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28. Arthritic K/B×N serum donors were bred by crossing asymptomatic KRN TCR tg mice on the C57Bl/6 or B10.BR background with NOD/Lt animals (*5*, *7*). The arthritic progeny were bled between 45 and 70 days of age for pools of arthritic serum. Control serum came from non-tg littermates.
29. We thank J. Hergueux and W. Manent for serum; P. Gerber and C. Ebel for help with ELISAs, purifications, and cultures; and V. Louerat, N. Zinck, and P. Michel for maintaining the mice. Supported by the Institut National de la Santé et de la Recherche Médicale, the Centre National de la Recherche Scientifique, the Hôpital Universitaire de Strasbourg (C.B. and D.M.), the Association pour la Recherche sur la Polyarthrite, the Naito Foundation (I.M.), and the Japanese Rheumatism Foundation (I.M.).

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Proapoptotic Bcl-2 Relative Bim Required for Certain Apoptotic Responses, Leukocyte Homeostasis, and to Preclude Autoimmunity

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Apoptosis can be triggered by members of the Bcl-2 protein family, such as Bim, that share only the BH3 domain with this family. Gene targeting in mice revealed important physiological roles for Bim. Lymphoid and myeloid cells accumulated, T cell development was perturbed, and most older mice accumulated plasma cells and succumbed to autoimmune kidney disease. Lymphocytes were refractory to apoptotic stimuli such as cytokine deprivation, calcium ion flux, and microtubule perturbation but not to others. Thus, Bim is required for hematopoietic homeostasis and as a barrier to autoimmunity. Moreover, particular death stimuli appear to activate apoptosis through distinct BH3-only proteins.

Apoptosis is essential for normal development, tissue homeostasis, and immune function; its altered regulation can trigger cancer, autoimmunity, and degenerative disorders (*1*). Dismantling of the cell is carried out by cysteine proteases (caspases) (*2*), but initiation of many apoptotic responses is regulated by members of the Bcl-2 protein family (*3*). They appear to govern the activity of adapter proteins such as Apaf-1 required to activate apical caspases such as caspase-9 (*3*). Some members, like Bcl-2 itself, promote cell survival, but two other subfamilies instead promote cell death. Those such as Bax exhibit considerable sequence homology with Bcl-2, possessing three of the four Bcl-2 homology (BH) domains, but the others, which include EGL-1 of *Caenorhabditis elegans* and at least six mammalian proteins (Bad, Bik, Blk, Hrk, Bid, Bim), share only the short (9- to 16-residue) BH3 domain with the Bcl-2 family (*3*). This domain allows them to bind to the prosurvival Bcl-2-like molecules and neutralize their function. Several BH3-only proteins are present in healthy cells but are maintained, by different mechanisms, in a latent form until unleashed by cytotoxic signals (*4*–*6*). For example, after engagement of death receptors such as Fas/APO-1 (CD95), activated caspase-8 cleaves Bid, generating a more proapoptotic form (*4*). On the other hand, the potent proapoptotic molecule Bim (*7*), the subject of this study, is expressed in many cell types but normally sequestered to the microtubular dynein motor complex by interaction with dynein light-chain LC8 (*6*). Apoptotic stimuli provoke release of Bim and LC8, allowing Bim to associate with Bcl-2-like proteins.

Little has been established about the physiological roles of the mammalian BH3-only proteins, but the finding that EGL-1 of *C. elegans* is required to initiate all its developmental cell death (*8*) argues that they may be critical triggers of apoptosis. For example, Bid appears to be important in the death of hepatocytes after Fas engagement (*9*). To identify the biological roles of Bim, we disrupted its gene in the mouse (*10*). The targeting vector replaces the exon encoding BH3, which is essential for proapoptotic function (*7*), with a *neo* cassette flanked by LoxP sites (Fig. 1A). Chimeric mice generated from two independent targeted embryonic stem (ES) cell clones (263 and 266) were crossed with C57BL/6 mice to produce animals heterozygous for the *bim* mutation, and crosses with Cre-expressing deleter mice (*11*) yielded a progeny line lacking the *neo* cassette (266 Del). ES cells and mice were genotyped by Southern blot and polymerase chain reaction (PCR) analysis (Fig. 1B). When $bim^{-/-}$ mice were generated, as expected their spleen cells contained no full-length Bim protein (Fig. 1C). A truncated BH3-less polypeptide anticipated from the mutant allele appears to be unstable, because it represented <5% of wild-type (WT) Bim. The mutant allele behaves as a null mutation, because the truncated polypeptide, even when highly expressed in transfected cells, does not affect apoptosis (*12*).

Bim appears to have an important, albeit unidentified, role in embryonic development. Although interbreeding of $bim^{+/-}$ mice produced healthy and fertile $bim^{-/-}$ offspring, their number was less than half that of +/+ progeny (Fig. 1D). The marked deficit in mutant progeny ($P < 0.001$) is not attributable to adventitious mutations introduced during ES cell manipulation, because it appeared in both independent *bim* mutant strains and persisted after deletion of the *neo* cassette (Fig. 1D). A preliminary analysis indicated that $bim^{-/-}$ fetuses die before embryonic day 10 (E10). However, the penetrance of the embryonic lethality appears to be strongly affected by genetic background (*13*), as found with mice lacking certain other cell death regulators (*14*). Hence, we are placing the mutation on inbred 129 and C57BL/6 backgrounds to permit studies of the basis for the embryonic deaths.

Bim is expressed in many hematopoietic cell types (*7*), and its loss markedly affected homeostasis in that compartment (Table 1). Although the number of red cells was normal, blood leukocytes were elevated severalfold because of two- to fourfold increases ($P < 0.05$) in B cells, mature T cells ($CD4^+8^-$ and $CD4^-8^+$), granulocytes, and monocytes (Table 1). Surprisingly, although megakaryocyte numbers were normal, platelets were half the normal amount (Table 1). This substantial drop may indicate that their shedding from megakaryocytes depends on Bim-dependent mechanisms akin to apoptosis. Like the blood, the spleen and lymph nodes of young adult $bim^{-/-}$ mice contained two to three times as many leukocytes as WT littermates, mostly because of elevated B and T cells (Table 1). The frequency of hematopoietic progenitors in bone marrow was normal, however, and most of the leukocytes were small, noncycling cells. Hence, their accumulation probably reflects extended cell survival rather than excessive proliferation.

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Bim deficiency perturbed thymic T cell development. As expected for loss of a proapoptotic molecule, the numbers of both the $CD4^-8^-$ pro-T cells and the mature T cells ($CD4^+8^-$ and $CD4^-8^+$) were two- to threefold higher than in WT littermates (Fig. 2A and Table 1). Surprisingly, however, the $CD4^+8^+$ pre-T cells, the predominant thymic subpopulation, were only half the normal level. These abnormalities occurred in both $bim^{-/-}$ strains studied and remained after removal of the *neo* cassette from the mutated *bim* locus. At birth, normal mice have very few mature T cells in the thymus, and the thymocyte composition of newborn $bim^{-/-}$ mice was normal (Fig. 2B). Hence, the reduction in pre-T cells in the adults may result from a negative feedback mechanism triggered by the abnormal accumulation of mature T cells. If the deficit involves apoptosis of $CD4^+8^+$ thymocytes, it seemed likely to require a pathway largely independent of control by Bcl-2, such as that from death receptors (*15*). This possibility could be excluded, however, because transgenic expression of a dominant-interfering mutant of FADD/MORT1, which blocks death receptor–induced apoptosis (*16*), did not affect thymus cellularity or subset composition in adult $bim^{-/-}$ mice (Fig. 2A). The deficit of $CD4^+8^+$ pre-T cells may therefore reflect either another cell death mechanism, such as one mediated by Bax, which can kill cells independent of Bcl-2 (*17*), or perhaps reduced proliferation of the precursors, because Bcl-2 family members can affect cell cycle entry (*3*).

The role of Bim in apoptosis signaling was investigated by comparing the sensitivity of lymphocytes from WT, $bim^{+/-}$, and $bim^{-/-}$ mice to eight diverse death stimuli: cytokine deprivation, γ-irradiation or treatment with the glucocorticoid dexamethasone, ionomycin (which elevates intracellular Ca^{2+} ion concentrations), phorbol 12-myristate 13-acetate (PMA), the topoisomerase-inhibitor etoposide, the microtubule-stabilizer taxol, or Fas ligand. Because Bim is thought to function by inactivating prosurvival Bcl-2 family members, we also compared lymphocytes from *bcl-2* transgenic mice, which are refractory to all these agents except Fas ligand (*15, 18*).

Bim appears to be required for certain apoptotic responses but not others. Remarkably, when cultured without cytokines (no treatment) or treated with ionomycin or taxol, purified $bim^{-/-}$ pre-T cells survived 10 to 30 times better than WT cells, almost as well as those expressing the *bcl-2* transgene (Fig. 3A). Indeed, Bim must normally be limiting for those pathways, because a gene dosage effect was revealed by the heterozygous cells, which reproducibly died at a rate intermediate between WT and $bim^{-/-}$ pre-T cells (Fig. 3A). The $bim^{-/-}$ pre-T cells were also more refractory than WT cells to dexamethasone and γ-irradiation, albeit less so than the *bcl-2* transgenic cells. They were, however, essentially as sensitive as the WT cells to treatment with PMA or Fas ligand (Fig. 3A) or etoposide. In most respects, $bim^{-/-}$ purified pre-B cells behaved similarly to the $bim^{-/-}$ thymocytes (Fig. 3B). They were resistant to culture without cytokines and to treatment with ionomycin and somewhat refractory to dexamethasone but they remained sensitive to etoposide. Unlike the thymocytes, however, the mutant pre-B cells showed no enhanced resistance to γ-radiation (Fig. 3B). Thus, a given apoptotic stimulus may be processed distinctly in different cell types. Because quiescent cells and those in cycle may differ in apoptotic signaling, we compared the responses of mature resting T and B cells with those of activated lymphoblasts to culture without cytokines. The absence of Bim augmented survival of the resting T and B cells severalfold and markedly protected both the B and T cell blasts against cytokine withdrawal (Fig. 3C). In summary, Bim is essential for responses to certain of the apoptotic stimuli that can be antagonized by Bcl-2 but is largely dispensable for others.

With age, Bim deficiency led to progressive lymphadenopathy and a systemic autoimmune disease that mirrors that described for transgenic mice expressing *bcl-2* in their B lympho-

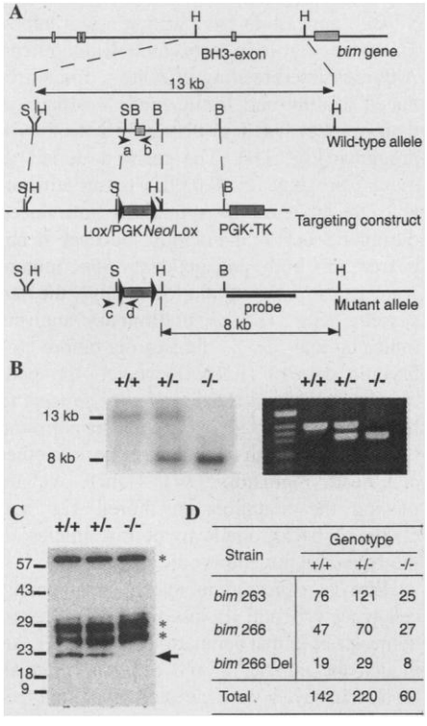

Fig. 1. Generation of Bim-deficient mice. **(A)** Mouse *bim* locus showing exons (shaded boxes) and restriction enzyme sites used to construct the targeting vector and diagnose homologous recombination. Targeting vector replaces the exon encoding the BH3 domain with the neomycin resistance cassette (Lox/pGKNeo/Lox). The external 3′ flanking probe is indicated, as are the positions of PCR primers for detecting the WT allele (A and B) and mutant allele (C and D). S, Sac I; B, Bgl II; H, Hind III. **(B)** Southern blot (left) and PCR analysis (right) of genomic DNA from WT $bim^{+/-}$ and $bim^{-/-}$ mice. **(C)** Immunoprecipitation and immunoblot analysis of proteins extracted from spleen cells of WT, $bim^{+/-}$, and $bim^{-/-}$ mice (*22*). Bim_L is indicated, and asterisks mark immunoglobulin chains and protein G from the immunoprecipitation reagents. **(D)** Genotype of offspring from crosses of $bim^{+/-}$ mice of three strains.

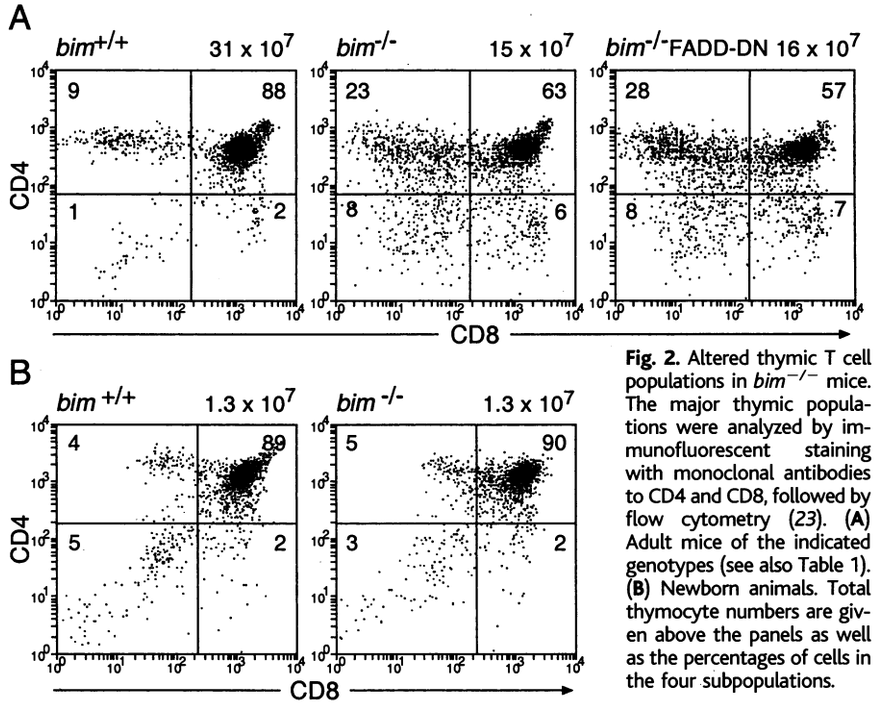

Fig. 2. Altered thymic T cell populations in $bim^{-/-}$ mice. The major thymic populations were analyzed by immunofluorescent staining with monoclonal antibodies to CD4 and CD8, followed by flow cytometry (*23*). **(A)** Adult mice of the indicated genotypes (see also Table 1). **(B)** Newborn animals. Total thymocyte numbers are given above the panels as well as the percentages of cells in the four subpopulations.

cytes (*19*). The spleen of many older *bim*$^{-/-}$ mice was enlarged five- to tenfold. Plasma cells were the cell population most dramatically increased. Although immunoglobulin M (IgM)–secreting cells were elevated about fourfold, the IgG secretors reached concentrations 30 to 200 times higher than those in WT mice (Fig. 4A). Accordingly, serum immunoglobulin increased. In *bim*$^{-/-}$ mice 6 to 12 months old, IgM was 3 times higher than normal, IgG was 10 times higher, and IgA was 3 times higher. Heterozygous mice showed smaller increases. By 1 year of age 55% of *bim*$^{-/-}$ mice had become terminally ill, and even 35% of the heterozygotes had succumbed (Fig. 4B), consistent with the notion that the amount of Bim is critical for normal immune regulation. All sick *bim*$^{-/-}$ and *bim*$^{+/-}$ mice had abnormally elevated titers of IgG autoantibodies to nuclear antigens and ~20% had antibodies to double-stranded DNA. Of the sick animals, 85% had a fatal kidney disease, diagnosed as immune complex glomerulonephritis, and 20% also exhibited histologic evidence of cardiac infarction or vasculitis. Glomerular disfunction was indicated by high serum concentrations of urea (15 to >100 mM versus the normal 9 ± 2 mM). Histological examination revealed glomerular hypercellularity, increased mesangial matrix, and eosinophilic deposits in dilated tubules (Fig. 4C). Deposits of immune complexes were implicated in the pathology by the strong granular reaction in

Table 1. Altered numbers of hematopoietic cells in *bim*$^{-/-}$ mice. The cellular composition of blood, spleen, and thymus for young adult *bim*$^{-/-}$ mice and their WT littermates was determined by cell counting and immunofluorescence staining with monoclonal antibodies specific for leukocyte subsets, followed by flow cytometry (*23*). All differences shown are statistically significant ($P < 0.05$).

Cell type	No. of cells per milliliter × 10^{-6}	
	bim$^{+/+}$	bim$^{-/-}$
Blood		
Red blood cells	7600 ± 700	8400 ± 1000
Total leukocytes	6.3 ± 0.9	22 ± 7
B cells	3.1 ± 0.5	10.7 ± 4
CD4$^+$8$^-$	1.0 ± 0.4	2.2 ± 0.7
CD4$^-$8$^+$	0.6 ± 0.2	2.2 ± 0.7
Monocytes	0.7 ± 0.2	3.0 ± 1.2
Granulocytes	0.7 ± 0.3	1.7 ± 0.5
Platelets	880 ± 100	460 ± 60
Spleen		
Total cells	160 ± 40	380 ± 110
B cells	97 ± 10	220 ± 100
CD4$^+$8$^-$	26 ± 2	69 ± 10
CD4$^-$8$^+$	13 ± 2	32 ± 5
Granulocytes	10 ± 1	32 ± 5
Thymus		
Total cells	200 ± 40	120 ± 50
CD4$^-$8$^-$	5 ± 2	12 ± 8
CD4$^+$8$^+$	170 ± 33	70 ± 23
CD4$^+$8$^-$	13 ± 4	30 ± 11
CD4$^-$8$^+$	4 ± 1	12 ± 7

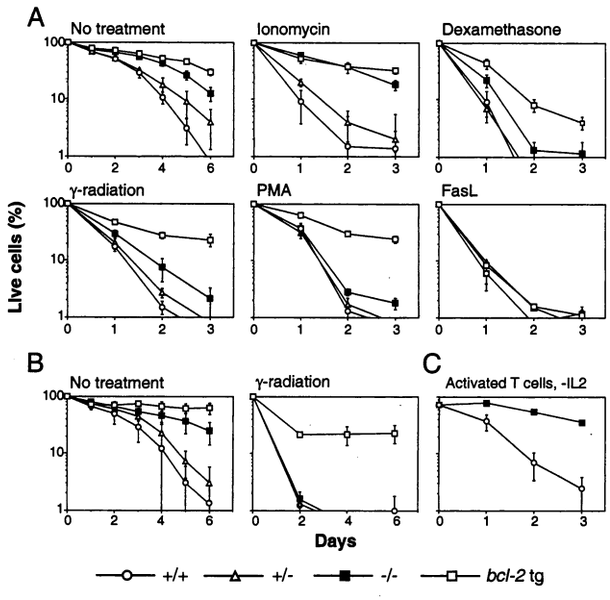

Fig. 3. Bim-deficient lymphocytes are resistant to certain apoptotic stimuli. (**A**) Tests on CD4$^+$8$^+$ pre-T cells. FACS-sorted CD4$^+$8$^+$ thymocytes from WT, *bim*$^{+/-}$ and *bim*$^{-/-}$ mice or Eμ-*bcl-2* transgenic mice were cultured in simple medium (no treatment) or treated with ionomycin (1 μg/ml), dexamethasone (10 nM), γ-irradiation (1000 rad), PMA (2 ng/ml), or cross-linked Fas ligand (100 ng/ml). (**B**) Tests on pre-B cells. FACS-sorted bone marrow pre-B cells (B220$^+$sIg$^-$CD43$^-$) were cultured in simple medium or treated with γ-irradiation (500 rad). (**C**) Test on activated T cells. Activated T lymphoblasts, prepared by culturing spleen cells for 3 days with Concanavalin A plus interleukin-2 (IL-2) and washing them free of these mitogens, were cultured for 3 days in simple medium without IL-2. (No cell death occurred in parallel cultures with IL-2.) Cell survival was quantified daily by PI staining and flow cytometric analysis. Data represent means ± SD of cells from three or four mice of each genotype.

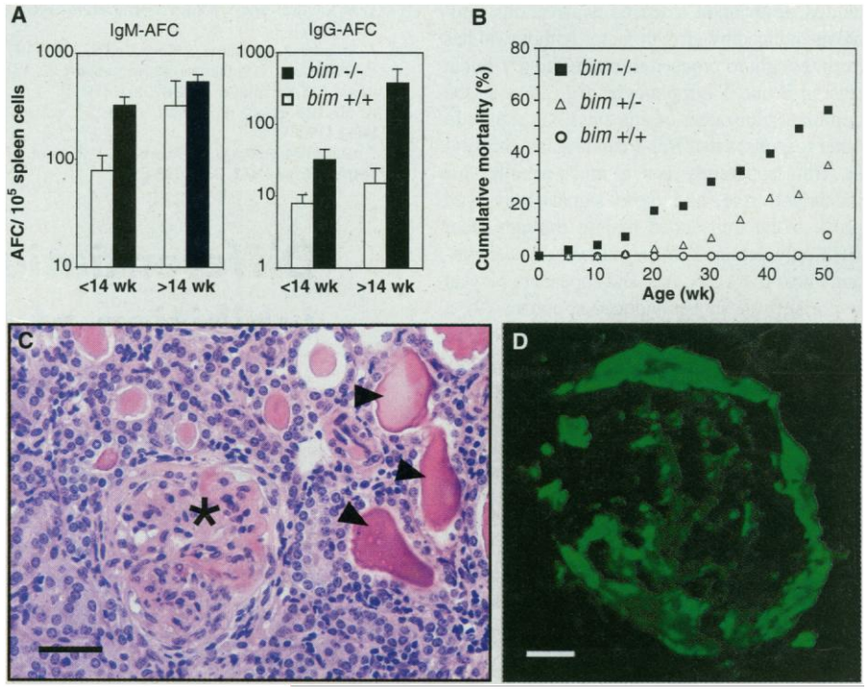

Fig. 4. Older Bim-deficient mice develop plasmacytosis and autoimmune kidney disease. (**A**) Spleen cell suspensions from four *bim*$^{-/-}$ mice (open bars) and four WT littermates (filled bars) in the indicated age categories were assayed for antibody-forming cells (AFC) by the ELISPOT technique. Data are presented as means + upper limit of standard deviation. (**B**) Cumulative mortality with age among *bim*$^{-/-}$ mice ($n = 93$) and *bim*$^{+/-}$ ($n = 94$) and WT littermates ($n = 26$). One of the latter died of unknown cause. Data from males and females were pooled, as no difference in disease incidence was apparent. (**C**) High-power view of the renal cortex of an ill *bim*$^{-/-}$ mouse showing an abnormal glomerulus (asterisk) with hypercellularity, proliferation of capsular epithelial cells, and dilated tubules containing (arrowed) eosinophilic casts (bar = 50 μm). (**D**) The presence of IgG-containing immune complexes in glomeruli, revealed by staining frozen kidney sections with FITC-labeled goat anti-mouse IgG antibodies (bar = 10 μm).

glomeruli with antibodies to IgG and the intense staining of tubular casts (Fig. 4D). Thus, the perturbed lymphocyte homeostasis produced by lack of Bim often culminates in a fatal systemic autoimmune disease.

The findings reported here indicate that, at least within the hematopoietic compartment, the BH3-only protein Bim is a key trigger of apoptosis and a critical physiological regulator of homeostasis. It influences T cell development and also may play a major role in terminating the normal immune response, because copious plasma cells accumulated in its absence, and renal failure often ensued due to a systemic autoimmune disease that has similarities to the human disorder systemic lupus erythematosus. Many of these effects, including the autoimmune disease (*19*), are highly reminiscent of those produced by overexpression of Bcl-2. These marked similarities argue that, at least for hematopoietic cells, Bim may well be the major physiological antagonist of the prosurvival proteins.

It might have been expected that a BH3-only protein would serve as the sentinel for damage to a single cellular component, but several disparate cytotoxic signals proved to be funneled through Bim. As well as microtubule perturbation (taxol) and Ca^{2+} flux, these included death upon cytokine deprivation. Apoptosis induced by growth factor withdrawal has been thought to proceed through Bad (*5*), but at least in B and T lymphocytes Bim must be the dominant transducer of this cytotoxic signal. In other responses that Bcl-2 can regulate, however, Bim apparently has a much smaller (or redundant) role, and those signals may well prove to be transduced mainly through other BH3-only proteins. In hepatocytes, but apparently not other cell types, Bid appears to play an important role in Fas-induced apoptosis (*9*), a response in which Bim appears to play no role. It thus appears likely that different BH3-only proteins are required to execute particular death responses in individual cell types.

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13. Some $bim^{-/-}$ males mated with $bim^{+/-}$ females routinely produced 50% $bim^{-/-}$ pups, but others sired hardly any $bim^{-/-}$ offspring. Because of the incomplete penetrance of the lethality, an inbred genetic background and large numbers of fetuses of different gestation ages will be required to identify the developmental defect imposed by Bim deficiency.
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22. Lysates precleared with an isotype-matched antibody (Ter119) were precipitated with rat anti-Bim monoclonal antibody 5E5 or 14A8 (*21*) and protein G–Sepharose. The washed immune complexes were fractionated electrophoretically on SDS–polyacrylamide gels and proteins transferred onto polyvinylidene difluoride membranes (Millipore). Bim protein was detected with 5E5 or 14A8, followed by a mouse anti-rat immunoglobulin reagent and ECL reaction (Amersham Pharmacia).
23. Peripheral blood erythrocytes and leukocytes were enumerated in a hemocytometer or with a ZM model Coulter counter and platelets were counted in a Sysmex NE8000 counter (TOA, Kobe, Japan). Suspensions of cells from thymus, spleen, lymph nodes, bone marrow, or blood were stained with cell type-specific monoclonal antibodies that had been purified on protein G–Sepharose and conjugated with fluorescein isothiocyanate (FITC), R-phycoerythrin (PE), or biotin (Molecular Probes), the latter detected with PE-streptavidin (Caltag). Viable [excluding propidium iodide (PI)] $CD4^+8^+$ thymocytes and B220$^+$/surface (s)IgM^-/$sIgD^-$/$CD43^-$ pre-B cells were purified in a FACStar+ or a modified FACS II cell sorter (Becton-Dickinson).

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Differentiation Stage–Specific Inhibition of the Raf-MEK-ERK Pathway by Akt

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Extracellular signals often result in simultaneous activation of both the Raf-MEK-ERK and PI3K-Akt pathways (where ERK is extracellular-regulated kinase, MEK is mitogen-activated protein kinase or ERK kinase, and PI3K is phosphatidylinositol 3-kinase). However, these two signaling pathways were shown to exert opposing effects on muscle cell hypertrophy. Furthermore, the PI3K-Akt pathway was shown to inhibit the Raf-MEK-ERK pathway; this cross-regulation depended on the differentiation state of the cell: Akt activation inhibited the Raf-MEK-ERK pathway in differentiated myotubes, but not in their myoblast precursors. The stage-specific inhibitory action of Akt correlated with its stage-specific ability to form a complex with Raf, suggesting the existence of differentially expressed mediators of an inhibitory Akt-Raf complex.

The Raf-MEK-ERK and PI3K-Akt signaling pathways are often simultaneously activated in response to growth factors and hormones. In some systems, the small guanine nucleotide binding protein Ras acts as an upstream positive effector of both the Raf-MEK-ERK pathway and the PI3K-Akt pathway (*1, 2*). However, it has also been proposed that these two pathways

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[2] **Caspases: Enemies Within**

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[3] **The Bcl-2 Protein Family: Arbiters of Cell Survival**

Jerry M. Adams; Suzanne Cory

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[19] **Enforced BCL2 Expression in B-Lymphoid Cells Prolongs Antibody Responses and Elicits Autoimmune Disease**

Andreas Strasser; Senga Whittingham; David L. Vaux; Mary L. Bath; Jerry M. Adams; Suzanne Cory; Alan W. Harris

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