The Action of Bax and Bcl-2 on T Cell Selection

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Summary

T cell development and selection in the thymus are shaped by the induction of apoptosis. However, a direct role in T cell development and selection for any of the molecules known to regulate apoptosis has remained controversial. We have studied the effect of *bax* and *bcl-2* transgenes in recombination activation gene 1–deficient (RAG-$1^{-/-}$) mice transgenic for the major histocompatibility complex class I–restricted F5 T cell receptor. Overexpression of a *bax* transgene in the thymus seriously impairs the production of mature T cells, whereas *bcl-2* overexpression greatly promotes it. The effect of *bax* and *bcl-2* overexpression on antigen-induced negative selection was studied using fetal thymic organ cultures. This analysis showed that Bcl-2 strongly inhibits negative selection, whereas Bax does not affect it. Our data directly show that Bcl-2 family members have specific roles in T cell selection and also lend support to the hypothesis that Bax and Bcl-2 can antagonize each other's action in a certain apoptosis pathway while in another they can be functionally nonreciprocal.

Key words: Bax • Bcl-2 • thymic selection • apoptosis • fetal thymic organ cultures

The development of T cells in the thymus is characterized by the production of large numbers of immature thymocytes that are then subjected to stringent selection criteria. Only those thymocytes expressing a TCR capable of interacting with self-peptides presented by MHC molecules on thymic epithelial cells are positively selected and exit into the periphery (1–4). However, thymocytes with receptors that bind to self-peptide–MHC complexes with high affinity are negatively selected and die (1, 5, 6). Although negative selection of thymocytes has been shown to be mediated by the induction of apoptosis (7), any direct role that molecules known to regulate apoptosis play in the outcome of this process has remained controversial.

Many different molecules that can regulate apoptosis have now been identified. Of particular interest with regards to T cell development are the members of the Bcl-2 family (for review see reference 8). This family consists of proteins that enhance cell survival, such as Bcl-2 or Bcl-X_L, and those that promote cell death, such as Bax or Bad. Bax was isolated by virtue of its interaction with Bcl-2 with which it forms heterodimers (9). Thymocytes from mice expressing a *bcl-2* transgene in their T cells are protected against apoptosis induced by dexamethasone (10, 11). Conversely, thymocytes from mice expressing a *bax* transgene in their T cells show accelerated cell death in response to treatment with dexamethasone (12). In the thymus the Bcl-2 protein is uniformly expressed in medullary thymocytes but only in a small fraction of cortical thymocytes (13, 14). This lack of Bcl-2 correlates with the fact that most cortical thymocytes undergo programmed cell death. Furthermore, nearly all $CD4^+$ and $CD8^+$ single positive (SP)[1] thymocytes express Bcl-2, whereas the vast majority of $CD4^+CD8^+$ double positive (DP) thymocytes do not (15–18). DP thymocytes are the stage of T cell development during which the processes of positive or negative selection operate. These observations all serve to suggest that Bcl-2 upregulation in the final steps of thymocyte development may play a role in T cell selection.

Given that T cell maturation is contingent on positive and negative selection, efforts have been made to study the regulation of these processes by Bcl-2 with regard to negative selection in particular. Initial studies examined the effect of a *bcl-2* transgene on thymocytes with receptors that recognize self-superantigens. In some studies, Bcl-2 was found to inhibit superantigen-mediated deletion of autoreactive thymocytes (11, 19), whereas another study failed to demonstrate any effect of Bcl-2 on this process (10). Subsequently, two further studies sought to address the effect of Bcl-2 on negative selection using TCR transgenic mice

[1]*Abbreviations used in this paper:* 7AAD, 7-aminoactinomycin; DP, double positive; FTOC, fetal thymic organ culture; NP, nucleoprotein; RAG-1, recombination activation gene 1; SP, single positive.

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also expressing a *bcl-2* transgene (20, 21). Both used mice transgenic for a TCR reactive with the male self-antigen H-Y in the context of H-2D^b. Recognition of the H-Y antigen in male TCR transgenic mice of the H-2^b haplotype results in the deletion of most DP thymocytes (5). Strasser et al. (20) found an approximately fourfold increase in the total number of thymocytes in H-Y TCR × *bcl-2* double transgenic male mice over H-Y TCR male mice alone. They concluded that Bcl-2 reduces the efficiency of negative selection. However, Tao et al. (21) reached the opposite conclusion since although they also found an increase in the total number of thymocytes in double transgenic male mice, a large fraction of the surviving DP thymocytes expressed endogenous TCR-α chains and not the α chain of the H-Y TCR. This is at variance with Strasser et al. (20), who showed that in their mice the majority of surviving thymocytes did express the transgenic H-Y TCR and that their survival could not be explained by the expression of endogenous TCR genes.

Further doubt has been cast on the significance of *bcl-2* family members in negative selection from work using *bcl-X_L* transgenic mice (22, 23). Bcl-X_L is a close homologue of Bcl-2 that has similar protective effects against apoptotic stimuli (24) and a role in the maturation of T cells as judged from the block in T cell development found in *bcl-$X_L^{-/-}$* chimeric mice (25). T cells from *bcl-X_L* transgenic mice are protected against exposure to a variety of apoptosis-promoting stimuli. However, analysis of superantigen-mediated clonal deletion in *bcl-X_L* transgenic mice, as well as H-Y TCR × *bcl-X_L* double transgenic male mice, suggests that Bcl-X_L has no effect on negative selection (22, 23, 26).

We sought to clarify the role of *bcl-2* family members in thymic negative selection by studying the effects of *bcl-2* and *bax* transgenes on peptide-induced negative selection in fetal thymic organ culture (FTOC). The FTOCs were derived from mice transgenic for the F5 TCR (27, 28), which recognizes an influenza virus nucleoprotein (NP)-derived nonamer peptide (NP68) in the context of MHC class I H-2D^b (29). To exclude any effects due to endogenous TCR rearrangements, the mice used were also deficient for the recombination activation gene 1 (RAG-1). Negative selection of immature thymocytes in the FTOC was induced by addition of the cognate peptide and directly quantitated by measuring DNA fragmentation in the thymocytes. This approach has the advantage of allowing the direct quantitation of antigen-induced negative selection in isolation from other thymic selection events, effects on cell survival, and peripheral T cell activation.

Our study has shown that the apoptotic regulatory molecules Bax and Bcl-2 can have profound effects upon the selection of mature T cells bearing a single TCR. Bax impairs the production of mature SP T cells, whereas Bcl-2 enhances their production. We have also shown that Bcl-2 strongly inhibits negative selection, whereas Bax has no effect on this process. Therefore, for the first time we directly show that Bcl-2 can inhibit antigen-mediated negative selection and that Bax and Bcl-2 can be functionally nonreciprocal, as suggested by recent genetic studies (30).

Materials and Methods

Mice. The F5 RAG-1$^{-/-}$ mice with the *bax* or *bcl-2* transgenes were generated by serial crossing of F5 TCR transgenics, RAG-1$^{-/-}$ mice, and *bax* or *bcl-2* transgenics. The mice transgenic for the αβ-TCR from the F5 cytotoxic T cell clone were generated as previously reported (28). Mice deficient in the RAG-1 gene were obtained from Dr. Eugenia Spanopoulou (31). The *bax* transgenic mice were as described previously (12) and the bcl-2 transgenics were as also previously described (32). Breedings were done so as to obtain F5 RAG-1$^{-/-}$ mice with *bax* or *bcl-2* transgenes and homozygous for either MHC class I H-2^b or H-2^q after backcrossing with inbred C57 BL/10 mice for H-2^b and inbred SWR for H-2^q. Antibodies specific for H-2^b and H-2^q were used to determine the MHC haplotypes present. The genotyping of mice for the F5 TCR, *bax* or *bcl-2* transgenes and the presence of the RAG-1 gene was done by either Southern analysis or PCR.

Reagents and Antibodies. The nonamer peptide NP68, from the nucleoprotein of influenza virus A/NT/60/68 (NP366-374: amino acid sequence, ASNENMDAM), was synthesized on a peptide synthesizer (Applied Biosystems 430A; PE Applied Biosystems, Foster, CA). The dexamethasone used was purchased from Sigma Chemical Co. and anti-CD95 (anti-Fas) (Jo2) was purchased from PharMingen (San Diego, CA). The following mAbs and second layer reagents were used for flow cytometric analysis: FITC-conjugated YTS169.4 (anti-CD8α; reference 33), FITC-conjugated anti-mouse MHC class I H-2^q (PharMingen), phycoerythrin conjugated anti-CD4 (Sigma Chemical Co., St. Louis, MO), biotin conjugated anti-mouse MHC class I H-2^b (PharMingen), biotin conjugated KT11 (anti-V_β11; reference 34), biotin conjugated anti-CD69 (PharMingen), and streptavidin-RED 670 (GIBCO BRL, Gaithersburg, MD).

Fetal Thymic Organ Culture. Fetal thymic lobes were isolated from day 15 embryos, obtained from timed matings of homozygous F5/RAG-1$^{-/-}$ females with RAG-1$^{-/-}$ male mice heterozygous for the *bax* or *bcl-2* transgene. The presence of the *bax*/*bcl-2* transgene was determined by PCR analysis on DNA isolated from each individual embryo. The fetal thymic lobes were transferred onto nucleopore polycarbonate filters (Costar Corporation, Cambridge, MA) and cultured at 37°C, 5% CO_2 in RPMI 1640 (GIBCO BRL) supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine and antibiotics. After 4 d, the filters were transferred to medium alone or containing 10 μM NP68 peptide, 0.5 μM dexamethasone, or 1 μg/ml anti-CD95 (Jo2) antibody and cultured for 11 h. Thymocytes were then harvested for analysis by gently disrupting the thymic lobes mechanically in 1.5-ml Eppendorf tubes.

Flow Cytometry. For flow cytometric analysis, 1–5 × 10^5 cells were stained with various combinations of mAbs in PBS containing 1% BSA (Sigma Chemical Co.) and 0.1% sodium azide. For analysis of DNA content, cells were stained with 7-aminoactinomycin D (7AAD; Sigma Chemical Co.) in PBS containing 0.3% saponin (Sigma Chemical Co.), 2% FCS, and 0.1% sodium azide. Stained cells were analyzed on a FACScan® flow cytometer (Becton Dickinson, Mountain View, CA) using Cellquest software (Becton Dickinson).

Results

Effects of Bax on T Cell Development. To definitively study the effects of apoptosis regulatory molecules on T cell development we generated mice having either a *bax* (12) or a *bcl-2* (32) transgene, an F5 TCR transgene (27) on a RAG-

$1^{-/-}$ background (31), as well as being homozygous for the selecting H-2^b or nonselecting H-2^q MHC haplotypes. These mice remove all problems of interpretation due to endogenous TCR rearrangements since all the DP and SP thymocytes in these mice express only the F5 TCR.

First, we studied F5/RAG-$1^{-/-}$/*bax* transgenics on the H-$2^{b/b}$ background. As shown in Fig. 1 and Table 1, upregulated *bax* expression causes a dramatic decrease in total thymus cellularity as well as mature SP T cell production. Thymocytes were analyzed by three-color flow cytometry with antibodies against CD4, CD8, and $V_\beta11$ (recognizing the F5 TCR-β chain) or CD69 (Fig. 1). The presence of *bax* in the F5/RAG-$1^{-/-}$ mice results in a fall in total thymocyte numbers from 1.90×10^8 to 0.39×10^8 and decreases $CD4^-CD8^{hi}$ cell numbers more than sixfold from 1.26×10^7 to 0.20×10^7 (Table 1). This is also reflected in a fall in the percentage of CD8 SP ($CD4^-CD8^{hi}$) cells produced (Fig. 1). In the thymus of F5/RAG-$1^{-/-}$ mice, TCR expression is upregulated ($V_\beta11^{hi}$) during the transition from $CD4^{hi}CD8^{hi}$, through $CD4^{lo}CD8^{hi}$, to $CD4^-CD8^{hi}$ thymocytes, as shown by the shaded region in Fig. 1. However, the presence of the *bax* transgene greatly diminishes this upregulation, particularly in the transitional $CD4^{lo}CD8^{hi}$ population, though even in the $CD4^{hi}CD8^{hi}$ DP thymocytes the small proportion of $V_\beta11^{hi}$ cells is lost. A reduced level of $V_\beta11$ expression on $CD4^-CD8^{hi}$ is also found on the *bax* transgenic mice. The observed transient upregulation of CD69 (35, 36), as thymocytes pass through the $CD4^{lo}$ $CD8^{hi}$ compartment, is almost completely abolished in F5/RAG-$1^{-/-}$ mice expressing the *bax* transgene (Fig. 1).

To determine whether the reduction in total thymocyte numbers in F5/RAG-$1^{-/-}$/*bax* mice was dependent on

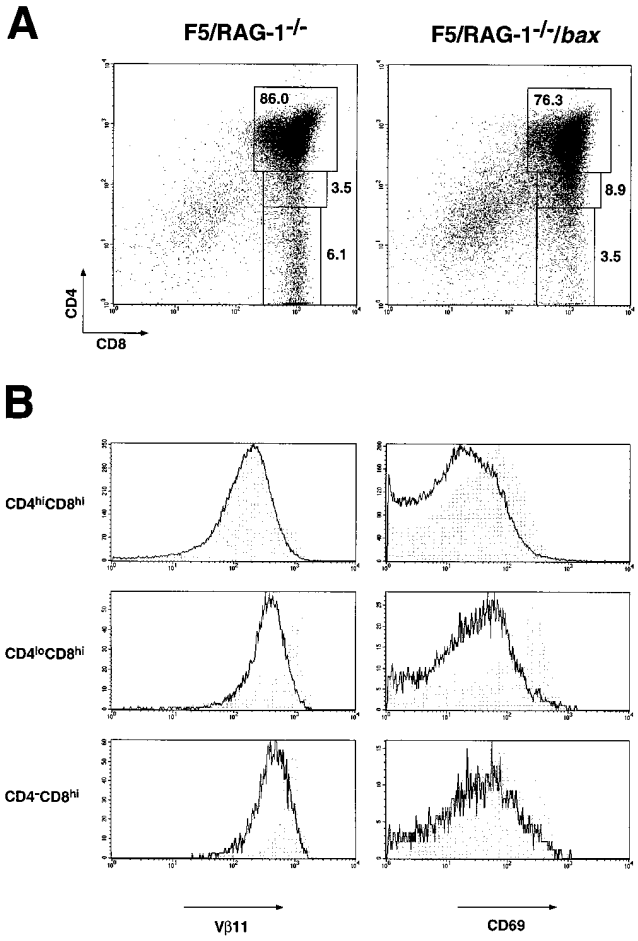

Figure 1. Flow cytometric analysis of T cell development in the thymus of F5/RAG-$1^{-/-}$/*bax* H-$2^{b/b}$ mice and littermate controls lacking the *bax* transgene. Thymocytes were isolated from F5/RAG-$1^{-/-}$/*bax* H-$2^{b/b}$ mice with or without the *bax* transgene then stained for CD4 (PE), CD8 (FITC), and either $V_\beta11$ or CD69 (both biotinylated). (*A*) Dot plots of CD4- and CD8-stained thymocytes with gates on the $CD4^{hi}CD8^{hi}$, $CD4^{lo}CD8^{hi}$, and $CD4^-CD8^{hi}$ populations. The numbers represent the percentage of the total number of thymocytes in each gate. (*B*) Histograms represent $V_\beta11$ or CD69 expression on the thymocytes in each gate. The shaded region represents F5/RAG-$1^{-/-}$ mice, whereas the black line represents the same mice plus the *bax* transgene.

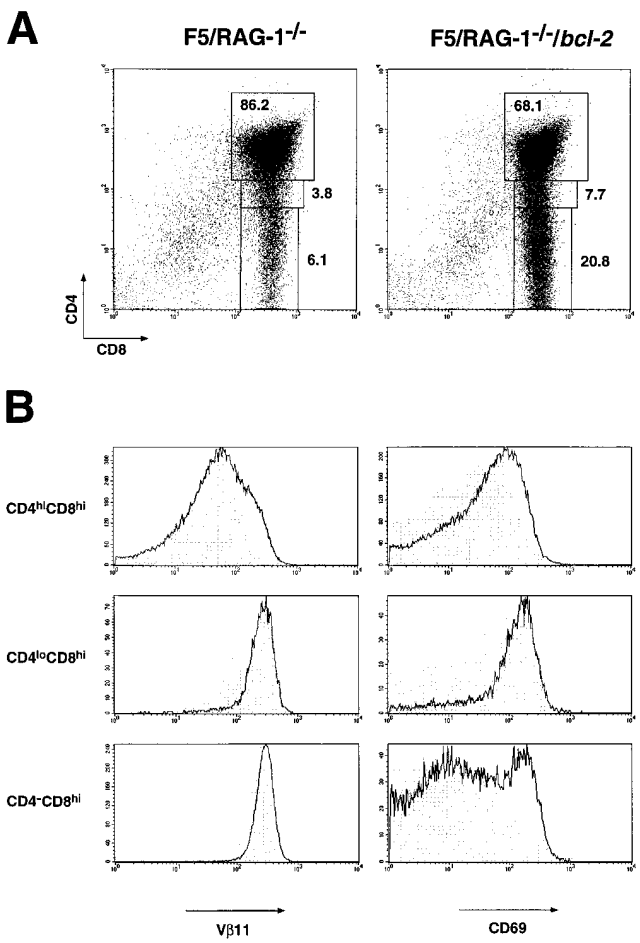

Figure 2. Flow cytometric analysis of T cell development in the thymus of F5/RAG-$1^{-/-}$/*bcl-2* H-$2^{b/b}$ mice and littermate controls lacking the *bcl-2* transgene. Thymocytes were isolated from F5/RAG-$1^{-/-}$/*bcl-2* H-$2^{b/b}$ mice with or without the *bcl-2* transgene then stained for CD4, CD8, and either $V_\beta11$ or CD69. (*A*) Dot plots of CD4- and CD8-stained thymocytes with gates on the $CD4^{hi}CD8^{hi}$, $CD4^{lo}CD8^{hi}$, and $CD4^-$ $CD8^{hi}$ populations. The numbers represent the percentage of the total number of thymocytes in each gate. (*B*) Histograms represent $V_\beta11$ or CD69 expression on the thymocytes in each gate. The shaded region represents F5/RAG-$1^{-/-}$ mice, whereas the black line represents the same mice plus the *bcl-2* transgene.

Table 1. *Effect of bax Transgene and MHC on Thymocyte Number in F5/RAG-1$^{-/-}$ Mice*

	F5/RAG-1$^{-/-}$ H-2$^{b/b}$		F5/RAG-1$^{-/-}$ H-2$^{q/q}$	
	−*bax*	+*bax*	−*bax*	+*bax*
Total thymocytes	1.90 ± 0.32	0.39 ± 0.14	2.03 ± 0.40	0.80 ± 0.16
×10^8	*n* = 6	*n* = 6	*n* = 4	*n* = 4
CD8 SP thymocytes	1.26 ± 0.25	0.20 ± 0.11	—	—
×10^7	*n* = 6	*n* = 6		

Total thymocyte numbers were calculated for age-matched and gender-matched littermates with or without the *bax* transgene. The total number of CD8 SP thymocytes was determined after FACS® analysis after staining with CD4 and CD8 antibodies. The values represent the mean of four or six (*n*) mice and the errors represent their standard deviation.

the presence of selecting MHC molecules we crossed these mice onto the H-2$^{q/q}$ background. The H-2^q MHC haplotype is nonselecting for the F5 TCR and T cell development is blocked at the DP thymocyte stage in F5/RAG-1$^{-/-}$/H-2$^{q/q}$ mice (37). The total number of thymocytes in F5/RAG-1$^{-/-}$/H-2$^{q/q}$ mice was reduced from 2.03 × 10^8 to 0.80 × 10^8 in the presence of the *bax* transgene (Table 1) showing that *bax* has an effect on the survival of thymocytes, which is independent of thymocyte selection.

Therefore, *bax* decreases the survival of thymocytes in the absence of positive selection and also has a direct effect on the selection of mature T cells expressing the MHC class I–restricted F5 TCR. The *bax* transgene leads to a great reduction in the expression levels of molecules (TCR and CD69) usually upregulated during positive selection and a substantial decrease in the number of mature T cells produced. We conclude that *bax* can directly affect the process of T cell selection.

Effects of Bcl-2 on T Cell Development. Our next question was to determine whether Bcl-2 could act in the opposite fashion to Bax and thus enhance T cell selection. Therefore, we examined the effect of a *bcl-2* transgene on F5/RAG-1$^{-/-}$ mice with either the H-2$^{b/b}$ or H-2$^{q/q}$ MHC background. As seen in Fig. 2 and Table 2, upregulated *bcl-2* expression has a profound effect on the production of mature SP T cells. The *bcl-2* transgene in F5/RAG-1$^{-/-}$/H-2$^{b/b}$ mice results in a more than fourfold increase in the total number of CD4$^-$CD8^{hi} cells from 2.64 × 10^7 to 11.37 × 10^7 (Table 2). Table 2 also shows that the presence of *bcl-2* increases the total number of thymocytes from 2.48 × 10^8 to 4.98 × 10^8. The *bcl-2* transgene also leads to a greater than twofold increase in the percentage of CD8 SP (CD4$^-$CD8^{hi}) thymocytes produced (Fig. 2).

In contrast to *bax* transgenic mice, the *bcl-2* transgene leads to enhanced upregulation of TCR and CD69 expression during T cell development (Fig. 2). A higher percentage of CD4^{hi}CD8^{hi} cells in the *bcl-2* transgenic mice have high levels of V_β11 and CD69 expression. Similarly, virtually all the CD4^{lo}CD8^{hi} thymocytes are V_β11^{hi} and CD69^{hi} in the *bcl-2* transgenic mice as opposed to the control F5/RAG-1$^{-/-}$ mice. However, mature T cells in both control and *bcl-2* transgenic mice express similar levels of the F5 TCR.

The effect of the *bcl-2* transgene was also determined on the nonselecting H-2^q MHC haplotype. The total number of thymocytes was increased in the F5/RAG-1$^{-/-}$/*bcl-2* transgenic mice from 1.78 × 10^8 to 3.60 × 10^8 (Table 2). Bcl-2 can thus increase the survival of DP thymocytes even in the absence of positive selection. However, Bcl-2 also increases the number of mature SP T cells produced when

Table 2. *Effect of bcl-2 Transgene and MHC on Thymocyte Number in F5/RAG-1$^{-/-}$ Mice*

	F5/RAG-1$^{-/-}$ H-2$^{b/b}$		F5/RAG-1$^{-/-}$ H-2$^{q/q}$	
	−*bcl*-2	+*bcl*-2	−*bcl*-2	+*bcl*-2
Total thymocytes	2.48 ± 0.36	4.98 ± 1.15	1.78 ± 0.22	3.60 ± 0.52
×10^8	*n* = 4	*n* = 4	*n* = 4	*n* = 4
CD8 SP thymocytes	2.64 ± 0.65	11.37 ± 2.18	—	—
×10^7	*n* = 4	*n* = 4		

Total thymocyte numbers were calculated for age-matched and gender-matched littermates with or without the *bcl*-2 transgene.The total number of CD8 SP thymocytes was determined following FACS® analysis after staining with CD4 and CD8 antibodies. The values represent the mean of four or six (*n*) mice and the errors represent their standard deviation.

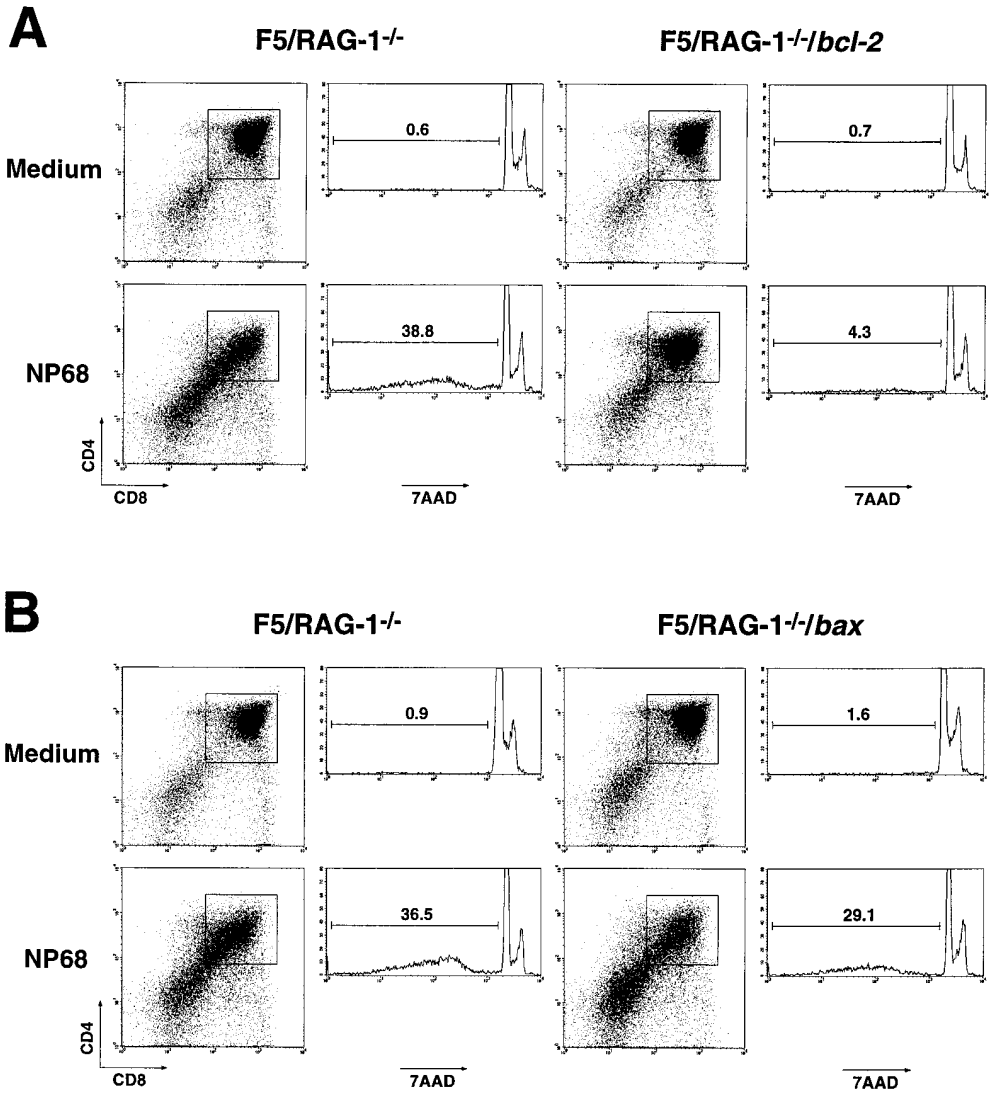

Figure 3. Direct measurement of the level of apoptosis caused by antigen induced negative selection in fetal thymic organ culture. FTOCs from F5/RAG-$1^{-/-}$ mice with or without the *bax* or *bcl-2* transgene were cultured for 4 d in vitro. 10 μM NP68 peptide, the cognate antigen for the F5 TCR, was then added to the culture. 11 h later the FTOCs were harvested and stained for CD4 and CD8 expression as well as 7AAD to determine DNA content. (*A*) Dot plots show CD4 and CD8 expression on single cell suspensions of thymocytes from F5/RAG-$1^{-/-}$ mice–derived FTOCs with or without *bcl-2* transgene, with or without addition of the NP68 peptide. Histograms show the DNA content of the DP thymocytes as determined by 7AAD staining. The marker and accompanying figure indicate the percentage of hypodiploid DNA and hence, give a direct measure of the level of apoptosis in each sample. (*B*) The dot plots and histograms refer to FTOCs from F5/RAG-$1^{-/-}$ mice with or without the *bax* transgene.

positive selection does take place, in mice of the H-2^b MHC haplotype. This latter effect is associated with elevated levels of TCR and CD69 expression during T cell development. Thus, *bcl-2* can directly affect the process of T selection. Since thymocyte selection involves both positive and negative selection, the question arises whether Bax and Bcl-2 are affecting either or both of these processes. To address this, we analyzed the effect of these molecules on negative selection in isolation from other thymic selection events.

Direct Quantification of Negative Selection. As described above, previous studies have provided contradictory evidence as to the influence of Bcl-2 on negative selection. We wanted to unambiguously determine the role of apoptotic regulatory molecules such as Bax and Bcl-2 on negative selection so we chose to study negative selection in FTOC. The use of FTOCs to study antigen-induced negative selection has been previously described (38). We have modified this system in order to directly quantitate the extent of apoptosis induced in DP thymocytes after acute exposure to antigenic peptide or other apoptotic stimuli. Fetal thymic lobes from F5/RAG-$1^{-/-}$ mice, with *bax* or *bcl-2* transgenes, were taken at day 15 of gestation. The genotype of the thymic lobes was determined by PCR analysis of DNA from the individual embryos from which they originated. Thymic lobes were cultured for 4 d in vitro, after which the apoptotic stimuli were added to the cultures and 11 h later the percentage of DP thymocytes undergoing apoptosis was determined (Fig. 3).

Since at this stage of the culture most of the thymocytes have differentiated into immature DP cells, the FTOC enabled us to analyze responses of DP thymocytes at early stages of thymic differentiation. Administration of antigen to adult TCR transgenic mice leads to thymocyte apoptosis as an indirect result of mature T cell activation as well as recognition of the antigenic peptide by the thymocytes themselves (39, 40). At day 4 of culture the thymic lobes were virtually devoid of mature T cells, therefore, apoptosis of thymocytes was solely due to recognition of the anti-

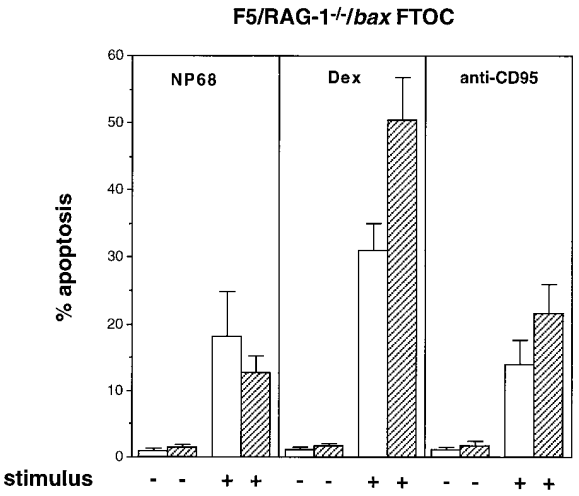

Figure 4. Bar chart showing the percentage of apoptosis in FTOCs derived from F5/RAG-$1^{-/-}$ mice plus or minus the *bax* transgene. The FTOCs were cultured for 4 d before addition of an apoptotic stimulus (+) either 10 μM NP68 peptide, 0.5 μM dexamethasone, or 1 μg/ml anti-Fas antibody (Jo2). After 11 h further culture the FTOCs were harvested and stained with CD4, CD8, and 7AAD. The percentage of apoptosis as determined by hypodiploid DNA content is shown for sets of individual thymic lobes with (+) or without (−) apoptotic stimulus. Each bar represents data from four to six thymic lobes and the error bar represents their standard deviation. The data shown is for a single complete experiment with similar or identical results to four other independent experiments. (*Open bars*) F5/RAG-$1^{-/-}$; (*striped bars*) F5/RAG-$1^{-/-}$/*bax*.

genic peptide. Although FTOCs are performed in vitro, they create the possibility to analyze the responses of thymocytes in an intact thymic microenvironment, which is lost in suspension culture systems (41, 42). Another important benefit of the FTOC approach is that we can discard the contribution of survival effects on thymocyte viability in culture to the overall level of apoptosis. The differentiated FTOCs are grown under optimal conditions in vitro and show very low levels (<1%) of apoptosis, regardless of the expression of the *bax* or *bcl-2* transgenes. Therefore, the levels of apoptosis found are directly due to the stimuli and the influence of *bax* or *bcl-2* on the pathways they invoke and not to factors associated with culture conditions. Thus, we compared thymocyte apoptosis due to antigen, dexamethasone, and treatment with anti-CD95 (anti-Fas) antibody, and the influence of Bax and Bcl-2 in each of these apoptotic pathways.

Bax and Negative Selection. First, we examined negative selection in FTOCs generated from F5/RAG-$1^{-/-}$ H-$2^{b/b}$ mice with or without the *bax* transgene. Thymocyte apoptosis was examined in response to NP68 (antigenic peptide) as well as dexamethasone and anti-CD95 antibody (Jo2). As illustrated in Fig. 3, after staining with 7AAD as well as anti-CD4 and CD8, FACS® was used to determine the percentage of DP thymocytes containing hypodiploid DNA and thus undergoing apoptosis. To obtain statistically significant results, four to six individual lobes were assayed for each value shown in Fig. 4. These experiments were repeated on four independent occasions and similar data were obtained each time. After negative selection induced by NP68, the level of apoptosis in F5/RAG-$1^{-/-}$/*bax* FTOCs was not higher than those from the FTOCs without *bax* transgene, in fact it was slightly reduced. The level of NP68 induced apoptosis in *bax* FTOCs was also examined at 4 and 6 h to confirm that no effect was detectable at earlier time points in the culture (data not shown). However, the thymocytes of *bax* FTOCs show significantly higher apoptosis than non-*bax* FTOCs after incubation with dexamethasone (Fig. 4) as previously seen in primary thymocytes from *bax* transgenics (12). The *bax* transgene has no significant effect on CD95-induced apoptosis, which also accords with previous data from primary *bax* thymocytes (43). We conclude that the *bax* transgene can act in FTOCs to increase apoptosis in response to dexamethasone but that in the case of cognate antigen induced negative selection it has no effect. This suggests that Bax has no role in the apoptotic pathway activated by antigen induced negative selection.

Bcl-2 and Negative Selection. We then studied the induction of apoptosis in thymocytes of FTOC from F5/RAG-$1^{-/-}$ mice, with or without the *bcl-2* transgene. After negative selection induced by NP68 the level of apoptosis in *bcl-2* FTOCs was greatly reduced, four- to fivefold, over those without *bcl-2*. This demonstrates a strong protective effect of Bcl-2 on antigen-induced negative selection. The *bcl-2* transgene also caused a threefold reduction in dexamethasone induced apoptosis. Interestingly, we also found a moderate protective effect of Bcl-2 on CD95 induced apoptosis, as previously described for primary *bcl-2* thymocytes (44). However, in our system this could not be explained by enhanced survival of *bcl-2* transgenic thymocytes (see above). Thus, Bcl-2 can indeed partially inhibit CD95-induced apoptosis.

Our data show that Bcl-2 can strongly inhibit antigen-specific thymic negative selection, whereas Bax, an antagonist of Bcl-2, has no effect on negative selection.

Discussion

Apoptosis has been clearly demonstrated to be a key mechanism in negative selection (7). However, the role of individual molecules known to regulate apoptosis, in particular Bcl-2, has remained an area of controversy. Bcl-2 has been shown either to have no effect (10) or to inhibit (11, 19) superantigen-mediated deletion of autoreactive thymocytes. Studies on mice doubly transgenic for the H-Y TCR and *bcl-2* have reached different conclusions, namely that deregulated *bcl-2* expression reduces the efficiency of negative selection (20) or has no effect (21). Similar types of studies in *bcl-X_L* transgenic mice have concluded that Bcl-X_L has no effect on negative selection (22, 23). We sought to address the role of Bcl-2 in T cell development and negative selection using MHC class I–restricted F5 TCR transgenic mice on a RAG-$1^{-/-}$ background to produce mice whose T cells express a single TCR. We also studied the effect of a *bax* transgene within the same con-

text, as Bax can act antagonistically of Bcl-2 function and therefore, might be expected to have opposite functional consequences.

Expression of the *bax* transgene in F5/RAG-$1^{-/-}$ mice reduced the size of the thymus in the context of both selecting and nonselecting MHC. This suggests that Bax can exert a proapoptotic effect on thymocytes independently of thymic selection. However, with selecting MHC the number of CD8 SP thymocytes produced is greatly reduced and the upregulation of TCR and CD69 expression normally associated with maturation is also severely reduced. Precisely the converse is found with the *bcl-2* transgene, where thymus size is increased regardless of MHC. On the selecting MHC background, such mice exhibit increased numbers of thymocytes with upregulated TCR and CD69, coinciding with elevated numbers of CD8 SP thymocytes. These observations suggest that while Bax and Bcl-2 confer pro- and antiapoptotic signals, respectively, they also exert effects on the processes involved in T cell selection. Therefore, these findings unambiguously confirm previous suggestions about *bcl-2* and T cell selection that were complicated by a lack of complete allelic exclusion and the presence of endogenous TCR rearrangements.

Having established these effects on T cell selection the question arises as to how Bax and Bcl-2 exert their actions. Do they act on positive or negative selection separately or on both processes in opposite directions? We addressed this question from the perspective of negative selection using fetal thymic organ culture as our model system. FTOCs have numerous advantages for this type of study as outlined above. Our experiments show that upregulated *bax* expression while accelerating glucocorticoid-induced apoptosis, has no effect on negative selection (Fig. 4). However, increased *bcl-2* expression leads to substantial inhibition of negative selection, three- to fourfold, while also inhibiting glucocorticoid-induced apoptosis and having a marginal effect on anti-CD95–induced apoptosis (Fig. 5). The marginal inhibitory effect of *bcl-2* on CD95-induced apoptosis has been noted previously on primary thymocytes in culture (44) and attributed to a survival effect, i.e., that the *bcl-2* transgene simply confers greater viability in culture. However, as the FTOC system negates the survival effect of Bcl-2 in culture it would seem that Bcl-2 can partially inhibit anti-CD95 induced apoptosis. This may be by partially blocking one or more parts of the caspase cascade activated by anti-CD95 treatment (reviewed in reference 45).

Overall, our study suggests that Bax has no role in the apoptotic pathway activated by antigen-induced negative selection, whereas Bcl-2 can act to block this pathway. Several important points can be made as a result of this finding. First, it establishes that a specific apoptosis regulatory molecule, Bcl-2, can also regulate negative selection. It remains to be seen whether apoptosis regulatory molecules can similarly influence positive selection and this may be directly measurable using an FTOC approach. For instance, the *bax* transgene leads to a decline in mature SP T cell production but does not increase negative selection. Therefore, it may act to decrease positive selection, as suggested by the decreased expression of CD69 on intermediate $CD4^{lo}CD8^{hi}$ thymocytes of *bax* transgenic mice. Thus, Bax may directly inhibit positive selection or reduce its efficiency indirectly by reducing the viability of thymocytes (12), effectively reducing the number of thymocytes available for positive selection. In contrast the *bcl-2* transgene leads to an increase in the number of intermediate $CD69^{hi}$ $CD4^{lo}CD8^{hi}$ and mature CD8 SP thymocytes as well as an increase in the total number of thymocytes in the presence of selecting and nonselecting MHC molecules. These results suggest that Bcl-2 increases the efficiency of positive

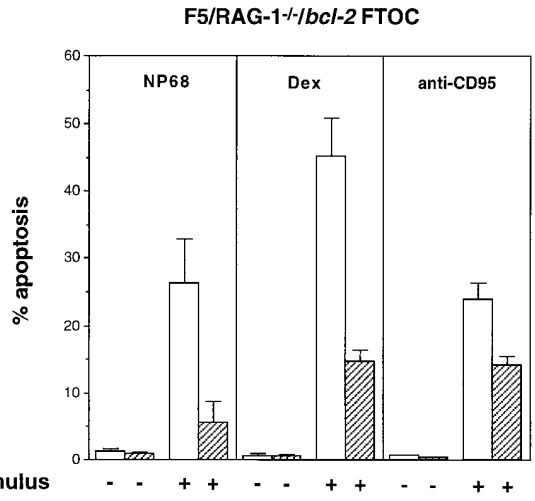

Figure 5. Bar chart showing the percentage of apoptosis in FTOCs derived from F5/RAG-$1^{-/-}$ mice plus or minus the *bcl-2* transgene. The FTOCs were cultured for 4 d before addition of an apoptotic stimulus (+) either 10 μM NP68 peptide, 0.5 μM dexamethasone, or 1 μg/ml anti-Fas antibody (Jo2). After 11 h further culture the FTOCs were harvested and stained with CD4, CD8, and 7AAD. The percentage apoptosis as determined by hypodiploid DNA content is shown for sets of individual thymic lobes with (+) or without (−) apoptotic stimulus. Each bar represents data from four to six thymic lobes. The data shown is for a single complete experiment with similar or identical results to four other independent experiments. (*Open bars*) F5/RAG-$1^{-/-}$; (*striped bars*) F5/RAG-$1^{-/-}$/*bcl-2*.

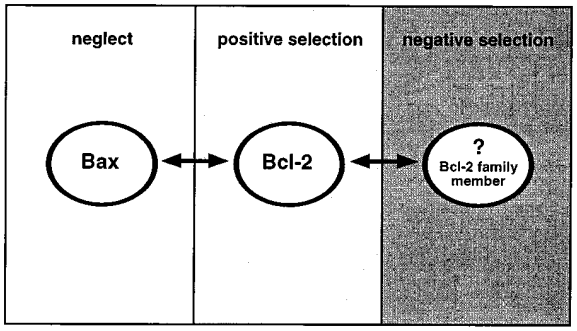

Figure 6. A model representing the properties of Bax and Bcl-2 in T cell selection. Bax and Bcl-2 have antagonistic effects on positive selection and death by neglect. However, Bax has no effect on negative selection, whereas Bcl-2 can inhibit it. This implies that an unidentified member of the Bcl-2 family can antagonize the effect of Bcl-2 on negative selection.

selection. Thus, in thymocytes Bax and Bcl-2 appear to be functionally antagonistic in the decision between death by neglect and positive selection, whereas Bcl-2 acts independently of Bax in inhibiting negative selection. A model summarizing these properties of Bax and Bcl-2 is shown in Fig. 6. Although Bax does not have a direct role in negative selection, it is very likely that other proapoptotic members of the Bcl-2 family do, in order to counteract Bcl-2 (Fig. 6). Therefore, a further task will be to determine which ones act in negative selection. Another aspect of the data is that whereas Bcl-2 has a strong inhibitory effect on negative selection it has very little effect on CD95-induced apoptosis. This would support the idea that the CD95 pathway has no great role in thymic negative selection (46, 47) rather than the view that CD95 modulation is an integral part of the negative selection of thymocytes (48).

Our study establishes peptide antigen-induced deletion in FTOCs as a model system to directly study apoptosis as a result of negative selection. Due to the intrinsically negligible levels of background cell death in the system it is also a very useful method to study other apoptotic stimuli in T cells. We hope to further develop this method to study apoptosis regulatory molecules in positive selection.

Recently, it has been proposed that, although Bax and Bcl-2 can form heterodimers and act to antagonize each other's function, they also regulate apoptosis independently of one another (30, 49). We further validate this hypothesis by showing Bax and Bcl-2 do not have reciprocal effects within the same system. Bax and Bcl-2 exert their opposing effects on glucocorticoid-induced apoptosis, whereas Bcl-2 inhibits negative selection and Bax has no effect. Hence, Bax and Bcl-2 can function independently of their physical interaction with each other in the cell (30).

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