculated in triplicate by PI exclusion. (B) Lysates from splenic T cells from the indicated genetic backgrounds left untreated or restimulated with platebound anti-CD3 for 6 h were immunoblotted for Bim isoform expression. β-actin serves as a loading control; asterisk indicates non-specific band. (C, D) CD4<sup>+</sup> and CD8<sup>+</sup> T cells were purified from activated WT, lpr, or bim<sup>−/−</sup> splenocytes and restimulated with platebound anti-CD3 for 24 h. Percent cell loss was calculated in triplicate by PI exclusion. (E) Splenic T cells from WT or lpr mice were stimulated for 48 h with platebound anti-CD3/anti-CD28, washed, and transfected with NS or Bim-specific siRNA. Three days post-transfection, cells were restimulated with 100 ng/ml platebound anti-CD3; percent cell loss was calculated in triplicate by PI exclusion. Differences in apoptosis sensitivity (relative to NS-treated WT cells) were statistically significant (p < 0.04). Lysates made from cells three days post-transfection were assessed for Bim knockdown by immunoblotting, right.

We hypothesized that loss of Bim from development, through germline gene ablation, may permit T cells to "compensate" accordingly via enhanced expression or
function of pro-apoptotic molecules. Therefore, we acutely silenced Bim using RNAi in activated WT and lpr T cells. Knockdown of Bim significantly protected activated WT and lpr T cells from apoptosis induced by 100 ng/ml anti-CD3 stimulation (Fig. 9E, left panel), demonstrating that Bim can play a prominent role in this apoptosis pathway. This effect was also noted in purified CD4+ and CD8+ T cell populations (data not shown), even though loss of Fas alone reduced sensitivity only in CD4+ T cells again as expected. Control blots showed that Bim siRNA effectively suppressed Bim protein expression in both WT and lpr T cells (Fig. 9E, right panel). This protective effect was less pronounced at higher doses of anti-CD3 stimulation (data not shown), suggesting stronger restimulation may override Bim siRNA effects and/or trigger alternative death effector pathways. Thus, our data suggests that Fas or Bim may partially compensate for the loss of one or the other from development in murine T cells during development.

**Relative BIM expression correlates with sensitivity to TCR-induced death in ALPS patients**

Based on our aforementioned results, we revisited TCR restimulation-induced apoptosis in PBL derived from several ALPS patients. Similar to controls, PBL cultures from ALPS Ia patients were primarily comprised of CD8+ T cells (data not shown). Surprisingly, we found that PBL from several ALPS Ia patients displayed normal or slightly more death in response to OKT3 titration compared to normal controls, despite impaired apoptosis upon direct Fas crosslinking. (Fig. 10A). Similarly, T cells derived from an ALPS Ib patient harboring a dominant interfering mutation in FASL32 were also killed effectively upon TCR restimulation (Fig. 10B). Consistent with defective FASL function, TCR-induced apoptosis was unaffected by Fas blockade. These results exposed a glaring contradiction in the concept that FAS mediates most or all TCR-induced death.
Figure 10. Suppression of Bim expression in ALPS type IV patient causes resistance to TCR-induced death. (A) Activated human PBL from normal control donors (NC1, NC2), or 6 ALPS type Ia patients were restimulated with increasing doses of OKT3 for 24 h. Percent cell loss was calculated in triplicate by PI exclusion. (B) Activated human PBL from an ALPS type Ib patient or a normal control donor (NC) were treated as in (A). Percent cell loss was calculated in triplicate by PI exclusion. (C) Activated human PBL from normal control donors (NC1, NC2), or 6 ALPS type Ia patients restimulated with 100 ng/ml OKT3 for 0 (-) or 8 h (+), lysed and immunoblotted for BIM. β-actin serves as a loading control. Spot densitometry analysis of the ratio of BIM (EL isoform) to β-actin (normalized to NC1 untreated, dashed line) is plotted below. (D) Activated human PBL from normal control donors (NC1, NC2), an ALPS type Ia patient, and an ALPS Type IV patient (P58) were restimulated with OKT3 for 24 h. Percent cell loss was calculated in triplicate by PI exclusion. Differences in apoptosis sensitivity (relative to NC1 or NC2) were statistically significant (p < 0.01). (E) Activated human PBL as in (D) were restimulated with 100 ng/ml OKT3 for 0 (-) or 8 h (+), lysed and immunoblotted for BIM. β-actin serves as a loading control.
We next assessed the relative expression of BIM before and after restimulation of PBL in ALPS Ia patients. In general, we noted higher BIM protein expression in restimulated ALPS Ia T cells relative to controls (Fig. 10C). In 4/6 ALPS Ia patients, steady-state BIM expression was also elevated relative to controls. Using spot densitometry, we estimated that ALPS Ia T cells had between 30–80% more BIM protein than normal controls both before and after TCR ligation (Fig. 10C, bottom panel). BIM siRNA treatment did not result in a significantly greater rescue of TCR-induced death in ALPS Ia cells compared to normal controls (data not shown), perhaps due to incomplete depletion of BIM or compensation by other mediators (e.g. PUMA). Nevertheless, elevated BIM levels in cycling T cells with defective FAS function may suggest that these T cells are "primed" for apoptotic deletion through a compensatory increase in BIM expression.

Finally, we tested TCR-induced apoptosis in T cells derived from an ALPS Type IV patient (P58) with a gain-of-function, germline NRAS mutation that constitutively activates ERK and suppresses BIM expression. We recently demonstrated that P58 T cells are resistant to apoptosis induced by IL-2 withdrawal due to BIM suppression\(^{12}\). Remarkably, P58 T cells displayed partial resistance to TCR-induced death when compared to normal donor and ALPS Ia cells, despite comparable expression of FAS on the cell surface (Fig. 10D, Fig. 11). Moreover, BIM expression was attenuated in P58 T cells and could not be rescued by TCR restimulation (Fig. 10E), providing stronger evidence that BIM serves a physiologically relevant role in the restimulation apoptosis pathway, especially for CD8\(^+\) T cell homeostasis. Moreover, our data implies that relative BIM expression may represent an important determinant of TCR-induced apoptosis sensitivity, independently of FAS. However, we concede that NRAS/ERK dysregulation in P58 could alter TCR-induced death through BIM-independent mechanisms as well. Indeed, pharmacological ERK inhibitors actually provided a small but reproducible rescue of TCR-induced death in both normal and P58 T cells.
Figure 11. ALPS Type IV patient T cells express functional FAS. (A) Activated PBL from a normal control donor (NC, open histogram), an ALPS type Ia patient (gray), or ALPS type IV (P58, black) were stained with FITC-conjugated anti-CD95 or isotype control Ab (dashed line) and analyzed by flow cytometry. (B) Activated PBL from a normal donor (NC), an ALPS type IV patient (P58) and an ALPS type Ia patient were treated with 20 or 200 ng/ml APO1.3 mAb plus 200 ng/ml Protein A. Percent cell loss was calculated in triplicate by PI exclusion.

The physiological function of Bim was originally revealed from characterization of Bim-deficient mice, from which T cells were profoundly resistant to lymphokine withdrawal death\(^8\). The pro-apoptotic function of Bim also enforces immune tolerance through thymocyte negative selection, CD8\(^+\) T cell cross tolerance, and the regulation of antigen presenting cells including B cells and dendritic cells\(^23,33-35\). Here we demonstrate that BIM also plays a significant role in TCR-induced death of activated human T cells, working in tandem with FAS signaling as a separate signal to kill T cells. This provides a new mechanism besides the cleavage of BID for an extrinsic signal to activate the intrinsic mitochondrial death program. This paradigm may be distinct from Bim-dependent "activated T cell death" described by Hildeman et al. in mice challenged with a single dose of superantigen\(^36\), which may be interpreted as predominantly cytokine withdrawal apoptosis, not restimulation-induced death with repeated Ag dosing. On the other hand, the marked accumulation and persistence of Bim-deficient murine CD8\(^+\) T cells in chronic viral infection models could be connected to failed deletion in response to repeated TCR stimulation\(^37,38\).
Our results show that direct signals from the TCR program T cells to die through Bim, which is fundamentally different from the secretion of death cytokines such as FasL that engage external death receptors. This has some interesting implications. First, it may be advantageous in conditions where Fas may not be effective. For example, Bim has a greater influence in CD8+ T cells that can utilize FasL:Fas as a calcium-independent cytolytic mechanism against infected target cells and therefore may be inured to its lethal effects. Second, the direct molecular connection inside the cell may make the Bim pathway more efficient. Careful investigation of the temporal effects of killing after TCR engagement may reveal differences between Fas and Bim effectiveness. Third, as Bim expression is extensively regulated post-translationally, the fact that translation inhibitors only partially block TCR-induced death could indicate there is a direct death pathway entrained to TCR restimulation that does not require new protein synthesis. Finally, pro-apoptotic mediators like Bim or Puma acting at the convergence of TCR and CWA may help to restrain these pathways at a focal point for tight control of those T cells escape death and emerge as memory T cells.

Recently, three groups reported that loss of both Bim and Fas in mice results in massive lymphadenopathy/splenomegaly, early onset of SLE-like autoimmune manifestations, and even greater accumulation of antigen-specific CD8+ T cells upon chronic viral infection. These experiments reprised earlier work that obtained very similar results when transgenic Bcl-2 overexpressing mice were crossed onto an lpr background. However, their general conclusions still emphasized the traditional model, reiterated in an accompanying review, that Fas and Bim control T cell homeostasis through two distinct pathways: restimulation-driven versus IL-2 withdrawal-induced apoptosis, respectively. Our study illustrates that death of activated T cells via Fas or Bim are not mutually exclusive pathways, as both can operate in IL-2 dependent TCR-induced apoptosis. During infections this combination of potent extrinsic and intrinsic signals may act to ensure rapid and efficient killing of hyper-responsive or cross-reactive autoimmune T cells upon repeated antigen encounter, thus preventing immunotoxicity and maintaining peripheral tolerance. Another intriguing possibility relates to the potential of Bim and Fas to partially compensate for one another in driving TCR-induced apoptosis. This applies to situations where either gene function is lost from...
development, such as in \( lpr \) or \( bim^{-} \) mice, and may explain why only acute knockdown of Bim resulted in significant reduction of TCR-induced apoptosis in murine T cells in vitro. The idea that Bim participates in ensuring T cell homeostasis both during and after effector T cell responses may also explain why Bcl-2 Tg \( lpr \) mice described years ago have strikingly worse lymphocyte accumulation compared to either Bcl-2 Tg or \( lpr \) mice alone. Our results provide a new interpretation of the mouse studies by revealing that the infection-induced derangement of T cell homeostasis caused by Bim-deficiency could be accounted for by an impairment of both intrinsic and extrinsic apoptosis. It is also notable that ALPS patients show wide variability in conventional CD3\(^+\) T cell numbers, with a substantial fraction showing no increases. By contrast, the fraction and absolute number of "double negative" (CD4\(^-\)CD8\(^-\)) \( \alpha/\beta \) T cells are invariably elevated. This may reflect that alternative effectors such as BIM could preserve equipoise in the conventional T cell compartment.

In humans, our data are consistent with previous studies suggesting that TCR-induced death involves multiple effector molecules, and clearly includes components other than FAS or BIM that remain to be elucidated. We have previously noted a role for tumor necrosis factor-alpha (TNF-\( \alpha \)) in this process for murine CD8\(^+\) T cells; however, blockade of this pathway in human T cells had little demonstrable effect (data not shown), which requires further exploration. A recent paper from Mateo et al. implicated perforin and cytotoxic granules in the execution of TCR-induced death, particularly for ALPS Ia patient cells. Other inputs implicated in control of AICD sensitivity, including NF-\( \kappa \)B regulation through HPK-1 or generation of reactive oxygen species (ROS), likely relate more to the regulation of FasL or Bim expression. However, we are studying patients with impaired TCR-induced apoptosis despite normal induction of FASL and BIM, and normal apoptosis triggered by FAS ligation or IL-2 deprivation (A.L. Snow, unpublished data). Insights gleaned from such patients may further advance our understanding of the biochemical complexity and physiological relevance of apoptosis in different immune cell compartments. Nevertheless, our current findings further elucidate FAS-independent signals for the restimulation-induced death of activated T cells via BIM induction.
CONCLUSION

Although Fas-FasL signaling is often considered synonymous with TCR restimulation-induced death, the data provided herein show it has a quantitatively lesser role than previously acknowledged, and support a critical role for BIM induction in the execution of antigen-driven "extrinsic" apoptosis. Increased BIM expression following TCR restimulation, even with a surfeit of IL-2, works in parallel to FAS signaling in driving mitochondrial depolarization, caspase 9 activation, and eventual apoptosis. Like FAS blockade, suppression of BIM induction via RNAi or increased NRAS activity in an ALPS variant patient results in partial resistance to TCR-induced death. These data build upon previous work from Sandalova and colleagues by demonstrating that BIM is indispensable for maximum sensitivity to restimulation-induced apoptosis of human T cells. More importantly, our findings revise previous models in showing that FAS and BIM both participate in eliminating activated T cells through this IL-2 dependent, propriocidal death pathway.

References

1. Strasser A, Pellegrini M. T-lymphocyte death during shutdown of an immune response. Trends Immunol. 2004;25:610-615.
2. Boehme SA, Lenardo MJ. Propriocidal apoptosis of mature T lymphocytes occurs at S phase of the cell cycle. Eur J Immunol. 1993;23:1552-1560.
3. Arnold R, Brenner D, Becker M, Frey CR, Krammer PH. How T lymphocytes switch between life and death. Eur J Immunol. 2006;36:1654-1658.
4. Lenardo MJ. Fas and the art of lymphocyte maintenance. J Exp Med. 1996;183:721-724.
5. Lenardo M, Chan KM, Hornung F, et al. Mature T lymphocyte apoptosis--immune regulation in a dynamic and unpredictable antigenic environment. Annu Rev Immunol. 1999;17:221-253.
6. Lenardo MJ. Interleukin-2 programs mouse alpha beta T lymphocytes for apoptosis. Nature. 1991;353:858-861.
7. Green DR. Fas Bim boom! Immunity. 2008;28:141-143.
8. Bouillet P, Metcalf D, Huang DC, et al. Proapoptotic Bcl-2 relative Bim required for certain apoptotic responses, leukocyte homeostasis, and to preclude autoimmunity. Science. 1999;286:1735-1738.
9. Erlacher M, Labi V, Manzl C, et al. Puma cooperates with Bim, the rate-limiting BH3-only protein in cell death during lymphocyte development, in apoptosis induction. J Exp Med. 2006;203:2939-2951.
10. Fischer SF, Belz GT, Strasser A. BH3-only protein Puma contributes to death of antigen-specific T cells during shutdown of an immune response to acute viral infection. Proc Natl Acad Sci U S A. 2008;105:3035-3040.

11. Fletcher JI, Huang DC. Controlling the cell death mediators Bax and Bak: puzzles and conundrums. Cell Cycle. 2008;7:39-44.

12. Oliveira JB, Bidere N, Niemela JE, et al. NRAS mutation causes a human autoimmune lymphoproliferative syndrome. Proc Natl Acad Sci U S A. 2007;104:8953-8958.

13. Puthalakath H, Strasser A. Keeping killers on a tight leash: transcriptional and post-translational control of the pro-apoptotic activity of BH3-only proteins. Cell Death Differ. 2002;9:505-512.

14. Green DR, Droin N, Pinkoski M. Activation-induced cell death in T cells. Immunol Rev. 2003;193:70-81.

15. Alderson MR, Tough TW, Davis-Smith T, et al. Fas ligand mediates activation-induced cell death in human T lymphocytes. J Exp Med. 1995;181:71-77.

16. Brunner T, Mogil RJ, LaFace D, et al. Cell-autonomous Fas (CD95)/Fas-ligand interaction mediates activation-induced apoptosis in T-cell hybridomas. Nature. 1995;373:441-444.

17. Dhein J, Walczak H, Baumler C, Debatin KM, Krammer PH. Autocrine T-cell suicide mediated by APO-1/(Fas/CD95). Nature. 1995;373:438-441.

18. Ju ST, Panka DJ, Cui H, et al. Fas(CD95)/FasL interactions required for programmed cell death after T-cell activation. Nature. 1995;373:444-448.

19. Bidere N, Su HC, Lenardo MJ. Genetic disorders of programmed cell death in the immune system. Annu Rev Immunol. 2006;24:321-352.

20. Oliveira JB, Fleisher T. Autoimmune lymphoproliferative syndrome. Curr Opin Allergy Clin Immunol. 2004;4:497-503.

21. Zheng L, Fisher G, Miller RE, Peschon J, Lynch DH, Lenardo MJ. Induction of apoptosis in mature T cells by tumour necrosis factor. Nature. 1995;377:348-351.

22. Davidson WF, Haudenschild C, Kwon J, Williams MS. T cell receptor ligation triggers novel nonapoptotic cell death pathways that are Fas-independent or Fas-dependent. J Immunol. 2002;169:6218-6230.

23. Chen M, Wang YH, Wang Y, et al. Dendritic cell apoptosis in the maintenance of immune tolerance. Science. 2006;311:1160-1164.

24. Stranges PB, Watson J, Cooper CJ, et al. Elimination of antigen-presenting cells and autoreactive T cells by Fas contributes to prevention of autoimmunity. Immunity. 2007;26:629-641.

25. Brunet A, Bonni A, Zigmond MJ, et al. Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. Cell. 1999;96:857-868.

26. Ley R, Balmanno K, Hadfield K, Weston C, Cook SJ. Activation of the ERK1/2 signaling pathway promotes phosphorylation and proteasome-dependent degradation of the BH3-only protein, Bim. J Biol Chem. 2003;278:18811-18816.

27. Matsui H, Asou H, Inaba T. Cytokines direct the regulation of Bim mRNA stability by heat-shock cognate protein 70. Mol Cell. 2007;25:99-112.
28. Sandalova E, Wei CH, Masucci MG, Levitsky V. Regulation of expression of Bcl-2 protein family member Bim by T cell receptor triggering. Proc Natl Acad Sci U S A. 2004;101:3011-3016.
29. Sandalova E, Hislop AD, Levitsky V. T-cell receptor triggering differentially regulates bim expression in human lymphocytes from healthy individuals and patients with infectious mononucleosis. Hum Immunol. 2006;67:958-965.
30. Lopes AR, Kellam P, Das A, et al. Bim-mediated deletion of antigen-specific CD8 T cells in patients unable to control HBV infection. J Clin Invest. 2008;118:1835-1845.
31. Redmond WL, Wei CH, Kreuwel HT, Sherman LA. The apoptotic pathway contributing to the deletion of naive CD8 T cells during the induction of peripheral tolerance to a cross-presented self-antigen. J Immunol. 2008;180:5275-5282.
32. Bi LL, Pan G, Atkinson TP, et al. Dominant inhibition of Fas ligand-mediated apoptosis due to a heterozygous mutation associated with autoimmune lymphoproliferative syndrome (ALPS) Type Ib. BMC Med Genet. 2007;8:41.
33. Bouillet P, Purton JF, Godfrey DI, et al. BH3-only Bcl-2 family member Bim is required for apoptosis of autoreactive thymocytes. Nature. 2002;415:922-926.
34. Davey GM, Kurts C, Miller JF, et al. Peripheral deletion of autoreactive CD8 T cells by cross presentation of self-antigen occurs by a Bcl-2-inhibitable pathway mediated by Bim. J Exp Med. 2002;196:947-955.
35. Enders A, Bouillet P, Puthalakath H, Xu Y, Tarlinton DM, Strasser A. Loss of the pro-apoptotic BH3-only Bcl-2 family member Bim inhibits BCR stimulation-induced apoptosis and deletion of autoreactive B cells. J Exp Med. 2003;198:1119-1126.
36. Hildeman DA, Zhu Y, Mitchell TC, et al. Activated T cell death in vivo mediated by proapoptotic bcl-2 family member bim. Immunity. 2002;16:759-767.
37. Grayson JM, Weant AE, Holbrook BC, Hildeman D. Role of Bim in regulating CD8+ T-cell responses during chronic viral infection. J Virol. 2006;80:8627-8638.
38. Pellegrini M, Belz G, Bouillet P, Strasser A. Shutdown of an acute T cell immune response to viral infection is mediated by the proapoptotic Bcl-2 homology 3-only protein Bim. Proc Natl Acad Sci U S A. 2003;100:14175-14180.
39. Hughes PD, Belz GT, Fortner KA, Budd RC, Strasser A, Bouillet P. Apoptosis regulators Fas and Bim cooperate in shutdown of chronic immune responses and prevention of autoimmunity. Immunity. 2008;28:197-205.
40. Hutcheson J, Scatizzi JC, Siddiqui AM, et al. Combined deficiency of proapoptotic regulators Bim and Fas results in the early onset of systemic autoimmunity. Immunity. 2008;28:206-217.
41. Weant AE, Michalek RD, Khan IU, Holbrook BC, Willingham MC, Grayson JM. Apoptosis regulators Bim and Fas function concurrently to control autoimmunity and CD8+ T cell contraction. Immunity. 2008;28:218-230.
42. Reap EA, Felix NJ, Wolthusen PA, Kotzin BL, Cohen PL, Eisenberg RA. bcl-2 transgenic Lpr mice show profound enhancement of lymphadenopathy. J Immunol. 1995;155:5455-5462.
43. Strasser A, Harris AW, Huang DC, Krammer PH, Cory S. Bcl-2 and Fas/APO-1 regulate distinct pathways to lymphocyte apoptosis. Embo J. 1995;14:6136-6147.
44. Straus SE, Sneller M, Lenardo MJ, Puck JM, Strober W. An inherited disorder of lymphocyte apoptosis: the autoimmune lymphoproliferative syndrome. Ann Intern Med. 1999;130:591-601.
45. Mateo V, Menager M, de Saint-Basile G, et al. Perforin-dependent apoptosis functionally compensates Fas deficiency in activation-induced cell death of human T lymphocytes. Blood. 2007;110:4285-4292.
46. Brenner D, Golks A, Becker M, et al. Caspase-cleaved HPK1 induces CD95L-independent activation-induced cell death in T and B lymphocytes. Blood. 2007;110:3968-3977.
47. Kaminski M, Kiessling M, Suss D, Krammer PH, Gulow K. Novel role for mitochondria: protein kinase Ctheta-dependent oxidative signaling organelles in activation-induced T-cell death. Mol Cell Biol. 2007;27:3625-3639.