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Bcl-2 Allows Effector and Memory CD8+ T Cells To Tolerate Higher Expression of Bim

Sema Kurtulus,* Pulak Tripathi,* Maria E. Moreno-Fernandez,† Allyson Sholl,* Jonathan D. Katz,‡ H. Leighton Grimes,* and David A. Hildeman*

As acute infections resolve, most effector CD8+ T cells die, whereas some persist and become memory T cells. Recent work showed that subsets of effector CD8+ T cells, identified by reciprocal expression of killer cell lectin-like receptor G1 (KLRG1) and CD127, have different lifespans. Similar to previous reports, we found that effector CD8+ T cells reported to have a longer lifespan (i.e., KLRG1low/CD127high) have increased levels of Bcl-2 compared with their shorter-lived KLRG1high/CD127low counterparts. Surprisingly, we found that these effector KLRG1low/CD127high CD8+ T cells also had increased levels of Bim compared with KLRG1high/CD127low cells. Similar effects were observed in memory cells, in which CD8+ central memory T cells expressed higher levels of Bim and Bcl-2 than did CD8+ effector memory T cells. Using both pharmacologic and genetic approaches, we found that survival of both subsets of effector and memory CD8+ T cells required Bcl-2 to combat the proapoptotic activity of Bim. Interestingly, inhibition or absence of Bcl-2 led to significantly decreased expression of Bim in surviving effector and memory T cells. In addition, manipulation of Bcl-2 levels by IL-7 or IL-15 also affected expression of Bim in effector CD8+ T cells. Finally, we found that Bim levels were significantly increased in effector CD8+ T cells lacking Bax and Bak. Together, these data indicate that cells having the highest levels of Bim are selected against during contraction of the response and that Bcl-2 determines the level of Bim that effector and memory T cells can tolerate. The Journal of Immunology, 2011, 186: 000–000.

During acute viral infection, naive CD8+ T cells expand vigorously and generate a population of effector T cells. Shortly after viral clearance, most of the effector T cells die, whereas some remain and become memory T cells. Although initial work suggested that Fas/Fas ligand signaling was critical for the death of activated T cells (1–5), more recent work suggests a dominant role for members of the Bcl-2 family (6). More specifically, the proapoptotic molecule Bim and its ability to signal through the downstream proapoptotic executors Bax and Bak are essential for driving the contraction of T cell responses in vivo (7–10). Failure to eliminate effector T cells in Bim-deficient animals leads to significantly enhanced T cell memory (11) and protective immunity (11). However, mechanism(s) by which some cells succumb to, whereas others resist, Bim-driven death remain unclear.

The effector CD8+ T cell response to acute viral infection is heterogeneous, consisting of two major subsets, identified largely by the reciprocal cell-surface expression of killer cell lectin-like receptor G1 (KLRG1) and IL-7Rα (CD127) (1, 12, 13). Effector CD8+ T cells with increased expression of CD127, but decreased expression of KLRG1, survived more during contraction of the response, and have been referred to as memory precursor effector CD8+ T cells. Conversely, effector CD8+ T cells with decreased expression of CD127, but increased expression of KLRG1, survive less during contraction of the response and have been called short-lived effector cells (12). The longer lifespan of KLRG1low/CD127high CD8+ T cells has been attributed to their increased expression of the antiapoptotic molecule Bcl-2, although this has yet to be tested experimentally.

Similar to effector T cells, heterogeneity also exists in the memory compartment, as two major subpopulations of memory T cells have been described (14). CD8+ effector memory T cells (TEm) lack CD62L and CCR7 expression, and they reside in peripheral tissues (e.g., liver, lung, etc.) and the spleen. CD8+ central memory T cells (TCM) expressing lymph node-homing receptors CD62L and CCR7 are mostly found in the secondary lymphoid organs. Although IL-7 and IL-15 critically control survival and homeostatic turnover of overall memory T cells (15, 16), the role of Bcl-2 in the survival of memory T cells is somewhat controversial. For example, Y449 in the cytoplasmic domain of IL-7Rα was shown to be critical for survival of memory T cells but not for IL-7–driven Bcl-2 upregulation (17), suggesting that antiapoptotic signals other than Bcl-2 control memory T cell survival. In contrast, using a gene-knockout approach, we showed that Bcl-2 is critical for IL-7– and IL-15–driven survival in vitro and for overall memory T cell survival in vivo (18, 19). However, the requirement of Bcl-2 in the survival of different subsets of memory CD8+ T cells was not determined in either of the previous studies (17, 18). Thus, the role of Bcl-2 in maintaining subsets of memory T cells and the degree to which Bcl-2 combats Bim in such cells remain unclear.

In naive T cells, most Bim is complexed to Bcl-2 (20), and we recently showed that this interaction is critical to promote naive

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Abbreviations used in this article: CHX, cycloheximide; Foxo3a, forkhead box subclass O transcription factor 3a; gp33-3p, gp33-specific; KLRG1, killer cell lectin-like receptor G1; LCMV, lymphocytic choriomeningitis virus; LCMV-sp, lymphocytic choriomeningitis virus–specific; MFI, mean fluorescence intensity; p.i., postinfection; TCM, central memory T cell; TEm, effector memory CD8+ T cell.

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T cell survival (18). Following activation, levels of Bcl-2 within activated T cells are decreased, which is correlated with enhanced death of effector T cells (21). Recent work, however, has suggested a potentially more complex interplay between Bim and Bcl-2 (22), as it was shown that genetic ablation of Bim leads to decreased levels of Bcl-2 protein, whereas overexpression of Bcl-2 promotes increased expression of Bim mRNA and protein within activated T cells (21, 22). These data suggested that Bim and Bcl-2 could reciprocally affect the other’s expression in T cells, although the mechanism(s) underlying such phenomenon remain unclear.

In this study, we examined the role of Bim and Bcl-2 in both effector and memory subsets of CD8+ T cells using both pharmacologic and genetic approaches. We found that both effector and viral-specific memory CD8+ T cells require Bcl-2 to combat the proapoptotic effects of Bim and, by doing so, determine the levels of Bim CD8+ T cells can tolerate and survive.

Materials and Methods

Mice and viral infection

C57BL/6 mice were either purchased from The Jackson Laboratory or Taconic Farms. Bim−/− mice were a gift from P. Bouillet and A. Strasser (Walter and Eliza Hall Institute, Melbourne, Australia) and have been backcrossed to C57BL/6 mice for at least 14 generations. Breeding pairs of Bcl-2−/− mice were obtained from The Jackson Laboratory and were mated to Bim−/− mice to generate Bim−/− Bcl-2−/−, Bim−/−/Bcl-2−/−, and Bim−/− Bcl-2−/− mice. Lck-Cre Bax−/−Bak−/− mice were a gift from Dr. Stanley Korsmeyer. IL-15-deficient mice on a C57BL/6 background were purchased from Taconic Farms. ABT-737 was a generous gift of Abbott Laboratories, dissolved in DMSO, and diluted in 35% polyethylene glycol, 5% Tween-80, and 65% of dextrose in water solution. Mice were injected i.p. with 2× 106 cells were stained with different combinations of the following cell-surface Abs: anti-CD8, CD44, CD62L, KLRG1, and CD127 Abs. CD44 + CD62L− KLRG1 high CD127 low or CD44 + CD62L− KLRG1 low CD127 high CD8+ T cells from C57BL/6 or Lck-Cre Bax−/−Bak−/− mice were sorted with FACS Aria (BD Biosciences), and RNA was isolated from cells using Qiagen’s RNeasy Mini Isolation kit (Qiagen) and converted into cDNA using SuperScript II Reverse transcriptase (Invitrogen, CA).

Results

Levels of Bcl-2 and Bim are correlated in subpopulations of effector CD8+ T cells

Although the increased survival of KLRG1lowCD127high effector CD8+ T cells was attributed to their increased expression of Bcl-2, this was not tested (12). In addition, as Bcl-2 is critical to combat Bim to ensure naive T cell survival (18), and Bim is critical for contraction of T cell responses (10, 21), it is possible that decreased levels of Bim could contribute to enhanced survival of KLRG1 low CD127 high effector CD8+ T cells. Using a mouse model of LCMV infection, we examined the levels of Bim and Bcl-2 within LCMV-specific (LCMV-sp) effector CD8+ T cell subsets by intracellular flow cytometry using specific Abs (23). First, we found that the levels of Bim and Bcl-2 were largely correlated in both KLRG1 low CD127 high effector CD8+ T cell subsets by intracellular flow cytometry using specific Abs (23). First, we found that the levels of Bim and Bcl-2 were largely correlated in both KLRG1 low CD127 high effector CD8+ T cells at the peak of the response (Fig. 1A). Next, we found that Bcl-2 levels were significantly higher in KLRG1lowCD127high cells compared with KLRG1highCD127low effector D3gp33-specific (gp33-sp) CD8+ T cells at the peak of the response (Fig. 1B, 1C). Surprisingly, we also found that Bim levels were significantly increased in KLRG1lowCD127high effector CD8+ T cells (Fig. 1B, 1C). Thus, both Bim and Bcl-2 are more highly expressed in effector CD8+ T cells with more potential to develop into long-lived memory cells.

Bcl-2 is critical to combat Bm for survival of KLRG1low CD127 high effector CD8+ T cells

We next used both genetic and pharmacologic approaches to determine whether Bcl-2 is critical for the survival of effector CD8+ T cells following LCMV infection. First, we took advantage of Bim−/− Bcl-2−/− mice, as loss of a single allele of Bim has previously been shown to prevent the early lethality and kidney disease of Bcl-2 deficiency (24). We infected groups of C57BL/6 and Bim−/− Bcl-2−/− mice with LCMV and analyzed the effector CD8+ T cell subsets 10 and 23 d later. As expected, 75–80% of KLRG1 high CD127 low and 30–40% of KLRG1 low CD127 high
effector gp33-sp CD8+ T cells were lost between days 10 and 23 p.i. (Fig. 2A). Interestingly, deficiency in Bcl-2 did not exacerbate the loss of KLRG1highCD127low effector CD8+ T cells, but it significantly enhanced the loss of KLRG1lowCD127high cells (Fig. 2A). This greater loss of effector CD8+ T cells in the absence of Bcl-2 was largely alleviated by the additional loss of the remaining allele of Bim (Fig. 2A). These data show that Bcl-2 is critical to counteract Bim in KLRG1 low CD127 high effector CD8+ T cells.

As the lymphopenic environment and potential developmental abnormalities in T cells from Bim+/− or Bcl-2−/− mice could have impacted the above results, we took advantage of a synthetic Bcl-2 antagonist, ABT-737, that inhibits Bcl-2, Bcl-xL, and Bcl-w (25). Our previous data showed that ABT-737 kills naive T cells in C57BL/6 mice in a Bim-dependent manner (18). To determine the role of Bcl-2 in effector T cell survival, we treated C57BL/6 mice with ABT-737 or vehicle between days 10 and 23 after LCMV infection. Pharmacological inhibition of Bcl-2 resulted in a significant reduction in the numbers of gp33-sp KLRG1lowCD127high effector CD8+ T cells, as 90% of these cells were lost during the contraction phase compared with an ~45% reduction in vehicle-treated mice (Fig. 2B). In contrast, loss of KLRG1highCD127high cells was slightly exacerbated by Bcl-2 inhibition (Fig. 2B). Thus, similar to our genetic studies, pharmacologic inhibition of Bcl-2 also resulted in a significant loss of effector CD8+ T cells, particularly affecting KLRG1lowCD127high cells.

Bcl-2 is critical for the generation and survival of memory CD8+ T cells

As KLRG1lowCD127high CD8+ effector T cells contribute to the long-term memory cell pool (12, 13), we next determined how inhibition of Bcl-2 during the contraction phase would affect memory development. Again, we treated C57BL/6 mice with ABT-737 between day 10 and 20 after LCMV infection and analyzed gp33-sp CD8+ memory T cells 110 d later. We found that the overall numbers of TEM and TCM were decreased in ABT-737–treated mice compared with those treated with vehicle (Fig. 2C).
Thus, these results suggest that generation of normal numbers of memory CD8\(^+\) T cells critically depends upon the function of Bcl-2 during the contraction of the response.

Although inhibiting Bcl-2 at the effector stage can have a long-term impact on CD8\(^+\) T cell memory, we next determined the roles and expression levels of Bim and Bcl-2 in memory T cells after they were formed. First, we measured Bim and Bcl-2 levels in gp33\(^{sp}\) TEM and TCM CD8\(^+\) T cells by flow cytometry. Similar to subsets of effector CD8\(^+\) T cells, the levels of Bim and Bcl-2 were directly correlated in both TEM and TCM subsets of CD8\(^+\) gp33\(^{sp}\) memory cells (Fig. 3A). Further, levels of both Bim and Bcl-2 were significantly increased in TCM compared with TEM within the CD8\(^+\) gp33\(^{sp}\) population (Fig. 3B, 3C). Thus, similar to KLRI\(^{low}\)/CD127\(^{high}\) cells, gp33\(^{sp}\) TCM CD8\(^+\) T cells exhibited high expression of both Bim and Bcl-2.

Next, we determined the role of Bcl-2 in combating Bim to maintain LCMV-sp memory CD8\(^+\) T cells. We infected C57BL/6 and Bim\(^{-/-}\) mice with LCMV and, 90 d later, treated them with ABT-737 or vehicle control for 10 d and analyzed gp33\(^{sp}\)-memory cell subsets by flow cytometry. In C57BL/6 mice, ABT-737 led to a \(~3\)-fold loss of gp33\(^{sp}\) CD8\(^+\) TEM and a \(~2\)-fold loss of TEM cells (Fig. 4A). As expected, numbers of TEM and TCM in vehicle-treated Bim-deficient mice were significantly increased compared with vehicle-treated C57BL/6 mice (Fig. 4A). Further, gp33\(^{sp}\) TEM and TCM in Bim-deficient mice were not significantly decreased by ABT-737 (Fig. 4A), suggesting that a Bim/Bcl-2 balance is also critical for the maintenance of LCMV-sp memory cells. As ABT-737 can cause moderate lymphopenia (18) that can in turn cause homeostatic proliferation, we assessed in vivo T cell proliferation by guest on April 13, 2017 http://www.jimmunol.org/ Downloaded from using BrdU. gp33\(^{sp}\) CD8\(^+\) TEM and, to a lesser extent, TCM in ABT-737-treated C57BL/6 mice underwent significantly greater proliferation in vivo (Fig. 4B). ABT-737 did not increase proliferation of gp33\(^{sp}\) CD8\(^+\) T cells in Bim\(^{-/-}\) mice (Fig. 4B), as Bim\(^{-/-}\) mice are resistant to ABT-737-induced lymphopenia (18). Thus, the actual dependence of viral-specific memory T cells upon Bcl-2 is likely underestimated because of the lymphopenia-induced proliferation following ABT-737 treatment.

**FIGURE 3.** Expression of Bim and Bcl-2 in memory CD8\(^+\) T cell subpopulations. C57BL/6 mice were infected i.p. with LCMV. Mice (n = 6 mice/group) were sacrificed 100 d p.i. and splenocytes were stained with D\(^\text{D}\)gp33 tetramers and Abs against CD8, CD44, CD62L, and intracellularly against Bim or Bcl-2, and data were acquired on a flow cytometer and analyzed using FACS Diva software after gating on TEM (CD8\(^+\)CD44\(^{low}\)CD62L\(^{low}\)) (TEM) or TCM (CD8\(^+\)CD44\(^{high}\)CD62L\(^{high}\)) populations. A, Dot plots for Bim and Bcl-2 in TEM and TCM after gating on CD8\(^+\)gp33\(^{sp}\) cells. Histograms (B) and bar graphs (C) for Bim and Bcl-2 MFI in TEM or TCM CD8\(^+\) T cells are shown. Graphs are representative of three independent experiments. \(*p \leq 0.05, **p \leq 0.01.\)

**FIGURE 4.** Bcl-2 is critical for survival of memory CD8\(^+\) T cells. Groups of C57BL/6 and Bim\(^{-/-}\) mice (n = 5 mice/group) were infected i.p. with LCMV, and after 90 d, they were treated with either vehicle or ABT-737 (1 mg/mouse/d) for 10 d and sacrificed. Mice were also injected with BrdU (0.7 mg/mouse i.p.) for 2 d before sacrifice. Spleen cells were stained with D\(^\text{D}\)gp33 tetramers and Abs against CD8, CD44, and CD62L, and analyzed by flow cytometry. A, Bar graphs indicate the total numbers \pm SEM values of TEM (CD8\(^+\)CD44\(^{low}\)CD62L\(^{low}\)) or TCM (CD8\(^+\)CD44\(^{high}\)CD62L\(^{high}\)) subsets. B, Splenocytes were stained with the above MHC tetramer and surface stains along with an Ab against BrdU according to the manufacturer’s instructions. The percent of TEM and TCM that were BrdU\(^+\) \pm SEM are shown. C, Splenocytes were stained with the above MHC tetramer and surface stains along with an Ab against Bim. Bar graphs show Bim MFI \pm SEM values in TEM and TCM subsets. Graphs are representative of three independent experiments. D, Groups of C57BL/6, Bim\(^{-/-}\), and Bim\(^{-/-}\)Bcl-2\(^{-/-}\) mice (n = 4 mice/group) were infected i.p. with LCMV and sacrificed 100 d.p.i. Splenocytes were stained with MHC tetramers and surface stains as above and intracellularly for Bim. Bar graphs show Bim MFI \pm SEM values in TEM and TCM subsets. \(*p \leq 0.05, **p \leq 0.01.\)
Bcl-2 determines the level of Bim in effector and memory CD8+ T cells

As Bim and Bcl-2 levels within effector and memory CD8+ T cells are correlated and have been shown to influence expression of each other (21, 22), we next determined the role of Bcl-2 in maintaining Bim expression in memory T cells. To do this, we first treated C57BL/6 mice infected with LCMV 3 mo previously with ABT-737 or vehicle. Interestingly, we found that Bim levels were significantly decreased in both memory CD8+ subsets from ABT-737–treated compared with controls (Fig. 4C). We also assessed the influence of Bcl-2 on Bim levels in gp33–sp CD8+ T cells in groups of LCMV-infected C57BL/6, Bim+/– Bcl-2+/–, and Bim+/– Bcl-2+/– mice. Bim levels in CD8+ T cells from both Bim+/– Bcl-2+/– and Bim+/– Bcl-2+/– mice should be ~50% of that found in C57BL/6 mice, unless the lack of Bcl-2 has an effect on Bim levels. Similar to the results with ABT-737, we found that the levels of Bim within both gp33–sp TEm and Tcm subpopulations of memory cells from Bim+/– Bcl-2+/– mice were significantly decreased compared with cells from either C57BL/6 or Bim+/– Bcl-2+/– mice (Fig. 4D). Thus, using both pharmacologic and genetic approaches, we found that in Tcm, and to a lesser extent TEM, Bcl-2 is critical to maintain significant expression of Bim.

As Bim levels were decreased in TEm and Tcm in Bim+/– Bcl-2+/– mice and because these mice have a substantial loss of effector T cells, we next inquired whether the low levels of Bim in their memory cells were apparent earlier, during either the peak or after contraction of the response. To do this, we examined the levels of Bim within gp33–sp effector CD8+ T cells on days 10 and 23 after LCMV infection. In C57BL/6 mice, expression of Bim within gp33–sp CD8+ KLRG1highCD127low cells was significantly decreased from days 10–23 p.i. (Fig. 5A). Thus, using both pharmacologic and genetic approaches, we found that in Tcm, and to a lesser extent TEM, Bcl-2 is critical to maintain significant expression of Bim.

Next, we determined the role of Bcl-2 in influencing the levels of Bim in gp33–sp effector CD8+ T cell subsets. Using LCMV-infected C57BL/6 mice, Bim+/– Bcl-2+/–, and Bim+/– Bcl-2+/– mice, we assessed Bim levels within effector CD8+ subsets. On day 10 p.i., the levels of Bim in both KLRG1highCD127low and KLRG1lowCD127high effector cells in both Bim+/– Bcl-2+/– and Bim+/– Bcl-2+/– mice were roughly half of that observed in C57BL/6 mice (Fig. 5A, 5B). Interestingly, on day 23 p.i., the levels of Bim decreased slightly in KLRG1highCD127low cells from Bim+/– Bcl-2+/– mice, but decreased to a significantly greater extent in cells from Bim+/– Bcl-2+/– mice (Fig. 5A, 5B). Moreover, in KLRG1lowCD127high cells, the levels of Bim were only significantly reduced in mice that completely lacked expression of Bcl-2 (Fig. 5A, 5B). Similar decreases in Bim in effector CD8+ T cells were observed when C57BL/6 mice were treated with ABT-737 during contraction of the response (data not shown). Thus, Bcl-2 is required to maintain normal levels of Bim within effector CD8+ T cells.

IL-7 and IL-15 availability can influence the levels of Bim in effector CD8+ T cells

It is well known that IL-7 and IL-15 (as well as other common γ-chain cytokines) can increase expression of Bcl-2 within effector T cells (15, 26–28). Therefore, we predicted that manipulation of IL-7 and/or IL-15 levels during contraction of the CD8+ T cell response would affect their levels of Bim in a manner that correlates with Bcl-2 expression. First, we examined Bim levels in effector T cells from LCMV-infected C57BL/6 and IL-15–deficient mice that were treated with either isotype control Ab or anti–IL-7 neutralizing Ab between days 10 and 20 p.i. We found that, in both KLRG1high and KLRG1low cells, neutralization of IL-7 or loss of IL-15 led to decreased levels of Bim, which was slightly, albeit nonsignificantly, decreased by neutralization of IL-7 in IL-15–/– mice (Fig. 5C). Recently, we showed that IL-7 and IL-15 are critical for maintaining expression of Bcl-2 in effector CD8+ T cells (19). Thus, IL-7 and IL-15 are critical to maintain expression of both Bcl-2 and Bim in effector CD8+ T cells.

Conversely, we reasoned that administration of IL-7 or IL-15, either of which can increase Bcl-2 levels in effector T cells (19, 26, 29), would also increase their expression of Bim. To test this,
we infected groups of C57BL/6 mice with LCMV and administered PBS, human IL-7/anti–IL-7, or IL-15/IL-15Rα complexes on days 8, 10, 12, and 14 p.i., sacrificed them on day 16 p.i., and measured Bim levels within effector CD8+ T cells. In contrast to inhibition of IL-7 and IL-15, administration of either IL-7 or IL-15 significantly increased levels of Bim within effector CD8+ T cells (Fig. 5D). Thus, enhancing IL-7 or IL-15 availability increases the levels of both Bim and Bcl-2 in effector CD8+ T cells.

Posttranslational control of Bim by Bcl-2
As Bcl-2 is complexed to Bim in naive T cells (20), it is possible that Bcl-2 influences the levels of Bim through protein–protein interactions that increase the stabilization of Bim (30, 31). We next measured Bim turnover in effector CD8+ T cells by culturing splenocytes from either uninfected or day 10 LCMV-infected C57BL/6 mice with the inhibitor CHX and assessed Bim protein levels over time in culture. We found that 6 h was a reasonable time to assess Bim turnover because: 1) there was a significant loss of Bim at this time point; and 2) there was little cell death occurring during this time (data not shown). After culture with CHX, levels of Bim decreased moderately in naive T cells and substantially in effector T cells (Fig. 6A). Further, the CHX-induced loss of Bim protein was similar between KLRG1 highCD127low and KLRG1 lowCD127high effector CD8+ T cells (Fig. 6B). We next used Bim+/-Bcl-2-/- mice to test the role of Bcl-2 in stabilization of Bim protein. We found that the lack of Bcl-2 slightly, albeit significantly, exacerbated the CHX-induced loss of Bim in KLRG1 highCD127high effector cells compared with C57BL/6 and Bim+/- controls (Fig. 6B). In contrast, the absence of Bcl-2 did not accelerate the loss of Bim in KLRG1 highCD127low effector cells. Thus, Bcl-2 likely contributes somewhat to Bim stabilization, at least in KLRG1 highCD127high effector cells.

Lack of cell death allows effector T cells to tolerate higher expression of Bim
Another potential, and not mutually exclusive, explanation is that a threshold level of Bcl-2 is critical to protect effector CD8+ T cells from toxic levels of Bim. A prediction of this model is that inhibition of apoptosis should lead to increased expression of Bim. We tested this prediction using two separate approaches. First, we determined whether overexpression of Bcl-2, which protects activated T cells from death (21), would affect Bim levels in activated T cells. Similar to a recent report (22), retroviral overexpression of Bcl-2 led to significantly increased levels of Bim (Fig. 6C).
Bcl-2 within the cell determines the amount of Bim that T cells require for survival. A major role for Bcl-2 is to inhibit cell death and that the level of Bcl-2 is critical for the survival of particular subsets of effector and memory T cells (18). In this study, we show that Bcl-2 is important to prevent death signaling apparatus downstream of Bim. Interestingly, we found that the levels of Bim were dramatically higher in both KLRG1<sup>high</sup>CD127<sup>low</sup> and KLRG1<sup>low</sup>CD127<sup>high</sup> effector CD8<sup>+</sup> T cells in LckCre<sup>+</sup>Bax<sup>−/−</sup>Bak<sup>−/−</sup> mice compared with littermate Cre<sup>−</sup> or C57BL/6 controls (Fig. 6C). Bim mRNA levels were also increased in effector CD8<sup>+</sup> T cells from LckCre<sup>+</sup>Bax<sup>−/−</sup>Bak<sup>−/−</sup> mice (Fig. 6D). Importantly, increased levels of Bim were not accompanied by an increase in Bcl-2; in fact, Bcl-2 levels were significantly decreased in effector CD8<sup>+</sup> T cells from Cre<sup>−</sup>Bax<sup>+</sup>Bak<sup>−/−</sup> mice compared with C57BL/6 mice (Fig. 6E). Moreover, we found that the total numbers of KLRG1<sup>high</sup>CD127<sup>low</sup> and KLRG1<sup>low</sup>CD127<sup>high</sup> effector CD8<sup>+</sup> T cells were significantly increased in the absence of Bax and Bak (Fig. 6F). Finally, turnover of Bim was not significantly affected by the loss of Bax and Bak (data not shown). Together, these data strongly suggest that a major role for Bcl-2 is to inhibit cell death and that the level of Bcl-2 within the cell determines the amount of Bim that T cells can tolerate and survive.

Discussion

We and others previously showed that most effector CD8<sup>+</sup> T cells have decreased expression of Bcl-2 at the peak of the response (21, 26, 32). Further, our previous data in a superantigen model showed that decreased expression of Bcl-2 correlated with susceptibility to Bim-driven death of effector T cells (21). More recently, it was found that subsets of effector T cells differ in their expression of Bcl-2 (13, 33). In addition, it is well documented that most memory T cells express high levels of Bcl-2 (32). However, in none of these reports was the actual role of Bcl-2 in the survival of effector and memory T cells tested. We recently found that Bcl-2 was critical for the survival of most naive and some memory T cells (18). In this study, we show that Bcl-2 is critical for the survival of particular subsets of effector and memory T cells and, in doing so, allowed these cells to tolerate higher levels of Bim and survive.

A recent report showed that Bim and Bcl-2 can reciprocally affect the expression of each other (22), although the underlying mechanism(s) were unclear. Similar to our results, Marrack’s group (22) found that retroviral overexpression of Bcl-2 led to increased levels of Bim mRNA and protein within activated T cells. In this study, we found that Bcl-2 was important to prevent the loss of Bim protein in effector T cells, likely acting at two levels. At one level, Bcl-2 likely stabilizes Bim, a normally unstructured protein (34), and likely protects it from proteasomal degradation. At another level, a consequence of Bcl-2 binding to Bim is the prevention of cell death, which allows cells with higher levels of Bim to survive. Combined, these data suggest a model in which, following T cell activation, Bim levels are transcriptionally increased, but most effector T cells do not maintain sufficient levels of Bcl-2 to protect these T cells from death. The few cells that do maintain Bcl-2 at high levels also appear to have the highest levels of Bim; elimination or inhibition of Bcl-2 in these cells leads to rapid death, and only cells having the lowest expression of Bim survive. Conversely, inhibition of apoptosis downstream of Bim (i.e., in effector T cells that lack Bax and Bak) allowed cells to survive despite having substantially higher Bim mRNA and protein levels. This Bim/Bcl-2 balance in the effector stage is absolutely critical to memory T cell development, as disruptions to this balance affect the generation of normal numbers of memory CD8<sup>+</sup> T cells.

Although the loss of Bcl-2 in effector T cells is likely a significant initiating apoptotic event, our data also suggest an activation-induced increase in Bim expression; this has been difficult to observe previously, because the cells die. Indeed, when this death was eliminated by genetic deletion of Bax and Bak, we saw a significant increase in Bim mRNA and protein in both subsets of effector CD8<sup>+</sup> T cells. Interestingly, even though death was alleviated, and Bim levels were increased in both effector CD8<sup>+</sup> T cell subsets, they were not increased to the same overall level (i.e., Bim levels were still lower in KLRG1<sup>high</sup>CD127<sup>low</sup> cells). It is unlikely that different levels of Bcl-2 in the different subsets of CD8<sup>+</sup> T cells in Bax/Bak double-deficient mice contribute to this differential expression of Bim, because in both effector subsets, Bcl-2 levels are significantly lower than those observed in C57BL/6 animals. In contrast, the transcriptional profiles of KLRG1<sup>high</sup>CD127<sup>low</sup> and KLRG1<sup>low</sup>CD127<sup>high</sup> cells have been reported to be different (12). It is possible that Bim expression is controlled by distinct mechanisms in KLRG1<sup>high</sup>CD127<sup>low</sup> and KLRG1<sup>low</sup>CD127<sup>high</sup> cells.

We and others have recently shown that KLRG1<sup>high</sup>CD127<sup>low</sup> cells are predominantly maintained by IL-15, whereas IL-7 and IL-15 act redundantly to maintain KLRG1<sup>low</sup>CD127<sup>high</sup> cells (19, 35). We also found that Bcl-2 is a critical antiapoptotic molecule induced/maintained by IL-7 and IL-15 in both of these subsets (19). In this study, we show that Bim levels are also regulated by the availability of IL-7 and IL-15, perhaps because of their ability to maintain Bcl-2. In contrast to previous models in the literature, which suggest that Bim levels are increased in response to cytokine withdrawal (36), we find just the opposite: that Bim levels are positively correlated with cytokine availability. In the cytokine withdrawal models, the forkhead box subclass O transcription factor 3a (Foxo3a) has been shown to be a major transcriptional regulator of Bim (37). The data suggest that Foxo3a is maintained in the cytosol by cytokine signaling through Akt, and upon cytokine withdrawal, Foxo3a translocates to the nucleus and drives expression of Bim (37, 38). However, in primary T cells, the role for Foxo3a in controlling Bim expression is less clear. For example, one report suggested that Bim levels were not affected in primary T cells from Foxo3a-deficient mice (39). Recently, another group reported that levels of phosphorylated (and hence transcriptionally inactive) Foxo were actually higher in KLRG1<sup>low</sup>CD127<sup>high</sup>CD8<sup>+</sup> T cells relative to KLRG1<sup>high</sup>CD127<sup>low</sup>CD8<sup>+</sup> T cells when stimulated with IL-15 (40). As KLRG1<sup>low</sup>CD127<sup>high</sup>CD8<sup>+</sup> T cells have significantly higher levels of Bim compared with KLRG1<sup>high</sup>CD127<sup>low</sup> T cells, these data suggest that Foxos are not a significant regulator of Bim in KLRG1<sup>low</sup>CD127<sup>high</sup>CD8<sup>+</sup> T cells. In contrast, another group showed that TCR and cytokine signaling control expression of Bim in human memory CD4<sup>+</sup> T cell subsets through an Akt/Foxo3a-dependent mechanism (41). Although this study showed decreased Bim expression in CD4<sup>+</sup> T<sub>CM</sub>, our data clearly show that Bim levels in CD8<sup>+</sup> T<sub>CM</sub> are increased. Whether these differences are due to the differences between humans and mice or between CD4<sup>+</sup> and CD8<sup>+</sup> T cells remain to be determined. Nonetheless, more work is necessary to further understand the regulation of Bim in effector and memory T cell subsets.

The similarity in Bim and Bcl-2 expression between effector and memory CD8<sup>+</sup> T cell subsets is intriguing. At first glance, the simplest explanation for our data would be that CD8<sup>+</sup> T<sub>EM</sub> cells are derived largely from KLRG1<sup>high</sup>CD127<sup>low</sup> cells, whereas KLRG1<sup>low</sup>CD127<sup>high</sup> cells give rise to CD8<sup>+</sup> T<sub>CM</sub>. Indeed, the few viral-specific CD62L<sup>+</sup> cells that are present shortly after the contraction of the response are located within the KLRG1<sup>low</sup>CD127<sup>high</sup> cell population (data not shown and Ref. 42). In addition, adoptive
transfer studies have suggested that CD8+ T cells are derived from KLRG1lowCD127high cells (12). However, the origin of CD8+ TEM is a little less clear. The ability to track and monitor effector CD8+ T cells based on their level of Bim and/or Bcl-2 we think would be helpful in determining the origins of memory T cell subsets. Unfortunately, as Bim and Bcl-2 are intracellular molecules, their levels cannot be detected while maintaining cell viability without reporter mice.

Our results also suggest that the regulation of effector CD8+ T cell responses is dictated by a dynamic interplay carefully controlled by the levels of pro- and antia apoptotic Bcl-2 family members. Why would this balance be so critical? There are few circumstances in nature whereby a quiescent cell is stimulated to undergo 15–20 rounds of vigorous division and then return to quiescence. During an immune response, expanded naive T cells bear many hallmarks of neoplasia: rapid proliferation, generation of self-renewing cells, and massive epigenetic changes. This balance of a tumor suppressor (Bim) with a proto-oncogene (Bcl-2) is likely part of a highly conserved mechanism to prevent effector T cells from turning into T cell lymphomas. As such, we have found that a highly effective anticancer therapeutic can dramatically curtail effector and memory T cell responses in vivo. Further, we found that the effects of ABT-737 on effector T cell responses are long-lived, impacting the memory compartment long after cessation of the drug. Thus, we envision one potential application of Bcl-2 antagonists could be to target and eliminate autoreactive T cells in autoimmune disease or eliminate allorreactive T cells based on their level of Bim and/or Bcl-2 we think would offer substantial promise as a way to delete populations of unwanted, disease-causing T cells.

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Disclosures

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References


