The Journal of Immunology

Functional Roles of Fas and Bcl-2-Regulated Apoptosis of T Lymphocytes

Luk Van Parijs, Andre Biuckians and Abul K. Abbas

J Immunol 1998; 160:2065-2071; ;
http://www.jimmunol.org/content/160/5/2065

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Functional Roles of Fas and Bcl-2-Regulated Apoptosis of T Lymphocytes[1]

Luk Van Parijs,[2] Andre Biuckians, and Abul K. Abbas[3]

Apoptotic cell death is an important mechanism for maintaining homeostasis in the immune system and for regulating the fates of lymphocytes following encounters with self and foreign Ags. To study the physiologic roles of the proapoptotic Fas pathway and the antiapoptotic protein, Bcl-2, in T cell maturation and homeostasis, a TCR transgene has been bred into mice lacking functional Fas and mice that express Bcl-2 constitutively. In vitro, Fas-deficient T cells are resistant to activation-induced cell death, whereas Bcl-2-overexpressing T cells are resistant to death induced by withdrawal of growth factors. In vivo, Bcl-2-overexpressing mice accumulate T cells in the thymus and peripheral lymphoid tissues in the absence of Ag, but these cells are deleted normally after Ag administration. In contrast, Fas-deficient mature T cells are present in normal numbers in the absence of Ag, but are resistant to Ag-induced deletion. Both Fas-deficient and Bcl-2 overexpressing thymocytes are deleted when exposed to transgene-encoded circulating self Ag, indicating that the pathways of apoptosis controlled by these proteins are not critical for negative selection of developing thymocytes. Moreover, deficiency of Fas, but not Bcl-2 overexpression, results in the accumulation of autoreactive T cells in peripheral lymphoid tissues. These results demonstrate that Fas and Bcl-2 regulate different pathways of apoptosis that may serve distinct functions in lymphocyte homeostasis and in the maintenance of T cell tolerance. ***The Journal of Immunology,*** **1998, 160: 2065–2071.**

Apoptosis plays an important role in shaping the repertoire of lymphocytes and in regulating the size of the mature lymphocyte pool. In the T cell compartment, nonfunctional cells as well as self-reactive cells are eliminated by apoptosis. The same process regulates the number of lymphocytes in peripheral lymphoid organs following Ag exposure, when the rapid expansion of Ag-specific cells is followed by a reduction in cell number when the Ag is cleared (1–4). The best-defined regulators of apoptosis in T cells are members of the Fas and Bcl families. Fas (CD95) induces apoptosis in activated T cells when they are repeatedly stimulated by Ag and functions to maintain T cell tolerance by deleting autoreactive cells (reviewed in 5, 6). The Bcl family proteins, particularly Bcl-2 and Bcl-x_L, prevent T cells from undergoing apoptosis when they are deprived of activating stimuli or growth factors and when they are exposed to ionizing radiation (reviewed in 7, 8).

The functional roles of Fas and Bcl proteins in the immune system have been examined in a variety of experimental models, including cell lines, as well as transgenic and mutant mice (e. g., 9–13). The results of such studies support the idea that the Fas/FasL pathway is critically involved in the elimination of mature, self-reactive lymphocytes, and Bcl proteins are important for lymphocyte survival in response to growth factors and various activating stimuli (reviewed in 14). However, whether these pathways serve distinct or overlapping functions is an issue of considerable uncertainty. Importantly, little has been done to explore the functions of these pro- and antiapoptotic proteins in the physiologic responses of homogeneous lymphocyte populations specific for defined Ags. To address this issue, we have bred a TCR transgene into *lpr* mice, which lack functional Fas (5, 6), and into mice that constitutively express Bcl-2 in T cells (11). We have analyzed the responses of these T cells to their cognate Ag in vivo, and to the Ag expressed as a "self" protein in transgenic mice. Our results support the hypothesis that Fas and Bcl-2 regulate pathways of apoptosis that are largely distinct and serve different physiologic functions.

Materials and Methods

Generation of TCR transgenic mice

All mice used were 6 to 10 wk old and were maintained in accordance with the guidelines of the Committee on Animals of Harvard Medical School and those prepared by the Committee of Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council. Mice expressing the transgenic 3A9 TCR, specific for HEL^{46-61} + $I\text{-}A^k$ (15), were kindly provided by Dr. M. Davis (Stanford University, Palo Alto, California). The 3A9 transgene was expressed on a B10.BR ($H\text{-}2^k$) background. MRL-*lpr/lpr* mice were obtained from The Jackson Laboratory, Bar Harbor, Maine. To generate TCR transgenic mice that lacked functional Fas, 3A9 TCR transgenic mice were backcrossed with MRL*lpr/lpr* mice for four to six generations. Mice expressing human Bcl-2 under the control of the EμPμ promoter element were also obtained from The Jackson Laboratory. This strain of Bcl-2 transgenic mice constitutively expresses human Bcl-2 in all immature and mature T cells (11), on a C57BL/6J ($H\text{-}2^b$) background. To generate TCR transgenic mice that carry a Bcl-2 transgene, 3A9 TCR transgenic mice ($H\text{-}2^k$) were initially crossed directly with the commercially available Bcl-2 transgenic mice ($H\text{-}2^b$). It was discovered that the T cells bearing the 3A9 TCR transgene do not mature normally in mice that are heterozygous ($H\text{-}2^{k/b}$) at the MHC locus, and $CD4^+$ T cells derived from 3A9 ($H\text{-}2^{k/b}$) mice respond poorly to HEL^{46-61} peptide (L.V.P., unpublished data). Therefore, the Bcl-2 transgene was first backcrossed onto an $H\text{-}2^k$ background (B10.BR) for two generations, until $H\text{-}2^k$ homozygous Bcl-2 transgenic mice were available to mate with 3A9 TCR transgenic mice. 3A9 mice that expressed the *lpr* mutation (3A9/*lpr*) or the *Bcl-2* transgene (3A9/Bcl-2), or were wild-type

Immunology Research Division, Department of Pathology, Brigham & Women's Hospital and Harvard Medical School, Boston, Ma 02115

Received for publication September 22, 1997. Accepted for publication November 4, 1997.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by National Institutes of Health Grants AI32531 and AI35297 to A.K.A.

[2] Current address: Department of Biology, Room 68-380, Massachusetts Institute of Technology, 77 Massachusetts Ave., Cambridge, MA 02139.

[3] Address correspondence and reprint requests to Dr. A.K. Abbas, LMRC-521, 221 Longwood Avenue, Boston, MA 02115.

0022-1767/98/$02.00

at both loci (3A9/+), and all homozygous for the H-2^k haplotype were used in the experiments presented here.

To generate TCR transgenic mice that also expressed a "self " Ag, 3A9 TCR transgenic mice were crossed with mice that expressed high serum concentrations (approximately 20 ng/ml) of soluble[4] hen egg white lysozyme (sHEL, ref. 16). sHEL transgenic mice were kindly provided by Dr. C. Goodnow (John Curtin School of Medicine, Canberra, Australia). Since the sHEL transgene was expressed on a C57BL/6 (H-2^b) background, sHEL mice were first backcrossed four generations with MRL +/+ or MRL-*lpr/lpr* mice, to generate mice that were homozygous for H-2^k, and either wildtype (sHEL/+) or Fas-mutant (sHEL/*lpr*). To generate TCR transgenic mice expressing a "self " Ag, which lacked functional Fas or expressed a Bcl-2 transgene, 3A9/lpr mice were bred with sHEL/*lpr* mice, and 3A9/Bcl-2 mice were bred with sHEL/+ mice. H-2^k homozygous 3A9 mice that expressed both HEL and mutant Fas (3A9 × HEL/*lpr*), or a Bcl-2 transgene (3A9 × HEL/Bcl-2), or control mice (3A9 × HEL/+) were used in the experiments presented here.

Genotyping TCR transgenic mice

The genotype of all mice used here was determined by PCR analysis of tail DNA. The following PCR primers were used to identify the various transgenes and mutant genes studied: 3A9 (15), 5′ GCA GTC ACC CAA AGC CCA AG 3′ and 5′ CCC CAG CTC ACC TAA CAC TG 3′, yielding a PCR product of 371 bp; lpr (13), 5′ GTA AAT AAT TGT GCT TCG TCA G 3′ and 5′ TAG AAA GCT GCA CGG GTG TG 3′, yielding PCR fragments of 212bp (*lpr*) and 184bp (wildtype); Bcl-2 (17), 5′ GCA ACT GAT GAA TGG GAG CAG TGG 3′ and 5′ GCA GAC ACT CTA TGC CTG TGT GG 3′, yielding a PCR fragment of 381 bp; sHEL (18), 5′ GAG CGT GAA CTG CGC GAA GA 3′ and 5′ TCG GTA CCC TTG CAG CGG TT 5′, yielding a PCR product of 160 bp; I-$A^{k/b}$ (19), 5′ CAT GGG CAT AGA AAG GGC AGT CTT TGA ACT 3′, 5′ CAT AGC CCC AAA TGT CTG ACC TCT GGA GAG 3′, and 5′AGT CTT CCC AGC CTT CAC ACT CAG AGG TAC 3′, yielding PCR products of 155 bp (I-A^b), as well as 210 bp and 780 bp (I-A^k).

Phenotypic analysis of TCR transgenic mice

The numbers of lymphocytes of various types present in the 3A9 mice were calculated by multiplying the total number of viable cells found in lymphoid organs by the percentage of the total cells that each lymphocyte population represented in that organ. The frequency and phenotype of lymphocytes in 3A9 mice were determined by staining an aliquot of 10^6 viable cells. The mAbs used were FITC-conjugated anti-Vβ8 and anti-CD3; phycoerythrin (PE)-conjugated anti-CD4, anti-CD8, and anti-B220; and CyChrome C (CyC)-conjugated anti-CD4 (all from PharMingen, San Diego, California). Analyses were performed on a FACScan flow cytometer (Becton Dickson, San Francisco, California).

In vitro cell death assays

To assay the induction of apoptotic cell death induced by growth factor withdrawal, naive $CD4^+$ T cells, purified using CD4 Dynabeads (Dynal A. S., Oslo, Norway) were cultured in 1 ml of RPMI 1640 supplemented with 1 mM L-glutamine, penicillin, streptomycin, nonessential amino acids, sodium pyruvate, and HEPES (all from Life Technologies, Grand Island, New York) for 1 to 3 days. At daily intervals, the level of apoptotic cell death was assessed by propidium iodide staining (20). Activation-induced cell death (AICD) was studied in T cells that had been activated by culturing 2×10^5 naive $CD4^+$ T cells in 1 ml in the presence of 2×10^6 syngeneic APCs and 1 μg/ml HEL^{46-61} peptide. After 3 days, the activated cells were collected and cultured overnight in medium containing 50 U/ml IL-2. To assay AICD, these cells were cultured with 50 U/ml IL-2 in the presence or absence of plate-bound anti-CD3 (1 μg/ml) for 24 h, and apoptosis was assessed by propidium iodide staining.

In vivo T cell deletion assay

Mice were injected i.p. with PBS or 100 μg of HEL^{46-61} peptide in a volume of 200 μl. Peptide injections were repeated daily for a total of three doses. The thymus, spleen, and lymph nodes of treated and control mice were harvested for staining and flow cytometric analysis 4 or 8 days after the first injection.

[4] Abbreviations used in this paper: s, soluble; AICD, activation-induced cell death; HEL, hen egg white lysozyme.

In vitro assay for autoreactive T cells

Graded numbers of purified $CD4^+$ T cells ($0–1 \times 10^6$) were cultured with 2×10^6 syngeneic APCs in 1 ml of media supplemented with 50 U/ml of IL-2. After 3 to 4 days, 200-μl aliquots of cells were pulsed for 6 h with [^{3}H]TdR to assay proliferation.

Results

Lymphocyte populations in TCR transgenic mice

We first asked whether Fas deficiency or Bcl-2 overexpression would alter the maturation of T cells, by comparing the numbers and phenotypes of T cells in the thymi and peripheral lymphoid tissues of 3A9/+, 3A9/*lpr*, and 3A9/Bcl-2 mice. Vβ8 staining was used to estimate the numbers of T cells expressing the transgenic TCR. Phenotypic analysis showed that 3A9/*lpr* mice have a normal distribution of immature and mature subsets in the thymus. Since the 3A9 TCR is class II MHC restricted, $CD4^+$ cells are preferentially selected in the 3A9/+ (and 3A9/*lpr*) transgenic mice. 3A9/Bcl-2 mice, in contrast, show markedly increased numbers of total thymocytes, with a significant increase in the numbers of V$\beta8^+CD4^+$ cells, as well as V$\beta8^+CD8^+$ single-positive cells (Fig. 1 and Table I). Both V$\beta8^+CD4^+$ and V$\beta8^+CD8^+$ thymocytes in these mice express the 3A9 TCR, as determined by staining with a clonotype-specific mAb (data not shown). This suggests that Bcl-2 promotes the survival of thymocytes that are not positively selected because they do not express CD4, the appropriate T cell coreceptor. It is possible that these thymocytes express a second (class I-restricted) TCR, which would allow them to complete maturation. We have not detected expression of other Vβ elements on Bcl-2 transgenic V$\beta8^+CD8^+$ thymocytes (data not shown), but they may express an endogenous TCRα-chain (20). V$\beta8^+CD8^+$ T cells are also found in significantly increased numbers in the peripheral lymphoid tissues of 3A9/Bcl-2 mice (Fig. 1 and Table I). More than 80% of these cells express the 3A9 TCR (data not shown), as well as surface markers characteristic of mature, naive T cells (L-selectin^{high}, CD69, and CD25^{low}).

The numbers and distribution of T cells expressing Vβ8 and CD4 in the peripheral lymphoid tissues is identical in both 3A9/+ and 3A9/*lpr* (Fig. 1 and Table I). In 3A9/Bcl-2 mice the numbers of these cells is significantly increased. However, in all 3A9 mouse strains, more than 80% of V$\beta8^+CD4^+$ cells also express the 3A9 TCR, and these T cells exhibit identical proliferative responses and cytokine secretion to Ag in vitro (data not shown). These findings suggest that Bcl-2 promotes the survival of T cells in the absence of antigenic stimulation because overexpression of this molecule leads to dysregulation of T cell maturation and homeostasis in vivo, but does not alter the response of T cells to Ag in vitro.

Bcl-2 and Fas regulate distinct pathways of T cell apoptosis in vitro

Before analyzing the response of 3A9 T cells to Ag in vivo, we examined whether a deficiency of Fas or overexpression of Bcl-2 would affect apoptosis induced under different conditions. T cells undergo passive cell death when they are cultured without activation signals or growth factors (21). The assay we have used for this type of cell death is to culture naive T cells in medium, without adding Ag or cytokines, and to measure apoptosis by propidium iodide staining at daily intervals. As shown in Figure 2*A*, naive 3A9/+ and 3A9/*lpr* T cells undergo apoptosis, but 3A9/Bcl-2 T cells remain viable for 3 days in culture under these conditions, demonstrating that Bcl-2 makes T cells resistant to passive cell death. Activated T cells can be induced to undergo AICD by exposing them to high concentrations of Ag or anti-TCR/CD3 Ab in vitro (13, 21–24). As shown in Figure 2*B*, activated 3A9/*lpr* T cells remain viable when cultured with high concentrations of anti-CD3,

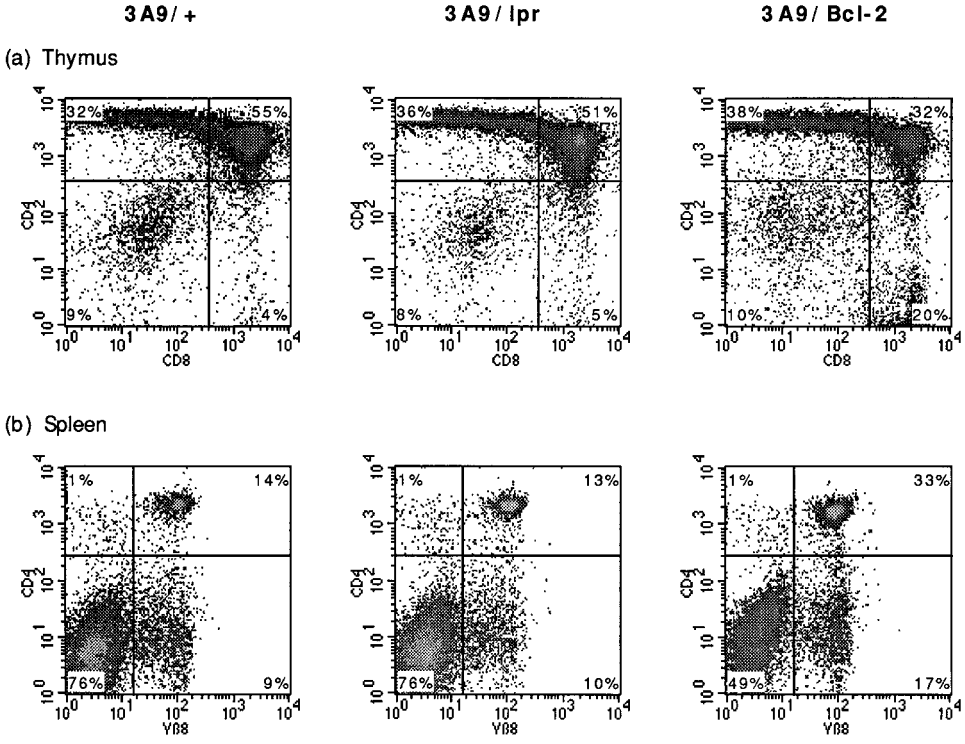

FIGURE 1. Phenotypes of T cells in the thymus and spleen of 3A9/+, 3A9/*lpr*, and 3A9/Bcl-2 mice. Thymus and spleen cells were harvested from 8-wk-old 3A9/+, *lpr*, and Bcl-2 mice, and stained with mAbs to Vβ8, CD4, and CD8. The percentage of cells in each quadrant of the dot plots is indicated.

confirming that they are resistant to AICD (13, 21). In contrast, both 3A9/+ and 3A9/Bcl-2 T cells become apoptotic in this assay, demonstrating that AICD is not blocked by Bcl-2 expression. Furthermore, 3A9/+ and 3A9/Bcl-2 T cells become apoptotic when cultured with an anti-Fas Ab, while Fas-deficient 3A9/*lpr* T cells remain viable (data not shown). These results indicate that Fas and Bcl-2 regulate different pathways of apoptosis. Therefore, TCR transgenic T cells lacking Fas or constitutively expressing Bcl-2 provide models for studying the consequences of disrupting either of these two death pathways, and thus for elucidating the functions of these death pathways in vivo.

Ag-induced deletion of mature T cells in vivo is regulated by Fas, but not Bcl-2

To compare the roles of Fas and Bcl-2 in Ag-induced deletion of specific T cells in vivo, we first studied T cell deletion in 3A9/*lpr* and 3A9/Bcl-2 mice treated with high doses of HEL^{46-61} peptide i.p. We, and others, have previously shown that this protocol leads to the deletion of the majority of TCR transgenic T cells in the thymus, spleen and lymph nodes of wild-type mice (13, 25). In 3A9 mice treated with peptide, most TCR transgenic T cells in the spleen and lymph nodes are activated by day 3 (data not shown). In wild-type mice, these peripheral Ag-specific cells are subsequently deleted by day 8 (Fig. 3). The deletion of mature Ag-specific cells is greatly reduced in the spleen and lymph nodes of 3A9/*lpr* mice. This is consistent with earlier studies showing that peripheral T cell deletion is defective in Fas-deficient mice (13). In contrast, mature Ag-specific T cells are deleted in Bcl-2 transgenic mice, demonstrating that Bcl-2 does not inhibit this process (Fig. 3). The extent of deletion in 3A9/Bcl-2 mice is typically less than that seen in wild-type mice. A significant fraction of the surviving T cells express activation markers (20–60% in Ag-treated 3A9/Bcl-2 mice, 0–30% in similarly treated 3A9/+ mice; data not shown), indicating that they have responded to Ag and are not new thymic emigrants. These cells may represent activated T cells that are not programmed to undergo AICD, and are instead eliminated by passive cell death when Ag is cleared. Because Bcl-2 plays a major role in protecting T cells from passive cell death (Fig. 2, refs. 8, 14), more activated cells would be expected to survive after peptide treatment in Bcl-2 transgenic mice than in wildtype mice.

Table I. *Thymocyte and spleen cell populations in 3A9 TCR wild-type, Fas-deficient, and Bcl-2 transgenic mice*[a]

	Thymus						Spleen		
	Total	$CD4^+CD8^+$ Cells	Total $CD4^+$ Cells	$V\beta8^+CD4^+$ Cells	Total $CD8^+$ Cells	$V\beta8^+CD8^+$ Cells	Total	$V\beta8^+CD4^+$ Cells	$V\beta8^+CD8^+$ Cells
3A9/+	93.4 ± 15.0	50.4 ± 12.3	32.2 ± 6.2	29.9 ± 7.2	4.1 ± 0.6	1.8 ± 0.9	86.1 ± 3.8	12.1 ± 3.4	7.7 ± 1.3
3A9/*lpr*	89.3 ± 12.8	46.4 ± 11.9	34.9 ± 8.9	32.4 ± 4.8	3.4 ± 1.0	1.8 ± 0.4	97.7 ± 5.1	12.6 ± 2.6	9.7 ± 3.7
3A9/Bcl-2	215.0 ± 38.2	68.8 ± 9.3	83.9 ± 14.5	76.2 ± 12.1	46.7 ± 10.4	44.0 ± 9.2	127.6 ± 29.6	41.9 ± 8.3	18.5 ± 4.4

[a] Thymi and spleens were harvested from 6- to 10-wk-old wild-type (3A9/+, $n = 12$), *lpr* (3A9/*lpr*, $n = 12$), and Bcl-2 transgenic (3A9/Bcl-2, $n = 12$) mice that expressed the 3A9 TCR transgene alone (3A9) or in conjunction with a transgene for HEL protein (3A9 × HEL). The total cell counts obtained were multiplied by the frequencies of the relevant cell populations, as determined by FACS, in order to obtain cell counts for individual cell populations. All values are $\times 10^{-6}$, and the error represents the SD of the mean obtained by averaging the results for all mice analyzed.

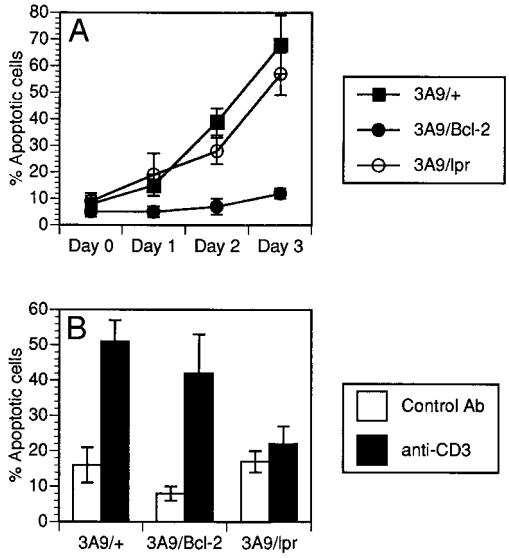

FIGURE 2. Susceptibility of 3A9/+, 3A9/*lpr*, and 3A9/Bcl-2 T cells to passive cell death and AICD. *A*, Passive cell death: naive 3A9/+, *lpr*, and Bcl-2 T cells were cultured in vitro in medium for the indicated time. *B*, AICD: activated 3A9/+, *lpr*, and Bcl-2 T cells were cultured for 24 h with anti-CD3 or control Ab in the presence of IL-2. In both cultures, apoptotic cell death was assessed by staining an aliquot of cells with propidium iodide. The results represent the average of three independent experiments, and the error bars depict the SDs calculated.

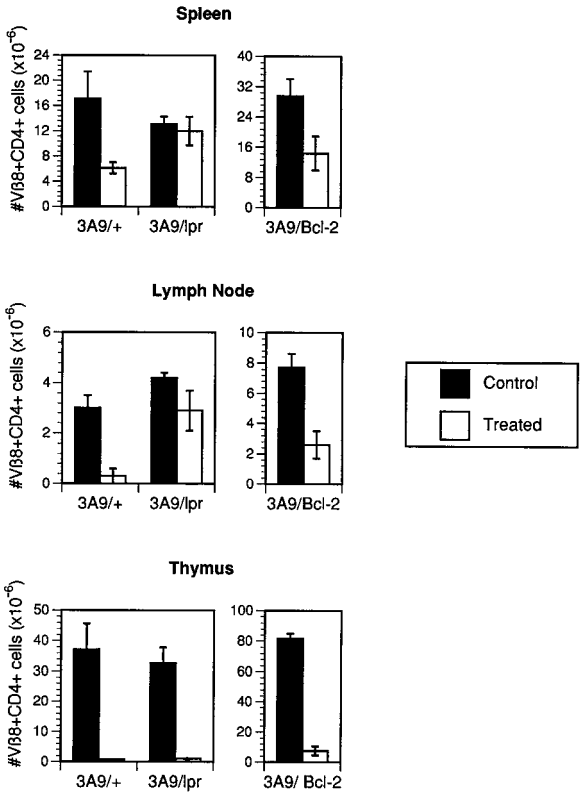

FIGURE 3. Peripheral T cell deletion is defective in 3A9/*lpr*, but not 3A9/Bcl-2, mice. 3A9/+, *lpr*, and Bcl-2 mice were treated with 100 μg HEL^{46-61} peptide or PBS i.p. once daily for 3 days. The total number of cells in the thymi, spleens, and lymph nodes of experimental mice was determined at 8 days after the first injection, and the percentage of $V\beta8^+CD4^+$ cells was assessed by flow cytometry. Total numbers of $V\beta8^+CD4^+$ cells were obtained by multiplying total cell counts by $V\beta8^+CD4^+$ percentages. Data were averaged from three experiments, and the error bars depict the SDs calculated. A total of 12 3A9/+ (6 per experimental condition), 8 3A9/*lpr* (4 per experimental condition), and 4 3A9/Bcl-2 (2 per experimental condition) mice were analyzed.

Alternatively, Bcl-2 expression may play a minor role in protecting mature T cells from AICD. This interpretation cannot be excluded but is not consistent with in vitro data with primary murine T cells (Fig. 2, ref. 26). Also, developing thymocytes are deleted by peptide administration in 3A9/+, 3A9/*lpr*, and 3A9/Bcl-2 mice, suggesting that Fas and Bcl-2 are not involved in negative selection in the thymus.

Role of Fas and Bcl-2 in thymic selection induced by an endogenous "self" Ag

A limitation of this approach is that administering a peptide Ag in multiple large doses is not a physiologic way of mimicking selection induced by a self Ag, which is present throughout the development of the animal. To circumvent this problem we have bred our HEL-specific 3A9/+, *lpr*, and Bcl-2 mice with mice that express soluble HEL (approximately 20 ng/ml) in the serum (16). A quarter of the offspring from these crosses express both the 3A9 TCR and HEL protein, and are called 3A9 × HEL mice. In these mice, HEL is effectively a self protein and leads to the deletion of the majority of TCR-bearing thymocytes. As shown in Table II, negative selection of HEL ("self" Ag)-reactive 3A9 T cells occurs equivalently in the thymus of 3A9 × HEL/+ and 3A9 × HEL/*lpr* mice. This confirms previous results reported by us and others that Fas is not required for negative selection in the thymus (13, 21, 27). The total number of thymocytes in 3A9 × HEL/Bcl-2 mice is also significantly reduced (Table II). However, the thymus in these mice is still much larger than in 3A9 × HEL wild-type or *lpr* mice. This is not due to a Bcl-2-induced block in the deletion of TCR transgenic T cells, because the reduction in $CD4^+$ T cells in the Bcl-2 transgenic mice is equivalent to that seen in wild-type and Fas-deficient mice. The fraction of surviving $CD4^+$ cells that express Vβ8 is significantly reduced after Ag exposure, suggesting that most of these $CD4^+$ cells are not HEL^{46-61} specific (Table II). An equally striking reduction in $V\beta8^{hi}$-expressing cells is seen in the double positive thymocyte population in 3A9 × HEL mice, again suggesting that the surviving cells are not Ag responsive. The increased cellularity seen in the thymi of 3A9 × HEL/Bcl-2 transgenic mice compared with wild-type and *lpr* mice results from the accumulation of a large number of thymocytes that are $CD4^-8^-$ or $CD8^+$. Presumably, these cells do not respond to HEL^{46-61} and are, therefore, not negatively selected. The increased number of double-negative thymocytes found in HEL-transgenic 3A9/Bcl-2 mice compared with nontransgenic 3A9/Bcl-2 mice is probably not Ag-dependent because these cells are mostly $V\beta8^-$ (data not shown). Instead, their increased numbers may result from their enhanced survival, due to Bcl-2 expression, as well as other compensatory mechanisms. These results indicate that Bcl-2 overexpression promotes the survival of developing thymocytes that do not recognize Ag (see also Fig. 1, Table I), but does not protect from negative selection. Since introducing the cognate Ag as a circulating protein leads to deletion of T cells in the thymus, these mice do not contain mature 3A9 T cells. Therefore, they cannot be used for studying AICD of peripheral T cells.

Autoreactive T cells and lymphoproliferation in Fas-deficient and Bcl-2-overexpressing mice

It is believed that one consequence of the failure of peripheral T cell deletion in lpr mice is the accumulation of autoreactive T cells,

Table II. *Negative selection of thymocytes in 3A9 × HEL wild-type, Fas-deficient, and Bcl-2-overexpressing mice*[a]

	Total	$CD4^+$ (% $V\beta8^+$)	$CD8^+$	$CD4^+CD8^+$ (%$V\beta8^+$)	$CD4^-CD8^-$
3A9/+	93.4 ± 15.0	32.2 ± 6.2 (93.7%)	4.1 ± 0.6	50.4 ± 12.3 (43.5%)	7.8 ± 1.5
3A9 × HEL/+	6.7 ± 3.2	1.2 ± 0.2 (36.7%)	0.4 ± 0.1	1.0 ± 0.2 (19.6%)	4.3 ± 1.9
3A9/*lpr*	89.3 ± 12.8	34.9 ± 8.9 (94.8%)	3.4 ± 1.0	46.4 ± 11.9 (39.5%)	7.4 ± 3.7
3A9 × HEL/*lpr*	9.5 ± 4.1	3.0 ± 2.1 (33.9%)	0.8 ± 0.4	1.6 ± 0.1 (12.4%)	4.8 ± 1.0
3A9/Bcl-2	215.0 ± 38.2	83.9 ± 14.5 (95.3%)	46.7 ± 10.4	68.8 ± 9.3 (42.3%)	19.2 ± 4.1
3A9 × HEL/Bcl-2	112.3 ± 34.2	12.5 ± 7.6 (46.1%)	38.6 ± 5.6	18.7 ± 2.6 (22.3%)	50.4 ± 16.3

[a] Thymi were harvested from 6- to 10-wk-old wild-type (3A9/+, $n = 12$; 3A9 × HEL/+, $n = 12$), *lpr* (3A9/*lpr*, $n = 12$; 3A9 × HEL/*lpr*, $n = 12$), and Bcl-2 transgenic (3A9/Bcl-2, $n = 12$; 3A9 × HEL/Bcl-2, $n = 6$) mice that expressed the 3A9 TCR transgene alone (3A9), or in conjunction with a transgene for HEL protein (3A9 × HEL). The total cell counts obtained were multiplied by the frequencies of the relevant cell populations, as determined by FACS, in order to obtain cell counts for individual cell populations. All values are $\times 10^{-6}$, and the error represents the SD of the mean obtained by averaging the results for all mice analyzed. The percentage of thymocytes that express $V\beta8$ was determined by triple staining with Abs to $V\beta8$, CD4, and CD8, and gating on the appropriate populations. The values given are the averages for the mice analyzed.

which rapidly expand in vitro upon culture without overt stimulation (28). Since the breedings for the mice, described in experimental procedures, also resulted in animals not expressing the 3A9 transgene, it was possible to compare the *lpr* and Bcl-2-transgenic mice for the presence of autoreactive T cells. Culture of $CD4^+$ T cells from *lpr* mice with syngeneic APCs results in the rapid outgrowth of autoreactive T cells, but this is not seen with Bcl-2 transgenic T cells (Fig. 4). Expression of the 3A9 TCR transgene prevents the expansion of autoreactive T cells in *lpr* mice (Fig. 4), presumably because of allelic exclusion of endogenous TCRs, including those that could be self-reactive. This is the likely reason why the 3A9/lpr mice do not produce anti-dsDNA autoantibodies and do not develop autoimmune disease (data not shown). These results demonstrate that Fas, but not Bcl-2, regulates the survival and/or expansion of autoreactive T cells in vivo.

One of the manifestations of autoimmunity in *lpr* mice is lymphoproliferation, due to the accumulation in peripheral lymphoid tissues of double-negative ($CD4^-CD8^-$) T cells that aberrantly express the B cell marker, B220. As shown in Table III, by 8 to 12 wk of age, *lpr* mice (not expressing the 3A9 TCR transgene) contain $CD3^+B220^+$ cells in peripheral lymphoid tissues. Although Bcl-2-overexpressing mice have enlarged lymph nodes and spleens, this is not due to an accumulation of abnormal $CD3^+B220^+$ cells. This is additional evidence that Bcl-2 overexpression does not result in manifestations of autoimmunity. Also, as seen with autoreactive T cells (Fig. 4), introducing the 3A9 TCR transgene eliminates the accumulation of double-negative cells and the lymphoproliferation of *lpr* mice (Table III). In contrast, the presence of the 3A9 TCR transgene does not prevent the accumulation of T cells in Bcl-2 transgenic mice, indicating that this expansion is not Ag-dependent.

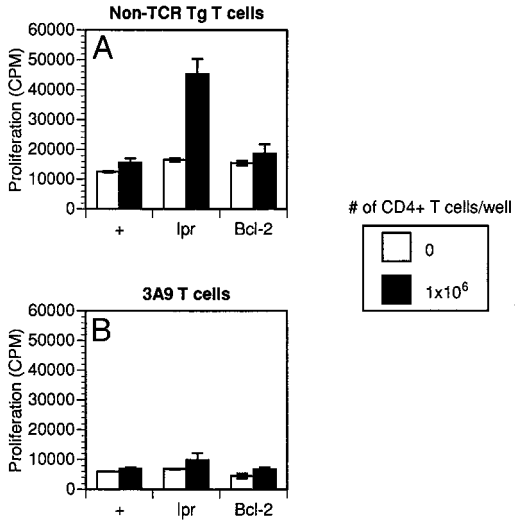

FIGURE 4. Purified $CD4^+$ T cells from *lpr*, but not Bcl-2, mice are autoreactive. Naive $CD4^+$ T cells were purified from the spleens of 3A9 and non-TCR transgenic/+, *lpr*, and Bcl-2 mice, and cultured with syngeneic APCs. Autoproliferation of non-TCR transgenic (*A*) and 3A9 (*B*) T cells was assayed by [^{3}H]TdR incorporation at 72 h. The results represent the average of triplicate wells, and the error bars depict the SDs calculated. Data shown are from one representative experiment of two performed.

Discussion

The goal of this study was to examine the regulation of apoptosis by Fas and Bcl-2 in normal T cells and to establish the roles of these proteins in vivo. This was achieved by breeding the lysozyme (HEL)-specific 3A9 TCR transgene into mice that were Fas deficient or that expressed human Bcl-2 as a transgene. Analysis of the T cells from these mice showed that, in vitro, Bcl-2 prevents T cells from undergoing passive cell death, while Fas is involved in AICD (Fig. 2). Thus, Fas and Bcl-2 regulate pathways of apoptosis that are induced by different stimuli and are presumably biochemically distinct as well. The question of whether Fas-induced and Bcl-2-regulated pathways of apoptosis are discrete or intersecting is controversial and not fully resolved. Some experiments with transfected lymphoid cell lines, and studies of hepatocytes in Bcl-2 transgenic mice, have shown that Bcl-2 can inhibit Fas-mediated apoptosis (29–31). Other experiments, however, show that Bcl-2 does not block apoptosis induced by the Fas pathway (17, 32, 26), and our results support this conclusion. It is possible that the effects of Bcl-2 depend on the levels of expression or vary in different cell types. However, our results, and those of others (17, 26), show that under conditions where Bcl-2 is functional in untransformed lymphocytes, because it blocks passive cell death, it does not prevent AICD. It is also possible that different members of the Bcl family, e.g. Bcl-2 and Bcl-x_L, may have different functional effects in T cells. However, these molecules perform identical functions when they are expressed as transgenes in T cells (33, 34).

Regardless of the mechanistic relationships between the Fas pathway and Bcl proteins, the approach we have used allows us, for the first time, to compare the biologic roles of these proteins in an Ag-specific T cell population whose activation status and functional responses can be readily manipulated by physiologic stimuli. Our studies using TCR transgenic mice with defects in Fas or overexpression of Bcl-2 reveal distinct functions of the two pathways of apoptosis. Fas-dependent AICD, which is disrupted in *lpr* mice, appears to be uniquely involved in deleting mature $CD4^+$ T cells that are stimulated by large doses of Ags (Fig. 3). Previous studies using superantigens and TCR transgenic mice are all consistent with this conclusion (e. g., 13, 21, 27). The accumulation of autoreactive T cells seen in untreated *lpr* mice (Fig. 4 and Table

Table III. *Spleen cell populations in normal and 3A9 TCR wild-type, Fas-deficient, and Bcl-2-overexpressing mice*[a]

	Total	$CD3^+$ cells	$B220^+$ cells	$CD3^+$ $B220^+$ cells
Normal mice				
MRL/+	75.1 ± 7.8	13.9 ± 2.3	63.7 ± 14.7	3.1 ± 2.2
MRL/*lpr*	111.1 ± 28.0	27.3 ± 8.1	88.5 ± 10.0	18.7 ± 5.9
Bcl-2 transgenic	117.6 ± 14.3	42.1 ± 13.2	70.7 ± 19.6	1.7 ± 1.1
3A9 TCR transgenic mice				
3A9/+	94.3 ± 12.6	22.8 ± 5.2	70.5 ± 5.1	2.7 ± 1.9
3A9/lpr	107.0 ± 19.7	26.6 ± 8.3	76.5 ± 7.8	2.4 ± 1.6
3A9/Bcl-2	153.6 ± 18.4	67.1 ± 12.7	75.5 ± 14.5	3.1 ± 2.9

[a] Spleens were harvested from 8- to 12-wk-old wild-type (MRL/+, $n = 8$; 3A9/+, $n = 12$), *lpr* (MRL/*lpr*, $n = 8$; 3A9/*lpr*, $n = 12$), and Bcl-2 transgenic (Bcl-2 transgenic, $n = 8$; 3A9/Bcl-2, $n = 6$) mice that expressed the 3A9 TCR transgene (3A9), or from transgene-negative littermates. The total cell counts obtained were multiplied by the frequencies of the relevant cell populations, as determined by FACS, in order to obtain cell counts for individual cell populations. All values are $\times 10^{-6}$, and the error represents the SD of the mean obtained by averaging the results for all mice analyzed.

III) suggests that Fas-mediated AICD also controls responses to certain self Ags. It is likely that these self Ags are abundantly and widely expressed, since AICD is induced only when numerous T cells respond coordinately to high concentrations of Ag. The pathologic consequences of defects in the Fas pathway are autoimmune reactions directed against such abundant self Ags, presumably triggered by class II-restricted autoreactive helper T cells.

In contrast to the Fas pathway of AICD, which controls responses to persistent Ag stimulation, the Bcl-2-regulated pathway of apoptosis appears to be most important for promoting the survival of lymphocytes, even cells that do not receive adequate activating stimuli. Thus, constitutive expression of Bcl-2 results in enhanced survival of naive T cells cultured without Ag (Fig. 2) and increased accumulation of T cells that are not positively selected in vivo (Fig. 1 and Table I). However, Ag-induced deletion of mature $CD4^+$ T cells is not blocked by Bcl-2, and this is reflected in the absence of autoreactive T cells in the Bcl-2 transgenic mice (Fig. 4) and the lack of an autoimmune phenotype in such mice (Table III, and data not shown).

Finally, the results presented here show that Fas and Bcl-2 do not control deletion of $CD4^+CD8^+$ thymocytes induced by high doses of Ag or by the transgenic expression of a "self" Ag (Fig. 2 and Table II). Previous studies have shown that Bcl-2 fails to inhibit thymic deletion of self-reactive $CD8^+$ T cells, as well as of thymocytes that react with endogenous superantigens (10). It has also been shown that Fas is not required for deletion of thymocytes induced by administration of cognate Ag or superantigens (13, 21, 27), although there may be particular timepoints or thymocyte subpopulations in which negative selection is Fas dependent (35, 36). Acute exposure to a peptide administered at high doses may not be the physiologic counterpart of exposure to a self Ag. Our results with a transgene-encoded endogenous "self" Ag are closer to the physiologic situation of negative selection by self Ags than the studies in which peptides, superantigens, or polyclonal activators are administered at high doses. Based on our findings (Table II), we feel it is unlikely that Fas plays an obligatory role in the self Ag-induced negative selection of the majority of thymocytes.

The identification of pathways of apoptosis in mature T cells with distinct induction and control mechanisms, and different biologic roles, leads to predictions about the pathophysiologic consequences and practical implications of disrupting or augmenting these pathways. It is likely that Bcl proteins will be the key to prolonging lymphocyte survival, which may be useful for enhancing protective immunologic memory. Modulating the Fas pathway may, on the other hand, be most useful for controlling autoreactivity.

References

1. Cohen, J. J., R. C. Duke, V. A. Fadok, and K. S. Sellins. 1992. Apoptosis and programmed cell death in immunity. *Annu. Rev. Immunol. 10:267.*
2. Green, D. R., and D. W. Scott. 1994. Activation-induced apoptosis in lymphocytes. *Curr. Opin. Immunol. 6:476.*
3. Lenardo, M. J., S. Boehme, L. Chen, B. Combadiere, G. Fisher, M. Freedman, H. McFarland, C. Pelfrey, and L. Zheng. 1995. Autocrine feedback death and the regulation of mature T lymphocyte antigen responses. *Intern. Rev. Immunol. 13:115.*
4. Goodnow, C. C. 1996. Balancing immunity and tolerance: deleting and tuning lymphocyte repertoires. *Proc. Natl. Acad. Sci. USA 93:2264.*
5. Nagata, S., and P. Golstein. 1995. The Fas death factor. *Science 267:1449.*
6. Van Parijs, L., and A. K. Abbas. 1996. Role of Fas-mediated cell death in the regulation of immune responses. *Curr. Opin. Immunol. 8:355.*
7. Yin, X. M., Z. N. Oltvai, D. J. Veis-Novack, G. P. Linette, and S. J. Korsmeyer. 1994. Bcl-2 gene family and the regulation of programmed cell death. *Cold Spring Harbor Symp. Quant. Biol. 59:387.*
8. Cory, S. 1995. Regulation of lymphocyte survival by the Bcl-2 gene family. *Annu. Rev. Immunol. 13:513.*
9. Vaux, D. L., S. Cory, and J. M. Adams. 1988. *Bcl-2* gene promotes haemopoietic cell survival and cooperates with *c-myc* to immortalize pre-B cells. *Nature 335:440.*
10. Sentman, C. L., J. R. Shutter, D. Hockenbery, O. Kanagawa, and S. J. Korsmeyer. 1991. bcl-2 inhibits multiple forms of apoptosis but not negative selection in thymocytes. *Cell 67:879.*
11. Strasser, A., A. W. Harris, and S. Cory. 1991. *bcl-2* transgene inhibits T cell death and perturbs thymic self-censorship. *Cell 67:889.*
12. Boise, L. H., M. González-García, C. E. Postema, L. Ding, T. Lindsten, L. A. Turka, X. Mao, G. Nùñez, and C. B. Thompson. 1993. *bcl-x*, a *bcl-2*-related gene that functions as a dominant regulator of apoptotic cell death. *Cell 74:597.*
13. Singer, G. G., and A. K. Abbas. 1994. The fas antigen is involved in peripheral but not thymic deletion of T lymphocytes in T cell receptor transgenic mice. *Immunity 1:365.*
14. Strasser, A. 1995. Life and death during lymphocyte development and function: evidence for two distinct killing mechanisms. *Curr. Opin. Immunol. 7:228.*
15. Ho, W. Y., M. P. Cooke, C. C. Goodnow, and M. M. Davis. 1994. Resting and anergic B cells are defective in CD28-dependent costimulation of naive $CD4^+$ T cells. *J. Exp. Med. 179:1539.*
16. Goodnow, C. C., J. Crosbie, S. Adelstein, T. B. Lavoie, S. J. Smith-Gill, R. A. Brink, H. Pritchard-Briscoe, J. S. Wotherspoon, R. H. Loblay, K. Raphael, R. J. Trent, and A. Basten. 1988. Altered immunoglobulin expression and functional silencing of self-reactive B lymphocytes in transgenic mice. *Nature 334:676.*
17. Strasser, A., A. W. Harris, D. C. S. Huang, P. H. Krammer, and S. Cory. 1995. Bcl-2 and Fas/APO-1 regulate distinct pathways to lymphocyte apoptosis. *EMBO J. 14:6136.*
18. Hartley, S. B., M. P. Cooke, D. A. Fulcher, A. W. Harris, S. Cory A. Basten, and C. C. Goodnow. 1993. Elimination of self-reactive B lymphocytes proceeds in two stages: arrested development and cell death. *Cell 72:325.*
19. Freeman, G. J., F. Borriello, R. J. Hodes, H. Reiser, K. S. Hathcock, G. Laszlo, A. J. McKnight, J. Kim, L. Du, D. B. Lombard, G. S. Gray, L. M. Nadler, A. H. Sharpe. 1993. Uncovering of functional alternative CTLA-4 counter-receptor in B7-deficient mice. *Science 262:907.*
20. Padovan, E., G. Casorati, P. Dellabona, S. Meyer, M. Brockhaus, and A. Lanzavecchia. 1993. Expression of two T cell receptor alpha chains: dual receptor T cells. *Science 262:422.*
21. Van Parijs, L., A. Ibraghimov, and A. K. Abbas. 1996. The roles of costimulation and Fas in T cell apoptosis and peripheral tolerance. *Immunity 4:321.*
22. Brunner, T., R. J. Mogil, D. LaFace, N. J. Yoo, A. Mahboubi, F. Echeverri, S. J. Martin, W. R. Force, D. H. Lynch, C. F. Ware, and D. R. Green. 1995.

Cell-autonomous Fas (CD95)/Fas-ligand interaction mediates activation-induced apoptosis in T cell hybridomas. *Nature 373:441.*
23. Dhein, J., H. Walczak, C. Baumler, K.-M. Debatin, and P. H. Krammer. 1995. Autocrine T-cell suicide mediated by APO-1/(Fas/CD95). *Nature 373:438.*
24. Ju, S.-T., D. J. Panka, H. Cui, R. Ettinger, M. El-Kharib, D. H. Sherr, B. Z. Stanger, and A. Marshak-Rothstein. 1995. Fas(CD95)/FasL interactions required for programmed cell death after T-cell activation. *Nature 373:441.*
25. Liblau, R. S., R. Tisch, K. Shokat, X. D. Yang, N. Dumont, C. C. Goodnow, and H. O. MeDevitt. 1996. Intravenous injection of soluble antigen induces thymic and peripheral T-cell apoptosis. *Proc. Natl. Acad. Sci. USA 93:3031.*
26. Moreno, M. B., S. A. Memon, and C. M. Zacharchuk. 1996. Apoptosis signaling pathways in normal T cells. *J. Immunol. 157:3845.*
27. Mogil, R. J., L. Radvanyi, R. Gonzalez-Quintial, R. Miller, G. Mills, A. N. Theofilopoulos, D. R. Green. 1995. Fas (CD95) participates in peripheral T cell deletion and associated apoptosis in vivo. *Int. Immunol. 7:1451.*
28. Weston, K. M., S.-T. Ju, C. Y. Liu, and M.-S. Sy. 1988. Autoreactive T cells in MRL/MP-*lpr*/*lpr* mice: characterization of the lymphokines produced and analysis of antigen-presenting cells required. *J. Immunol. 141:1941.*
29. Itoh, N., Y. Tsujimoto, and S. Nagata. 1993. Effect of bcl-2 on Fas antigen-mediated cell death. *J. Immunol. 151:621.*
30. Rodriguez, I., K. Matsuura, K. Khatib, J. C. Reed, S. Nagata, and P. Vassalli. 1996. A bcl-2 transgene expressed in hepatocytes protects mice from fulminant liver destruction but not from rapid death induced by anti-Fas antibody injection. *J. Exp. Med. 183:1031.*
31. Armstrong, R. C., T. Aja, J. Xiang, S. Gaur, J. F. Krebst, K. Hoang, X. Bai, S. J. Korsmeyer, D. S. Karanewsy, L. C. Fritz, and K. J. Tomaselli. 1996. Fas-induced activation of the cell death protease CPP32 is inhibited by Bcl-2 and Ice family protease inhibitors. *J. Biol. Chem. 271:16850.*
32. Memon, S. A., M. B. Moreno, D. Petrak, and C. M. Zacharchuk. 1995. Bcl-2 blocks glucocorticoid- but not Fas- or activation-induced apoptosis in a T cell hybridoma. *J. Immunol. 155:4644.*
33. Chao, D. T., G. P. Linette, L. H. Boise, L. S. White, C. B. Thompson, and S. J. Korsmeyer. 1995. Bcl-X_L and Bcl-2 repress a common pathway of cell death. *J. Exp. Med. 182:821.*
34. Grillot, D. A. M., R. Merino, and G. Nùñez. 1995. Bcl-x_L displays restricted distribution during T cell development and inhibits multiple forms of apoptosis but not clonal deletion in transgenic mice. *J. Exp. Med. 182:1973.*
35. Castro, J. E., J. A. Listman, B. A. Jacobson, Y. Wang, P. A. Lopez, S. Ju, P. W. Finn, and D. L. Perkins. 1996. Fas modulation of apoptosis during negative selection of thymocytes. *Immunity 5:617.*
36. Kishimoto, H., and J. Sprent. 1997. Negative selection in the thymus includes semimature T cells. *J. Exp. Med. 185:263.*