The T Cell Receptor Repertoire of $CD4^-8^+$ Thymocytes Is Altered by Overexpression of the *BCL-2* Protooncogene in the Thymus

By Wen Tao,* Soo-Jeet Teh,* Ian Melhado,‡§ Frank Jirik,‡§ Stanley J. Korsmeyer,‖ and Hung-Sia Teh*§

*From the *Department of Microbiology and Immunology, the ‡Department of Medicine, the §Biomedical Research Centre, University of British Columbia, Vancouver, Canada V6T 1Z3; and the ‖Departments of Medicine and Pathology, Howard Hughes Medical Institute, Washington University, St. Louis, Missouri 63130*

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 $CD4^-8^+$ lineage. The change involving $CD4^-8^+$ 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 ($H\text{-}2^b$) or nonselecting ($H\text{-}2^d$) environments. Furthermore, a large proportion of $CD4^-8^+$ thymocytes produced in $H\text{-}2^b$ 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 $CD4^-8^+$ thymocytes expressing TCRs that are otherwise nonselectable. However, the *bcl-2* transgene did not protect $CD4^+8^+$ 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 $CD4^-8^-$ thymocytes and in mature $CD4^+8^-$ thymocytes. There is a dramatic downregulation of *bcl-2* mRNA in $CD4^+8^+$ thymocytes, particularly those expressing a low level of TCR. $CD4^+8^+$ 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 $CD4^+8^+$ thymocytes, which express a low level of the TCR-α/β (2). $CD4^+8^+$ thymocytes are produced daily in large numbers and have an average life span of 3.5 d (3). Since the TCR repertoire expressed by $CD4^+8^+$ 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 $CD4^+8^+$ thymocytes are predicted to express nonselectable TCR and

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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 salvation 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 *lck* tyrosine kinase gene (lck^{Pr}) (19). In these lck^{Pr}-*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_β 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 lck^{Pr}-*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 $H\text{-}2D^b$ class I molecule (22, 23). We found that in doubly transgenic female lck^{Pr}-*bcl-2*/H-Y TCR mice of the $H\text{-}2^b$ (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 $H\text{-}2^b$ 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). $H\text{-}2^b$ H-Y TCR, $H\text{-}2^d$ H-Y TCR, and lck^{Pr}-*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-$H\text{-}2D^b$ (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 β chain, F23.1, and the transgenic TCR α chain, T3.70, were prepared as previously described (23–25).

Flow Cytometry. Two-color staining of cells with FITC-labeled anti-CD8 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 β chain, thymocytes were incubated with biotinylated anti-CD8, PE-labeled anti-CD4, and FITC-labeled anti-F23.1 mAbs followed by Streptavidin Cychrome (PharMingen, distributed by Cedarlane Laboratories). Three-color sorting was performed with the FACStar Plus® cell sorter.

[1] *Abbreviations used in this paper:* AICD, activation-induced cell death; lck^{Pr}, proximal promoter of *lck*; PCD, programmed cell death.

mRNA Quantitation by PCR. RNA was extracted from 3 × 10^5–2 × 10^6 purified thymocyte subpopulations using the guanidinium isothiocynate 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 evaluation by quantitative reverse transcriptase PCR using p(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′-CCTGTGCCACCATGTGTCCATC-3′ and 5′-GCTGAGAACAGGGTCTTCAGAGAC-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 ^{32}P-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^b* transgenic mice, the transgenic TCR is positively selected by thymic epithelial cells that present H-2D^b 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 *lck^{Pr}-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^b doubly transgenic mice are 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.

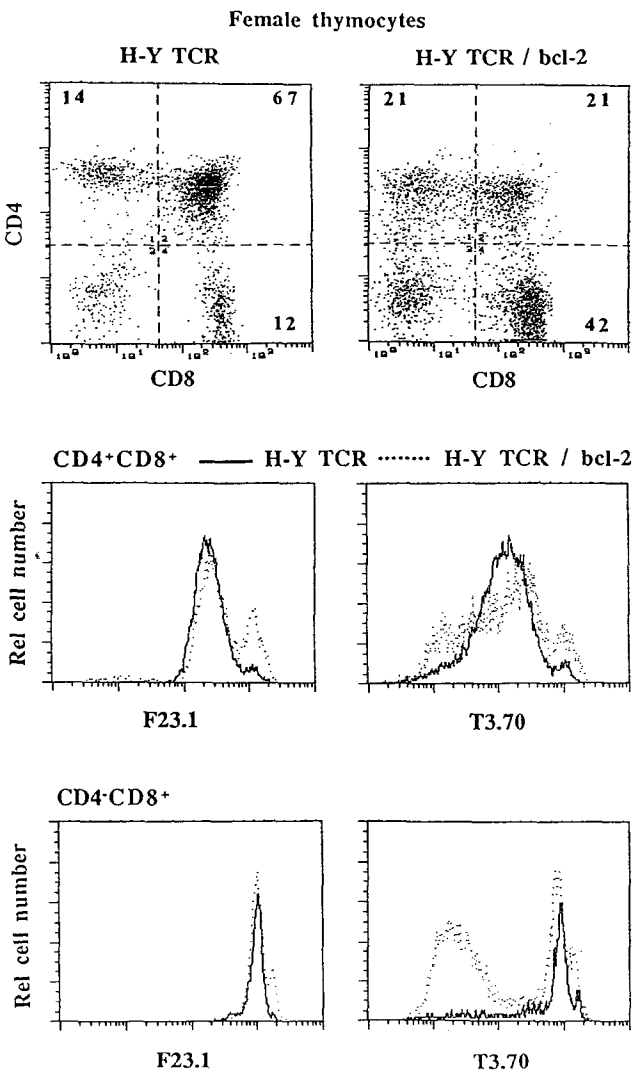


Figure 1. Alteration of the repertoire of CD4$^+$8$^+$ thymocytes and CD4$^-$8$^+$ thymocytes in H-Y TCR/*bcl-2* doubly transgenic mice. Thymocytes from female *H-2^b* H-Y TCR or female *H-2^b* H-Y TCR/*bcl-2* doubly transgenic mice were harvested and triple-stained with the indicated mAbs. The level 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.

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^b* 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

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%).

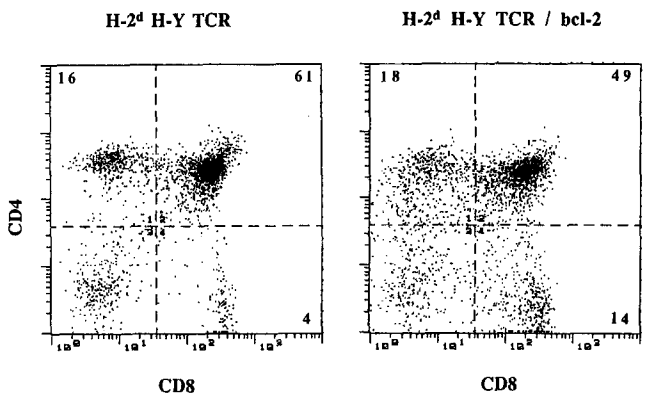
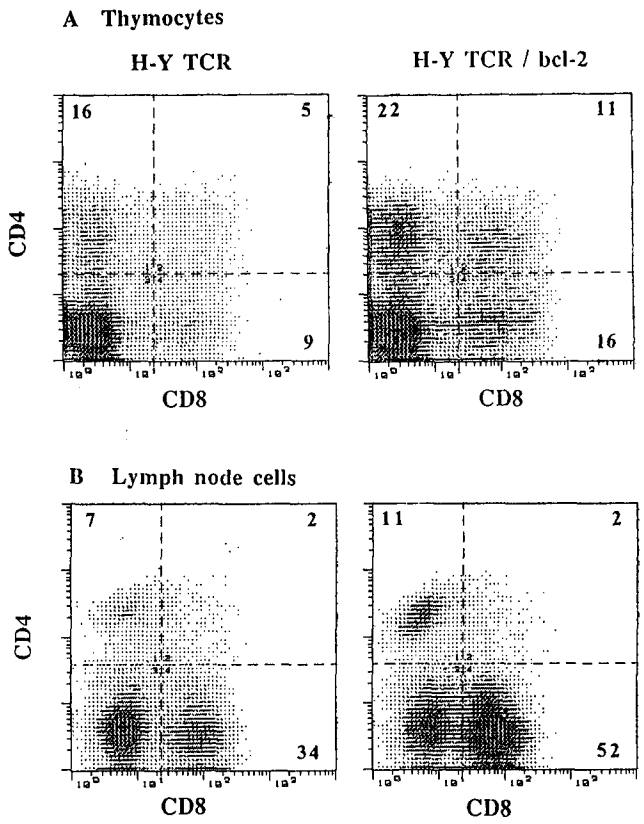


Figure 2. Overproduction of $CD4^-8^+$ thymocytes in *H-2*^{d} H-Y TCR/*bcl-2* doubly transgenic mice. *H-2*^{d} H-Y TCR/*bcl-2* doubly transgenic mice were produced by crossing *bcl-2* transgenic mice and H-Y TCR transgenic mice onto a *H-2*^{d} background. The lack of H-2D^b expression by these mice was confirmed by staining with the anti-H-2D^b mAb (data not shown). Thymocytes from these mice were double-stained with PE-labeled anti-CD4 and FITC-labeled anti-CD8 mAbs and analyzed in the FACScan® flow cytometer.

We next determined whether this skewing towards the $CD4^-8^+$ lineage in the doubly transgenic mice 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 *lck*^{pr}*-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 ($19 \times 10^6 \pm 6 \times 10^6$, n = 6 for H-Y TCR males versus $45 \times 10^6 \pm 5 \times 10^6$, 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 $CD4^-8^{lo}$ 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 ($112 \times 10^6 \pm 11 \times 10^6$, n = 3). However, a large fraction of surviving $CD4^+8^+$ thymocytes from doubly transgenic males 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-2ᵇ* H-Y TCR transgenic mice, or normal *H-2ᵇ* 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

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-2ᵇ* H-Y TCR and male *H-2ᵇ* H-Y TCR/*bcl-2* doubly transgenic mice were collected, double-stained with anti-CD4 and anti-CD8 mAbs, and analyzed in the FACScan®.

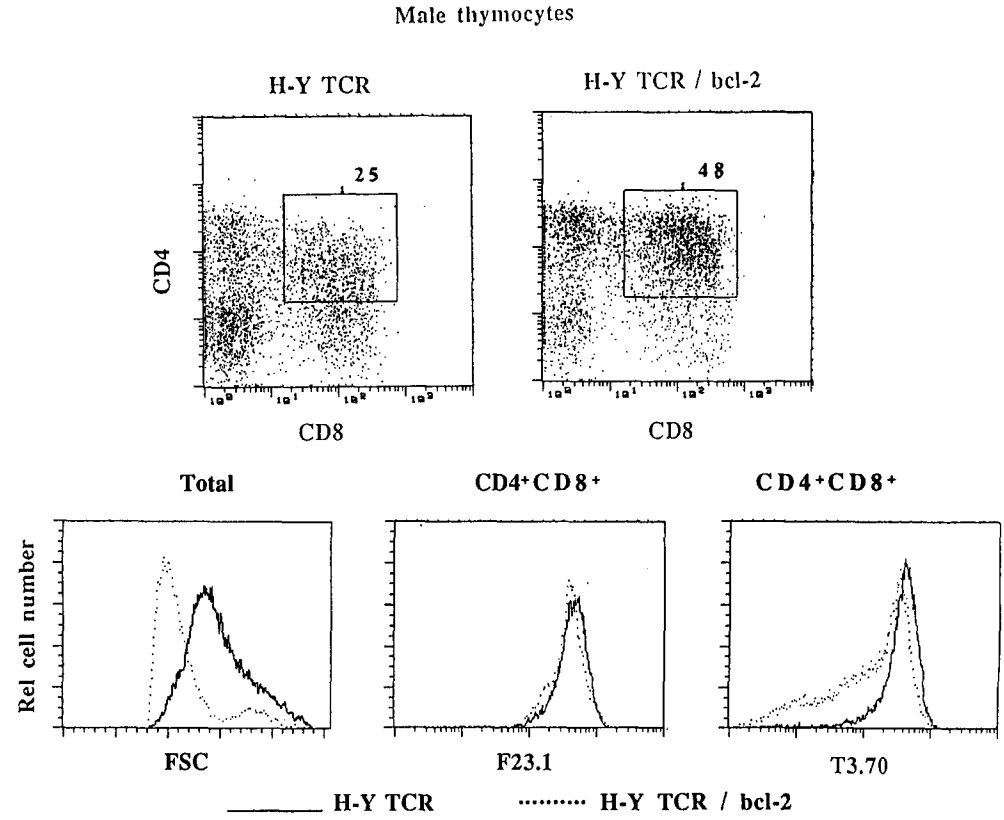


Figure 4. A large proportion of CD4⁺8⁺ thymocytes escaping negative selection in H-Y TCR/*bcl-2* doubly transgenic male mice express endogenous TCR α chains. Thymocytes from male *H-2ᵇ* H-Y TCR transgenic mice and male *H-2ᵇ* H-Y TCR/*bcl-2* doubly transgenic mice were collected and triple-stained with mAbs specific for CD4, CD8, and either the transgenic TCR β or the transgenic TCR α chain and analyzed in the FACScan®.

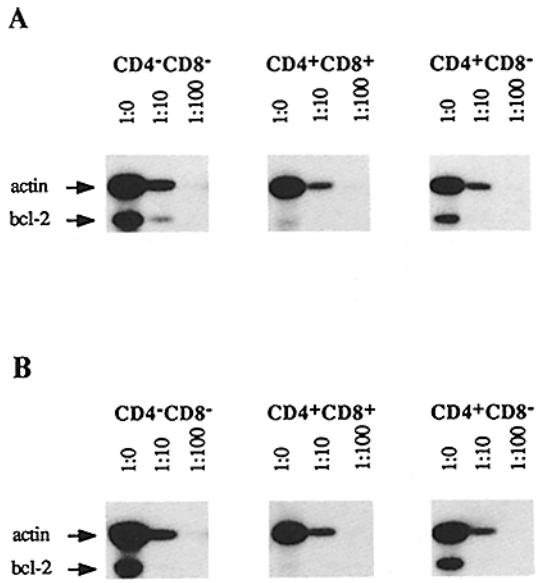
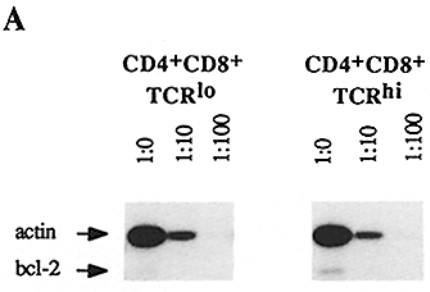


Figure 5. Differential expression of *bcl-2* by immature $CD4^-8^-$, $CD4^+8^+$, and mature $CD4^+8^-$ thymocytes. $CD4^-8^-$ thymocytes of ≥ 98% purity were prepared as described in Materials and Methods. $CD4^+8^+$ and $CD4^+8^-$ thymocytes of ≥95% purity were obtained by two-color staining with anti-CD4 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 ^{32}P-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-2*^{b} H-Y TCR transgenic mice. (*B*) Purified thymocyte subpopulations from C57Bl/6 mice.

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-2*^{b} 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.

Discussion

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-2*^{b} or a nonselecting *H-2*^{d} 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-2*^{b} H-Y TCR/*bcl-2* doubly transgenic female mice expressed endogenous TCR α chains rather than the transgenic TCR α chain. The bias towards the CD8 lineage is not as apparent in peripheral T



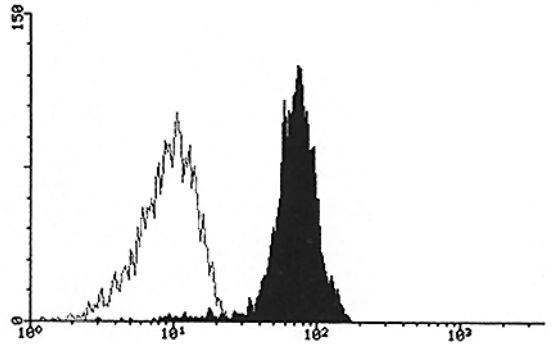


Figure 6. Coordinate upregulation of TCR expression and *bcl-2* mRNA by $CD4^+8^+$ thymocytes. Thymocytes from *H-2*^{b} H-Y TCR transgenic mice were harvested and triple stained with anti-CD4, anti-CD8, and anti-transgenic TCR β chain mAbs. $CD4^+8^+$ thymocytes expressing either a low or high level 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^{hi} and TCR^{lo} $CD4^+8^+$ 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 $p56^{lck}$ than does the cytoplasmic domain of CD8. This less efficient association of $p56^{lck}$ with CD8 may contribute to a reduction in efficiency of positive selection of $CD4^-8^+$ thymocytes expressing MHC class I–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-2*^{b} 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 endogenous 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 superantigen-reactive T cells in *lck*^{Pr}-*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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Address correspondence to Dr. Hung-Sia Teh, The Department of Microbiology and Immunology, The University of British Columbia, 6174 University Boulevard, Vancouver, BC, Canada V6T 1Z3.

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