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10. To ensure T cell–specific transgene expression, we constructed the $CD8^{CA}$ transgene so that it was expressed by the CD3δ promoter and enhancer (*24*). In the transgene construct, we mutated cytoplasmic cysteine residues 200 and 202 (*7*) to alanine by the polymerase chain reaction (PCR) overlap-extension method (*25*) using Lyt2.1 (*26*) as the template. Fertilized eggs from $(C57BL/6 \times C3H)F_1$ females mated to BALB/c males (Harlan Sprague-Dawley) were microinjected with $CD8^{CA}$ transgene DNA. Mice were screened for inheritance of the transgene by PCR and Southern (DNA) hybridization analysis of tail DNA. To confirm the nucleotide substitutions corresponding to the two amino acid changes, we amplified the DNA region containing the mutation by PCR from the tail DNA of the $CD8^{CA}$ founders, subcloned it into pBluescript (Stratagene), and sequenced the region by the dideoxynucleotide termination method (USB). Five founder lines were generated and two were subsequently followed. Lines 14 and 28 were bred to CD8-deficient mice (*3*) and 2C TCR transgenic mice (*13*) to generate $CD8^{CA}8^{-/-}$ ($H\text{-}2^b$) or $2C^+$ $CD8^{CA}$-$8^{-/-}$ ($H\text{-}2^b$ or $H\text{-}2^{d/b}$) mice.
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27. Thymocytes (3×10^7 to 4×10^7) from $CD8^{+/+}$, $CD8^{-/-}$, and $CD8^{CA}8^{-/-}$ mice were lysed in nonionic detergent Brij 96 buffer [1% Brij 96 (w/v) (Sigma), 20 mM tris (pH 8.0), 150 mM NaCl, 50 mM NaF, 100 μM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, leupeptin (10 μg/ml), and aprotinin (10 μg/ml)] and precleared with protein A–Sepharose CL-4B beads (Sigma). Precleared thymocyte lysates were added to protein A–Sepharose beads preadsorbed with rabbit antibodies to rat immunoglobulin G (Kappel) along with 5 μg of antibody to CD4 (anti-CD4) (GK1.5, purified from rat ascites or clone RM-4-4, PharMingen), 5 μg of antibody to CD8 (anti-CD8) (clone 53-6.7, PharMingen), or no specific antibody and allowed to form complexes. After extensive washing, the immunoprecipitates were incubated in 30 μl of kinase buffer [25 mM Hepes (pH 7.3), 0.1% Brij 96 (w/v), 10 mM $MgCl_2$, 3 mM $MnCl_2$, 30 μM Na_3VO_4, and 10 μM adenosine triphosphate (ATP) with 10 μCi of [γ-^{32}P]ATP (3000 μCi/mmol, Amersham)] for 45 min at *RT* to allow Lck autophosphorylation. For kinase assays in the presence of 10 μg of acid-denatured enolase (Boehringer Mannheim), one-fourth of the immunoprecipitates (from lysates of 3×10^7 thymocytes) were incubated in 15 μl of kinase buffer for 15 min at room temperature with constant vortexing. We stopped the kinase reactions by adding 2× sample loading buffer to the mixture, boiling it, and applying it to 10% SDS–polyacrylamide gel electrophoresis. Rainbow ^{14}C-methylated protein molecular weight markers (Amersham) were used to estimate the size of the phosphorylated bands. Protein gels were dried and exposed to XAR film (Kodak).
28. Care of mice used in the experiments was in accordance with the institutional guidelines of the Washington University School of Medicine.
29. We cultured 2×10^7 splenocyte effectors from 6- to 12-week-old mice with 2×10^7 irradiated stimulator splenocytes in 20 ml of mixed lymphocyte reaction (MLR) medium [RPMI with 10% heat-inactivated fetal calf serum and 5×10^{-5} M β-mercaptoethanol supplemented with Hepes (pH 7.3), penicillin-streptomycin, L-glutamine, nonessential amino acids, and sodium pyruvate] at 37°C for 5 days. The target splenocytes (5×10^6) were stimulated with concanavalin A (3 μg/ml) in 5 ml of MLR medium at 37°C for 2 days, purified on Histopaque 1077 (Sigma), and washed. The targets (2×10^6 to 5×10^6) were labeled with 0.5 mCi of $Na_2{}^{51}CrO_4$ (Amersham) in 1 ml of MLR medium containing 0.1 M α-methyl D-mannoside (USB) for 90 min at 37°C, washed with 0.1 M α-methyl D-mannoside in MLR medium, and left at 25°C for 30 min for spontaneous release. We plated 10^4 labeled targets in 96-well round-bottom plates (Costar). Effectors were purified on Histopaque 1077, washed, and plated at the indicated effector-to-target ratios in 200-μl reactions. For antibody blocking studies, effectors were preincubated for 30 min with either 2 μg of purified antibody to CD8α (anti-CD8α) (clone 53-6.7, Becton Dickinson) or MLR medium at 37°C before addition of labeled targets. Samples were centrifuged at 500*g* for 3 min, incubated for 3 hours at 37°C for cytolytic activity, and harvested with a supernatant collection system (Skatron). We measured ^{51}Cr release for each sample by γ-counting. The average of duplicate samples was determined and the percent of specific ^{51}Cr release was calculated from the following formula: 100 × (experimental − spontaneous release) × (total − spontaneous release)$^{-1}$. The data are representative of two experiments.
30. We thank N. Lee and E. Lacy for providing the pNeZ vector and the Lyt2.1 clone; A. Shaw for advice on enolase assays; L. Dustin and L. Fields for reagents; L. Blackburn for animal husbandry; members of the Loh laboratory for helpful discussions; and A. Cheng, L. Dustin, and K.-I. Nakayama for valuable comments. Supported by the Howard Hughes Medical Institute, NIH grant AI 155322-13, the Human Frontier Science Program (D.Y.L. and T.W.M.) and Medical Scientist Training Program training grants (I.T.C. and E.D.B.).

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Disappearance of the Lymphoid System in Bcl-2 Homozygous Mutant Chimeric Mice

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The *bcl-2* proto-oncogene can prevent the death of many cell types. Mice were generated that were chimeric for the homozygous inactivation of *bcl-2*. Lymphocytes without Bcl-2 differentiated into phenotypically mature cells. However, in vitro, the mature T cells that lacked Bcl-2 had shorter life-spans and increased sensitivity to glucocorticoids and γ-irradiation. In contrast, stimulation of CD3 inhibited the death of these cells. T and B cells with no Bcl-2 disappeared from the bone marrow, thymus, and periphery by 4 weeks of age. Thus, Bcl-2 was dispensable for lymphocyte maturation, but was required for a stable immune system after birth.

The *bcl-2* proto-oncogene was initially isolated from the breakpoint of the t(14;18) chromosomal translocation found in over 60% of human follicular lymphomas (*1*). As a result of the translocation, Bcl-2 expression in B cells is aberrantly increased (*2*). The deregulated Bcl-2 promotes cell survival and prevents programmed cell death in many, but not all, types of cells (*3*). The topographical distribution of Bcl-2 protein in tissues characterized by apoptotic cell death also suggests a potential role for Bcl-2 in programmed cell death (*4*). Cells derived from transgenic mice that have deregulated and overexpressed *bcl-2* displayed extended B and T cell survival (*5*, *6*). These transgenic studies suggested that Bcl-2 may play an important role in B cell and T cell differentiation as well as in the maintenance of B cell memory (*6*, *7*).

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Human *bcl-2* has been shown to rescue cells normally destined to die by programmed cell death in the roundworm *Caenorhabditis elegans* (8). The genotypic and phenotypic characteristics of the *ced*-9 mutant in *C. elegans* appear similar to that of mammalian *bcl-2* (9). These findings implicate the general importance of Bcl-2 or its homolog throughout phylogeny. Because all of the mammalian studies on *bcl-2* derive from descriptive studies of Bcl-2 expression or from forced inappropriate expression, the *bcl-2* loss-of-function studies were performed to address the physiological function of Bcl-2 in mammals.

To evaluate the function of Bcl-2 on lymphocyte development and function, we adopted the "double-knockout" method (*10, 11*). This approach may permit the avoidance of possible early lethality resulting from homozygous mutations because of the presence of blastocyst-derived wild-type cells. Homozygous null mutations of the two *bcl-2* alleles were introduced in embryonic stem (ES) cells by sequential homologous recombinations (Fig. 1, A to C) (*12*). Homologous recombination events were screened by polymerase chain reaction (PCR) and verified by Southern (DNA) blot analysis (Fig. 1D) (*12*). Three independent ES cell lines (from mouse strain 129) bearing homozygous mutations at the *bcl-2* locus (*bcl-2*$^{-/-}$) from two distinct parental ES cell lines, E14 and D3, were obtained. We injected the mutant ES cells (*bcl-2*$^{-/-}$) or parental ES cells (*bcl-2*$^{+/+}$) into mouse strain C57BL/6 (B6) blastocysts (*bcl-2*$^{+/+}$) and generated chimeric mice (designated as knockout chimera or control chimera, respectively). Blastocyst-derived cells can provide an experimental internal control from the same animal as long as they are phenotypically distinguishable from the ES-derived cells. The ES cell– and blastocyst-derived T and B lymphocytes in the chimeric mice could be distinguished with a monoclonal antibody (mAb) to Ly-9.1, a cell-surface marker that is found on 129, but not C57BL/6, strain cells (*10, 13*). In order to independently confirm our findings, *bcl-2*$^{-/-}$ ES cells were injected in some experiments into RAG-2–deficient blastocysts in which only the ES-derived lymphocytes may contribute to generation of T and B cells in the chimeric mice (*11*). In the Southern blot analysis shown in Fig. 1D, purified Ly-9.1^{+} thymocytes show a pattern identical to that of the injected ES cells (*bcl-2*$^{-/-}$), indicating that the Ly-9.1^{+} cells were derived from the injected ES cells (*bcl-2*$^{-/-}$). To confirm that the Ly-9.1^{+} cells do not have *bcl-2* transcripts, RNA prepared from purified Ly-9.1^{+} cells was subjected to reverse-transcribed PCR (*14*). Compared with signal from Ly-9.1^{-} thymocytes, the *bcl-2* transcript in Ly-9.1^{+} cells could not be detected, whereas amounts of control transcript (CD28 transcript) were equivalent (Fig. 1E).

Cells from lymphoid organs and bone marrow of the chimeric mice (2 weeks old) were stained for three-color flow cytofluorometric analysis with antibodies to CD4, CD8, αβ T cell receptor (TCR), pgp-1, heat-stable antigen (HSA), interleukin-2 receptor α-chain (p55), B220, immunoglobulin (Ig) M or IgD, and with antibodies to Ly-9.1. Figure 2 shows a comparison of the Ly-9.1 expression pattern on thymocytes from a C57BL/6 mouse, a 129 mouse, and a representative chimera. Ly-9.1^{-} (B6 blastocyst–derived; *bcl-2*$^{+/+}$) and Ly-9.1^{+} (ES cell–derived; *bcl-2*$^{-/-}$) cells from thymus, spleen, lymph node, peripheral blood, and bone marrow of the 2-week-old knockout chimeric mice showed almost identical staining patterns for CD4/CD8 and B220/IgM (*15*). In addition, there was no difference in expression of TCRαβ, pgp-1, HSA, p55, or IgD (*15*). These findings were also confirmed by using the chimeric mice produced with *bcl-2*–deficient ES cells and RAG-2–deficient blastocysts in which only ES-derived lymphocytes can mature (*11, 15*). These results suggest that normal phenotypic maturation of T and B cells do not require Bcl-2, although studies that used transgenic mice that overexpressed Bcl-2 postulated its participation in lymphocyte development (*3, 5–7*). Because the thymocyte subpopulation distribution appears grossly normal, it is unlikely that the absence of Bcl-2 plays a significant role in positive and negative thymocyte selection. We conclude that the up-regulation of Bcl-2 expression observed in the mature T cells is a result and not the cause of thymocyte development.

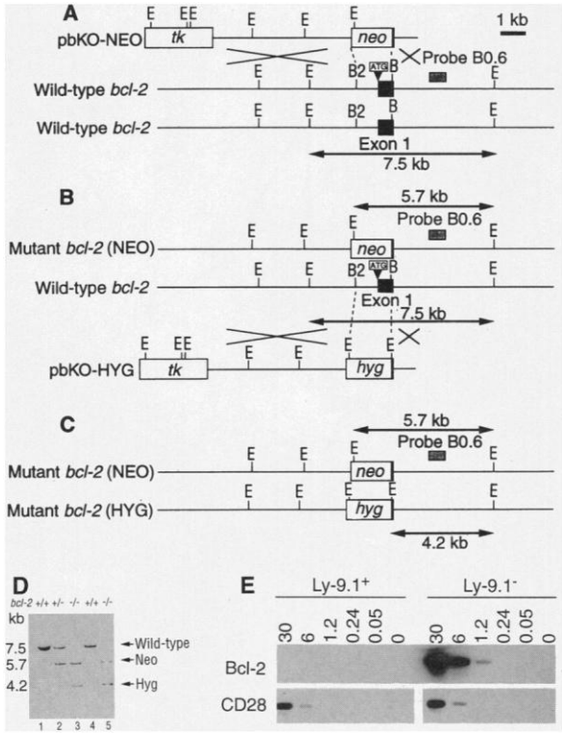

Fig. 1. Gene targeting of the mouse *bcl-2* gene in ES cells. (**A**) Genomic structure surrounding exon 1 of mouse *bcl-2*, and structure of the pbKO-NEO targeting vector. (**B**) Predicted structure of the singly targeted *bcl-2* locus, and structure of the pbKO-HYG targeting vector. (**C**) Predicted structure of the doubly targeted *bcl-2* locus. Arrowheads indicate the translation initiation site. The location of the hybridization probe (B0.6), a 0.6-kb Xba I–Eco RI fragment (the Eco RI was derived from the arm of λ FIX II phage), is shown. Expected sizes of the Eco RV fragments that hybridize with the B0.6 probe are indicated. E, Eco RV; B, Bam HI; B2, Bgl II. The restriction map of Bam HI is not complete. (**D**) Southern blot analysis of ES cells (lanes 1 to 3) and lymphocytes from the chimeric mice (lanes 4 and 5) with the B0.6 probe. Genomic DNA was digested by Eco RV. Lane 1, E14 (*bcl-2*$^{+/+}$); lane 2, EC1401 (*bcl-2*$^{+/-}$); lane 3, ECG331 (*bcl-2*$^{-/-}$); lane 4, Ly-9.1^{-} thymocytes (*bcl-2*$^{+/+}$); lane 5, Ly-9.1^{+} thymocytes (*bcl-2*$^{-/-}$) (*14*). Expected sizes for wild-type *bcl-2*, mutant *bcl-2* by pbKO-NEO, and mutant *bcl-2* gene by pbKO-HYG are shown at left. (**E**) Southern blot analysis of reverse-transcribed PCR products (*14*). Approximate numbers of cells (× 10^{-3}) used for PCR amplification are indicated above each lane. The *bcl-2* transcripts (upper) and the control CD28 transcripts (lower) in Ly-9.1^{+} or Ly-9.1^{-} thymocytes are shown.

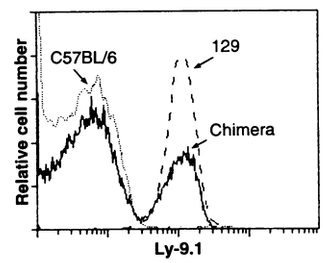

Fig. 2. Flow cytometric analysis of Ly-9.1 expression. Thymocytes from C57BL/6 (fine dotted line), 129 (coarse dotted line), and a representative chimera (solid line) were stained with fluorescein isothiocyanate (FITC)–conjugated anti–Ly-9.1 (PharMingen, San Diego, California). Flow cytometric analysis was performed by FACScan (Becton Dickinson, Mountain View, California).

Murine mature T cells have a relatively long life-span (half-life, 10 months), whereas the half-life of immature T cells has been estimated to be 3.5 days (*16*). Among T cells, Bcl-2 protein is predominantly expressed in mature T cells, but not in immature T cells (*4*). To test if Bcl-2 contributes to the longevity and resistance to apoptosis of mature lymphocytes, spleen cells from a knockout or a control chimeric mouse were placed in in vitro culture (Fig. 3). Blastocyst-derived T and B cells (*bcl-2*$^{+/+}$) from the two kinds of chimeric mice showed no significant differences in survival (*15*). In contrast, *bcl-2*$^{-/-}$ ES-derived T cells had a shorter life-span than *bcl-2*$^{+/+}$ ES-derived T cells or blastocyst-derived T cells. A significant increase of DNA fragmentation was accompanied with the loss of *bcl-2*$^{-/-}$ T cells, suggesting that these cell deaths occurred by apoptosis (*15*). Similar results were obtained from the analysis of TCR^{hi} (mature) thymocytes, whereas there was virtually no difference in the survival of TCR$^{lo/med}$ (immature) thymocytes (*15*). In contrast, no obvious in vitro survival disadvantage for *bcl-2*$^{-/-}$ B cells was found (Fig. 3).

Transgenic mouse models have suggested that susceptibility to apoptosis is developmentally regulated, such that mature cells are relatively resistant compared to the immature cells (*6*). To test if Bcl-2 expression correlates with the acquisition of resistance to apoptosis, thymocytes and spleen T cells from the chimeric mice were exposed to glucocorticoid or γ-irradiation, whose effect is blocked by overexpression of Bcl-2 (*6*). The TCR^{hi} thymocytes and spleen T cells derived from *bcl-2*$^{-/-}$ ES cells were much more sensitive to dexamethasone or γ-irradiation than those derived from C57BL/6 or *bcl-2*$^{+/+}$ ES cells (Fig. 4). The sensitivity of the *bcl-2*$^{-/-}$ mature T cells to these treatments was similar to that of immature T cells. These data demonstrate that the presence of Bcl-2 in mature T cells is indispensable for the resistance to apoptosis induced by glucocorticoid and γ-irradiation.

Mature T cells have been shown to be relatively resistant not only to glucocorticoid and γ-irradiation but also to TCR stimulation by mAb to CD3 (anti-CD3). We tested the effect of anti-CD3 on *bcl-2*$^{+/+}$ or *bcl-2*$^{-/-}$ peripheral T cells. Anti-CD3 treatment of *bcl-2*$^{-/-}$ mature T cells inhibited the acceleration of death, whereas anti-CD3 induced the marginal loss of *bcl-2*$^{+/+}$ T cells (Fig. 5). The prevention of death by TCR stimulation suggested the presence of a novel mechanism to inhibit cell death by TCR signaling. Such signaling may be of importance in the prevention of programmed cell death as the thymocytes undergo positive selection during development. In addition, our findings indicated that the characteristics of the induction of cell death to immature T cells by glucocorticoid, γ-irradiation, or anti-CD3 stimulation are distinct, based on the observation from p53 and Bcl-2 knockout mice (*17*).

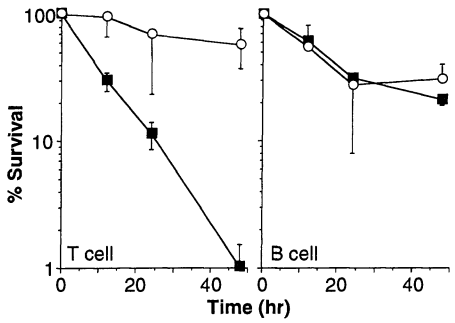

Fig. 3. In vitro cell survival experiment using spleen cells from knockout and control chimeras. Spleen cells from each chimeric mouse were placed in in vitro culture for the indicated times (*18*). Viable cells were counted and analyzed by fluorescence-activated cell sorting to obtain profiles of Ly-9.1–FITC/TCRαβ–phycoerythrin (PE) and Ly-9.1–FITC/B220-PE staining. The cell number of each population was calculated as (total viable cell number) × (percentage of the population in viable cells). The cell number at time 0 hours was defined as 100. The results for T and B cells from control chimeras (open circles) and from knockout chimeras (closed squares) are shown. Experiments were done using three knockout and three control chimeras, from which means (shown as symbols) and standard deviations (bars) were calculated. Experiments were performed three times using different independent ES cell lines (n = 9) and control parental ES cell lines (n = 9) with similar results.

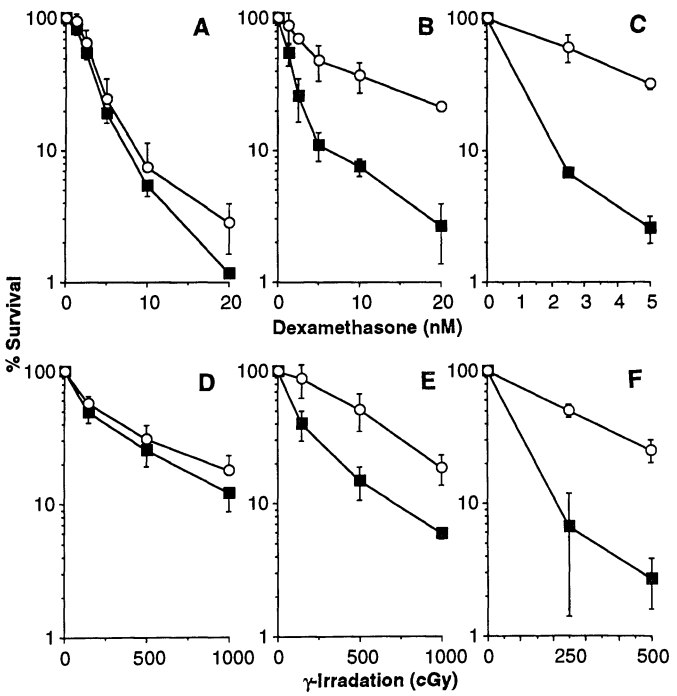

Fig. 4. Sensitivity of *bcl-2*$^{+/+}$ and *bcl-2*$^{-/-}$ T cells to dexamethasone or γ-irradiation. (**A** to **C**) Cells were exposed for 20 hours to dexamethasone at the indicated concentrations. (**D** to **F**) Cells from knockout and control chimeras were subjected to ionizing γ-irradiation at the indicated doses and then placed in culture for 20 hours. Open circles and closed squares show T cells derived from *bcl-2*$^{+/+}$ and *bcl-2*$^{-/-}$ ES cells, respectively. The cell number at 20 hours without dexamethasone or γ-irradiation was defined as 100. The percent survivals of TCR$^{lo/med}$ immature thymocytes (A and D), TCR^{hi} mature thymocytes (B and E), and spleen T cells (C and F) are shown. Experiments were done using three knockout and three control chimeras, from which means (shown as symbols) and standard deviations (bars) were calculated. Experiments were performed three times using different independent ES cell lines (n = 9) and control parental ES cell lines (n = 9) with similar results.

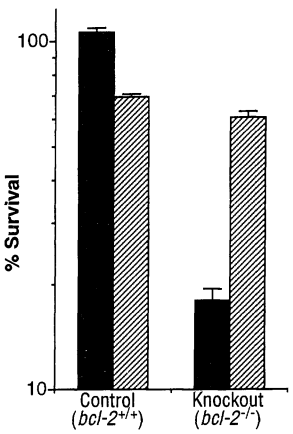

Fig. 5. Inhibition of cell death of *bcl-2*$^{-/-}$ mature T cells by anti-CD3 stimulation. Lymph node cells from control and knockout chimeras were placed in culture for 20 hours with (striped columns) or without (solid columns) 1 μg/ml of anti-CD3 (145-2C11, PharMingen). Experiments were done using three knockout and three control chimeras, from which means (shown as columns) and standard deviations (bars) were calculated. Experiments were performed three times using different independent ES cell lines (n = 9) and control parental ES cell lines (n = 9) with similar results.

To test whether *bcl-2*$^{-/-}$ lymphocytes showed any abnormalities in vivo, we initially examined the ratio of *bcl-2*$^{-/-}$ (Ly-9.1$^+$) to *bcl-2*$^{+/+}$ (Ly-9.1$^-$) cells in the peripheral blood at 2, 3, and 4 weeks of age. At 2 weeks, peripheral lymphoid organs appeared normal in size and morphology in chimeric mice. However, the percentage of the *bcl-2*$^{-/-}$ T and B cells rapidly decreased between 2 and 4 weeks postnatally (Fig. 6). In contrast, the percentage of Ly-9.1$^+$ T and B cells derived from *bcl-2*$^{+/+}$ ES cells did not change significantly (Fig. 6). In addition, we examined the content of Ly-9.1$^+$ cells in bone marrow, thymus, and lymph node from young (2 weeks old) and old (6 to 8 weeks old) knockout and control chimeras (Table 1). There was no significant difference in the percentage of Ly-9.1$^+$ cells, including immature and mature thymocytes, peripheral T cells, pre-B cells, and B cells from young and old control chimeras. In contrast, the percentage of Ly-9.1$^+$ cells from knockout chimeras decreased with time. Not only did mature *bcl-2*$^{-/-}$ T and B cells vanish, but *bcl-2*$^{-/-}$ immature thymocytes and pre-B cells also disappeared. Therefore, the abnormality among the *bcl-2*$^{-/-}$ cells must also affect the generation of lymphoid precursor cells.

Taken together with the in vitro findings, the disappearance of *bcl-2*$^{-/-}$ T cells appears to be a combination of a shortened life-span for peripheral T cells and the decreased production of the precursor cells. Because shortening of the in vitro life-span in *bcl-2*$^{-/-}$ B cells was not obvious, the decreased B cell population may be attributable solely to the decreased production of the precursor cells. This may explain the finding that the decrease of the *bcl-2*$^{-/-}$ peripheral B cells in the knockout chimera was not as dramatic as in the T cells (Fig. 6).

Most recently, the chimeric mice that carry the inactivated *bcl-2* alleles were bred to generate mice with only the mutated *bcl-2* alleles in their germline. Gross examination of these *bcl-2*$^{-/-}$ mice revealed normal development, though smaller, for at least the first 4 weeks of life. At 2 weeks of age, phenotypically mature lymphocytes were present, although they had increased sensitivity to death in the vitro assays discussed above. The number of lymphocytes in vivo decreased with kinetics similar to those observed in the chimeric mice. These preliminary findings confirm the results obtained with our chimeric mice.

It has been suggested that Bcl-2 may be expressed by hematopoietic stem cells (*4*). Therefore, it is possible that such cells are severely affected by the absence of Bcl-2, the results of which only become apparent with time. Further studies on the life-span and the ability to generate lymphoid progeny of the hematopoietic stem cells will be required to delineate the exact mechanism of apparent bone marrow and thymic failure.

Table 1. Change in percentages of Ly-9.1$^+$ cells with age at different developmental stages.

Age (weeks)	*n*	T cell			B cell		
		Thymus		Lymph node	Bone marrow		Lymph node
		TCR$^{lo/med}$	TCR^{hi}		B220$^+$sIg$^-$	B220$^+$sIg$^+$	
Control chimeras							
2	12	33.0 (11.5)	38.4 (16.4)	42.4 (16.5)	42.0 (17.9)	45.5 (16.7)	36.7 (14.2)
6 to 8	7	28.9 (18.7)	40.5 (23.7)	55.1 (24.0)	49.4 (8.1)	61.8 (6.0)	48.7 (11.1)
Knockout chimeras							
2	8	34.8 (9.9)	45.8 (12.1)	44.3 (15.3)	39.3 (8.4)	55.5 (8.8)	53.0 (11.3)
6 to 8	15	0.2 (0.1)	0.4 (0.2)	0.4 (0.3)	0.2 (0.1)	1.6 (0.4)	0.9 (0.3)

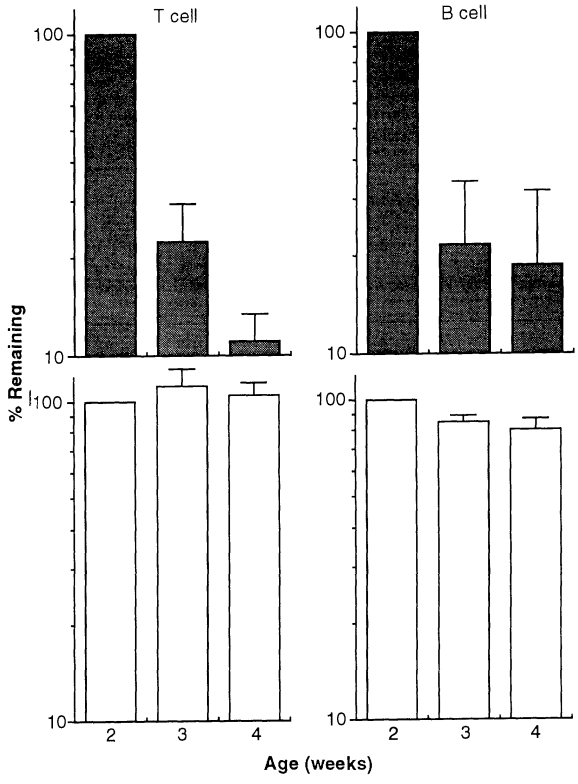

Fig. 6. Rapid decrease of *bcl-2*$^{-/-}$ peripheral blood lymphocytes in vivo. Percentage of Ly-9.1$^+$ cells at 2 weeks of age was defined as 100. Percent remaining was calculated as (percentage of the population at the indicated time)/(percentage of the population at 2 weeks of age). Percent change of Ly-9.1$^+$ T and B cells from knockout (**upper**) and control (**lower**) chimeras is shown. Experiments were done using seven knockout and four control chimeras, from which means (shown as columns) and standard deviations (bars) were calculated.

REFERENCES AND NOTES

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12. Cloned genomic DNA corresponding to the *bcl-2* locus was isolated from a library of strain 129 mouse DNA (Stratagene, La Jolla, CA). The targeting vectors, pbKO-NEO and pbKO-HYG, were constructed by replacement of a 1.4-kb Bgl II–Bam HI fragment containing most of the *bcl-2* coding region [M. Negrini, E. Silini, C. Kozak, Y.

Tsujimoto, C. M. Croce, *Cell* **49**, 455 (1987)] with either a PGK-*neo*-polyadenylate [poly(A)] cassette derived from pKJ-1 [V. L. Tybulewicz, C. E. Crawford, P. K. Jackson, R. T. Bronson, R. C. Mulligan, *Cell* **65**, 1153 (1991)] or a PGK-hyg-poly(A) cassette (*11*), respectively. The targeting vectors contained 6.5 kb of homology 5′ and 1.1 kb 3′ of the drug-resistance marker. The PGK-*tk*-poly(A) cassette (V. L. Tybulewicz *et al.*, as above) was ligated into a restriction site in a vector polylinker at the 5′ end of the insert. E14 and D3 ES cell lines were maintained as described [E. J. Robertson, *Embryo-Derived Stem Cell Lines: Teratocarcinomas and Embryonic Stem Cells: A Practical Approach*, E. J. Robertson Ed. (IRL Press, Oxford, 1987)]. A transfection was performed using pbKO-NEO, then homologous recombinants were further transfected with pbKO-HYG. G418 selection (300 μg/ml; Gibco BRL, Gaithersburg, MD) and gancyclovir selection (2 μM) at the first-round transfection, or G418, hygromycin B (150 μg/ml; Sigma, St. Louis, MO), and gancyclovir selection at the second-round transfection was started 24 hours later. Cell lysate of the clones was used as a template for PCR amplifications with a *bcl-2* flanking primer (5′-ATTCGTTCTCTTTATACTACCAAGG-3′) and a PGK-promoter–specific primer (5′-TGCTAAAGCG-CATGCTCCAGACTG-3′). Frequency of the homologous recombinants was 25.7% with pbKO-NEO at first-round transfection and 20.0% with pbKO-HYG at second-round transfection.

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14. Thymocytes (4×10^7) from a knockout chimera or from a C57BL/6 mouse (Ly-9.1$^-$, negative control) were mixed with magnetic beads coated with sheep antibodies to rat IgG (Dynal, Great Neck, NY) for 30 min on ice. After magnetic separation, unbound thymocytes were mixed with anti-Ly-9.1 (rat IgG_{2a} mAb; PharMingen, San Diego, CA) for 30 min on ice, washed twice, mixed with magnetic beads coated with sheep anti-rat IgG for 30 min on ice, then magnetically separated. Unbound cells were further processed as Ly-9.1$^-$ cells. The bound cells were resuspended and separated three more times. The purity was estimated as >99%. DNA was prepared from the 3×10^6 purified Ly-9.1$^+$ thymocytes or from the Ly-9.1$^-$ thymocytes [T. Maniatis, E. F. Fritsch, J. Sambrook, *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1982)]. RNA was extracted from 3×10^6 purified Ly-9.1$^+$ thymocytes or Ly-9.1$^-$ thymocytes by the APCG single-step method [P. Chomczynski and N. Sacchi, *Anal. Biochem.* **162**, 156 (1987)]. The resulting RNA preparation was then used as a template in a cDNA synthesis reaction using $p(dN)_6$ random primers (Invitrogen, San Diego, CA). Serial dilutions (fivefold) of this cDNA were then used as templates for PCR amplifications, with primers specific for the *bcl-2* and the CD28 control. The PCR products were detected by hybridization of Southern transfers with *bcl-2* or CD28-specific oligonucleotides (5′-CAGCCTGA-GAGCAACCCAATG-3′ or 5′-GTGGTAGATAGC-AACGAGGT-3′, respectively).
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18. Assay was performed in 96-well, V-bottom plates (Costar, Cambridge, MA) with 200 μl of RPMI-1640 that contained 10% fetal calf serum (in triplicate). After the cells were harvested, they were counted and stained for flow cytofluorometric analysis.
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Immunoglobulin V_H3 Gene Products: Natural Ligands for HIV gp120

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Infection with human immunodeficiency virus–type 1 (HIV-1) depletes T cells expressing CD4 and B cells expressing immunoglobulin (Ig) V_H3 gene products. A subpopulation of normal B cells from non–HIV-infected individuals was shown to bind to HIV gp120 by means of membrane Ig; most of these B cells expressed V_H3 family Ig. Serum V_H3 IgM from uninfected individuals also avidly bound gp120. Finally, gp120 selectively induced Ig secretion by V_H3 B cells, indicating that the binding of gp120 functionally activated these cells. These results indicate that naturally occurring V_H3 Ig is a second ligand for gp120 and a candidate superantigen for V_H3 B cells.

Acquired immunodeficiency syndrome (AIDS) is characterized by dysfunction and depletion of immune cells including CD4$^+$ T cells and B cells (*1*, *2*). CD4$^+$ T cells are selectively deleted as a result of a series of events initiated by the binding of HIV to the CD4 molecule by means of the viral envelope glycoprotein gp120 (*3*). The exact mechanism by which binding leads to deletion of this population is unknown. However, several models have been proposed, including direct cytotoxicity (*4*), gp120-induced polyclonal apoptosis (*5*), and oligoclonal superantigen-mediated deletion (*6*).

We reported a clonal deficit of B cells bearing rearranged Ig V_H3 genes in individuals with AIDS (*7*). V_H3 is the largest family of Ig heavy chain variable regions consisting of 30 or more related gene segments (*8*). Analogous to the situation with CD4$^+$ T cells, a model for V_H3 B cell depletion could involve direct binding of HIV to this B cell population. We therefore examined whether HIV gp120 bound to a subpopulation of normal B cells. Mononuclear cells were obtained from fresh tonsil tissue from subjects aged 2 to 14 years as described (*9*). All of these patients were seronegative for HIV. Tonsil cells were incubated with recombinant gp120 from HIV-1_{SF2} [expressed in Chinese hamster ovary (CHO) cells], stained with antisera to HIV-1_{SF2} gp120 and lineage-specific markers, and analyzed by flow cytometry (*10*). In all eight subjects, 3 to 6% of the B cell population (CD19$^+$) bound gp120 (Fig. 1A). T cells in the tonsil served as an internal control for gp120 binding. As expected, 68% of the T cell (CD19$^-$, CD3$^+$) population bound gp120, consistent with the known abundance of CD4$^+$ T cells (Fig. 1A). In some experiments, tonsils were initially depleted of T cells by erythrocyte rosetting (*9*). This procedure did not diminish the population of CD19$^+$ cells binding gp120.

Although normal B cells do not express CD4, it was conceivable that this small population of B cells might express this molecule. However, we were unable to detect CD4 on the surface of CD19$^+$ tonsil cells (*11*). Furthermore, pretreatment of tonsil cells with Leu3a, an antibody to CD4 (anti-CD4) that blocks the binding of gp120 to CD4 (*12*), completely inhibited gp120 binding by tonsil T cells (Fig. 1B). In contrast, Leu3a did not inhibit binding of gp120 to the subpopulation of CD19$^+$ cells (Fig. 1B), demonstrating that gp120 did not bind to the B cells by means of CD4. Consistent with these results, we observed that a similar B cell subpopulation also bound nonglycosylated HIV-1_{SF2} gp120 produced in yeast (*11*), a form of gp120 that does not bind to CD4 (*13*).

We postulated that gp120 bound to these normal B cells by means of membrane Ig. To address this idea, we selectively removed cell surface Ig by pretreating tonsil cells with antibodies to κ and λ Ig light chain (anti-κ and anti-λ) before the gp120 binding assay. Endocytosis of membrane Ig with antibody to Ig decreases surface Ig by >90% (*14*); in the present experiments, such treatment removed 94% of membrane IgD (Fig. 1C). After removal of membrane Ig, a corresponding amount of gp120 binding was lost (Fig. 1D), indicating that gp120 binding was associated with membrane Ig.

To determine whether the B cells that

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