The T Cell Receptor Repertoire of CD4<sup>+</sup> Thymocytes Is Altered by Overexpression of the BCL-2 Protooncogene in the Thymus

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Summary

The bcl-2 gene encodes an intracellular, membrane-associated protein that protects immature cortical thymocytes from a wide variety of apoptotic stimuli, including glucocorticoids, radiation, and anti-CD3 treatment. Since cortical thymocytes are the primary target cells for thymic positive and negative selection processes, and since these processes are associated with cell death, we evaluated the role of bcl-2 in T cell development in two ways. In the first approach, transgenic mice expressing high levels of Bcl-2 in cortical thymocytes were mated with H-Y T cell receptor (TCR) transgenic mice, the latter being a well-defined system for the study of positive and negative selection of T cells. We found that the bcl-2 transgene had a dramatic effect on positive selection. This was manifested by a greatly increased production of mature thymocytes that were highly skewed towards the CD4<sup>-</sup>8<sup>+</sup> lineage. The change involving CD4<sup>-</sup>8<sup>+</sup> thymocytes occurred not only in bcl-2 transgenic mice, but was also observed in H-Y TCR/bcl-2 doubly transgenic mice, regardless of whether the H-Y TCR was expressed in the selecting (H-2<sup>b</sup>) or nonselecting (H-2<sup>d</sup>) environments. Furthermore, a large proportion of CD4<sup>-</sup>8<sup>+</sup> thymocytes produced in H-2<sup>b</sup> H-Y TCR/bcl-2 doubly transgenic female mice expressed endogenous TCR α chains rather than the transgenic TCR α chain. These observations are consistent with the model that high expression of Bcl-2 in cortical thymocytes overrides the normal apoptotic pathway. This then allows the selection of CD4<sup>-</sup>8<sup>+</sup> thymocytes expressing TCRs that are otherwise nonselectable. However, the bcl-2 transgene did not protect CD4<sup>+</sup>8<sup>+</sup> thymocytes expressing the male-specific TCR from deletion in male doubly transgenic mice. In the second approach, we determined the level of bcl-2 mRNA expression in populations of thymocytes defined by their CD4/CD8 phenotypes using quantitative reversed transcriptase PCR techniques. Our results indicate that bcl-2 mRNA was expressed at a high level in immature CD4<sup>-</sup>8<sup>-</sup> thymocytes and in mature CD4<sup>-</sup>8<sup>-</sup> thymocytes. There is a dramatic downregulation of bcl-2 mRNA in CD4<sup>+</sup>8<sup>+</sup> thymocytes, particularly those expressing a low level of TCR. CD4<sup>+</sup>8<sup>+</sup> thymocytes that upregulated their TCR, likely as a result of receiving positive selection signals, also upregulated bcl-2 mRNA. This observation suggests that rescue of immature thymocytes from the programmed cell death pathway by positive selection signals is accompanied by the upregulation of bcl-2 mRNA.

The development of T cells in the thymus is associated with the production of large numbers of immature thymocytes that are subjected to thymic selection processes (1). Positive selection refers to the development of a mature T cell repertoire that recognizes foreign antigenic peptides which are presented by self-MHC molecules. Negative selection refers to the elimination of T cells that are immunocompetent against self-antigenic peptides presented by self-MHC molecules. The primary target for positive and negative selection appears to be the immature CD4<sup>+</sup>8<sup>+</sup> thymocytes, which express a low level of the TCR-α/β (2). CD4<sup>+</sup>8<sup>+</sup> thymocytes are produced daily in large numbers and have an average life span of 3.5 d (3). Since the TCR repertoire expressed by CD4<sup>+</sup>8<sup>+</sup> thymocytes is generated in a random manner, only a very minor fraction of these cells is expected to express TCR of sufficient affinity for positively and negatively selecting ligands. Thus, the vast majority of CD4<sup>+</sup>8<sup>+</sup> thymocytes are predicted to express nonselectable TCR and
are postulated to undergo a form of programmed cell death (PCD)\(^1\) (1, 2). Cell death induced by negative selection is a TCR-mediated process and occurs rapidly since CD4\(^+\)8\(^-\) thymocytes expressing a TCR of defined specificity are deleted within hours of interacting with the deleting ligand (4–6). This form of cell death is dependent upon TCR–ligand interaction and it has also been referred to as activation-induced cell death (AICD). It is clear from these considerations that interference with the death pathways of thymocytes, particularly those associated with PCD and AICD, are likely to influence the outcome of thymic selection processes.

The bcl-2 protooncogene, which encodes an intracellular membrane protein, was discovered as a result of its translocation to the Ig locus in human follicular center B cell lymphoma (7–12). This t(14; 18) chromosomal translocation spares the coding region of the bcl-2 gene and appears to deregulate its expression. Enforced bcl-2 expression delays the death of certain hematopoietic cell lines deprived of growth factors (13). Moreover, transgenic mice expressing a bcl-2 gene under the control of the Ig H chain enhancer contain a large excess of B cells that are adapted for long-term survival in vitro and in vivo (14–17). The Bcl-2 protein is expressed in specific areas of the thymus, being present in medullary but not most cortical thymocytes (18). Since the majority of CD4\(^+\)8\(^-\) cortical thymocytes are destined for PCD the lack of Bcl-2 in these cells suggests that Bcl-2 may be involved in the survival of T cells.

To assess the role of bcl-2 in T cell development, transgenic mice were generated in which bcl-2 expression was redirected to the immature T cells in the thymic cortex using the proximal promoter of the kk tyrosine kinase gene (kk\(^p\)) (19). In these kk\(^p\)-bcl-2 transgenic mice, total thymocyte numbers were normal, suggesting that a substantial amount of cell death was still occurring (19). However, the expression of Bcl-2 in cortical thymocytes protected them from a wide variety of apoptotic stimuli including glucocorticoids, radiation, and anti-CD3 treatment (19–21). Examination of the V\(\beta\) repertoires in these mice indicated that deletion of T cells with reactivity towards self-superantigens still occurred, thus suggesting that negative selection was Bcl-2 independent (19). Furthermore, deregulated bcl-2 expression led to the production of larger numbers of T cells having a mature phenotype (19). Similar results were obtained in transgenic mice where the bcl-2 gene was regulated by the Ig H chain enhancer (E\(\mu\)). However, in E\(\mu\)-bcl-2 transgenic mice, a proportion of thymocytes with reactivity against self-superantigens appeared to have survived deletion in the thymus (20, 21).

To further evaluate the role of Bcl-2 in T cell development, the kk\(^p\)-bcl-2 transgenic mice were mated with the H-Y TCR transgenic mice, which expressed on a majority of their T cells a TCR that is specific for the male (H-Y) antigen presented by the H-2\(d\) class I molecule (22, 23). We found that in doubly transgenic female kk\(^p\)-bcl-2/H-Y TCR mice of the H-2\(d\) (selecting) haplotype, the repertoire of positively selected CD4\(^-\)8\(^-\) T cells was dramatically altered. In contrast, negative selection of CD4\(^+\)8\(^-\) H-Y TCR\(^+\) thymocytes was unaffected in H-2\(d\) doubly transgenic male mice. We also examined the normal developmentally regulated expression of the endogenous bcl-2 gene in thymocyte subpopulations and the effect of positive selection on bcl-2 expression.

### Materials and Methods

**Mice.** C57Bl/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). H-2\(^d\) H-Y TCR, H-2\(^d\) H-Y TCR, and kk\(^p\)-bcl-2 transgenic mice were produced as previously described (19, 22, 23).

**Antibodies.** PE-labeled anti-CD4 (GK 1.5), FITC-labeled, or biotinylated anti-CD8 (53-6.7) mAbs were purchased from Becton Dickinson (Mississauga, ON, Canada). Anti-H-2\(d\) (H141-31) mAb was purchased from Bioproducts for Science (Indianapolis, IN) and FITC-labeled sheep anti-mouse Ig was purchased from Silenus Laboratories (Hawthorne, Australia). mAbs specific for the transgenic TCR \(\beta\) chain, F23.1, and the transgenic TCR \(\alpha\) chain, T3.70, were prepared as previously described (23–25).

**Flow Cytometry.** Two-color staining of cells with FITC-labeled anti-CD4 and PE-labeled anti-CD4 mAb was performed as described (23). Three-color analysis was used to determine the expression of a specific cell surface marker by CD4\(^+\)8\(^-\) thymocytes as previously described (6). Briefly, thymocytes were first incubated with the mAb that is specific for the marker of interest. This was followed by incubation with FITC-labeled sheep anti-mouse Ig, normal mouse serum, PE-labeled anti-CD4 mAb, biotinylated anti-CD8 mAb, and finally, by Streptavidin-Tandem (Southern Biotechnology Associates, Birmingham, AL). The labeled cells were analyzed with a FACScan® flow cytometer equipped with a single argon laser (Becton Dickinson). For three-color analysis, 25,000 events were collected per sample and the data was analyzed using FACScan® Research Software (Becton Dickinson).

**Purification of Thymocyte Subpopulations.** CD4\(^+\)8\(^-\) thymocytes were isolated according to the following procedure. Thymocytes were incubated with IMDM plus 10% FCS with cytotoxic anti-CD4 (RL 172) (26) and anti-CD8 (HO 2.2) (27) mAbs for 40 min on ice. The cells were washed once and incubated with Low-Tox-M rabbit complement (Cedarlane Laboratories, Hornby, ON, Canada) 1:10 dilution for 45 min at 37°C. Dead cells were removed by centrifugation over Histopaque 1077 (Sigma Chemical Co., St. Louis, MO). Viable cells from the interface were collected, washed twice, and incubated in IMDM plus 10% FCS with the GK 1.5 (anti-CD4) mAb and the 53-6.7 (anti-CD8) mAb for 20 min on ice. Sheep anti-mouse IgG-coated Dynabeads (Dynal, Oslo, Norway) were then added to the cell suspension and incubated with rotation for 40 min at 4°C. Cells that were bound to Dynabeads were removed with a magnet. The purity of the remaining cells was determined by two-color staining with FITC-labeled anti-CD8 and PE-labeled anti-CD4 mAbs. CD4\(^+\)8\(^-\) and CD4\(^+\)8\(^-\) thymocytes were isolated by staining thymocytes with FITC-labeled anti-CD8 and PE-labeled anti-CD4 mAbs followed by two-color cell sorting using the FACStar Plus® cell sorter (Becton Dickinson). For the isolation of CD4\(^+\)8\(^-\) thymocytes expressing either low or high levels of the transgenic TCR \(\beta\) chain, thymocytes were incubated with biotinylated anti-CD8, PE-labeled anti-CD4, and FITC-labeled anti-F23.1 mAbs followed by Streptavidin Cyochrome (PharMingen, distributed by Cedarlane Laboratories). Three-color sorting was performed with the FACStar Plus® cell sorter.

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1. Abbreviations used in this paper: AICD, activation-induced cell death; kk\(^p\), proximal promoter of kk; PCD, programmed cell death.
mRNA Quantitation by PCR. RNA was extracted from 3 × 10^5 - 2 × 10^6 purified thymocyte subpopulations using the guanidinium isothiocyanate method as described (28). Serial dilutions (10-fold) of this RNA were used as templates for a cDNA synthesis reaction. bcl-2 and β-actin mRNA levels were then evaluated by quantitative reverse transcriptase PCR using (dN)6 random primers and the GeneAmp RNA PCR Kit (Perkin Elmer Cetus, Norwalk, CT). Reverse transcriptase PCR was performed according to the manufacturer's procedure. Primers used for amplification were oligonucleotides recognizing sequences in bcl-2 (5'-CCTGTCGCCACCTGTTCCATC-3' and 5'-GCTGAGAACAGGTCTTCCAGAGAC-3'), and β-actin (β-actin A and B) (29). All primers were used in the same PCR reaction mixture. Amplification conditions were 96°C for 1.5 min followed by 22 cycles of 96°C for 45 s, 58°C for 2 min, and 72°C for 1 min. After amplification, 10 μl of PCR products were run on 1.5% agarose gel and transferred to nylon membranes (Gene Screen Plus, Du Pont, Mississauga, ON, Canada). Radioactive probes were made using random priming (30), and the filters were hybridized with the mixture of 32P-labeled probes specific for bcl-2 and β-actin. The filters were then washed and autoradiographed with intensifying screens at −70°C.

Results

CD4^+8^+ Thymocytes from Doubly H-Y TCR/bcl-2 Transgenic Mice Possess an Altered TCR Repertoire. Transgenic mice in which a TCR of defined antigenic specificity is expressed by a majority of T cells have served as useful models for analyzing the mechanisms of positive and negative selection (2). We have used the H-Y TCR transgenic mice as our model system. In these mice the transgenic TCR, specific for the male (H-Y) antigen presented by H-2D^b, is under the transcriptional control of endogenous enhancers (2). Among immature CD4^+8^- and CD4^+8^+ thymocytes, the TCR products expressed are almost exclusively of transgenic origin. In female H-2^d transgenic mice, the transgenic TCR is positively selected by thymic epithelial cells that present H-2D^d class I molecules in association with self-peptides of undefined structure (23, 31, 32). The net result of this positive selection process is the overproduction of CD4^+8^- thymocytes that almost exclusively express the transgenic TCR. Whereas there is no suppression of production of mature CD4^+8^+ thymocytes, the production of these cells in female transgenic mice requires the use of endogenous TCR α chains (2, 23). To better understand the effect of the bcl-2 gene in positive selection, we mated the H-Y TCR transgenic mice with the lkb^+-bcl-2 transgenic mice to produce doubly transgenic mice. Positive selection in female doubly transgenic mice was followed by determining the percentages of thymocytes with the four CD4/CD8 phenotypes, as well as the composition of the TCR expressed by these thymocyte subsets. As shown in Fig. 1, 42% of the thymocytes in female H-2^d doubly transgenic mice were of the CD4^+8^- phenotype. This represents a 3.5-fold increase in CD4^+8^- thymocytes as compared with the single H-Y TCR transgenic littermate. In contrast, 21% of the thymocytes are of the CD4^+8^- phenotype in the doubly transgenic mice, compared with the 14% observed for single H-Y TCR transgenic littermates. Similar results were observed for three other sets of single H-Y TCR and doubly transgenic mice. Thus, a major effect of the bcl-2 transgene in doubly transgenic mice is the further skewing of thymocytes towards the CD4^+8^- lineage.

Three-color staining of thymocytes with mAbs specific for CD4, CD8, and either the transgenic TCR β (detected by the F23.1 mAb) or α (detected by the T3.70 mAb) chain allowed us to determine the level of transgenic TCR β or α chain expressed by the four thymocyte subsets defined on the basis of their CD4/CD8 phenotypes. As previously reported, and shown here in Fig. 1, CD4^+8^- thymocytes from female H-2^d TCR transgenic mice expressed primarily the transgenic TCR heterodimer. It can also be deduced from Fig. 1 that a small percentage of CD4^+8^- thymocytes from

Figure 1. Alteration of the repertoire of CD4^+8^- thymocytes and CD4^+8^- thymocytes in H-Y TCR/bcl-2 doubly transgenic mice. Thymocytes from female H-2^d H-Y TCR or female H-2^d H-Y TCR/bcl-2 doubly transgenic mice were stained with the indicated mAbs. The level of the transgenic TCR β chain (detected by the F23.1 mAb) or transgenic TCR α chain (detected by the T3.70 mAb) expressed by CD4^+8^- thymocytes and by CD4^+8^- thymocytes from these two types of mice are as indicated.
H-Y TCR transgenic mice expressed a high level of the transgenic TCR heterodimer (6, 33). CD4^-8^+ thymocytes from doubly transgenic mice also expressed primarily the transgenic TCR heterodimer. However, it is clear that although a greater proportion of CD4^-8^+ thymocytes from doubly transgenic mice expressed a high level of the transgenic TCR β chain, only a fraction of these cells also expressed a high level of the transgenic TCR α chain. Furthermore, a smaller proportion of CD4^-8^+ thymocytes from doubly transgenic mice was found to express intermediate levels of the transgenic TCR α chain when compared with single H-Y TCR transgenic littermates. This was accompanied by an increase in the proportion of CD4^-8^+ thymocytes that express very low levels of the transgenic TCR α chain. These cells presumably expressed higher levels of endogenous TCR α chains which were not stained by the T3.70 mAb. This bias towards endogenous TCR α usage by CD4^-8^+ thymocytes is also reflected in the TCR composition of mature CD4^-8^+ thymocytes in the doubly transgenic mice. As previously reported, CD4^-8^+ thymocytes from H-2^b^ female H-Y TCR transgenic mice primarily express the transgenic TCR heterodimer (23 and Fig. 1). In contrast, more than half of the mature CD4^-8^+ thymocytes from the doubly transgenic mice expressed transgenic TCR β chain in association with high levels of endogenous TCR α chains, which were not detected by the T3.70 mAb. This skewing towards the CD8 lineage was not as pronounced in the LN of doubly transgenic mice and was only reflected by a slight increase in the proportion of CD4^-8^+ T cells. The LN of these doubly transgenic mice contained 26.3 ± 0.4% (n = 3) CD4^-8^+ T cells compared with 17.0 ± 0.5% (n = 5) in H-Y TCR transgenic mice. However, the proportion of CD4^-8^+ T cells in the LN of H-Y TCR or doubly transgenic mice expressing high levels of the transgenic TCR was approximately the same (about 15%).

We next determined whether this skewing towards the CD4^-8^+ lineage in the doubly transgenic mouse was dependent on the interaction of the transgenic TCR with positively selecting ligands in the thymus. We have previously shown that the transgenic TCR confers no selective advantage in mice of the H-2^d_ haplotype (23). Doubly transgenic mice of the H-2^d_ haplotype were produced by backcrossing the bcl-2 and H-Y TCR transgenes onto a H-2^d_ background. As shown in Fig. 2, CD4^-8^+ thymocytes were also overproduced by 3.5-fold in female H-2^d_ doubly transgenic mice. Thus, overproduction of CD4^-8^+ thymocytes in doubly transgenic mice is independent of interaction of the transgenic TCR with its positively selecting ligand(s) in the thymus.

**Negative Selection Occurs Efficiently in H-Y TCR/bcl-2 Double Transgenic Male Mice.** It was previously shown that CD4^-8^+ thymocytes expressing the H-Y TCR were deleted efficiently in male H-2^b_ mice (22). To determine whether these cells were protected from deletion by Bcl-2, the H-Y TCR transgenic mice were mated with lkl^-/-_bcl-2 transgenic mice to produce doubly transgenic offspring. Thymocytes and LN cells were harvested from doubly transgenic male mice and their CD4/CD8 phenotypes determined by two-color analysis. Two phenotypes of doubly transgenic male mice were observed. The first phenotype is similar to that of the single H-Y TCR transgenic male mice and is shown in Fig. 3. Doubly transgenic males with this phenotype have a very prominent CD4^-8^+ population and poorly defined populations of CD4^-8^-, CD4^-8^+, and CD4^-8^+ thymocytes. Previous studies (22, 34) have determined that this phenotype is a consequence of the efficient deletion of CD4^-8^+ thymocytes expressing the H-Y TCR. The efficient deletion of CD4^-8^+ thymocytes is reflected in greatly reduced thymocyte cell yields (19 ± 6 × 10^6_ , n = 6 for H-Y TCR males versus 45 ± 5 × 10^6_ , n = 3 for doubly transgenic males). It is also reflected in the poor development of CD4^-8^+ T cells and the presence of a prominent CD4^-8^+ population in the LN (22, 34, and Fig. 3). The second phenotype is shown in Fig. 4. In this instance, deletion of CD4^-8^+ thymocytes in doubly transgenic males is incomplete. The number of thymocytes recovered from doubly transgenic males was similar to that of normal mice (112 ± 11 × 10^6_, n = 3). However, a large fraction of surviving CD4^-8^+ thymocytes from doubly transgenic mice did not express the transgenic TCR α chain despite having high and uniform expression of the transgenic TCR β chain (Fig. 4). Thymocytes from H-Y TCR transgenic males surviving deletion were large cells, as defined by the forward scatter parameter, and expressed high levels of the transgenic TCR heterodimer. In contrast, the vast majority of thymocytes recovered from doubly transgenic males were small cells and of these, a majority was not stained by the T3.70 mAb (Fig. 4).

Thymocytes and LN cells recovered from doubly transgenic male mice, with either of the phenotypes described in Figs. 3 and 4, are functionally tolerant to the male antigen as they do not respond to male H-2^b_ APC, even in the presence of an excess amount of exogenously added JL-2 (data not shown).
Positive Selection of CD4⁺8⁺ Thymocytes Is Associated with Increased bcl-2 Expression. It was previously shown that whereas bcl-2 is expressed at high levels by mature medullary thymocytes, only occasional cortical thymocytes express bcl-2 (18). It is not known whether immature CD4⁺8⁻ thymocytes express bcl-2, or whether the commitment of CD4⁺8⁺ thymocytes to the PCD pathway is associated with the cessation of Bcl-2 production. It is also not known whether the rescue of CD4⁺8⁺ thymocytes from the PCD pathway by positive selection signals is associated with the reexpression of bcl-2 in these cells. To address these questions, we determined bcl-2 mRNA levels in immature CD4⁺8⁻ and CD4⁺8⁺ thymocytes, as well as in mature CD4⁺8⁻ thymocytes from female H-2ª H-Y TCR transgenic mice, or normal H-2ª mice, by quantitative reversed transcriptase PCR. The results of one of three representative experiments are shown in Fig. 5. CD4⁺8⁻ thymocytes from both H-Y TCR transgenic and normal mice expressed a high level of the bcl-2 mRNA. Densitometric measurements indicate that this level of bcl-2 mRNA expression was downregulated by

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Figure 3. Efficient deletion of autospecific CD4⁺8⁺ thymocytes in H-Y TCR/bcl-2 doubly transgenic mice. Thymocytes and LN cells from male H-2ª H-Y TCR and male H-2ª H-Y TCR/bcl-2 doubly transgenic mice were collected, double-stained with anti-CD4 and anti-CD8 mAbs, and analyzed in the FACScan®.

Figure 4. A large proportion of CD4⁺8⁺ thymocytes escaping negative selection in H-Y TCR/bcl-2 doubly transgenic male mice express endogenous TCR α chains. Thymocytes from male H-2ª H-Y TCR transgenic mice and male H-2ª H-Y TCR/bcl-2 doubly transgenic mice were collected and triple-stained with mAbs specific for CD4, CD8, and either the transgenic TCR β or the transgenic TCR α chain and analyzed in the FACScan®.
Bcl-2 Participates in T Cell Development. Our results show that thymus-specific expression of a bcl-2 transgene favors the development of thymocytes of the CD4+8+ lineage. This skewing towards the CD4+8+ lineage occurs not only in bcl-2 transgenic mice but also in H-Y TCR/bcl-2 doubly transgenic mice, regardless of whether the H-Y TCR is expressed in a selecting H-2k or a nonselecting H-2d environment. Thus, the further skewing towards the CD4+8+ lineage observed in H-Y TCR transgenic mice appears to be independent of the antigen specificity of the transgenic TCR. This is consistent with the observation that a large proportion of CD4+8+ thymocytes produced in H-2d H-Y TCR/bcl-2 doubly transgenic female mice expressed endogenous TCR α chains rather than the transgenic TCR β chain. The bias towards the CD8 lineage is not as apparent in peripheral T cells as it is in the thymus.

Discussion

5.1- and 7.2-fold in CD4+8+ thymocytes from H-Y TCR transgenic mice and normal mice, respectively. Mature CD4+8+ thymocytes from both H-Y TCR transgenic mice or normal mice upregulated bcl-2 mRNA expression by 3.8- and 7.7-fold, respectively, when compared with immature CD4+8+ thymocytes. By normalizing the level of bcl-2 mRNA expressed by CD4+8+ thymocytes to that of β-actin mRNA, it was found that the level of bcl-2 mRNA expressed by these cells from H-Y TCR transgenic mice was about 1.6-fold higher as compared with the same thymocyte subpopulation from normal mice. It is conceivable that the slightly higher expression of bcl-2 mRNA in CD4+8+ thymocytes from H-Y TCR transgenic mice was due to the larger number of thymocytes in H-Y TCR transgenic mice that had received positive selection signals. To test this hypothesis, we triply stained thymocytes from female H-2d H-Y TCR transgenic mice with mAbs specific for CD4, CD8, and the transgenic TCR β chain, and sorted for CD4+8+ thymocytes expressing either low or high levels of the transgenic TCR β chain. As shown in Fig. 6, CD4+8+ thymocytes from H-Y TCR transgenic mice expressing high levels of the transgenic TCR, β chain expressed a level of bcl-2 mRNA that was 1.9-fold higher than CD4+8+ thymocytes expressing low levels of the transgenic TCR β chain. A 2.0-fold increase was observed in a repeat experiment.

Figure 5. Differential expression of bcl-2 by immature CD4-8-, CD4+8+, and mature CD4+8- thymocytes. CD4-8- thymocytes of ≥98% purity were prepared as described in Materials and Methods. CD4+8+ and CD4+8- thymocytes of >95% purity were obtained by two-color staining with anti-CD4 and anti-CD8 mAbs followed by cell sorting. Total cellular RNA was extracted from the indicated purified populations, and 10-fold dilutions of these RNAs were used as templates in a cDNA synthesis reaction. The coamplification was performed with mixed bcl-2 and β-actin primers, and the Southern blots of the PCR products were hybridized with the 32P-labeled probes specific for bcl-2 and β-actin. The sizes of the PCR coamplified transcript fragments of bcl-2 and β-actin are 405 bp and 570 bp, respectively. (A) Purified thymocyte subpopulations from female H-2d H-Y TCR transgenic mice. (B) Purified thymocyte subpopulations from C57Bl/6 mice.

Figure 6. Coordinate upregulation of TCR expression and bcl-2 mRNA by CD4+8+ thymocytes. Thymocytes from H-2d H-Y TCR transgenic mice were harvested and triple stained with anti-CD4, anti-CD8, and anti-transgenic TCR β chain mAbs. CD4+8+ thymocytes expressing either a low or high level of the transgenic TCR β chain were isolated by cell sorting. >95% of the sorted cells were of the CD4+8+ phenotype. (A) Quantitative reversed transcriptase PCR for bcl-2 mRNA expression by the TCR lo and TCR hi CD4+8+ thymocyte populations was determined as described in Materials and Methods and in Fig. 5. (B) (Open and filled histograms) Low and high expression, respectively, of the transgenic TCR β chain by the sorted CD4+8+ thymocytes.
cells of doubly transgenic mice. Although there is a slight increase in the proportion of mature CD4+8+ T cells, there is no evidence of increased usage of endogenous TCR α chains. However, this observation does not imply that CD4+8+ T cells selected under the influence of the bcl-2 transgene are abnormal since previous studies (2, 35) have shown that the peripheral T cell repertoire is shaped by mechanisms distinct from those operative in the thymus. In contrast, high levels of bcl-2 expression in CD4+8+ thymocytes expressing the H-Y TCR fails to protect them from deletion by deleting ligands present in male TCR transgenic mice. In doubly transgenic mice that have high thymocyte cell numbers, the increase in cell numbers was due to the increased production of small CD4+8+ thymocytes that did not express the male-specific TCR. We found that immature CD4+8+ thymocytes expressed a high level of bcl-2 mRNA. This is consistent with a recent report (36) where these cells were shown to express a high level of the Bcl-2 protein. The low level of bcl-2 mRNA in CD4+8+ thymocytes expressing a low TCR level and the higher level of bcl-2 mRNA in CD4+8+ thymocytes expressing a high level of TCR is consistent with the model that the commitment of CD4+8+ thymocytes to the PCD pathway is associated with the downregulation of bcl-2 mRNA, and that rescue from this PCD pathway by positive selection signals is associated with the upregulation of bcl-2 mRNA. However, this conclusion is in contrast with that obtained from a recent study with bcl-2 homozygous mutant chimeric mice where it was shown that bcl-2 was not required for T cell development (37). Thus, a more likely explanation of our data is that the developmentally regulated expression of bcl-2 is a result, and not the cause, of T cell developmental processes. Importantly, the expression of bcl-2 is required for the survival of mature T cells in peripheral lymphoid organs (37), a conclusion consistent with the reinduction of bcl-2 expression in positively selected CD4+8+ T cells observed in our study.

Model to Account for Bias Towards the CD4+8+ Lineage in bcl-2 Transgenic Mice. The observed bias towards the maturation of cells of the CD4+8+ lineage in the bcl-2 transgenic mice contrasts with T cell development in normal mice, where the development of CD4+8+ thymocytes is favored over that of CD4+8- thymocytes. We propose that this bias towards the CD4+8+ lineage in bcl-2 transgenic mice is due in part to differences in signaling efficiency between CD4 and CD8 coreceptor molecules during the positive selection process. In the H-Y TCR transgenic model system, we have shown that the CD8 molecule functions as a coreceptor in the positive selection of CD4+8- thymocytes expressing the H-Y TCR (38). Although it remains to be demonstrated, positive selection of CD4+8- thymocytes expressing MHC class II-restricted TCR may also be dependent on CD4 molecules that serve a coreceptor function. Previous studies (39) have shown that the cytoplasmic domain of CD4 interacts with a higher affinity with p56Lck than does the cytoplasmic domain of CD8. This less efficient association of p56Lck with CD8 may contribute to a reduction in efficiency of positive selection of CD4+8- thymocytes expressing MHC class II-restricted TCR. Since the life span of CD4+8+ thymocytes is increased in bcl-2 transgenic mice, this may compensate for the relatively inefficient selection of CD4+8+ thymocytes expressing MHC class I-restricted TCR. In contrast, as a result of efficient signaling mediated by the CD4 coreceptor molecule, CD4+8+ thymocytes expressing MHC class II-restricted TCR are probably selected at a higher efficiency, and the increased life span of CD4+8+ thymocytes as a result of transgenic bcl-2 expression is thus less likely to contribute to positive selection in these cells.

Explanation for the Preferential Usage of Endogenous TCR α Chains by CD4 Thymocytes. The preferential usage of endogenous TCR α chains by CD4+8+ thymocytes from female H-2k H-Y TCR/bcl-2 doubly transgenic mice may be related to the low affinity of the H-Y TCR for the positively selecting ligand. Experimental upregulation of the CD8 coreceptor and its effect on positive selection of immature CD4+8+ thymocytes expressing the H-Y TCR suggests that this is a low affinity TCR (40, 41). Recent studies (42, 43) suggest that CD4+8+ thymocytes can continue to rearrange their endogenous TCR α genes until they receive positive selection signals. As CD4+8+ thymocytes from doubly transgenic mice survive longer, it is likely that they will have a higher probability of generating TCR α chains able to pair with the transgenic TCR β chain more efficiently than the transgenic α chain. Some of these transgenic β/endogenous α TCR heterodimers may have a higher affinity for positively selecting ligands than the H-Y TCR, and thus have a selective advantage over the H-Y TCR. This would lead to the production of a large number of CD4+8+ thymocytes expressing high levels of endogenously expressed TCR α chains in the doubly transgenic females. The validity of this model can be tested further by mating bcl-2 transgenic mice with transgenic mice expressing TCR with putatively higher affinities for class I selecting ligands, or with transgenic mice that express a class II-restricted TCR.

Bcl-2 Overexpression Does Not Inhibit Negative Selection. Previous studies (19) have shown that Bcl-2 does not confer resistance to deletion of self-reactive T cells bearing certain Vβ regions that are specific for self-superantigens presented in the context of certain MHC class II molecules. However, depending on the promoters used to regulate expression of the bcl-2 transgene, some protection of T cells against deletion by self-superantigens was observed (20, 21). Superantigens differ from conventional peptide antigens in several ways. Conventional peptide antigens are processed and presented in the antigen-binding groove of MHC class I or II molecules, and are recognized by combinatorial elements of the TCR heterodimer (44). Superantigens, on the other hand, do not require conventional antigen processing, are not MHC haplotype restricted (although they require presentation by MHC class II), and interact primarily with the Vβ portion of the TCR (45, 46). Thus, superantigen-reactive T cells may differ from T cells that are reactive to conventional peptide-MHC complexes in terms of their activation or their negative selection, requirements. Furthermore, the deletion of T cell precursors specific for superantigens occurs...
at a later developmental stage, apparently after the CD4+8+ thymocytes have been committed to the CD4 or CD8 lineages (47). It was suggested that the lack of protection of super-antigen-reactive T cells in lck+ bcl-2 transgenic mice, when compared with other bcl-2 transgenic mice that used other promoters to drive the bcl-2 transgene, could be due to the downregulation of the activity of the proximal lck promoter in mature thymocytes (21). In this study, we show that CD4+8+ thymocytes expressing the H-Y TCR are deleted efficiently despite high levels of bcl-2 expression in these cells. This suggests that AICD that is brought about by negative selection signals occurs independently of Bcl-2. In contrast, high Bcl-2 levels in CD4+8+ thymocytes favor development along the CD8 lineage pathway. The differential sensitivity of these two developmental processes in the thymus to high levels of bcl-2 expression may provide a unique means to analyze the signaling mechanisms associated with these processes.

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