

Bim/Bcl-2 balance is critical for maintaining naive and memory T cell homeostasis

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We examined the role of the antiapoptotic molecule Bcl-2 in combating the proapoptotic molecule Bim in control of naive and memory T cell homeostasis using *Bcl-2*^{-/-} mice that were additionally deficient in one or both alleles of *Bim*. Naive T cells were significantly decreased in *Bim*^{+/-}*Bcl-2*^{-/-} mice, but were largely restored in *Bim*^{-/-}*Bcl-2*^{-/-} mice. Similarly, a synthetic Bcl-2 inhibitor killed wild-type, but not *Bim*^{-/-}, T cells. Further, T cells from *Bim*^{+/-}*Bcl-2*^{-/-} mice died rapidly *ex vivo* and were refractory to cytokine-driven survival *in vitro*. *In vivo*, naive CD8⁺ T cells required Bcl-2 to combat Bim to maintain peripheral survival, whereas naive CD4⁺ T cells did not. In contrast, *Bim*^{+/-}*Bcl-2*^{-/-} mice generated relatively normal numbers of memory T cells after lymphocytic choriomeningitis virus infection. Accumulation of memory T cells in *Bim*^{+/-}*Bcl-2*^{-/-} mice was likely caused by their increased proliferative renewal because of the lymphopenic environment of the mice. Collectively, these data demonstrate a critical role for a balance between Bim and Bcl-2 in controlling homeostasis of naive and memory T cells.

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Abbreviations used: LCMV, lymphocytic choriomeningitis virus; pfu, plaque-forming unit; SP, single-positive thymocyte.

Maintenance of T cell homeostasis is critical for normal functioning of the immune system. After thymocyte selection, T cells enter the periphery, where they are maintained as resting naive cells. Transient disruption of homeostasis occurs when naive T cells undergo antigen-driven expansion and acquire effector functions. Effector T cells then either undergo apoptosis or survive to become memory cells. This process resets T cell homeostasis, promotes protective immunity, and limits autoimmunity. Thus, T cell homeostasis is ultimately achieved through maintenance of distinct T cell populations (naive, effector, and memory), although the mechanisms that maintain homeostasis in each population are not fully understood.

Regulation of responsiveness to soluble cytokines and cytokine availability is one mechanism that maintains independent T cell populations. For example, whereas naive T cell homeostasis is mostly intact in the absence of IL-15 (1, 2), IL-15-deficient mice are defective in maintaining memory T cells over time (1–4). This is because the slow proliferative turnover that is crucial for the maintenance of memory, but not

naive, CD8⁺ T cells *in vivo* is IL-15-dependent (2–6). Differential responsiveness to IL-15 between naive and memory T cells is at least partly explained by differences in their IL-15R expression (7). IL-7, on the other hand, is critical for maintenance of both naive and memory T cell homeostasis (8, 9, 10–12). IL-7 regulation of molecules that promote or inhibit apoptosis is likely responsible for the effects of IL-7, inasmuch as overexpression of the antiapoptotic molecule Bcl-2 or genetic loss of the proapoptotic molecule Bim largely restores peripheral T cell homeostasis in the absence of IL-7R signaling (13–15). Thus, competition for available IL-7 limits total T cell numbers, whereas IL-15 allows proliferative renewal of memory CD8⁺ T cells without major effects on the naive T cell pool.

Recent experiments have begun to shed light on how proapoptotic Bcl-2 family members mechanistically regulate the cell death process. For example, genetic loss of the multidomain proapoptotic molecules Bax and Bak blocks the ability of BH3-only molecules, such as Bim, to cause apoptosis (16). Both multidomain and BH3-only proapoptotic molecules are prevented from initiating apoptosis through physical

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interactions with antiapoptotic Bcl-2 family members such as Bcl-2 and Mcl-1 (17–19). However, it remains unclear how particular antiapoptotic molecules target specific proapoptotic molecules to prevent cell death within lymphocytes.

IL-7 and -15 both increase Bcl-2 expression in naive and memory T cells (12, 20–22). Initial studies show that Bcl-2 is largely required for short-term naive T cell homeostasis (23–25), although studies on the role of Bcl-2 in long-term naive or memory T cell homeostasis have not been performed. This is largely because *Bcl-2*^{-/-} mice die between 2 and 6 wk of age (23–25). Thus, interpretation of observations that *Bcl-2*^{-/-} mice have defects in naive T cell homeostasis is complicated by the early lethality of these mice. However, a recent study showed that the additional loss of one or two alleles of *Bim* completely rescued *Bcl-2*^{-/-} mice from death (26). In contrast, naive T cells from *Bim*^{+/-}*Bcl-2*^{-/-} mice could not be rescued from death in vitro by IL-7 (26); although naive T cell homeostasis in these mice was not directly examined in vivo.

In this study, we took advantage of *Bim*^{+/-}*Bcl-2*^{-/-} mice to study the roles of Bcl-2 and Bim in controlling homeostasis of distinct T cell populations. We found that *Bim*^{+/-}*Bcl-2*^{-/-} mice have substantial defects in their numbers of peripheral T cells and that loss of the remaining allele of *Bim* largely restored peripheral T cell numbers. Thymectomy experiments show that Bcl-2 is needed to maintain peripheral CD8⁺ T cell survival by antagonizing Bim. We also found that *Bim*^{+/-}*Bcl-2*^{-/-} mice could generate fairly normal numbers of functional memory CD4⁺ and CD8⁺ T cells after lymphocytic choriomeningitis virus (LCMV) infection, but that these cells accumulate because of lymphopenia-driven proliferation. Collectively, these data suggest that a Bim/Bcl-2 balance is critical for controlling normal homeostasis of naive and memory T cells.

RESULTS

In *Bim*^{-/-} or *Bim*^{+/-} mice, T cells appear to develop normally in the absence of Bcl-2

Because loss of a single *Bim* allele prevents the lethality and kidney disease of Bcl-2 deficiency (26), we reasoned that *Bim*^{+/-}*Bcl-2*^{-/-} mice could be used to determine whether Bcl-2 is required for T cell survival in vivo when Bim expression is reduced by 50%. Further, study of *Bim*^{-/-}*Bcl-2*^{-/-} mice could determine the specific role of Bcl-2 in antagonizing Bim. As previously reported for *Bcl-2*^{-/-}*Bim*^{+/-} mice (23–25), T cell development in 28-d-old *Bim*^{+/-}*Bcl-2*^{-/-} mice was not grossly disturbed compared with *Bim*^{+/-}*Bcl-2*^{+/-} controls. *Bim*^{+/-}*Bcl-2*^{-/-} mice had increased percentages and total numbers of CD4 single-positive (SP) thymocytes, but no substantial difference in the percentages or numbers of CD8 SP thymocytes (Fig. 1, A–D). The slight increase in CD4 SP thymocytes was also observed in *Bim*^{+/-}*Bcl-2*^{+/-} (not depicted) and *Bim*^{-/-}*Bcl-2*^{-/-} mice (Fig. 1, A and C). There were no significant differences in percentages or numbers of double-negative (CD4⁻CD8⁻) or double-positive (CD4⁺CD8⁺) thymocytes in *Bim*^{+/-}*Bcl-2*^{+/-} and *Bim*^{-/-}

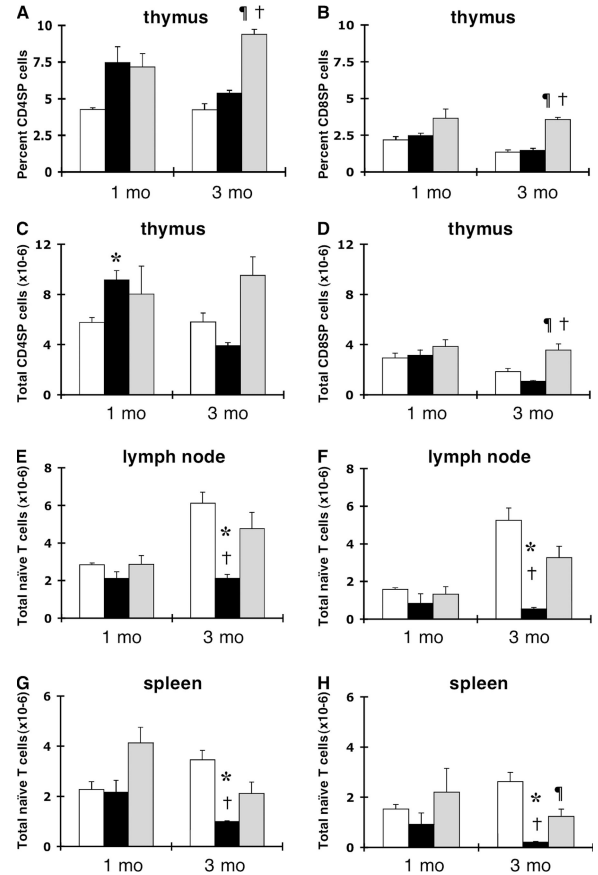


Figure 1. Dramatic loss of peripheral T cells, but not thymocytes, in *Bim*^{+/-}*Bcl-2*^{-/-} mice with age. Groups of *Bim*^{+/-}*Bcl-2*^{+/-} (*n* = 3 mice/time point; open bars), *Bim*^{+/-}*Bcl-2*^{-/-} (*n* = 4 mice/time point; shaded bars), or *Bim*^{-/-}*Bcl-2*^{-/-} (*n* = 4 mice/time point; gray bars) mice were killed at either 1 or 3 mo of age, and thymuses, spleens, and peripheral LNs were removed. Cells were stained with antibodies against TCR, CD44, CD4, and CD8. For thymus analysis, results show the percentage of CD4 (A) or CD8 (B) SP cells and the absolute number of CD4 (C) or CD8 (D) SP cells ± the SEM. For LN (E and F) and spleen (G and H) analysis, results show the absolute number of naive CD44^{lo} CD4⁺ T cells (E and G) or naive CD44^{lo} CD8⁺ T cells (F and H) ± the SEM. *, statistically significant difference between *Bim*^{+/-}*Bcl-2*^{-/-} and *Bim*^{+/-}*Bcl-2*^{+/-} mice (number of CD4SP cells in thymus, *P* ≤ 0.02; number of CD4s in LN and spleen, *P* ≤ 0.02; number of CD8s in LN and spleen, *P* ≤ 0.02); †, statistically significant difference between *Bim*^{+/-}*Bcl-2*^{-/-} and *Bim*^{-/-}*Bcl-2*^{-/-} mice (percentage of CD4SP cells in thymus, *P* ≤ 0.001; number of CD4SP cells in thymus, *P* ≤ 0.03; percentage of CD8SP cells in thymus, *P* ≤ 0.001; number of CD8SP cells in thymus, *P* ≤ 0.01; number of CD4s in LN, *P* ≤ 0.05; number of CD4s in spleen, *P* ≤ 0.04; number of CD8s in LN, *P* ≤ 0.02; number of CD8s in spleen, *P* ≤ 0.05); †+, statistically significant difference between *Bim*^{-/-}*Bcl-2*^{-/-} and *Bim*^{+/-}*Bcl-2*^{+/-} mice (percentage of CD4SP cells in thymus, *P* ≤ 0.001; percentage and number of CD8SP cells in thymus, *P* ≤ 0.001 and *P* ≤ 0.03; number of CD8s in spleen, *P* ≤ 0.03).

Bcl-2^{-/-} mice (unpublished data). Numbers and percentages of LN naive CD4⁺ and CD8⁺ T cells were, again, fairly normal in 28-d-old *Bim*^{+/-}*Bcl-2*^{-/-} mice, with only a slight decrease in numbers of naive CD8⁺ T cells compared with *Bim*^{+/-}*Bcl-2*^{+/-} or double-deficient mice (Fig. 1, E and F).

Similar results were obtained for spleen (Fig. 1, G and H). *Bim^{+/-}Bcl-2^{+/-}* mice had normal numbers of naive CD4⁺ and CD8⁺ T cells in the LN and spleen (unpublished data). Thus, initial T cell development and seeding of secondary lymphoid organs does not require Bcl-2, at least under conditions of Bim heterozygosity.

At 3 mo, the percentages of CD4 and CD8 SP thymocytes were similar for *Bim^{+/-}Bcl-2^{-/-}*, *Bim^{+/+}Bcl-2^{+/+}*, and *Bim^{+/-}Bcl-2^{+/-}* (not depicted) mice (Fig. 1, A–D), except for a less than twofold decrease in CD4 and CD8 SP thymocyte numbers in *Bim^{+/-}Bcl-2^{-/-}* mice (Fig. 1, A–D). In contrast, *Bim^{-/-}Bcl-2^{-/-}* mice had significantly increased percentages and numbers of CD4 and CD8 SP thymocytes (Fig. 1, A–D). In the periphery, *Bim^{+/-}Bcl-2^{-/-}* mice had significant decreases in the numbers of naive CD4⁺ (~2–3-fold decrease) and naive CD8⁺ (~10-fold decrease) T cells compared with wt or *Bim^{+/-}Bcl-2^{+/-}* mice (Fig. 1, E and F), whereas *Bim^{-/-}Bcl-2^{-/-}* mice had near normal numbers of peripheral T cell numbers in LN (Fig. 1, E and F) and spleen (Fig. 1, G and H). Again, *Bim^{+/-}Bcl-2^{+/-}* mice had normal numbers of naive CD4⁺ and CD8⁺ T cells in the LNs and spleen (unpublished data). The decreased numbers of mature naive T cells in the periphery of *Bim^{+/-}Bcl-2^{-/-}* mice in the face of their near normal numbers of SP thymocytes suggest that Bcl-2 promotes the survival of naive T cells. Further, the increase in SP thymocytes and the near normal number of peripheral T cells in *Bim^{-/-}Bcl-2^{-/-}* mice suggests that a single Bim allele controls T cell numbers in the periphery by promoting apoptosis and/or decreasing thymic production.

Bim rapidly kills T cells in the absence of Bcl-2

We used *in vitro* and *in vivo* approaches to further evaluate Bim and Bcl-2 effects on T cell homeostasis. We cultured naive peripheral T cells *in vitro* in the absence of growth and/or survival factors and measured cell survival over time. Both CD4⁺ and CD8⁺ T cells from *Bim^{+/-}Bcl-2^{-/-}* mice died very quickly *ex vivo* compared with either *Bim^{+/+}Bcl-2^{+/+}* or double-heterozygous controls (Fig. 2, A and B). Further, loss of the remaining Bim allele in double-deficient mice resulted in survival slightly greater than that observed with *Bim^{+/+}Bcl-2^{+/+}* mice (Fig. 2, A and B). Because activated T cells die much more readily than naive T cells *ex vivo* (27), the increased death of the *Bim^{+/-}Bcl-2^{-/-}* T cells could have been caused by the presence of an increased proportion of activated T cells. However, although there were slight increases in activated/memory phenotype T cells in *Bim^{+/-}Bcl-2^{-/-}* mice (8.18 ± 1.23% of CD4⁺ T cells were CD44^{hi} in *Bim^{+/+}Bcl-2^{+/+}* vs. 16.4 ± 2.61% in *Bim^{+/-}Bcl-2^{-/-}* mice), this increase could not account for the death of most *Bim^{+/-}Bcl-2^{-/-}* T cells in culture. Nonetheless, we directly examined the death of naive phenotype (CD44^{lo}) CD4⁺ and CD8⁺ T cells from *Bim^{+/-}Bcl-2^{-/-}* mice in culture and found that it was significantly increased compared with naive T cells from either *Bim^{+/+}Bcl-2^{+/+}* or *Bim^{+/-}Bcl-2^{+/-}* mice (Fig. 2, C and D). Thus, naive T cells from *Bim^{+/-}Bcl-2^{-/-}* mice die at an accelerated rate *in vitro* during

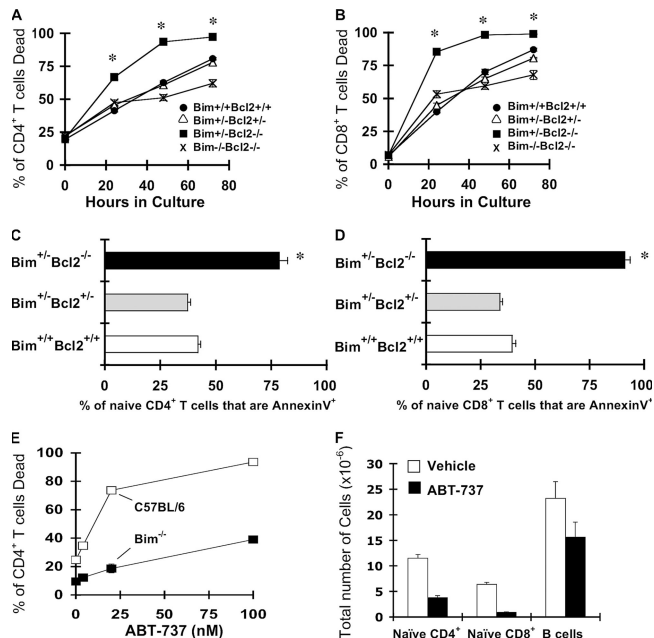


Figure 2. Naive Bcl-2-deficient T cells exhibit increased Bim-mediated death. LN cells from individual naive *Bim^{+/+}Bcl-2^{+/+}* (filled circles); *Bim^{+/-}Bcl-2^{+/-}* (open triangles); *Bim^{+/-}Bcl-2^{-/-}* (filled squares); or *Bim^{-/-}Bcl-2^{-/-}* mice (x marks; $n = 3$ mice/group) were cultured at 37°C and at various times were stained with antibodies against TCR- β and either CD4 or CD8 and propidium iodide, and cell death was assessed by flow cytometry. Results show the percentage of CD4⁺ (A) or CD8⁺ (B) T cells that were PI⁺ (dead) \pm the SEM. *, statistically significant difference in the death of CD4⁺ and CD8⁺ T cells from *Bim^{+/-}Bcl-2^{-/-}* mice compared with all other groups (for CD4s $P \leq 0.009$; for CD8s $P \leq 0.007$). At 48 and 72 h, the death of CD4⁺ and CD8⁺ T cells from *Bim^{-/-}Bcl-2^{-/-}* mice was significantly decreased compared with *Bim^{+/+}Bcl-2^{+/+}* mice (for CD4s $P \leq 0.02$; for CD8s $P \leq 0.05$). Similarly, LN cells were cultured at 37°C and stained with antibodies against TCR- β , CD44, CD4 or CD8, and AnnexinV-FITC. Cell death was assessed by flow cytometry. Results show the percentage of naive (CD44^{lo}) CD4⁺ (C) and CD8⁺ (D) T cells that were AnnexinV⁺ (apoptotic or dead) \pm the SEM. Death of naive CD4⁺ and CD8⁺ T cells from *Bim^{+/-}Bcl-2^{-/-}* mice was significantly increased compared with all other groups ($P \leq 0.01$). These data are representative of two independent experiments with similar results. (E) LN cells from either C57BL/6 (open squares) or *Bim^{-/-}* (filled squares) mice were cultured with the indicated concentrations of ABT-737 for 24 h. Results show the percentage of CD4⁺ T cells (dead) \pm the SEM (similar results were obtained with CD8⁺ T cells). (F) Groups of C57BL/6 mice ($n = 4$ mice/group) were injected i.p. with either vehicle (open bars) or ABT-737 (shaded bars) for 14 d and killed, and cell numbers were quantified by flow cytometric analysis. Results show the absolute numbers of CD4⁺ T cells, CD8⁺ T cells, and B cells present in the LN \pm the SEM. Similar results were obtained in the spleen.

withdrawal from survival factors that are normally present *in vivo*, and this rate decreases to wt levels with loss of the remaining Bim allele.

As an independent test of the roles of Bim and Bcl-2 in the maintenance of peripheral T cells, we used a synthetic Bcl-2 antagonist, which binds to Bcl-2, Bcl-xL, and Bcl-w, but not to Mcl-1 or A1 (28). Culture of wt T cells from naive

mice with ABT-737 led to substantial induction of cell death (Fig. 2 E). Further, ABT-737 required Bim for its ability to kill T cells (Fig. 2 E). Additionally, administration of ABT-737 in vivo led to a significant loss of CD4⁺ and CD8⁺ T cells (and, to a lesser extent, B cells) compared with vehicle-treated mice (Fig. 2 F). Because naive T cells normally express little Bcl-xL (29) and do not express Bcl-w (unpublished data), the effects of ABT-737 in vitro and in vivo are likely caused by its ability to bind to Bcl-2. Further, because ABT-737 requires Bim for its ability to kill T cells, these data suggest that Bcl-2 is critical to antagonize Bim to promote naive T cell survival, even in T cells that have developed in mice with normal amounts of Bim and Bcl-2.

Bcl-2 is required for cytokine-driven survival of T cells

We next tested whether cytokines that increase Bcl-2 expression and promote naive T cell survival in vitro and in vivo (9, 12, 30, 31) require Bcl-2 to promote survival. Groups of resting, untreated *Bim*^{+/+}*Bcl-2*^{+/+}, *Bim*^{+/-}*Bcl-2*^{+/-}, and *Bim*^{+/-}*Bcl-2*^{-/-} mice were killed, and their LN T cells were cultured with various doses of cytokines (IL-7, -4, -6, or -15) that can prevent naive T cell death in vitro (9, 12, 30–32). Both CD4⁺ and CD8⁺ T cells from unmanipulated *Bim*^{+/-}*Bcl-2*^{-/-} mice were significantly less responsive to rescue by IL-4 and -7 than those from either *Bim*^{+/+}*Bcl-2*^{+/+} or *Bim*^{+/-}*Bcl-2*^{+/-} mice (Fig. 3, A and C). On the other hand, Bcl-2 was not required for IL-6–driven survival of CD4⁺ T cells or for most CD8⁺ T cells (Fig. 3 B). As expected, IL-15 was unable to rescue CD4⁺ T cells from death, but largely required Bcl-2 to prevent the death of CD8⁺ T cells (Fig. 3 D). The effect of Bcl-2 loss was consistently greater for CD8⁺ than CD4⁺ T cells, which is consistent with the greater loss of CD8⁺ over CD4⁺ T cells in vivo observed in *Bim*^{+/-}*Bcl-2*^{-/-} mice (Fig. 3). Impaired cytokine-driven rescue was likely not caused by lack of cytokine receptor expression on the surface of the T cells in *Bim*^{+/-}*Bcl-2*^{-/-} mice because these cells express normal levels of CD127 (IL-7R α) and CD132 (cytokine receptor common γ chain [γ_c]; Fig. 3, E and F). Further, levels of CD122 (IL-2R β), CD124 (IL-4R α), and IL-15R α were not decreased on *Bim*^{+/-}*Bcl-2*^{-/-} T cells (unpublished data). Collectively, these data show that Bcl-2 is required downstream of several γ_c cytokines to optimally promote T cell survival.

Bim promotes naive CD8⁺ T cell death in the periphery

The restoration of naive T cell numbers in *Bim*^{-/-}*Bcl-2*^{-/-} mice (compared with *Bim*^{+/-}*Bcl-2*^{-/-} mice) could be caused by the absence of the proapoptotic effects of Bim on thymocytes, causing increased T cell production, or on mature peripheral naive T cells, causing increased survival. We performed two independent sets of experiments to differentiate between these possibilities. First, we injected groups of *Bim*^{+/+}*Bcl-2*^{+/+}, *Bim*^{+/-}*Bcl-2*^{-/-}, or *Bcl-2*^{-/-}*Bim*^{-/-} mice with neutralizing anti-IL-7 mAb to limit in vivo availability of IL-7 and determined peripheral T cell numbers 1 wk later, relative to mice treated with an isotype-matched control

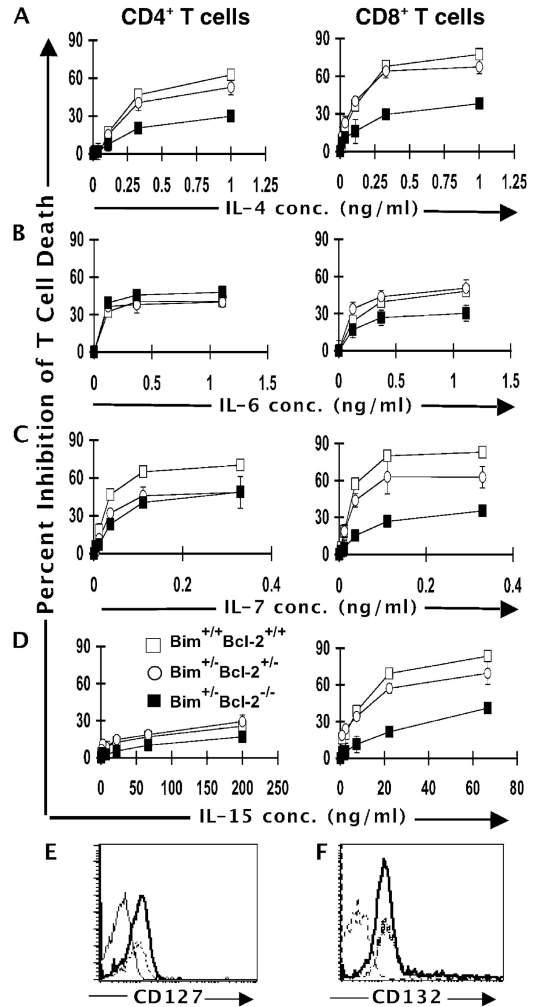


Figure 3. Bcl-2 is required for cytokine-mediated survival of naive T cells in vitro. LN cells from individual naive *Bim*^{+/+}*Bcl-2*^{+/+} (open squares), *Bim*^{+/-}*Bcl-2*^{+/-} (open circles), or *Bim*^{+/-}*Bcl-2*^{-/-} (filled squares) mice (*n* = 3 mice/group) were cultured at 37°C with IL-4 (A), -7 (B), -6 (C), or -15 (D) for 36 h at the concentrations shown. After culture, cells were stained with antibodies to TCR and CD8 and propidium iodide and cell death was assessed by flow cytometry. Cell death before culture was negligible. Results show the percentage of inhibition of cell death [(percentage of T cells dead in medium – percentage of T cells dead in cytokine) / percentage of T cells dead in medium] × 100 ± the SD, and they are representative of three independent experiments. LN cells from either *Bim*^{+/+}*Bcl-2*^{+/+} (thick line) or *Bim*^{+/-}*Bcl-2*^{-/-} (dotted line) mice were also assessed for expression of IL-7R α (CD127; E) and γ_c (CD132; F) by flow cytometry. Staining of cells with isotype control antibody is shown by the thin line). These data are representative of two independent experiments with similar results.

antibody. The efficacy of IL-7 neutralization in vivo for each mouse was confirmed by demonstrating the loss of immature (IgM⁺B220^{dull}) B cells in the BM (unpublished data) (33). After IL-7 neutralization, *Bim*^{+/+}*Bcl-2*^{+/+} mice had 65–70% of their peripheral naive CD4⁺ and CD8⁺ T cells remaining, whereas only 45–50% were left in *Bim*^{+/-}*Bcl-2*^{-/-} mice (Fig. 4, A and B). In contrast, there was essentially no decline in

the numbers of naive CD4⁺ or CD8⁺ T cells in *Bcl-2*^{-/-} *Bim*^{-/-} mice.

However, in vivo IL-7 neutralization can retard thymocyte development in addition to its effects on peripheral T cell survival (33, 34). Thus, IL-7-driven decreases in pre-T cell numbers could have contributed to the observed loss of peripheral T cells. In a second experiment, we studied the role of Bcl-2 in naive T cell survival in vivo without varying T lymphopoiesis by performing adult thymectomy to block new T cell production. Groups of *Bim*^{+/+}*Bcl-2*^{+/+}, *Bim*^{+/-}*Bcl-2*^{-/-}, and *Bim*^{-/-}*Bcl-2*^{-/-} mice were either thymectomized or sham thymectomized. 4 wk later, their peripheral naive T cells were analyzed by flow cytometry.

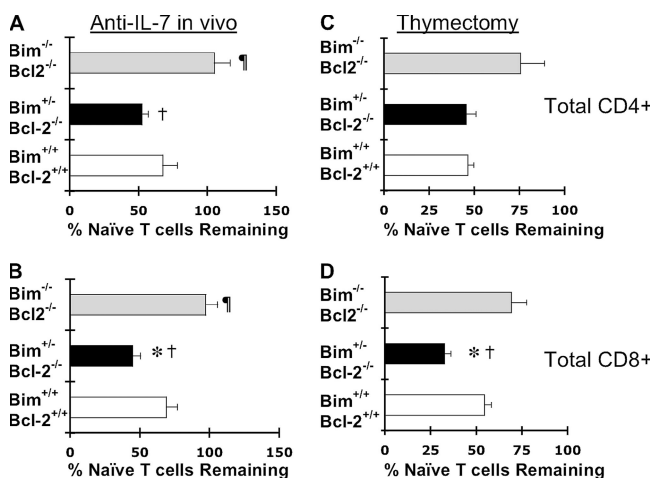


Figure 4. Bcl-2 is critical to prevent the proapoptotic effects of Bim in naive CD8⁺ T cells in vivo. (A and B) Groups of 6–9-wk-old *Bim*^{+/+}*Bcl-2*^{+/+} (*n* = 9 mice/group), *Bim*^{+/-}*Bcl-2*^{-/-} (*n* = 9 mice/group), or *Bim*^{-/-}*Bcl-2*^{-/-} (*n* = 9 mice/group) mice were injected i.p. with 3 mg of either control IgG (*n* = 4 mice/genotype) or M25 (*n* = 5 mice/genotype) 1 and 4 d before sacrifice. 7 d after the initial injection, LN and spleen cells were stained with antibodies against CD4, CD8, CD62L, and CD44, data were acquired by flow cytometry, and absolute numbers of naive CD44^{lo} CD62L^{hi} phenotype T cells in each organ were determined. Results show the percentage of naive CD44^{lo} CD62L^{hi} CD4⁺ (A) and CD8⁺ (B) T cells (combined LN and spleen) remaining in anti-IL-7-treated mice relative to control mAb-treated mice. *, statistically significant difference between *Bim*^{+/+}*Bcl-2*^{+/+} and *Bim*^{+/-}*Bcl-2*^{-/-} mice (*P* ≤ 0.04); †, statistically significant difference between *Bim*^{+/-}*Bcl-2*^{-/-} and *Bim*^{-/-}*Bcl-2*^{-/-} mice (CD4, *P* ≤ 0.008; CD8, *P* ≤ 0.002); ¶, statistically significant difference between *Bim*^{-/-}*Bcl-2*^{-/-} and *Bim*^{+/+}*Bcl-2*^{+/+} mice (CD4 and CD8, *P* ≤ 0.05). (C and D) Groups of 6–9-wk-old *Bim*^{+/+}*Bcl-2*^{+/+} (*n* = 3 mice/group), *Bim*^{+/-}*Bcl-2*^{-/-} (*n* = 8 mice/group), or *Bim*^{-/-}*Bcl-2*^{-/-} (*n* = 6 mice/group) mice were either thymectomized or sham thymectomized. 4 wk after thymectomy, spleens and LN cells were stained with antibodies against CD4, CD8, and CD44, and data were acquired by flow cytometry. Data are pooled from two independent experiments. Results show the percentage of naive T cells (CD44^{lo}; combined LN and spleen) remaining in thymectomized mice relative to sham-thymectomized controls ± the SEM. Similar results were observed at 2 wk after thymectomy. *, statistically significant difference between *Bim*^{+/+}*Bcl-2*^{+/+} and *Bim*^{+/-}*Bcl-2*^{-/-} mice (*P* ≤ 0.003); †, statistically significant difference between *Bim*^{+/-}*Bcl-2*^{-/-} and *Bim*^{-/-}*Bcl-2*^{-/-} mice (*P* ≤ 0.016).

When compared with their respective sham thymectomy controls, both *Bim*^{+/+}*Bcl-2*^{+/+} and *Bim*^{+/-}*Bcl-2*^{-/-} mice had ~50% of their naive CD4⁺ T cells remaining, whereas ~75% were left in *Bim*^{-/-}*Bcl-2*^{-/-} mice (Fig. 4 C). These data suggest that molecules other than Bcl-2 may antagonize Bim in CD4⁺ T cells. Conversely, for CD8⁺ T cells, *Bim*^{+/+}*Bcl-2*^{+/+} mice had ~50% of their naive CD8⁺ T cells remaining, slightly more (~65%) naive CD8⁺ T cells remained in *Bim*^{-/-}*Bcl-2*^{-/-} mice, whereas only ~25% were left in *Bim*^{+/-}*Bcl-2*^{-/-} mice (Fig. 4 D). These data suggest that, in vivo, a major function of Bcl-2 within naive CD8⁺ T cells is to antagonize Bim and that naive CD8⁺ T cells in *Bim*^{+/-}*Bcl-2*^{-/-} mice are largely maintained by thymic output rather than peripheral survival.

Bcl-2 is not required to generate or maintain memory T cells in *Bim*^{+/-}*Bcl-2*^{-/-} mice

Although the high levels of Bcl-2 on memory CD4⁺ and CD8⁺ T cells have been suggested to contribute to their survival in vivo (9–12, 35), the contribution of Bcl-2 to memory T cell survival in vivo has never been experimentally demonstrated. Consequently, the slightly increased numbers of memory phenotype T cells in the absence of exogenous antigenic stimulation in older *Bim*^{+/-}*Bcl-2*^{-/-} mice suggested that perhaps memory T cells do not require Bcl-2 for survival. To test this, we inoculated groups of *Bim*^{+/+}*Bcl-2*^{+/+}, *Bim*^{+/-}*Bcl-2*^{-/-}, and *Bim*^{-/-}*Bcl-2*^{-/-} mice with LCMV and used MHC class I and II tetramers to enumerate endogenously responding T cells 141 d later. The total numbers of D^bgp33⁺ memory CD8⁺ T cells were similar for *Bim*^{+/+}*Bcl-2*^{+/+}, *Bim*^{+/-}*Bcl-2*^{-/-}, and *Bim*^{-/-}*Bcl-2*^{-/-} mice 141 d after inoculation (Fig. 5 A). The number of D^bnp396⁺ CD8⁺ T cells was decreased by approximately twofold in *Bim*^{+/-}*Bcl-2*^{-/-} mice and increased by approximately twofold in *Bim*^{-/-}*Bcl-2*^{-/-} mice relative to *Bim*^{+/+}*Bcl-2*^{+/+} mice (Fig. 5 A). Despite the similar normal numbers of tetramer⁺ T cells for all three mouse lines, the percentage of CD8⁺ T cells that were tetramer⁺ was significantly increased in *Bim*^{+/-}*Bcl-2*^{-/-} mice compared with control *Bim*^{+/+}*Bcl-2*^{+/+} mice for both epitopes (Fig. 5 B). Numbers and percentages of D^bnp396-specific T cells were increased in *Bim*^{-/-}*Bcl-2*^{-/-} mice relative to control *Bim*^{+/+}*Bcl-2*^{+/+} mice (Fig. 5, A and B), likely reflecting the role of Bim in limiting the numbers of effector T cells during the response (36, 37). Similarly, *Bim*^{+/-}*Bcl-2*^{-/-} mice had a two- to threefold decrease in total numbers of memory CD4⁺ IA^bgp61-specific T cells compared with *Bim*^{+/+}*Bcl-2*^{+/+} mice, whereas *Bim*^{-/-}*Bcl-2*^{-/-} mice exhibited an intermediate phenotype (Fig. 5 A, inset). Thus, compared with their substantial reductions in naive T cells, *Bim*^{+/-}*Bcl-2*^{-/-} mice maintain relatively normal numbers of LCMV-specific memory T cells.

It was possible that, in *Bim*^{+/-}*Bcl-2*^{-/-} mice, an exaggerated expansion of T cells could have contributed to their increased numbers of memory T cells. To address this, we quantified LCMV-SP T cell responses at several times after LCMV infection in both *Bim*^{+/-}*Bcl-2*^{-/-} and wt mice. For CD8⁺ T cells,

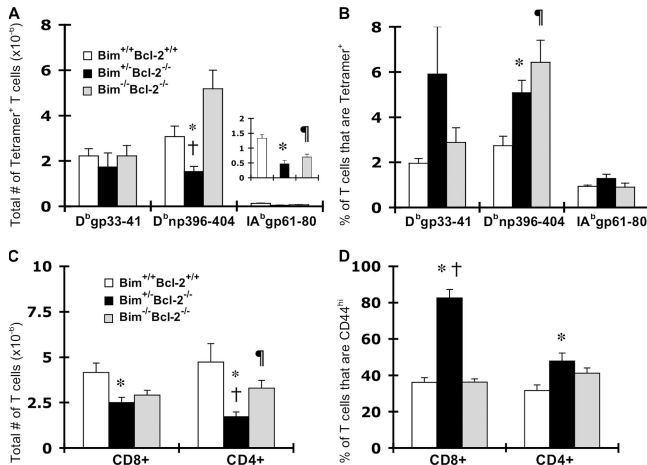


Figure 5. Bcl-2-independent generation of memory T cells. Groups of $Bim^{+/+}Bcl-2^{+/+}$, $Bim^{+/-}Bcl-2^{-/-}$, and $Bim^{-/-}Bcl-2^{-/-}$ mice ($n = 4-6$ mice/group) were injected i.p. with 2×10^5 pfu LCMV and killed 141 d later. Single spleen cell suspensions were stained with MHC tetrameric staining reagents and antibodies against CD16/32, CD62L, and either CD4 or CD8, and then analyzed by flow cytometry. Results show the numbers (A) and percentages (B) of LCMV-specific CD8⁺ (D^b gp33-41 and D^b np396-404) and CD4⁺ (I-A^bgp61-80; inset) T cells in the spleen ($10^6 \pm$ the SEM). Spleen cells were also stained with antibodies against CD4, CD8, and CD44 to assess the numbers of memory T cells across the genotypes. Results show the total numbers (C) and percentages (D) of CD44^{hi} (memory) CD8⁺ and CD4⁺ T cells \pm the SEM. *, statistically significant difference between $Bim^{+/-}Bcl-2^{-/-}$ and $Bim^{+/+}Bcl-2^{+/+}$ mice for D^b np396⁺ T cells (number and percentage of cells, $P \leq 0.02$); for total memory CD8s (number of cells, $P \leq 0.02$; percentage of cells, $P \leq 0.001$); for total memory CD4s (number of cells, $P \leq 0.02$; percentage of cells, $P \leq 0.03$). †, statistically significant difference between $Bim^{+/-}Bcl-2^{-/-}$ and $Bim^{-/-}Bcl-2^{-/-}$ mice, total number of D^b np396⁺ ($P \leq 0.02$); for total memory CD8s (percentage of cells, $P \leq 0.001$); for total memory CD4s (number of cells, $P \leq 0.03$). ¶, statistically significant difference between $Bim^{-/-}Bcl-2^{-/-}$ and $Bim^{+/+}Bcl-2^{+/+}$ mice, total number of IA^bgp61⁺ ($P \leq 0.005$); percentage of D^b np396⁺ ($P \leq 0.03$); for total memory CD8s (number of cells, $P \leq 0.05$). This experiment is representative of two independent experiments with similar results.

numbers of LCMV-SP T cells at the peak of the response was actually significantly decreased in $Bim^{+/-}Bcl-2^{-/-}$ mice compared with $Bim^{+/+}Bcl-2^{+/+}$ mice (Fig. S1, available at <http://www.jem.org/cgi/content/full/jem.20070618/DC1>). Interestingly, the overall contraction of the response was decreased in $Bim^{+/-}Bcl-2^{-/-}$ mice compared with $Bim^{+/+}Bcl-2^{+/+}$ mice, which was reflected by an increased percentage of tetramer-staining T cells amongst the CD8⁺ population in $Bim^{+/-}Bcl-2^{-/-}$ mice (Fig. S1). For IA^bgp61-SP CD4⁺ T cells, expansion and contraction of the response was minimally affected in $Bim^{+/-}Bcl-2^{-/-}$ mice (Fig. S1). Thus, the similar numbers of memory T cells in $Bim^{+/-}Bcl-2^{-/-}$ mice compared with $Bim^{+/+}Bcl-2^{+/+}$ mice was not caused by an exaggerated expansion in the former. Instead, the expansion of the response was actually less in $Bim^{+/-}Bcl-2^{-/-}$ mice.

We also examined the total numbers of all memory T cells in the 141-d-old LCMV-infected mice to determine whether

memory T cells accumulated at the expense of naive cells in $Bim^{+/-}Bcl-2^{-/-}$ mice. Total numbers of CD44^{hi} memory CD8⁺ T cells were reduced by less than twofold in both $Bim^{+/-}Bcl-2^{-/-}$ and $Bim^{-/-}Bcl-2^{-/-}$ mice (Fig. 5 C). However, as assessed on a percentage basis, memory T cells had nearly completely overtaken the spleen, as >80% of the CD8⁺ T cells expressed high levels of CD44 (Fig. 5 D). Numbers of memory CD4⁺ T cells were decreased by approximately two- to threefold in $Bim^{+/-}Bcl-2^{-/-}$ mice compared with $Bim^{+/+}Bcl-2^{+/+}$ mice and were intermediate in $Bim^{-/-}Bcl-2^{-/-}$ mice (Fig. 5 C). Percentages of activated/memory CD4⁺ T cells were also increased in $Bim^{+/-}Bcl-2^{-/-}$ mice compared with $Bim^{+/+}Bcl-2^{+/+}$ mice, although not as dramatically as CD8⁺ T cells (Fig. 5 D). Collectively, these results suggest that Bcl-2 is required less for the generation and/or maintenance of T cell memory than for the maintenance of naive T cells and is not absolutely required to combat Bim in memory T cells.

Accumulation of memory T cells in $Bim^{+/-}Bcl-2^{-/-}$ mice is caused by increased proliferation rather than increased survival or antigen persistence

It was also possible that increased viral persistence might contribute to memory T cell accumulation in $Bim^{+/-}Bcl-2^{-/-}$ mice. Thus, we assessed viral titers in the livers of mice at multiple times after inoculation. We were unable to detect virus in any mouse at any time by plaque assay (unpublished data). Further, because LCMV persistence is associated with decreased functionality of T cells (38, 39), we assessed the ability of splenic T cells from $Bim^{+/-}Bcl-2^{-/-}$ mice to produce IFN- γ after brief stimulation in vitro. Both CD4⁺ and CD8⁺ LCMV-specific T cells from $Bim^{+/-}Bcl-2^{-/-}$ mice were fully capable of producing IFN- γ and IL-2 after restimulation in vitro (unpublished data), suggesting that memory T cells in $Bim^{+/-}Bcl-2^{-/-}$ mice are functional. Thus, it is unlikely that viral persistence contributes to accumulation of memory T cells in $Bim^{+/-}Bcl-2^{-/-}$ mice.

We next examined whether memory T cell accumulation in $Bim^{+/-}Bcl-2^{-/-}$ mice was caused by increased survival or proliferation. To accomplish this, we evaluated the ability of IL-7 and -15 to promote endogenous memory T cell survival in vitro. LN cells from uninfected $Bim^{+/+}Bcl-2^{+/+}$ and $Bim^{+/-}Bcl-2^{-/-}$ mice were cultured with varying concentrations of IL-7 or -15, stained with anti-CD44 mAb to track endogenous activated/memory T cells and with Annexin V to assess apoptosis, and analyzed by flow cytometry. Although CD44^{hi} T cells from $Bim^{+/+}Bcl-2^{+/+}$ mice were efficiently rescued from death by either IL-7 or -15, survival of CD44^{hi} T cells from $Bim^{+/-}Bcl-2^{-/-}$ mice was barely affected by either cytokine (Fig. 6, A and B). These data suggest that, similar to their naive counterparts, endogenous memory T cells require Bcl-2 for cytokine-driven survival in vitro.

We next examined proliferative renewal of memory CD8⁺ T cells in $Bim^{+/+}Bcl-2^{+/+}$, $Bim^{+/-}Bcl-2^{-/-}$, and $Bim^{-/-}Bcl-2^{-/-}$ mice, using in vivo BrdU labeling. LCMV-infected mice from the experiment in Fig. 5 were injected daily with

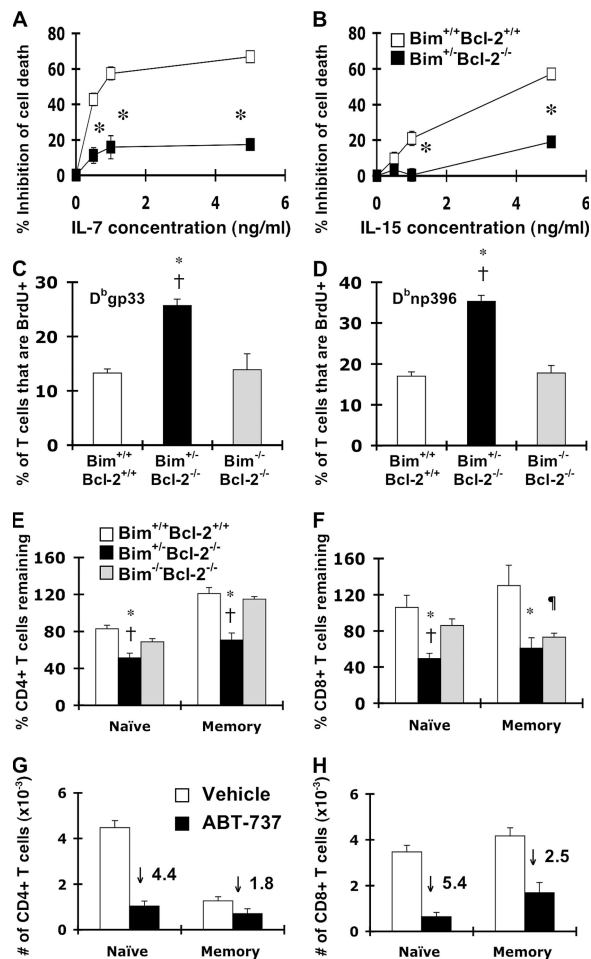


Figure 6. Accumulation of memory T cells in *Bim*^{+/-}*Bcl-2*^{-/-} mice is caused by increased proliferation rather than survival. Groups of *Bim*^{+/+}*Bcl-2*^{+/+} and *Bim*^{+/-}*Bcl-2*^{-/-} mice ($n = 3$ mice/group) were killed, and LN cells from individual mice were cultured with the indicated concentrations of IL-7 (A) or -15 (B) for 36 h at 37°C. After culture, cells were stained with antibodies against TCR- β , CD8, and CD44 to identify endogenous memory phenotype T cells and with Annexin V-FITC (R&D Systems) to identify cells that were dead, and then analyzed by flow cytometry. Results show the percentage of inhibition of cell death [(percentage of cells dead in medium - percentage of cells dead in cytokine) / percentage of cells dead in medium] $\times 100 \pm$ the SD. Cell death without cytokine was $50.9 \pm 2.5\%$ for *Bim*^{+/+}*Bcl-2*^{+/+} and $74.8 \pm 5.4\%$ for *Bim*^{+/-}*Bcl-2*^{-/-} mice. *, statistically significant difference between *Bim*^{+/+}*Bcl-2*^{+/+} and *Bim*^{+/-}*Bcl-2*^{-/-} mice for IL-7 ($P \leq 0.001$) and -15 ($P \leq 0.03$). (C and D) Groups of *Bim*^{+/+}*Bcl-2*^{+/+} ($n = 6$) and *Bim*^{+/-}*Bcl-2*^{-/-} mice ($n = 5$) were infected with LCMV. At day 126 after infection, mice were injected once daily with BrdU for 14 d. On day 141, mice were killed and BrdU accumulation within memory T cells was assessed by staining cells for CD8, CD62L, MHC tetramer, and intracellular BrdU. Results show the percentage of tetramer⁺CD62L⁺CD8⁺ T cells that are BrdU⁺ \pm the SEM. Percentages of D^bgp33⁺ (C) and D^bnp396⁺ (D) T cells that were BrdU⁺ were significantly increased in *Bim*^{+/-}*Bcl-2*^{-/-} mice compared with either *Bim*^{+/+}*Bcl-2*^{+/+} or *Bim*^{-/-}*Bcl-2*^{-/-} mice ($P \leq 0.002$ for both). (E and F) Adoptive transfer experiments. T cells were purified from LN and spleens of 2-mo-old *Bim*^{+/+}*Bcl-2*^{+/+}, *Bim*^{+/-}*Bcl-2*^{-/-}, and *Bim*^{-/-}*Bcl-2*^{-/-} mice. 2×10^6 T cells were transferred into Ly5.1 congenic recipient mice ($n = 12$ mice/group); on days 1

1 mg of BrdU during the 14 d before sacrifice. Mice were killed 141 d after LCMV inoculation, and the percentage of LCMV-specific T cells that were BrdU⁺ was assessed by intracellular immunofluorescence staining and flow cytometry. 13% of the CD62L^{hi} D^bgp33⁺ and 17% of the CD62L^{hi} D^bnp396⁺ T cells in *Bim*^{+/+}*Bcl-2*^{+/+} mice were BrdU⁺, whereas 26% of the CD62L^{hi} D^bgp33⁺ and 35% of the CD62L^{hi} D^bnp396⁺ T cells were BrdU⁺ in *Bim*^{+/-}*Bcl-2*^{-/-} mice (Fig. 6, C and D). Loss of the remaining *Bim* allele restored levels of proliferative renewal to normal in both the D^bgp33⁺ and D^bnp396⁺ memory T cell populations (Fig. 6, C and D). Thus, enhanced proliferation of LCMV-specific memory T cells in *Bim*^{+/-}*Bcl-2*^{-/-} mice also contributed to their maintenance at near normal levels.

The lymphopenic environment in *Bim*^{+/-}*Bcl-2*^{-/-} mice could have contributed to the enhanced proliferative renewal and subsequent accumulation of memory T cells in these mice. We addressed this issue in three independent experiments. First, we attempted to generate mixed BM chimeras in which lineage-depleted BM cells from either *Bim*^{+/+}*Bcl-2*^{+/+}, *Bim*^{+/-}*Bcl-2*^{-/-}, or *Bim*^{-/-}*Bcl-2*^{-/-} mice were mixed 50:50 with lineage-depleted BM from Ly5.1 congenic mice and transplanted into Ly5.1 congenic mice. This would allow us to track cells that had originated from the mutant mice based on their expression of Ly5.2. Although BM from *Bim*^{+/+}*Bcl-2*^{+/+} mice was able to establish chimerism in recipients, BM from *Bim*^{+/-}*Bcl-2*^{-/-} or *Bim*^{-/-}*Bcl-2*^{-/-} mice showed only a small degree of chimerism at 5 and 8 wk in recipient mice. (Fig. S2, available at <http://www.jem.org/cgi/content/full/jem.20070618/DC1>). Thus, under competitive conditions, *Bcl-2* is required for lymphoid reconstitution, which unfortunately precluded study of memory T cell homeostasis.

Second, we avoided complications of lymphopenia by transferring T cells from the various groups of mice into *Bim*^{+/+}*Bcl-2*^{+/+} Ly5.1 congenic recipients. As expected, significantly fewer naive CD4⁺ and CD8⁺ T cells remained in recipients of *Bim*^{+/-}*Bcl-2*^{-/-} cells compared with recipients of either *Bim*^{+/+}*Bcl-2*^{+/+} or *Bim*^{-/-}*Bcl-2*^{-/-} T cells (Fig. 6, E and F). Interestingly, similar to naive T cells, significantly fewer memory CD4⁺ and CD8⁺ T cells remained in recipients

($n = 6$ mice/group) and 10 ($n = 6$ mice/group), recipient mice were killed and donor T cells were quantified in their LNs and spleens by flow cytometry. Results show the percentage of donor naive and memory CD4⁺ (E) or CD8⁺ (F) T cells (combined LN and spleen) remaining on day 10, relative to the number present on day 1 after transfer \pm the SEM. (G and H) Purified T cells from 20-mo-old C57BL/6 mice were transferred into Ly5.1 congenic mice ($n = 8$). Recipient mice were treated with either vehicle ($n = 4$) or with ABT-737 ($n = 4$) for 10 d, and the number of donor naive and memory T cells was quantified by flow cytometry. Results show the total number of donor CD4⁺ (G) and CD8⁺ (H) naive versus memory T cells in mice treated with either vehicle (open bars) or ABT-737 (shaded bars) \pm the SEM. Numbers above the shaded bars indicate the fold decrease in cell number by ABT-737. Similar results were obtained in mice without cell transfer.

of *Bim*^{+/-}*Bcl-2*^{-/-} cells compared with recipients of *Bim*^{+/+}*Bcl-2*^{+/+} T cells (Fig. 6, E and F). Loss of the remaining allele of *Bim* increased the recovery of memory CD4⁺, but not CD8⁺ T cells (Fig. 6, E and F).

Third, to avoid complications of memory T cells arising in the various genetic backgrounds and potential minor antigenic differences in the KO mice that could influence the adoptive transfer experiments, we compared the in vivo effects of ABT-737 on naive versus memory T cells. Purified T cells from C57BL/6 mice were transferred into recipient mice, which were treated with either vehicle or with ABT-737 for 10 d, and then killed. The numbers of donor naive (CD44^{lo}) and memory (CD44^{hi}) T cells were quantified. Similar to our previous data, ABT-737 caused a substantial reduction in naive CD4⁺ and CD8⁺ T cells (Fig. 6, G and H). ABT-737 also decreased the numbers of transferred memory CD4⁺ and CD8⁺ T cells, but to a lesser extent than what we observed for naive T cells (Fig. 6, G and H). Collectively, these data suggest that naive T cells and, to a somewhat lesser extent, memory T cells depend on *Bcl-2* to combat *Bim* to maintain survival.

DISCUSSION

Our data demonstrate that *Bcl-2* is required to maintain naive T cell survival in vivo and does this largely by inhibiting the proapoptotic effects of *Bim*. Although *Bcl-2* is important for survival of T cell precursors (40), our studies suggest that the defects in naive T cell survival in *Bcl-2*-deficient mice are largely intrinsic to mature peripheral T cells and reflect unopposed *Bim* activity. First, naive peripheral T cells from *Bim*^{+/-}*Bcl-2*^{-/-} mice die readily in vitro, and this death is prevented by loss of the remaining *Bim* allele. Second, *Bim*^{+/-}*Bcl-2*^{-/-} mice have nearly normal numbers of thymocytes, and thymectomy results in greater loss of naive CD8⁺ T cells in *Bim*^{+/-}*Bcl-2*^{-/-} mice than in *Bim*^{+/+}*Bcl-2*^{+/+} mice. Both observations are more compatible with a defect in peripheral survival than a defect in thymic output. On the contrary, these data suggest that, in *Bim*^{+/-}*Bcl-2*^{-/-} mice, CD8⁺ T cells are maintained by continual thymic output rather than peripheral survival. A specific role for *Bcl-2* in suppressing *Bim*-induced apoptosis is supported by previous evidence that genetic loss of other proapoptotic *Bcl-2* family members, such as *Bax* or *Bik*, does not prevent the lethality or defects in peripheral T cell homeostasis observed in *Bcl-2*^{-/-} mice (41, 42). For example, the absence of *Bax* can mostly correct the loss of thymocytes in *Bcl-2*^{-/-} mice and IL-7R^{-/-} mice, but does not restore peripheral T cell numbers (43). Together with our observations, these data suggest that peripheral naive T cell survival requires suppression of *Bim* function either directly by IL-7 or by IL-7-driven induction of *Bcl-2*. This observation for peripheral naive T cells is consistent with previous observations that *Bim* drives the death of activated T cells (36) and that *Bim* and *Bcl-2* are bound to one another in resting T cells (44). *Bim* that is not complexed to *Bcl-2* would be free to promote apoptosis by activating *Bax* and/or *Bak*.

Although antagonism between *Bim* and *Bcl-2* is crucial for the regulation of T cell homeostasis, other *Bcl-2* family members also play roles. The following four observations indicate the importance of additional proapoptotic family members: a) T cell homeostasis is more severely disturbed in *Bax*/*Bak* double-deficient mice than in *Bim*^{-/-} mice; b) although naive *Bim*^{-/-}*Bcl-2*^{-/-} T cells die slower than *Bim*^{+/-}*Bcl-2*^{-/-} T cells in vitro, they still die quite readily; c) total loss of *Bim* (in *Bcl-2*^{-/-} mice) does not completely restore numbers of naive or memory T cells to normal; and d) *Bim*^{-/-}*Bcl-2*^{-/-} mice have significantly increased numbers of SP thymocytes, but normal or slightly decreased numbers of peripheral T cells, suggesting that other proapoptotic *Bcl-2* family members can limit the size of the naive T cell compartment. Our preliminary data indicate that *Bad* and *Noxa* mRNA levels appear to be increased in naive *Bim*^{-/-}*Bcl-2*^{-/-} T cells. It is possible that, in the absence of *Bcl-2*, either *Bad* or *Noxa* can limit peripheral naive T cell survival.

Our data showing the IL-7 dependence of some *Bim*^{+/-}*Bcl-2*^{-/-}, but not *Bim*^{-/-}*Bcl-2*^{-/-}, naive T cells suggests that IL-7 may have direct or indirect effects on combating *Bim*. One potential candidate for an indirect effect may be via IL-7's effects on *Mcl-1*, which also appears to be a critical mediator of naive T cell homeostasis (45). Deletion of *Mcl-1* results in substantial loss of both thymocytes and peripheral T cells. Physical interactions between *Mcl-1* and *Bak*, analogous to those between *Bcl-2* and *Bim*, prevent *Bak* homodimerization, mitochondrial pore formation, and spontaneous induction of apoptosis (18) that may bypass *Bim*. Alternatively, in the absence of *Bcl-2*, *Mcl-1* may interfere with *Bim* directly. Although *Bcl-xL*, which is up-regulated in activated T cells (46–49), may be another potential candidate molecule, recent work has shown that *Bcl-xL* is not normally required for memory T cell survival (50). Further, previous studies have shown that enforced expression of a human *Bcl-2* or *Bcl-xL* transgene were not sufficient to enhance survival of memory T cells after LCMV infection (51, 52). However, we have observed that, in the h*Bcl-2* transgenic mice used by Razvi et al. (51), the levels of endogenous *Bcl-2* are significantly decreased (unpublished data). Thus, potential effects of transgenes on endogenous molecules could have complicated the interpretation of these data.

In contrast to its crucial role in maintaining the survival of peripheral naive T cells, *Bcl-2* did not appear to be strictly required for the generation or maintenance of memory T cells in *Bim*^{+/-}*Bcl-2*^{-/-} mice. However, three key pieces of data argue for a prosurvival role for *Bcl-2* in memory T cells under normal conditions. First, IL-7 and -15 were unable to rescue the in vitro apoptosis of *Bim*^{+/-}*Bcl-2*^{-/-} memory T cells. Second, adoptive transfer studies showed that, similar to naive T cells, memory T cells from *Bim*^{+/-}*Bcl-2*^{-/-} mice were quickly lost when forced to compete with wt T cells in a lymphoreplete environment. Third, *Bcl-2* inhibition studies with ABT-737 in wt T cells show that although memory T cells are more resistant to the effects of ABT-737, they are still decreased by the drug. The apparent discrepancy between

the *in vivo* accumulation of memory T cells in *Bim*^{+/-}*Bcl-2*^{-/-} mice and their rapid loss in normal recipient mice is resolved by the increased proliferation of the LCMV-SP memory T cells in lymphopenic *Bim*^{+/-}*Bcl-2*^{-/-} mice. In addition, the increased proliferation of LCMV-SP T cells likely contributed to the lessening of the contraction of the T cell response in *Bim*^{+/-}*Bcl-2*^{-/-} mice. Thus, although memory T cells appear to be slightly less affected by the loss of *Bcl-2* than naive T cells, under normal conditions, *Bcl-2* is critical to maintain both memory and naive T cell homeostasis. In summary, we have investigated for the first time the involvement of *Bcl-2* in regulating survival of naive and memory T cell populations and have shown that *Bcl-2* promotes T cell survival by antagonizing *Bim*.

MATERIALS AND METHODS

Mice. *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 *Bcl2*^{Tm1sk} 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. Mice were genotyped by PCR of tail DNA (36). B6.SJL-Ptprc^a/BoAiTac (Ly5.1 congenic) mice used as recipients in adoptive transfer experiments were purchased from Taconic Farms or were bred in-house. All animal protocols were reviewed and approved by our Institutional Animal Care and Use Committee.

IL-7 depletion. M25 antibody was purified from ascitic fluid harvested from nude mice that had been injected with M25 hybridoma cells. Antibody was purified from ascitic fluid by precipitation with ammonium sulfate, followed by DEAE-cellulose column chromatography. Fractions containing mouse IgG2b were pooled, dialyzed against 0.15 M NaCl, and concentrated by pressure filtration. Mice were injected intraperitoneally every other day with 3 mg of M25 antibody or with MPC-11 isotype control.

Adult thymectomy. 6–9-wk-old mice were anesthetized with 200 mg/kg avertin and given 0.03 mg/kg atropine *i.p.* to reduce bradycardia. Under anesthesia, mice were immobilized on a surgical tray ventral side up. Using sterile technique, a small incision was made through the skin of the neck extending down to the middle of the ribcage with a sterile scalpel. The submandibular glands were pushed aside to expose the thin layer of tissue, which was cut to expose the thoracic cavity. A second midline incision was made to the level of the second rib. One side of the ribcage was lifted with a sterile forceps, and a suction bell constructed from a glass Pasteur pipette was inserted into the thoracic cavity. In sham-thymectomized mice, the suction bell was then removed from the thoracic cavity and the opening was sutured with surgical staples. In thymectomized mice, suction was applied to draw one lobe of thymus into the bell; this was repeated for the other thymic lobe. After removal of both thymic lobes, the skin was sutured with sterile surgical staples. Mice were then placed under a heat lamp and monitored until they awoke from anesthesia.

Adoptive transfer experiments. LN and spleen cells from 2-mo-old mice were purified by negative selection using a Pan T cell isolation kit and an autoMACS cell separator (Miltenyi Biotech). Purity of T cells using this technique was >95%. Approximately 2 × 10⁶ purified T cells were labeled *in vitro* with 5-(and-6)-carboxyfluorescein diacetate and 0.5 μM CFSE and transferred into Ly5.1 recipient mice *in vivo* injection. On days 1 and 10 after transfer, groups of recipient mice were killed and LNs (axillary, brachial, inguinal, and mesenteric) and spleens were removed and stained with antibodies against CD4 or CD8, Ly5.1, and CD44. Data were acquired on a FACSCalibur (BD Biosciences) flow cytometer and analyzed using CellQuest software (BD Biosciences). For

analysis, cells were gated on Ly5.1⁺CFSE⁺ (0–2 divisions) before analysis of CD44 expression.

Competitive BM reconstitution experiments. BM was harvested from the femurs of *Bim*^{+/+}*Bcl-2*^{+/+}, *Bim*^{+/+}*Bcl-2*^{-/-}, *Bim*^{-/-}*Bcl-2*^{-/-}, and B6.SJL-Ptprc^a/BoAiTac mice. Lineage-negative BM cells were purified using a lineage cell depletion kit and an AutoMacs Separator (Miltenyi Biotech). Lineage-depleted BM cells from either *Bim*^{+/+}*Bcl-2*^{+/+}, *Bim*^{+/+}*Bcl-2*^{-/-}, or *Bim*^{-/-}*Bcl-2*^{-/-} mice were mixed in a 50:50 ratio with lineage-depleted BM cells from B6.SJL-Ptprc^a/BoAiTac mice and 10⁶ cells were injected *i.v.* into lethally irradiated (2 × 500 rads) recipient B6.SJL-Ptprc^a/BoAiTac mice. At 5 and 8 wk after transfer, mice were bled and chimerism was tested by staining the cells with antibodies against CD4, CD8, Ly5.2, and Ly5.1.

Viruses. The Armstrong-3 strain of LCMV, a gift from R. Ahmed (Emory University, Atlanta, GA), was grown in BHK-21 cells; the number of plaque-forming units (pfu) was assayed on Vero cells as previously described (53). Mice were injected *i.p.* with 0.25 ml of 2 × 10⁵ pfu LCMV diluted in balanced salt solution.

Creation of MHC tetrameric staining reagents. Class II MHC tetrameric staining reagents were created as previously described (54, 55). In brief, recombinant baculoviral plasmids encoding I-A^b plus genetically linked peptide were transfected into SF-9 insect cells to produce baculoviral stocks. Baculoviral SF-9 supernatant was harvested and used to infect Hi-5 insect cells (Invitrogen). After infection, Hi-5 cells produced and secreted assembled monomeric I-A^b peptide complexes. Hi-5 cell supernatants were harvested and monomers were purified by affinity chromatography using anti-I-A^b antibody (M5-114) and eluted with a high pH buffer. After purification of monomeric MHC-peptide complexes, the complexes were buffer-exchanged into biotinylation buffer and biotinylated with the recombinant enzyme BirA (Avidity, Inc.). After biotinylation, unbound biotin was removed and buffer-exchanged into PBS using Centricon concentrators. Biotinylated monomers were coupled to streptavidin-phycoerythrin and subsequently subjected to size exclusion fast protein liquid chromatography on Superdex 200 (GE Healthcare) to separate large multimeric complexes from excess free monomeric complexes.

MHC I tetramers were provided by A. Zajac (University of Alabama, Birmingham, AL). The methodology for preparation of MHC class I tetramers was modified from the protocol described by Altman and coworkers (56). Recombinant MHC class I molecules fused to a BirA substrate peptide, and recombinant β₂-microglobulin were produced in *Escherichia coli* BL21 (DE3) cells as previously described (57). MHC-peptide complexes were generated by folding class I heavy chains *in vitro* with β₂-microglobulin and defined LCMV-derived peptide epitopes and enzymatically biotinylated with recombinant BirA enzyme. Biotinylated MHC monomers were purified over a Sepharose-G75 column (GE Healthcare), and fractions containing biotinylated class I-β₂-microglobulin-peptide complexes were pooled and exchanged into 20 mM Tris, pH 8.0, using centrifugal filtration devices (Millipore). The complexes were further purified by ion exchange chromatography using a QM-Sepharose column (GE Healthcare). Appropriate fractions were concentrated and dialyzed against PBS containing 2 mM EDTA, 1 μg/ml leupeptin, 1 μM pepstatin, and 200 μM PMSF (Sigma-Aldrich). The complexes were tetramerized by the step-wise addition of allophycocyanin-conjugated streptavidin (Invitrogen).

Flow cytometry. Spleens, LNs (axillary, inguinal, and brachial), and thymuses were harvested, and single-cell suspensions were counted with a Coulter Counter (model Z2; Beckman Coulter). 10⁶ cells were stained with fluorochrome-labeled antibodies (spleen and LNs [anti-CD4, -CD8, -CD44, and -CD62L]; thymus [anti-CD4, -CD8, -CD44, -CD25]), and data was acquired using a FACSCalibur flow cytometer. Data were analyzed with CellQuest software. To detect antigen-specific CD4⁺ T cells, 2 × 10⁶ LN or spleen cells/well was stained in triplicate with I-A^bgp61-80 tetrameric staining reagents for 2 h at 37°C. During the last 45 min of incubation, cells

were stained with anti-CD4-allophycocyanin, anti-CD16/32 biotin, and anti-CD44-FITC antibodies. Cells were washed twice, stained with SA-Cyochrome, washed twice again, and fixed with 2% paraformaldehyde. CD4⁺, CD16/32⁻ cells were gated and analyzed by dot plots that displayed CD44 versus tetramer to reveal small populations of LCMV-SP T cells. LCMV-SP. CD8⁺ T cells were detected by staining 2×10^6 LN or spleen cells/well in triplicate with D^bgp33, D^bnp396, or K^bnp205 tetrameric staining reagents for 90 min at 4°C. During the last 45 min of incubation, cells were stained with antibodies directed against various surface markers (CD8, CD44, CD62L, and CD127 from BD Biosciences, eBioscience, or produced in-house), washed, and fixed with 2% paraformaldehyde. Gates were drawn around tetramer⁺ populations based on background staining of uninfected animals (generally <0.05% for class II tetramer staining and <0.1% for class I tetramer staining). A minimum of 5×10^5 events were acquired on a FACSCalibur flow cytometer and analyzed using CellQuest software.

To assess naive T cell viability, spleen or LN cells were cultured in vitro in Kappler-Marrack complete tumor medium with or without the indicated concentrations of survival cytokines (IL-4, -6, -7, and -15, all purchased from R&D Systems) as previously described (31, 58). At the indicated times after culture, cells were stained with antibodies against TCR-β, CD4 or CD8, and propidium iodide (5 μg/ml), and cell viability was assessed by flow cytometry. To assess viability of memory versus naive T cells, cells were stained with antibodies against CD4, CD8, CD44, and AnnexinV-FITC (R&D Systems) and analyzed by flow cytometry.

Assessment of in vivo proliferation was performed using a BrdU Flow kit (BD Biosciences). In brief, mice were inoculated i.p. with 2×10^5 pfu LCMV and injected i.p. with 1 mg of BrdU daily for 14 d, beginning 127 d after inoculation. Mice were killed 141 d after inoculation, and single-spleen cell suspensions were surface stained with antibodies against CD8 and CD62L and MHC tetramers, followed by intracellular staining with anti-BrdU antibody according to the instructions in the kit. Stained cells were analyzed with a FACSCalibur flow cytometer and CellQuest software.

Intracellular cytokine staining was performed as previously described (57). In brief, spleen cells from LCMV-infected or -uninfected mice were cultured at 37°C for 4–5 h with or without various LCMV peptides (10 μg/ml GP61-80; 1 μg/ml GP33-41, NP396-404) and 10 μg/ml Brefeldin A. After culture, cells were stained for cell surface markers (CD4, CD8, CD44, and antibodies from BD Biosciences) for 45 min at 4°C. Cells were washed, fixed, and permeabilized with 0.03% saponin and stained intracellularly with PE-labeled anti-IFN-γ or anti-IL-2 antibody (BD Biosciences). A minimum of 5×10^5 events were acquired on a FACSCalibur flow cytometer and analyzed using CellQuest software.

Synthetic Bcl-2 inhibitor. ABT-737 was produced and provided by Abbott Laboratories, as previously described (28), and dissolved and diluted in 30% polyethylene glycol, 5% Tween-80, and 65% of a 5% dextrose in water solution. Mice were injected i.p. once a day with 75 mg/kg in a volume of 0.2 ml.

Statistical analyses. Statistical analyses were performed using a 2-sample Student's *t* test with Minitab for Windows Software (Release 14).

Online supplemental materials. Fig. S1 shows the kinetics of the LCMV-specific CD4⁺ and CD8⁺ T cell responses in *Bim*^{+/-}*Bcl-2*^{-/-} compared with *Bim*^{+/+}*Bcl-2*^{+/+} mice. Fig. S2 shows that Bcl-2 is critical to combat proapoptotic molecules other than Bim to allow BM reconstitution when done in a competitive manner. The online version of this article is available at <http://www.jem.org/cgi/content/full/jem.20070618/DC1>.

The authors thank Drs. Thomas Mitchell and Claire Chougnat for critical reading of the manuscript. The authors thank Abbott Laboratories for the generous gift of ABT-737. The authors also thank Dr. Alan Zajac for the class I MHC tetrameric staining reagents.

This work was supported by Public Health Service grants AI057753 (to D. Hildeman), CA105152 (to H.L. Grimes), and AI55848 (to F.D. Finkelman), and a Veteran's Association Merit award (to F.D. Finkelman).

The authors have no conflicting financial interests.

Submitted: 27 March 2007

Accepted: 4 June 2007

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