Combined loss of proapoptotic genes Bak or Bax with Bim synergizes to cause defects in hematopoiesis and in thymocyte apoptosis

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The proapoptotic members of the Bcl–2 family can be subdivided into members that contain several Bcl–2 homology (BH) domains and those that contain only the BH3 domain. Although it is known that BH3–only proteins and the multi–BH domain proteins, Bak and Bax, are essential for programmed cell death, the overlapping role of these two subgroups has not been examined in vivo. To investigate this, we generated Bak/Bim and Bax/Bim double deficient mice. We found that although $Bax^{-/-}Bim^{-/-}$, but not $Bak^{-/-}Bim^{-/-}$, mice display webbed hind and front paws and malocclusion of the incisors, both groups of mice present with dysregulated hematopoiesis. Combined loss of Bak and Bim or Bax and Bim causes defects in myeloid and B–lymphoid development that are more severe than those found in the single knock-out mice. $Bak^{-/-}Bim^{-/-}$ mice have a complement of thymocytes that resembles those in control mice, whereas $Bax^{-/-}Bim^{-/-}$ mice are more similar to $Bim^{-/-}$ mice. However, thymocytes isolated from $Bak^{-/-}Bim^{-/-}$ or $Bax^{-/-}Bim^{-/-}$ mice are markedly more resistant to apoptotic stimuli mediated by the intrinsic pathway as compared with thymocytes from single-knockout mice. These data suggest an essential overlapping role for Bak or Bax and Bim in the intrinsic apoptotic pathway.

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Abbreviations: 7-AAD, 7-amino–actinomycin D; BH, Bcl-2 homology; DKO, double KO; ES, embryonic stem.

Apoptosis in mammals proceeds through two distinct pathways, an "extrinsic" pathway, which transduces an apoptotic signal after the ligation of death receptors on the cell surface, and an "intrinsic" pathway, in which mitochondria play a critical role (1). The intrinsic pathway is regulated by the Bcl-2 protein family, which are divided into antiapoptotic (Bcl-2, Bcl-x_L, Mcl-1, A1/Bfl-1, Bcl-w) and proapoptotic (Bax, Bak, Bok/Mtd, Bcl-G, Bfk, Bad, Bim/Bod, Bik/Blk/Nbk, Bid, Hrk/DP5, Bmf, Noxa, Puma/Bbc3) members (2). Bcl-2–related proteins contain Bcl-2 homology (BH 1–4) domains, which are critical for homodimer and heterodimer formation between the family members (3). Although the antiapoptotic Bcl-2–like proteins contain all four (or in the case of Mcl-1 at least two) BH domains, the proapoptotic Bcl-2–related proteins can be subdivided into two categories: the multi-BH domain (BH1-3: e.g., Bax, Bak, Bok) and the BH3-only proteins (e.g., Bad, Bim; reference 4). Many Bcl-2 family members are localized to the mitochondrial outer membrane (and certain other intracellular membranes), suggesting that mitochondrial dys-

function is involved in apoptosis (2, 5, 6). During intrinsic apoptosis signaling, the integrity of the outer mitochondrial membrane is lost, leading to the dissipation of the transmembrane potential through the opening of mitochondrial permeability transition pores (6), and release of apoptogenic mitochondrial intermembrane proteins, such as cytochrome c (7, 8). In the cytoplasm, cytochrome c binds to the adaptor protein APAF-1 which causes aggregation and activation of the initiator caspase-9. Caspase-9 activates the effector caspases -3, -6 and -7 (9), which cause the downstream degradative events in apoptosis (10). Apoptosis signaling through the intrinsic pathway can be inhibited by overexpression of any of the Bcl-2–like prosurvival family members (11–13) or by loss of both proapoptotic multi-BH domain proteins, Bax and Bak (14–17).

Loss of any one of the antiapoptotic Bcl-2 family members, Bcl-2, Bcl-x_L, Mcl-1, or Bcl-w, causes severe developmental abnormalities (18, 19). In contrast, mice lacking single proapoptotic multi-BH domain proteins (Bax or Bak) survive to birth and reach adulthood (4). Bax-

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deficient mice appear normal but have increased numbers of sympathetic and motor neurons and developmental arrest in spermatogenesis (20), whereas no developmental or apoptotic defects have been reported in Bak$^{-/-}$ mice (17). However, the combined loss of Bax and Bak has dramatic consequences; most animals die in utero and the few mice that survive to birth display overt developmental apoptotic defects, and isolated cells from these mice are resistant to most, perhaps all, intrinsic, and in certain circumstances also some extrinsic, apoptotic stimuli (14–16, 21–25).

The proapoptotic BH3-only Bcl-2 family members are essential for the initiation of intrinsic apoptosis signaling (26, 27). In contrast to the multi–BH domain proteins, Bax and Bak, the BH3-only proteins display a restricted pattern of expression and stimulus-specific activation; thus, mice lacking a single BH3-only protein often show cell type– and apoptotic stimulus-specific defects in the intrinsic apoptotic pathway (26). The BH3-only protein, Bim, displays an expression pattern that overlaps at least partially with Bak or with Bax (28–30). Bim-deficient mice have defects in deletion of autoreactive thymocytes and B cells (31–33) and the shutdown of T lymphocyte immune responses (34, 35). Cultured Bim-deficient lymphocytes and granulocytes are resistant to apoptosis induced by growth factor deprivation or by treatment with the calcium ionophore, ionomycin (31–33, 36–38). The developmental defects that occur in Bcl-2–deficient mice are prevented by concomitant loss of Bim; even loss of a single allele abrogates fatal polycystic kidney disease (39). The rescue of the defects caused by Bcl-2 deficiency is unique to Bim, because deletion of Bik/Blk/Nbk, another BH3-only protein expressed in the kidney and hemopoietic cells, has no effect (40). Moreover, loss of Bax is insufficient to prevent polycystic kidney disease or melanocyte death that occurs in the Bcl-2$^{-/-}$ mice, and has only a minor effect on the apoptotic defects in Bcl-2$^{-/-}$ lymphocytes (41).

To examine the possible functional overlap between Bak and Bim or Bax and Bim, we generated mice that lack Bak plus Bim or Bax plus Bim. Bax/Bim, but not Bak/Bim, double KO (DKO) mice displayed webbed feet in hind and front paws, indicative of a defect in this programmed cell death during development. Additionally, the Bax/Bim DKO mice on a mixed background, but not on a pure C57BL/6 background, were runted and had malocclusions of the incisors that ultimately caused premature death. The hematopoietic system in Bak/Bim DKO or Bax/Bim DKO mice was dysregulated substantially, and showed an abnormal increase in the number of circulating leukocytes in peripheral blood and other defects in primary and peripheral lymphoid tissues. Moreover, thymocytes isolated from Bak/Bim DKO or Bax/Bim DKO mice were resistant to apoptosis induced by cytokine deprivation or treatment with etoposide. These data suggest that the defects in Bak/Bim DKO or Bax/Bim DKO mice are mediated by a resistance to intrinsic apoptotic stimuli.

RESULTS

Bax/Bim DKO mice display a defect in deletion of interdigital tissue

To examine the overlapping actions of Bak and Bim or Bax and Bim we intercrossed Bim$^{-/-}$ mice with Bak$^{-/-}$ or Bax$^{-/-}$ mice to yield progeny that lack Bak and Bim or Bax and Bim. Bak/Bim and Bax/Bim DKO mice were born at the expected Mendelian ratios from intercrosses of Bak$^{+/-}$Bim$^{+/-}$ or Bax$^{+/-}$Bim$^{+/-}$ mice (unpublished data). Similar to Bak/Bax DKO mice (17), Bax/Bim DKO mice were born with webbed hind and front paws (Fig. 1 A), indicative of a failure to delete the interdigital tissue between the toes. Bax$^{-/-}$, Bim$^{-/-}$, Bak$^{-/-}$, Bax$^{-/-}$Bim$^{+/-}$, Bax$^{+/-}$Bim$^{-/-}$, and Bak/Bim DKO mice did not exhibit such defects. Although Bax/Bim DKO mice kept on a C57BL/6 background (both strains backcrossed to C57BL/6 for >12 generations before inter-crossing) and Bax/Bim DKO with some contribution from the 129Sv background (Bax mutant mice backcrossed to C57BL/6 for only 6 generations before intercrossing with the Bim$^{-/-}$ mice) displayed defects in the deletion of the interdigital tissue, only Bax/Bim DKO mice with more 129Sv contribution exhibited a runted phenotype (Fig. 1 B) and malocclusions of the incisors (Fig. 1 C), and failed to survive past 28 d. The Bax/Bim DKO mice with significant 129Sv background contribution were able to grow normally and reach adulthood when their upper incisors were trimmed on a regular basis and they were fed powdered food. These data suggest that genes from the 129Sv background exacerbate the Bax/Bim DKO phenotype. Histologic analysis of nonhematopoietic tissues revealed that similar to Bax–deficient mice (20), the Bax/Bim DKO males were infertile. Thus, Bax and Bim, but not Bak and Bim, act synergistically to induce apoptosis in the interdigitating tissue and to ensure proper incisor development.
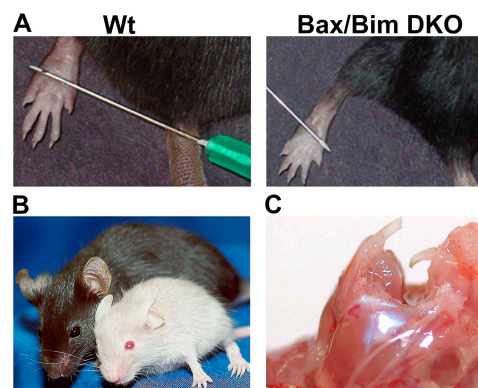

Figure 1.   Bax/Bim DKO mice display developmental defects.
(A) Photograph showing webbed feet in a Bax/Bim DKO (C57BL/6 background) mouse which is not seen in a WT mouse. (B) Photograph of Bax/Bim DKO runted mouse (white on a mixed C57BL/6/129sv background) compared with age- and sex-matched Bax$^{+/+}$Bim$^{-/-}$ control (black) mouse. (C) Photograph of Bax/Bim DKO mouse (white on a mixed C57BL/6/129sv background) showing deformed incisors.

Table I. Counts of bone marrow cells, peripheral blood leukocytes and platelets, splenocytes, and thymocytes

	WT	Bak$^{-/-}$	Bax$^{-/-}$	Bim$^{-/-}$	Bak/Bim DKO	Bax/Bim DKO
BMC, 1 × E7	1.4 ± 0.1	0.9 ± 0.1	1.2 ± 0.2	0.9 ± 0.1[a]	1.3 ± 0.1[b]	1.7 ± 0.2[b]
WBC, 1 × E7/ml	0.9 ± 0.04	0.9 ± 0.2	1.0 ± 0.1	2.4 ± 0.2[a]	3.2 ± 0.3[a,b]	3.5 ± 0.6[a]
Platelets, 1 × E8/ml	7.4 ± 0.3	12.4 ± 0.8[a]	8.7 ± 0.5[a]	5.4 ± 0.3[a]	7.6 ± 0.7[b]	7.6 ± 0.5[b]
RBC, 1 × E6/ml	9.7 ± 0.1	10.1 ± 0.2	9.7 ± 0.2	9.1 ± 0.1[a]	9.8 ± 0.2	10.2 ± 0.2
Splenocytes, 1 × E7	7.1 ± 0.9	9.0 ± 0.7	8.0 ± 0.9	12.9 ± 1.1[a]	12.2 ± 1.5[a]	11.7 ± 2.0[a]
Thymocytes, 1 × E7	7.1 ± 1.5	11.9 ± 1.4[a]	9.5 ± 1.2[a]	4.3 ± 1.0	8.6 ± 1.5[b]	5.6 ± 0.4

Quantitative analysis of total hematopoietic cellularity. Cell counts represent viable cells as determined by trypan blue exclusion. White blood cell (WBC), platelet, and red blood cell counts were obtained by an automated counter. All data are expressed as mean ± SE of at least eight mice/genotype, which were compared by Student's *t* test.
[a]$P < 0.05$ as compared with WT mice.
[b]$P < 0.05$ as compared with Bim$^{-/-}$ mice.
BMC, bone marrow cells.

Dysregulation of hematopoiesis in Bak/Bim DKO and Bax/Bim DKO mice

Although Bax and Bim are indispensable for the apoptosis of interdigital tissue, but not for other developmentally programmed cell deaths during mouse embryogenesis, the overlapping role of Bak or Bax and Bim during hematopoiesis is unknown. The total number of bone marrow cells in Bak/Bim or Bax/Bim DKO mice was comparable to that found in WT, Bax$^{-/-}$, or Bak$^{-/-}$ mice (Table I). Bim$^{-/-}$ mice displayed a 31% ($P < 0.05$), 47% ($P < 0.001$), and 36% ($P < 0.02$) decrease in bone marrow cells as compared with Bak/Bim DKO, Bax/Bim DKO, and WT mice, respectively (Table I). The decrease in bone marrow cells in Bim$^{-/-}$ mice may be related to the observed osteosclerosis (unpublished data; reference 42). In contrast to bone marrow, Bak/Bim DKO mice and Bax/Bim DKO mice exhibited a 3.7-fold ($P < 0.001$) and 3.9-fold increase ($P < 0.001$), respectively, in peripheral blood leukocytes as compared with WT mice (Table I). Bak/Bim DKO ($P < 0.05$) and Bax/Bim DKO ($P < 0.09$) mice had an elevated count of peripheral blood leukocytes as compared with Bim$^{-/-}$ mice. In support of a previous study (36), we observed that Bim$^{-/-}$ mice displayed a 2.7-fold increase ($P < 0.0001$) in peripheral blood leukocytes and a marked decrease in platelets compared with WT mice.

Bak/Bim DKO or Bax/Bim DKO mice had an elevated platelet number that was similar to WT mice (Table I) as compared with Bim$^{-/-}$ mice. There was a 36, 41, and 46% increase in total splenocytes in Bak/Bim DKO, Bax/Bim DKO, and Bim$^{-/-}$ mice, respectively, compared with WT mice (Table I). These data indicate that the development of splenomegaly occurs early in Bak/Bim DKO, Bax/Bim DKO, and Bim$^{-/-}$ mice. In contrast to spleen, the Bak/Bim DKO and Bax/Bim DKO mice had similar numbers of thymocytes as compared with WT mice. However, the Bak/Bim DKO mice had a twofold increase ($P < 0.05$) in the number of thymocytes as compared with Bim$^{-/-}$ mice. Similar to a previous report (36), Bim$^{-/-}$ mice showed a 30% decrease in thymocyte numbers as compared with WT mice. These data document that combined defects in Bak or Bax and Bim cause an overall dysregulation in hematopoietic development that is more severe than the defects that are observed in Bak$^{-/-}$, Bax$^{-/-}$, and Bim$^{-/-}$ mice.

Bak/Bim DKO and Bax/Bim DKO mice have abnormally decreased numbers of bone marrow myeloid precursors but markedly increased numbers of mature myeloid cells in the periphery

Because we observed defects in hematopoietic development in Bak/Bim DKO and Bax/Bim DKO mice that seemed to

Table II. Phenotypical analysis of peripheral blood leukocytes

1 × E5/ml	WT	Bak$^{-/-}$	Bax$^{-/-}$	Bim$^{-/-}$	Bak/Bim DKO	Bax/Bim DKO
	$n \geq 24$	$n = 13$	$n \geq 15$	$n = 25$	$n = 8$	$n = 12$
CD19$^+$	39.0 ± 1.5	36.7 ± 3.9	44.8 ± 4.3	112.5 ± 11.4[a]	177.0 ± 22.8[a,b]	199.4 ± 28.2[a,b]
CD3$^+$	20.3 ± 0.9	19.8 ± 1.4	22.5 ± 1.7	58.6 ± 4.1[a]	74.7 ± 9.7[a]	67.5 ± 10.4[a]
CD3$^+$CD4$^+$	12.1 ± 0.6	11.4 ± 0.8	14.3 ± 1.0	23.9 ± 1.8[a]	31.3 ± 3.8[a]	25.4 ± 3.5[a]
CD3$^+$CD8$^+$	7.2 ± 0.4	7.7 ± 0.6	7.0 ± 0.6	25.2 ± 1.7[a]	28.7 ± 4.0[a]	26.2 ± 3.7[a]
CD11b$^+$Gr-1$^-$CD62L$^-$	3.0 ± 0.2	2.1 ± 0.2[a]	5.5 ± 0.7[a]	8.0 ± 0.8[a]	10.0 ± 1.2[a]	16.0 ± 3.5[a,b]
CD11b$^+$Gr-1$^+$CD62L$^+$	2.4 ± 0.2	1.6 ± 0.2[a]	2.3 ± 0.2	4.1 ± 0.4[a]	6.7 ± 1.2[a]	6.3 ± 1.0[a]
CD11b$^+$Gr-1$^+$	5.4 ± 0.6	3.7 ± 0.6	4.5 ± 0.5	9.8 ± 1.6[a]	13.2 ± 3.8	11.4 ± 1.8[a]

Quantitative analysis of individual subsets of leukocytes in peripheral blood as determined by flow cytometry. All data are expressed as means ± SE, which were compared by Student's *t* test.
[a]$P < 0.05$ as compared with WT mice.
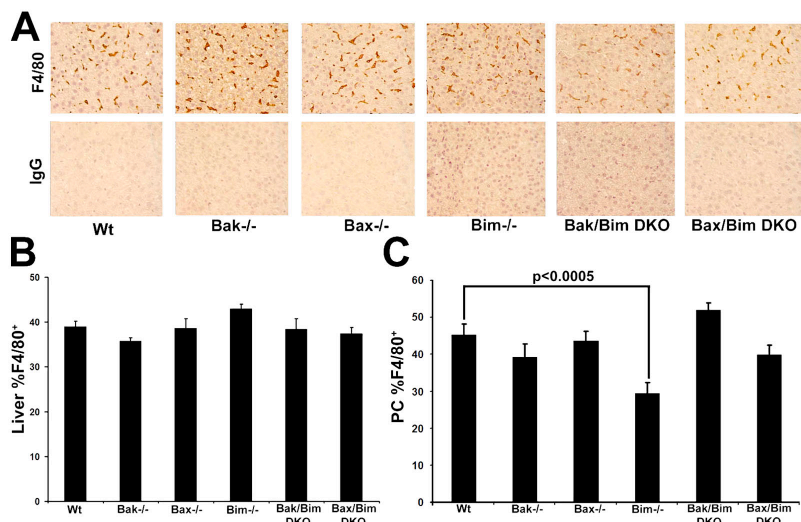[b]$P < 0.05$ as compared with Bim$^{-/-}$ mice.

Figure 2. Bak/Bim DKO and Bax/Bim DKO have normal numbers of tissue macrophages (A, B) Bak/Bim DKO and Bax/Bim DKO mice contain similar numbers of macrophages in their liver. Representative photomicrographs of F4/80-positive cells in the liver in WT ($n = 13$), Bak$^{-/-}$ ($n = 12$), Bax$^{-/-}$ ($n = 7$), Bim$^{-/-}$ ($n = 16$), Bak/Bim DKO ($n = 3$), and Bax/Bim DKO ($n = 7$) mice. Values represent the mean $\pm$ standard error. (C) Quantita-tive analysis of F4/80-positive macrophages in the peritoneum. Peritoneal cells from WT ($n = 23$), Bak$^{-/-}$ ($n = 12$), Bax$^{-/-}$ ($n = 9$), Bim$^{-/-}$ ($n = 15$), Bak/Bim DKO ($n = 4$), and Bax/Bim DKO ($n = 8$) mice were analyzed for F4/80 antigen expression. Values represent the mean $\pm$ standard error, which were compared by Student's t test.

mirror those found in Bim$^{-/-}$ mice, we sought to delineate the specific effects of the combined loss of Bak and Bim or Bax and Bim on the cellular subset composition of bone marrow, peripheral blood, spleen, and thymus. In bone marrow, Bax/Bim DKO mice exhibited a marked decrease in the total numbers of CD11b$^+$ bone marrow cells, which are precursors of peripheral blood monocytes or neutrophils, as compared with WT mice. In contrast, there were no differences in myeloid precursors in Bak/Bim DKO as compared with WT mice. The loss of Bax in Bim-deficient mice resulted in a 67% increase ($P < 0.05$) in the CD11b$^+$Gr-1$^-$ bone marrow cells as compared with Bim$^{-/-}$ mice. These numbers of CD11b$^+$Gr-1$^-$ bone marrow cells were equivalent to WT levels. The combined loss of Bak and Bim was able to rescue CD11b$^+$Gr-1$^-$ and CD11b$^+$Gr-1$^+$ populations of bone marrow cells as compared with Bim$^{-/-}$ mice to the levels found in WT mice. These data suggest that Bak and Bax seem to be differentially required but share some overlapping functions for maturation or survival of myeloid subpopulations in bone marrow of mice deficient for Bim.

Because Bak/Bim DKO and Bax/Bim DKO mice presented with reduced numbers of myeloid precursors in bone marrow, we examined blood and peripheral hematopoietic tissues to determine the effect of Bak plus Bim or Bax plus Bim deficiency on circulating monocytes and neutrophils. Bak/Bim DKO mice and Bax/Bim DKO mice had a 3.3-fold and 5.3-fold ($P < 0.003$) increase in the number of resident monocytes, respectively, as compared with WT mice. Bax/Bim DKO mice also had a twofold increase ($P < 0.05$) in resident monocytes as compared with Bim$^{-/-}$ mice. Bax$^{-/-}$ and Bim$^{-/-}$ mice exhibited only a 1.8-fold ($P < 0.003$) and 2.7-fold ($P < 0.0001$) increase in these cells, respectively (Table II). Additionally, Bak/Bim DKO and Bax/Bim DKO mice showed a 2.8-fold and 2.6-fold increase ($P < 0.002$) in inflammatory monocytes, respectively, compared with WT mice (Table II). Bax$^{-/-}$ mice had similar numbers of inflammatory monocytes and Bim$^{-/-}$ mice had a 1.7-fold increase ($P < 0.0007$) in inflammatory monocytes compared with WT mice (Table II). Moreover, Bak/Bim DKO and Bax/Bim DKO mice displayed a 2.4-fold and

Table III. Phenotypical analysis of bone marrow cells

1 × E6 cells	WT	Bak$^{-/-}$	Bax$^{-/-}$	Bim$^{-/-}$	Bak/Bim DKO	Bax/Bim DKO
	$n > 18$	$n = 12$	$n = 13$	$n = 14$	$n = 8$	$n = 10$
CD19$^+$	4.0 ± 0.6	2.6 ± 0.5	3.3 ± 0.4	3.0 ± 0.4	3.4 ± 1.0	7.7 ± 0.6^{a,b}
CD11b$^+$Gr-1$^-$	2.1 ± 0.3	1.5 ± 0.1	1.8 ± 0.2	1.5 ± 0.2	1.9 ± 0.2	2.5 ± 0.2^b
CD11b$^+$Gr-1$^+$	4.9 ± 0.6	2.2 ± 0.2^a	4.4 ± 0.6	2.9 ± 0.5^a	4.1 ± 0.6	2.0 ± 0.3^a

Quantitative analysis of B cells and myeloid cells in bone marrow as determined by flow cytometry. All data are expressed as mean ± SE, which were compared by Student's t test.
^{a}$P < 0.05$ as compared with WT mice.
^{b}$P < 0.05$ as compared with Bim$^{-/-}$ mice.

Table IV. Phenotypical analysis of splenic leukocytes

1 × E6	WT	Bak^{−/−}	Bax^{−/−}	Bim^{−/−}	Bak/Bim DKO	Bax/Bim DKO
	n > 18	n = 13	n = 12	n > 14	n = 8	n > 9
CD19^{+}	33.1 ± 3.1	32.9 ± 3.1	34.6 ± 2.3	54.5 ± 5.6[a]	63.7 ± 8.1[a]	57.3 ± 8.7[a]
CD3^{+}	20.8 ± 1.5	19.0 ± 1.7	17.8 ± 0.8	39.2 ± 3.2[a]	26.6 ± 3.9[b]	27.6 ± 3.9[b]
CD3^{+}CD4^{+}	11.8 ± 0.9	8.8 ± 0.9[a]	9.6 ± 0.8	17.2 ± 2.0[a]	11.4 ± 1.3[b]	10.8 ± 1.3[b]
CD3^{+}CD8^{+}	5.9 ± 0.6	6.1 ± 0.8	5.1 ± 0.5	13.6 ± 1.8[a]	8.2 ± 1.4[b]	8.8 ± 1.1[a,b]
CD11b^{+}Gr-1^{−}CD62L^{+/−}	1.5 ± 0.1	1.3 ± 0.1	1.4 ± 0.3	2.0 ± 0.3	2.5 ± 0.9	1.8 ± 0.4

Quantitative analysis of lymphocytes and myeloid cells in spleen as determined by flow cytometry. All data are expressed as mean ± SE, which were compared by Student's t test.
[a]P < 0.05 as compared with WT mice.
[b]P < 0.05 as compared with Bim^{−/−} mice.

2.1-fold increase (P < 0.008), respectively, in circulating neutrophils, whereas Bim^{−/−} mice had a 1.7-fold increase (P < 0.02) in neutrophils compared with WT mice (36). These data demonstrate a synergistic effect on accumulation of monocytes and neutrophils by the combined loss of Bak or Bax and Bim. Although Bax^{−/−}, Bim^{−/−}, Bak/Bim DKO, and Bax/Bim DKO mice have increased circulating monocytes, paradoxically, we did not observe an increase in macrophages in tissues. WT, Bak^{−/−}, Bax^{−/−}, Bim^{−/−}, Bak/Bim DKO, and Bax/Bim DKO mice displayed an equivalent complement of macrophages in liver (Fig. 2, A and B). Similarly, in the peritoneum there was a decrease in the numbers of F4/80-positive cells only in Bim^{−/−} mice (37%, P < 0.0005) as compared with WT mice (Fig. 2 C). These data suggest that recruitment of monocytes to tissue and their differentiation into a macrophage is independent of intrinsic apoptotic pathway.

B lymphocytes are increased abnormally in Bak/Bim DKO and Bax/Bim DKO mice

Mice lacking Bim have defects in deletion of autoreactive B cells (32); therefore, we examined the effect of deleting Bak and Bim or Bax and Bim on B cell development. There was a substantial increase in B lymphocytes in Bax/Bim DKO bone marrow compared with WT, Bax^{−/−}, Bak^{−/−}, Bim^{−/−}, or Bak/Bim DKO mice (Table III). These data demonstrate that the combined loss of Bax and Bim, but not Bak and Bim, alters B lymphocyte homeostasis in bone marrow more severely than loss of Bim alone. The B cell population increased 4.5-fold (P < 0.001) and 5.1-fold (P < 0.001) in blood and 1.9-fold (P < 0.01) and 1.7-fold (P < 0.02) in spleen within Bak/Bim DKO and Bax/Bim DKO mice, respectively, as compared with WT mice (Tables II and IV). Moreover, the combined loss of Bak or Bax in Bim^{−/−} mice led to higher numbers of circulating B cells in peripheral blood as compared
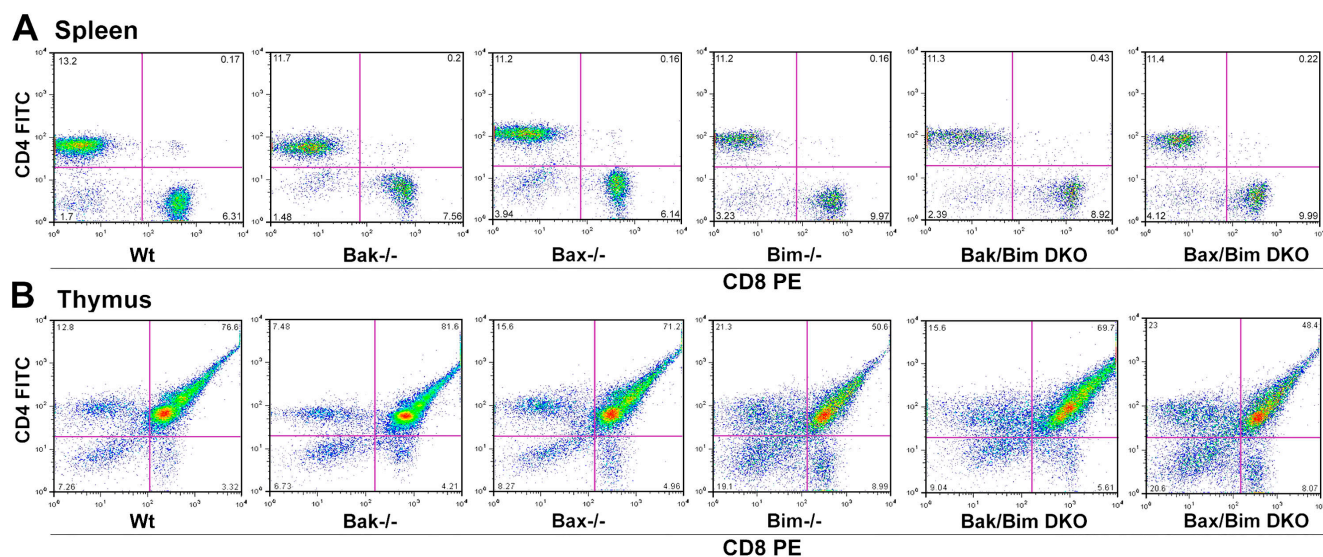

A Spleen

B Thymus

Figure 3. Bax/Bim DKO mice display an altered CD4^{+}8^{−}/CD4^{−}8^{+} T cell ratio resulting from abundant accumulation of mature CD4^{−}8^{+} T cells. (A) Bak/Bim DKO and Bax/Bim DKO mice display a 1:1 CD4^{+}8^{−}/CD4^{−}8^{+} T cell ratio in their spleen. Single-cell splenocyte suspensions were stained with the indicated antibodies and then analyzed by flow cytometry. Shown is a dot plot gated on the CD45^{+}CD3^{+} splenocytes. (B) Bax/Bim DKO and Bim^{−/−} mice exhibit reduced percentages and numbers of CD4^{+}8^{+} double positive thymocytes. Single-cell thymocyte suspensions were stained with the indicated antibodies and analyzed by flow cytometry.

with Bim$^{-/-}$ mice (Table II). Bim$^{-/-}$ mice showed a 2.9-fold (P < 0.0001) and a 1.6-fold (P < 0.003) increase in B cells in blood and spleen, respectively, as compared with WT mice; these data were consistent with previous reports (36). Because deficiency in Bak and Bim or Bax and Bim led to increased B cells in the periphery, these data suggest that loss of Bak or Bax in Bim$^{-/-}$ mice may prevent deletion of B cells leading to their abnormal accumulation in tissues.

Bim is essential for proper homeostasis of T lymphocytes

Deficiency in Bax (20) or Bim (36) were shown to affect the development of T lymphocytes. Bak/Bim DKO, Bax/Bim DKO, and Bim$^{-/-}$ mice exhibited a 3.7-fold, 3.4-fold (P < 0.002), and 2.9-fold (P < 0.0001) increase in T cells circulating in blood, respectively, as compared with WT mice, whereas Bak$^{-/-}$ and Bax$^{-/-}$ mice had normal numbers of T cells (Table II). In peripheral blood and in spleen, the CD4$^+$8$^-$/CD4$^-$8$^+$ T cell ratio was altered in Bak/Bim DKO and Bax/Bim DKO mice. Bak/Bim DKO and Bax/Bim DKO mice displayed an ~1:1 CD4$^+$8$^-$/CD4$^-$8$^+$ ratio (Fig. 3 and Tables II and IV), whereas WT mice showed a ratio of ~2:1. These data indicate that the change in the ratio of CD4$^+$8$^-$/CD4$^-$8$^+$ T cells may be due to the increased numbers of CD8 T cells in Bak/Bim DKO, Bax/Bim DKO, and Bim$^{-/-}$ animals (38). Although previously unreported, Bak$^{-/-}$ mice had a reduced CD4$^+$8$^-$/CD4$^-$8$^+$ ratio in blood and spleen. Similar to previous studies, Bim$^{-/-}$ mice exhibited a 1.8-fold increase (36), whereas Bak/Bim DKO and Bax/Bim DKO mice had only a marginal increase in splenic T cells as compared with WT mice (Table IV). Bak/Bim DKO and Bax/Bim DKO mice had fewer CD4$^+$ and CD8$^+$ T cells in spleen as compared with Bim$^{-/-}$ mice (Table IV).

Because Bak/Bim and Bax/Bim DKO mice displayed an altered CD4$^+$8$^-$/CD4$^-$8$^+$ T cell ratio similar to that found in Bim$^{-/-}$ mice, the thymus was examined to determine the effect of deleting Bak and Bim or Bax and Bim on thymic cell subset composition. Bak/Bim DKO mice displayed a different thymic profile as compared with Bax/Bim DKO or Bim$^{-/-}$ mice. Bak/Bim DKO mice had elevated double-negative population (CD4$^-$CD8$^-$) as compared with WT, Bim$^{-/-}$, and Bax/Bim DKO mice. Although Bax/Bim DKO and Bim$^{-/-}$ mice showed a similar decrease in CD4$^+$CD8$^+$ thymocyte numbers, Bak/Bim DKO CD4$^+$CD8$^+$

thymocytes were equivalent in number to WT mice (Table V). Moreover, Bak/Bim DKO mice had more CD4$^+$CD8$^-$ thymocytes as compared with WT, Bim$^{-/-}$, and Bax/Bim DKO mice. These data suggest that the accelerated maturation of thymocytes that occurs in mice deficient for Bim requires Bak, but not Bax.

Bak/Bim DKO and Bax/Bim DKO thymocytes are resistant to intrinsic apoptotic stimuli

Previous studies demonstrated that Bim-deficient thymocytes are abnormally resistant to certain apoptotic stimuli, such as cytokine withdrawal or treatment with the calcium ionophore, ionomycin (36). Deficiency in Bax and Bim substantially reduced the loss of mitochondrial membrane potential in response to cytokine deprivation or treatment with etoposide as compared with WT, Bax$^{-/-}$, Bim$^{-/-}$, Bak$^{-/-}$, and Bak/Bim DKO cells (Fig. 4 A). However, Bak/Bim DKO and Bax/Bim DKO thymocytes displayed more Rh123-posivity as compared with WT, Bak$^{-/-}$, Bax$^{-/-}$, and Bim$^{-/-}$ mice (Fig. 4 A). A similar trend was observed in Bax/Bim DKO and Bak/Bim DKO thymocytes that were stained with annexin V or with 7-amino–actinomycin D (7-AAD) after cytokine deprivation or treatment with etoposide (Fig. 4, B and C). Fewer Bax/Bim DKO and Bak/Bim DKO thymocytes were stained with annexin V or 7-AAD as compared with WT or single gene knock-out mice (Fig. 4, B and C). Although treatment with an extrinsic apoptotic stimulus, such as FasL, induced a similar staining pattern for Rh123, annexin V, and 7-AAD in WT, Bax$^{-/-}$, Bim$^{-/-}$, Bak$^{-/-}$, Bak/Bim DKO, and Bax/Bim DKO cells (Fig. 4, A–C), Bak/Bim DKO and Bax/Bim DKO cells were slightly more resistant to FasL–induced death. These data demonstrate that Bak or Bax and Bim are required for thymocyte apoptosis mediated by intrinsic death stimuli.

Because cytochrome C release generally occurs in cells that undergo apoptosis through the intrinsic pathway (43), and because caspase-9 requires cytochrome C to be activated (44, 45), we examined the activity of caspase-9 and caspase-3 in cells from WT, Bak$^{-/-}$, Bax$^{-/-}$, Bim$^{-/-}$, Bak/Bim DKO, and Bax/Bim DKO mice. There was marked reduction (>80%) in the activation of caspase-9 and –3 in Bak/Bim DKO and in Bax/Bim DKO cells as compared with cells from all other genotypes (Fig. 4, D

Table V. Phenotypical analysis of thymocytes

1 × E6	WT	Bak$^{-/-}$	Bax$^{-/-}$	Bim$^{-/-}$	Bak/Bim DKO	Bax/Bim DKO
	n = 24	n = 13	n = 14	n = 14	n = 8	n = 12
CD4$^-$CD8$^-$	4.1 ± 0.4	11.5 ± 1.6^a	7.4 ± 0.5^a	5.9 ± 0.6^a	11.0 ± 2.0^{a,b}	7.0 ± 1.5
CD4$^+$CD8$^+$	52.0 ± 4.5	73.4 ± 6.5^a	65.1 ± 4.8	19.3 ± 2.2^a	58.0 ± 11.3^b	19.8 ± 4.3^a
CD4$^-$CD8$^+$	3.3 ± 0.5	6.3 ± 0.8^a	4.7 ± 0.4^a	3.7 ± 0.4	4.3 ± 0.9	3.9 ± 1.0
CD4$^+$CD8$^-$	6.1 ± 0.8	7.1 ± 0.6	9.9 ± 0.7^a	7.3 ± 0.7	12.9 ± 2.0^{a,b}	6.2 ± 1.4

Quantitative analysis of thymocytes after analysis as determined by flow cytometry. All data are expressed as mean ± SE, which were compared by Student's t test.
^{a}P < 0.05 as compared with WT mice.
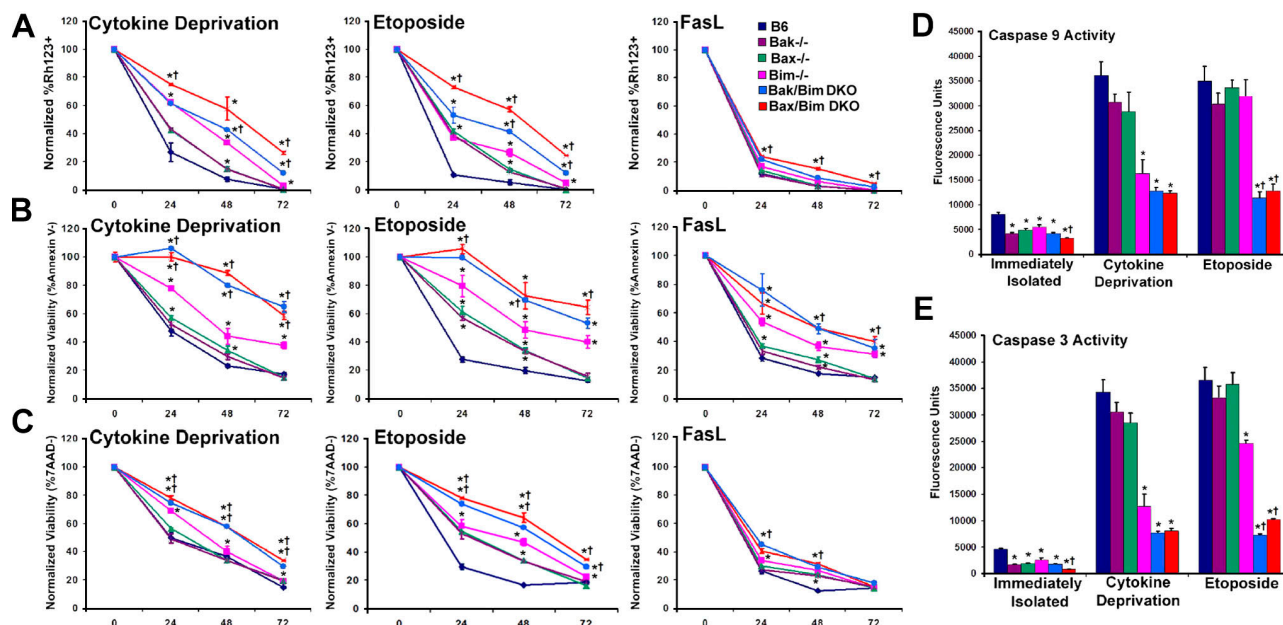^{b}P < 0.05 as compared with Bim$^{-/-}$ mice.

Figure 4. Bak/Bim DKO and Bax/Bim DKO thymocytes have abnor–mally low sensitivity to intrinsic apoptotic stimuli. Thymocytes iso-lated from WT, Bak$^{-/-}$, Bax$^{-/-}$, Bim$^{-/-}$, Bak/Bim DKO, and Bax/Bim DKO mice were plated in triplicate and left untreated (death by cytokine depri-vation) or were treated with etoposide (7.5 ng/ml) or FasL (20 ng/ml) and analyzed by flow cytometry. All data are expressed as percentages of (A) Rh123-positive, (B) annexin V-negative, or (C) 7AAD-negative cells nor-malized to the 0 time point. Bak/Bim DKO and Bax/Bim DKO thymocytes display reduced activation of caspase-9 (D) and (E) caspase-3. Thymocytes isolated from WT, Bak$^{-/-}$, Bax$^{-/-}$, Bim$^{-/-}$, Bak/Bim DKO, and Bax/Bim DKO mice were left untreated (cytokine deprivation) or were treated for 8 h with etoposide and then analyzed for the activity of caspase-9 and -3. Values represent the mean ± standard error, which were compared by Student's *t* test. *P < 0.05 as compared with WT. All data are representa-tive of two independently performed experiments. †P < 0.05 as compared with Bim$^{-/-}$.

and E). Deficiency in Bim resulted in a 54% reduction in the activity of caspase-9 in cells cultured in serum alone (cytokine deprivation), but had no effect on thymocytes treated with etoposide (Fig. 4 D). However, at lower doses of etoposide (<2.5 ng/ml), Bim$^{-/-}$ thymocytes had a marked decrease in caspase-9 activity compared with WT cells (unpublished data). Deficiency in Bim also resulted in a 63 and 32% reduction in the activity of caspase-3 as com-pared with WT cells after cytokine deprivation or treat-ment with etoposide, respectively (Fig. 4 E). WT, Bak$^{-/-}$, and Bax$^{-/-}$ thymocytes displayed similar levels of activity of caspase-9 and –3 after cytokine deprivation or treatment with etoposide. These data further indicate that deficiency in Bak and Bim or Bax and Bim reduces the activation of caspases after an insult mediated by the intrinsic apoptotic pathway (Fig. 4, D and E). Collectively, these data suggest that the loss of Bim confers protection at the mitochondrial membrane in a Bak- or Bax-dependent manner.

DISCUSSION

Members of the Bcl-2 protein family are essential for deter-mining cellular survival/death after an apoptotic insult through the intrinsic pathway (26). Loss of Bax or Bak alone, two proapoptotic multi–BH-domain proteins, has relatively little effect on mouse development, with the exception of the male infertility found in Bax$^{-/-}$ mice (17). Loss of BH3-

only genes results in increased cell accumulation and resis-tance to apoptosis, which often is cell type– or stimulus-spe-cific (46). For example, Bim is required for cytokine with-drawal and calcium flux–induced apoptosis (36), whereas Puma is essential for DNA damage–induced apoptosis trans-duced via the tumor suppressor p53 (47, 48). The combined loss of Bak and Bax has dramatic consequences for mouse development and apoptosis signaling through the intrinsic pathway (17). Although these data demonstrate the impact of deleting two multi–BH domain proapoptotic Bcl-2 family members, the consequences of removing a proapoptotic multi–BH domain family member plus a BH3-only gene have not been studied in vivo. To date, the only related study examined the in vitro effect of deleting Bax and the BH3-only protein, Noxa. Combined loss of Bax and Noxa conferred upon cultured fibroblasts increased protection against DNA damage–induced apoptosis as compared with loss of either protein alone (49). However, there was no syn-ergistic effect in Bax/Noxa DKO thymocytes that were UV irradiated. These data suggest that Bax and Noxa interact with each other in a cell type–specific manner. Here we demonstrate that Bax/Bim DKO mice display webbed paws, indicative of a developmental defect in apoptosis. A similar defect was observed in Bak/Bax DKO mice (17), although the role of Bim in the developing footpad of these mice was not examined. However, Bak/Bim DKO mice were born

with normal digits. These data indicate that Bax and either Bak or Bim are required for proper formation of the interdigital tissue. Moreover, these data suggest that in the absence of Bax and Bim, Bak and the remaining BH3-only proteins are not sufficient for the induction of programmed cell death in the developing footpad. However, in Bak/Bim DKO mice, Bax must be activated by a BH3-only protein, and this activation does not require the formation of higher-order complexes of Bax and Bak. Taken together, these data suggest that Bak and Bim may act at the same level in the developing footpad. Because BH3-only proteins function upstream of Bak/Bax in apoptosis signaling (15, 16, 27), this result indicates that Bim is the predominant activator upstream of Bak in programmed cell death of interdigitating cells.

Bax/Bim DKO mice with increased contribution of 129Sv background genes displayed a more severe phenotype, including growth retardation, malocclusions of the incisors, and premature death. Dramatic influence of genetic background on the phenotype of mice lacking apoptosis-regulatory genes was observed previously. For example, caspase-3–deficient mice are embryonic lethal on a mixed C57BL/6 × 129Sv background, but survive on an inbred C57BL/6 background (50, 51). Thus, one has to be cautious when interpreting small differences between KO and control mice that are kept on a mixed genetic background. Our work demonstrates clearly that Bax/Bim DKO mice display a novel phenotype compared with Bax$^{-/-}$, Bim$^{-/-}$, or Bak/Bax DKO mice; this indicates an overlapping function for Bax and Bim in the development of teeth.

Few studies have examined the role of the Bcl-2 protein family in the development of myeloid cells. A recent study (37) demonstrated that cultured granulocytes lacking Bim or overexpressing prosurvival Bcl-2 are resistant to cytokine withdrawal in culture. Our data suggest that the proapoptotic genes Bak and Bax are differentially required for myeloid progenitor cell survival/differentiation in the bone marrow of Bim$^{-/-}$ mice. Even though Bim is a proapoptotic gene, Bim$^{-/-}$ mice have normal myeloid progenitor potential (unpublished data), but they have an unexpected reduction in total numbers of CD11b$^+$Gr-1$^-$ and CD11b$^+$Gr-1$^+$ bone marrow cells. Bax, but not Bak, deficiency in Bim$^{-/-}$ mice is able to restore normal numbers of CD11b$^+$Gr-1$^-$ bone marrow cells through an unknown mechanism. In contrast, deficiency in Bak and Bim mice completely restored the CD11b$^+$Gr-1$^+$ population in Bim$^{-/-}$ mice to the levels found in WT mice. These data suggest that the functional redundancy displayed by Bak and Bax is cell type–specific. Moreover, loss of Bim negatively affects developing myeloid cells, but the combined loss of Bax and Bim paradoxically suppresses myeloid progenitor potential (unpublished data). Thus, in bone marrow, Bax, Bak, or Bim may function to promote the survival or differentiation of myeloid cells (52, 53). Together, these data indicate that expression of Bax or Bak differentially influences various myeloid cell fates in bone marrow of Bim$^{-/-}$ mice.

In contrast to bone marrow, Bak/Bim DKO and Bax/Bim DKO mice display a marked increase in circulating monocytes and neutrophils as compared with WT, Bak$^{-/-}$, and Bax$^{-/-}$ mice. Additionally, the myeloid populations in Bak/Bim DKO and Bax/Bim DKO mice resemble Bim$^{-/-}$ mice in peripheral blood. Although there is a decrease in the numbers of F4/80$^+$ peritoneal cells in Bim$^{-/-}$ mice as compared with WT, Bax$^{-/-}$, and Bax/Bim DKO mice, there is a concomitant increase in lymphocytes in the peritoneum of Bim$^{-/-}$ and Bax/Bim DKO mice (unpublished data). These data suggest that deficiency in Bim affects the ratio of macrophages/lymphocytes, at least in certain tissues. Similarly, in liver and spleen, Bak/Bim DKO and Bax/Bim DKO mice have normal numbers of tissue macrophages. These data suggest that Bak, Bax, or Bim do not affect extravasation or recruitment of monocytes into a subset of tissues. Thus, an increase in the monocyte pool through a decrease in apoptosis is not a prerequisite for abnormally elevated numbers of macrophages in tissues. Because in Bak/Bax DKO and Bax/Bim DKO mice there is a normal complement or reduction in myeloid precursor cells, respectively, and because there is no difference in the numbers of tissue or peritoneal macrophages, these data suggest that the increase in peripheral blood monocytes in Bak/Bim DKO, Bax/Bim DKO, or Bim$^{-/-}$ mice may be due to enhanced lifespan in peripheral blood. The abnormal increase in the number of monocytes in peripheral blood of Bak/Bim DKO, Bax/Bim DKO, or Bim$^{-/-}$ mice may result in a negative feedback loop that reduces the number of myeloid cells in bone marrow.

Previous studies demonstrated that Bim, but not Bax, single-gene KO mice have abnormally increased numbers of B lymphocytes in spleen (17, 36). In Bax/Bim DKO mice, the increased numbers of CD19$^+$ cells in bone marrow may be due to enhanced B cell production in bone marrow or increased survival of B cells within the marrow. Alternatively, it is possible that increased numbers of B cells in peripheral blood may recirculate into bone marrow. We did not observe an increase in the B cell population in bone marrow of Bak/Bim DKO or Bim$^{-/-}$ mice. In contrast, combined deficiency in Bak/Bim or Bax/Bim exert a greater effect on the number of B cells compared with WT, Bax$^{-/-}$, Bak$^{-/-}$, and Bim$^{-/-}$ mice in peripheral blood. However, the B cell population in Bak/Bim DKO and Bax/Bim DKO mice resemble Bim$^{-/-}$ mice. Collectively, these data suggest that the overlapping role of Bak and Bax may be dependent on individual cell types within a given tissue. Although not examined in this study, because Bim is critical for deletion of autoreactive B cells (32) and because mice lacking Bim develop systemic lupus erythematosus–like disease (at least on a mixed C57BL/6 × 129Sv genetic background; reference 36), it is possible that Bak/Bim DKO or Bax/Bim DKO mice may develop autoimmune disease, even on an inbred C57BL/6 background. Taken together, the increase in B cells observed in Bak/Bim and Bax/Bim DKO mice is further evidence that Bim is central for the maintenance of B cell homeostasis.

Although Bim may activate Bak or Bax to regulate the development of B cells and to maintain their homeostasis in the periphery, Bim seems to act preferentially on Bax, but not on Bak, in thymocytes. Defects in development of thymocytes in Bim$^{-/-}$ mice are not exacerbated by loss of Bax. Bim$^{-/-}$ mice and Bax/Bim DKO mice display fewer double-positive thymocytes than WT animals. This decrease in double-positive thymocytes may be attributed to enhanced maturation of thymocytes, which leave the thymus at a faster rate or it may be due to a negative feedback loop in the thymus triggered by the abnormally increased numbers of T cells in the periphery (54, 55). Conversely, mice lacking Bak and Bim show a thymic profile comparable to WT mice. Together, these data suggest that although the expression of Bak and Bax overlap in the thymus, their functions are not redundant in Bim$^{-/-}$ mice. In the thymus, Bak may not be activated by another BH3 protein or Bak is not sufficient to proceed with the process of negative selection in the absence of Bax and Bim. In contrast, to compensate for the loss of Bak, mice lacking Bak and Bim may show increased activation of Bax, which may be responsible for the normal thymic populations. In this situation, monomers of Bax or the increased levels of unbound Bcl–2–like prosurvival proteins may function to reduce the accelerated maturation observed in Bim$^{-/-}$ mice. However, the latter possibility seems to be unlikely because overexpression of Bcl-2 in thymocytes induces a phenotype that resembles that found in Bim$^{-/-}$ mice (56). It is still unclear why in the absence of Bim, Bax and Bak seem to be inactivated, whereas in mice deficient for Bak and Bim, Bax may be activated. One possibility may be that in the thymus, Bak may function as a prosurvival protein, which previously was shown to occur in neurons (53). Future studies are required to delineate the role of Bak and Bax in the selection and maturation processes of thymocytes in the absence of Bim.

The combined loss of Bak and Bax confers remarkable resistance to apoptosis induced by intrinsic apoptotic stimuli (26). This inhibition is sustained over time because thymocytes or lymphocytes from Bak/Bax DKO mice do not undergo cytokine withdrawal–induced death over a 4-d period (14, 17). During apoptosis, Bax or Bak become activated by BH3 proteins; this has been postulated to occur through a direct interaction between these two groups of proapoptotic Bcl-2 family members or through BH3 protein–mediated sequestering of prosurvival Bcl–2–like proteins (54). The activated Bax or Bak causes permeabilization of the outer mitochondrial membrane (directly or indirectly), where it oligomerizes into higher-order species; this results in the release of cytochrome c into the cytosol (43). Recent studies using KO mice provided insight into the functional interactions between BH3-only proteins, multi-BH domain proapoptotic Bcl-2 family members, and antiapoptotic Bcl–2–like proteins. Unlike deficiency in Bax (41) or in the BH3-only gene, Bik (40), deficiency in Bim is able to rescue the defects observed in mice lacking Bcl-2 (39).
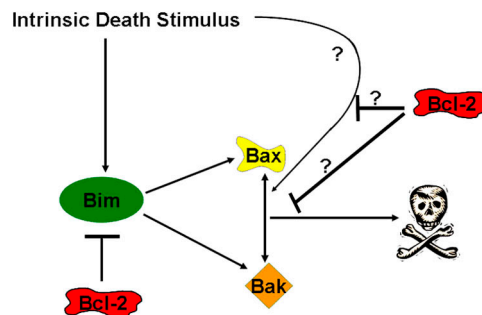

Figure 5. Summary of the genetic interactions of Bcl-2 family members. Previous studies have shown that deficiency in Bim, but not Bax, is sufficient to rescue Bcl-2$^{-/-}$ mice. These data suggest that, in part, Bcl-2 may function to inhibit Bim. Bcl-2 also may function to prevent the formation of higher-order complexes of Bak and Bax or function to prevent the activation of Bak or Bax. Because Bim$^{-/-}$ or Bcl-2/Bim DKO thymocytes undergo apoptosis in response to certain intrinsic apoptotic stimuli (e.g., DNA damage), these data suggest that additional pathways that are independent of Bim must exist. However, all of these intrinsic pathways require Bak and Bax for apoptosis.

These data suggest that Bcl-2 does not function solely to prevent the activation of Bax (but also must inactivate Bak function). These data also suggest that one of the roles of Bim may be to block the function of Bcl-2. Bcl–2/Bim DKO lymphocytes treated with certain intrinsic apoptotic stimuli (e.g., cytokine deprivation) undergo much less apoptosis than Bcl-2$^{-/-}$ cells.; the Bcl–2/Bim DKO lymphocytes are as resistant to apoptotic stimuli as Bim$^{-/-}$ lymphocytes (39). However, because Bcl–2/Bim DKO and Bim$^{-/-}$ cells still undergo apoptosis in response to certain apoptotic stimuli, such as DNA damage (39), these data indicate that factors in addition to Bim and Bcl–2 can regulate lymphocyte death (Fig. 5). In this scenario, other BH3-only proteins, such as Puma or Noxa (47), may activate Bak or Bax or inhibit Bcl–2–like prosurvival proteins, such as Bcl-x$_L$ or Mcl-1 (Fig. 5). Because deficiency in Noxa alone or Noxa plus Bax failed to protect thymocytes against Xray irradiation (49), these data indicate that Noxa may not cooperate with Bax in this cell death pathway. Here we show that Bim acts through Bak or Bax to induce thymocyte apoptosis induced by cytokine deprivation or treatment with etoposide. We show that thymocytes lacking Bak and Bim or Bax and Bim display a marked reduction in apoptosis, although not a complete inhibition as observed in thymocytes lacking Bak and Bax. These data demonstrate that in cultured thymocytes, Bak and Bax act redundantly in the apoptotic process. Our data does not preclude the possibility that the lack of Bak and Bim or Bax and Bim results in an increased level of Bcl-2 antiapoptotic proteins (54), but we have not found any evidence for this. Recent studies have shown that the BH3 peptide from Bim or Puma bind with very high affinity to all antiapoptotic Bcl–2–like proteins, whereas the BH3 domain regions from the other BH3-only proteins exhibited

more selective binding (57, 58). This may indicate that Bim and Puma activate Bak/Bax function indirectly through binding and neutralization of prosurvival Bcl-2 family members. Another recent study supports the possibility that Bim may act directly on Bak/Bax. Bim BH3 peptide, but not the Puma BH3 peptide, was sufficient to induce the conformational change, oligomerization, and activation of Bak and Bax to permeabilize mitochondrial membranes (58). Thus, future genetic studies (e.g., generation of mice lacking Bak, Bax, Bcl-2, or Bim) are necessary to determine whether Bim has an active role in apoptosis through activation of Bak or Bax or functions to sequester Bcl-2–like proteins.

MATERIALS AND METHODS

Mice. Bim-deficient mice (produced by gene targeting in W9.5 129Sv genetic background embryonic stem (ES) cells and originally kept on a mixed C57BL/6 $\times$ 129sv background) were described previously (36), and were backcrossed onto the C57BL/6 background for 13 generations. Bax-deficient mice (produced by gene targeting in RW-4 129Sv genetic background ES cells and originally kept on a mixed C57BL/6 $\times$ 129Sv background; reference 20), which were backcrossed for 12 generations, Bak-deficient mice (produced by gene targeting in R1 129Sv genetic background ES cells and originally kept on a mixed C57BL/6 $\times$ 129sv background), which were backcrossed for 5 generations (17), and congenic control (C57BL/6, B6) mice were purchased from the Jackson ImmunoResearch Laboratories. Bak/Bim and Bax/Bim DKO mice on the C57BL/6 background were established at Saint Louis University, whereas Bax/Bim DKO mice which had more contribution of 129Sv background genes (Bax mutant mice backcrossed to C57BL/6 only 6 times) were established at the Walter and Eliza Hall Institute of Medical Research. The Animal Care and Use Committee at Saint Louis University and the Walter and Eliza Hall Institute of Medical Research approved all experiments on mice.

Immunophenotyping. Peripheral blood was isolated from 5–8-wk-old mice by cardiac puncture after euthanasia. Single-cell suspensions of bone marrow, peritoneal cells, spleen, and thymus also were isolated. Nonspecific staining of cells with antibodies was prevented by incubation with anti–CD16/32 (24G2) monoclonal antibody (BD Biosciences). Cells were stained with fluorochrome-conjugated antibodies to CD45, CD11b, Gr-1, CD62L, CD19, CD3, CD4, CD8 (BD Biosciences), and F4/80 (Caltag), or isotype control antibodies for 30 min. For peripheral blood, red blood cells were lysed and cells were fixed with BD FACS lysing solution (BD Biosciences) after incubation with antibodies. For single-cell populations, red blood cells were lysed with PharmLyse (BD Biosciences) before incubation with antibodies and were fixed in 1% paraformaldehyde after incubation with antibodies. Data were acquired on a BD FACSCalibur (BD Biosciences) using CELLQuest Software at the St. Louis University Core Flow Cytometry Facility. All analysis was performed using FlowJo software (Tree Star Inc.). Total peripheral blood leukocyte numbers were determined on the automated hematology analyzer ABX Pentra 60, whereas cell numbers from single-cell suspensions of hemopoietic organs were determined by using a hemocytometer.

Immunohistochemistry. After euthanasia, mouse tissues were isolated, fixed in 10% neutral buffered formalin, embedded in paraffin, and sectioned. To stain for F4/80 expression, antigens were retrieved using the Dako target retrieval solution (DakoCytomation). After antigen retrieval, sections were blocked in hydrogen peroxide, incubated with anti-F4/80 (Caltag Laboratories; Clone BM8 or CI:A3-1) antibody or an isotype-match control antibody for 1 h, incubated with a secondary biotinylated rabbit anti–rat antibody (DakoCytomation), treated with streptavidin peroxidase conjugate (DakoCytomation), and the color was visualized with diaminobenzidine. All sections were counterstained with hematoxylin. F4/80-positive cells were scored by examining three fields/section at 400$\times$ by an observer blinded to the study. A mini-

mum of 75 cells was counted in each field. Photographs were taken on a Nikon microscope equipped with the Nikon digital camera DMX1200.

Cell culture. Thymocytes were isolated, counted, and plated in triplicate at 200,000 cells/well of a 48-well plate. Etoposide (7.5 ng/ml) or FasL (20 ng/ml; Qbiogene) was added to thymocytes in culture. Cells were harvested at 24-h intervals; stained with Rh123, 7-AAD, and annexin V-APC; and analyzed by flow cytometry. Death by cytokine deprivation was triggered by plating cells in 10% FBS/DMEM in the absence of added cytokines, and analyzing for apoptosis by flow cytometry every 24 h. To determine the activity of caspase-3 and -9, 2×10^6 thymocytes/well were plated in a 6-well plate in 1 ml DMEM and treated with etoposide (7.5 ng/ml) or left untreated. Immediately after processing, single-cell suspensions were collected (t = 0). After 8 h, cells were harvested and analyzed for caspase-3 (BD Biosciences) and caspase-9 (R&D Systems) activity as described by the manufacturer.

Statistical analysis. Results were expressed as the mean $\pm$ standard error. Differences between groups were analyzed using Student's t test.

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