CD8 Surface Levels Alter the Fate of α/β T Cell Receptor-expressing Thymocytes in Transgenic Mice

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Summary

The mature T cell receptor (TCR) repertoire is established on the basis of discriminative events involving binding of the TCR α and β chains and CD4 or CD8 on immature thymocytes to major histocompatibility complex (MHC)/self-peptide complexes expressed in the thymus. To ask whether the strength of the interaction between a CD8/TCR complex and a MHC/self-peptide ligand plays a pivotal role in deciding the fate of a maturing thymocyte, we generated lines of transgenic mice that express distinct and elevated levels of CD8α, approximately 2, 3, and 6–10 times. These lines were then crossed to a transgenic line expressing the class I–restricted TCR, 2C. We found that thymocytes expressing the 2C TCR in combination with the highest levels of CD8 were deleted on the H-2 K^b background that is normally positively selecting for the 2C TCR. In contrast, thymocytes coexpressing the 2C TCR and moderately elevated levels of CD8 were selected for maturation. These results demonstrate directly that CD8 levels can affect the developmental fate of a maturing thymocyte and argue in support of an affinity model for thymocyte selection.

Mature T lymphocytes recognize foreign Ag in the form of processed peptides bound to MHC molecules. The recognition of the peptide-MHC ligand is mediated by the α/β TCR complex and CD4 or CD8. Modeling studies indicate that the variable regions of the TCR simultaneously contact the antigenic peptide and the polymorphic α helices that flank the peptide binding groove of MHC molecules (1–3). CD4 and CD8, on the other hand, contact monomorphic determinants on MHC class II and class I molecules, respectively (4–8), and are believed to be intimately involved with the TCR in corecognition of Ag-MHC complexes (9, 10). The cytoplasmic domains of both CD4 and CD8 are physically associated with the lymphocyte-specific tyrosine kinase, $p56^{lck}$ (11–14), and thus, the coreceptor functions of CD4 and CD8 may include collaboration with the TCR complex to transduce transmembrane signals upon recognition of antigen (15, 16). Functional studies on CTL have demonstrated a direct correlation between the CD8 dependency of CTL clones and the density of the peptide Ag–class I MHC determinants on a target cell (17–19). Since the TCR and CD8 on CTL have been shown to interact with the same antigenic class I molecule (9, 10), these findings suggest that the CD8 coreceptor may actually contribute to the affinity of a TCR for its Ag–class I MHC ligand.

Mature $CD4^+CD8^-$ and $CD4^-CD8^+$ T cells differentiate in the thymus from fetal liver or adult bone marrow–derived stem cells that express neither the α/β TCR nor CD4 and CD8 (reviewed in reference 20). The major intermediate in the T cell differentiation pathway is the double-positive thymocyte, which expresses both CD4 and CD8 and low levels of the α/β TCR. Two selective forces are known to operate during thymocyte maturation to choose the α/β TCR repertoire expressed on mature T cells. Positive selection rescues from cell death those thymocytes that will be able, as mature T cells, to engage self-MHC molecules that have bound foreign antigen; negative selection either eliminates or inactivates thymocytes bearing autoreactive α/β TCRs (reviewed in references 20–22). Consequently, only a subset of the TCR specificities expressed on immature thymocytes are found on mature peripheral T lymphocytes.

Studies on T cell selection using bone marrow and thymus chimeras (23) and class II transgenic mice (24, 25) suggest that positive and negative selection may be separable events mediated largely by distinct subsets of thymic stromal cells, radiation-resistant thymic epithelial cells in the case of positive selection, and bone marrow–derived APC and medullary epithelial cells in the case of negative selection (26). However, both positive and negative selection appear to depend on the formation of a complex between the α/β TCR and MHC molecules bound to self-peptides (27–30). Conse-

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quently, a major unresolved issue in T cell development is how the positively selected thymocytes escape clonal deletion or anergy (20, 21).

The affinity model has been proposed to resolve this paradox. This model suggests that the fate of a maturing thymocyte is determined by the strength of the interaction between a MHC/self-peptide ligand and a multimeric complex containing the α/β TCR and CD4 or CD8. Thymocytes bearing low affinity receptors are selected for maturation, whereas thymocytes bearing intermediate or high affinity receptors are subject to negative selection, and are either clonally inactivated or deleted. One prediction of the affinity model is that increasing the affinity of an α/β TCR for a selecting MHC/self-peptide ligand should convert a positively selecting interaction into one that is negatively selecting. To test this prediction, we attempted to change the avidity of thymocyte/stromal cell interactions by increasing the surface levels of CD8 on maturing thymocytes. Transgenic lines expressing elevated levels of murine CD8α on all subsets of thymocytes and mature T cells were generated and then crossed, on what is a normally positively selecting background, to a transgenic line expressing a class I–restricted TCR. We find that thymocytes coexpressing the TCR transgene and moderately elevated levels of CD8 are selected for maturation, whereas thymocytes expressing the TCR transgene and markedly enhanced levels of CD8 are deleted; thus, our results support the affinity model for thymocyte selection.

Materials and Methods

Construction of the pNeZCD8α.1 Plasmid. The CD8α.1 allele of CD8α was isolated from a CBA/J genomic library prepared from MboI partially digested liver DNA in the λ vector EMBL3. To distinguish between transcripts of the CD8α.1 transgene and the endogenous CD8α.2 gene, a 130-bp fragment from the 3′ untranslated region of the sea urchin actin gene CyIIIa (31) was inserted into a SacI site in the 3′ untranslated region of CD8α.1. To prepare the pNeZCD8α.1 plasmid, sequences 5′ of the PvuII site lying ~125 bp upstream from the initiating ATG (32) were deleted. This promoterless CD8α.1/actin gene was cloned into a CD3-δ cassette. The pNeZ cassette was prepared in pNNO3 (a gift from R. Tizzard, Biogen, Cambridge, MA) by first inserting a 0.6 kb NarI-EcoRI restriction fragment carrying the murine CD3-δ enhancer (33) into a BamHI site using BamHI linkers. A second fragment containing the BclI-BclI promoter region of CD3-δ (33) was cloned into the SmaI and HindIII sites of this plasmid.

Generation of CD8α.1 Transgenic Mice. The plasmid pNeZCD8α.1 was digested with NotI to excise the CD3-δ/CD8α.1 sequences from the plasmid vector. The DNA was isolated from an agarose gel by electrophoresis onto a dialysis membrane, phenol extracted, and ethanol precipitated. The purified DNA was injected at a concentration of 1–5 ng/μl in 5 mM Tris-HCl, pH 7.4, 5 mM NaCl, 0.1 mM EDTA into fertilized eggs of the C57BL/6J inbred strain (The Jackson Laboratory, Bar Harbor, ME). To generate line 2761, the pNeZD8α.1 fragment was coinjected with a 5.5-kb BamHI-XbaI restriction fragment containing the human CD2 enhancer (a gift from D. Kioussis, National Institute for Medical Research, London; 34). DNA purified from cut tail tips was digested and analyzed for integration of the transgene by hybridization with a CD8α cDNA probe (a gift of D. Littman, University of California, San Francisco).

Purification and Staining of Lymphocytes. Tissues were homogenized by crushing between two frosted glass slides in PBS with 1% BSA, 0.1% sodium azide followed by filtration through Nitex mesh (no. 3-40/26; Tetko Inc., Elmsford, NY). Red blood cells were lysed in 0.15 M ammonium chloride, 10 mM potassium bicarbonate, 0.1 mM EDTA for 5 min on ice. Lymphocytes were washed and ~10^6 cells per sample were stained with saturating concentrations of antibody. Cells were washed again and stained with secondary reagents as indicated in the figure legends. Splenic T cells were prepared by labeling splenocytes with anti-Ia^p (mouse IgG2A; Accurate Chemical & Scientific Corp., Westbury, NY) followed by depletion with goat anti–mouse IgG–coated magnetic beads (Advanced Magnetics, Cambridge, MA). Stained cells were fixed in PBS with 1% formaldehyde, 0.1% sodium azide and analyzed on a FACScan® flow cytometer using FACScan® Research Software (Becton Dickinson & Co., Mountain View, CA). The three-color analyses using anti-CD8α.1, anti-CD8α.2, and anti-1B2 were accomplished by staining first with anti-CD8α.1 (clone 01MLeuKO3) and anti-CD8α.2 (clone 2.43), followed by staining with FITC-labeled goat anti–mouse Ig (Becton Dickinson & Co.) and PE-labeled goat anti–rat Ig (Tago Inc., Burlingame, CA). The washed cells were treated with purified mouse Ig (Sigma Chemical Co., St. Louis, MO), then with biotinylated anti-1B2, followed by staining with streptavidin-red 613. Dead cells were eliminated from the analysis on the basis of forward and sideways light scatter. 5,000 cells were analyzed for each plot for two-color analysis, and 15,000 cells for three-color analysis.

Antibodies. The following mAbs were used for staining: anti-CD8α, clone 53-6.7; anti-CD4, clone GK1.5; anti-Thy-1.2, clone 30-H12 were from Becton Dickinson & Co. Anti-CD8α.1, clone 01MLeuKO3 and anti-Ia^p, clone 4-16.17 were from Accurate Chemical & Scientific Corp. Anti-CD8α.1, clone 116-13.1 and anti-CD8α.2, clone 19/178 were gifts from Drs. S. Kimura and U. Hammerling (Sloan-Kettering Institute). Anti-CD8α.2, clone 2.43 was a gift from Dr. J. Nikolic-Zugic (Sloan-Kettering Institute). Anti-TCR-α/β, clone 57–597 and anti-Lyt-3 (CD8β) were from Pharmingen (San Diego, CA). The anti-2C TCR antibody is 1B2 (35). Anti CD3ϵ, clone 145-2C11 was from Boehringer Mannheim Biochemicals, Indianapolis, IN. Several secondary antibodies and reagents were used. Streptavidin-PE, avidin-FITC, and goat anti–mouse Ig-FITC were obtained from Becton Dickinson & Co; goat anti–rat Ig F(ab′)-PE and goat anti–mouse Ig F(ab′)-FITC and -PE were from Tago Inc.; goat anti–rat Ig-FITC was from Southern Biotechnology Associates (Birmingham, AL); streptavidin-red 613 was from Bethesda Research Laboratories (Gaithersburg, MD); and purified mouse Ig was from Sigma Chemical Co.

Results

Generation of CD8α.1 Transgenic Mice. To direct expression of a CD8α transgene to all subclasses of thymocytes and peripheral T cells, the murine CD8α.1 gene was inserted downstream of the murine CD3-δ promoter and enhancer in the construct pNeZCD8α.1 (Fig. 1). The murine CD3-δ enhancer is located in a DNase I hypersensitive area at the 3′ end of the CD3-δ gene and functions in transient transfection assays as a position- and orientation-independent regulatory element that targets the transcription of heterologous genes specifically to T cells (33). Therefore, we predicted that the CD3-δ enhancer should act as a T cell–specific regulatory element in transgenic mice and target transgene expression to both the CD4 and CD8 T cell subsets. The

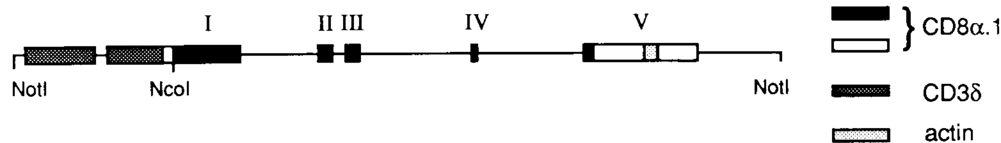

Figure 1. Diagram of the pNeZCD8α.1 expression plasmid. The components of this plasmid include the murine CD3-δ sequences necessary for T cell–specific expression, as indicated by dark stippling: a 0.6-kb NarI-EcoRI restriction fragment containing the CD3-δ enhancer sequences and a 0.5-kb BclI-BclI restriction fragment comprising the 5′ upstream regulatory sequences (33, 64). Exons 1–5 of the murine CD8α.1 gene, as reported by Liaw et al. (32), are indicated by black boxes; the 5′ and 3′ nontranslated regions, by white boxes. The lightly stippled box within the 3′ nontranslated region denotes a 130-bp fragment of the sea urchin actin gene, pCyIIIa (31), which was inserted to provide a distinction between endogenous and transgene transcripts. A NotI restriction digest was performed to sever the plasmid vector pNNO3 from the sequences microinjected into fertilized mouse eggs.

pNeZCD8α transgene was microinjected into fertilized eggs derived from the C57BL/6J (B6) strain. This strain expresses the CD8α.2 allele; thus, transgene expression can be distinguished from endogenous gene expression by surface staining with antibodies that distinguish between the α.1 and α.2 alleles of CD8. Five transgenic lines were generated. These lines were initially screened for transgene expression by assaying for the presence of mature peripheral $CD4^+CD8^+$ double-positive T cells. Splenic T cells were analyzed by two-color FACS® analyses using antibodies specific for CD4 and both alleles of CD8. We found that two of the five lines, lines 151 and 140, contained significant numbers of double-positive peripheral T cells and expressed elevated levels of CD8 on both splenocytes and thymocytes (Fig. 2). An additional four lines were generated by coinjecting the pNeZCD8α.1 transgene and a fragment containing the T cell–specific enhancer from the human CD2 gene (34). One of these lines, 2761, also expressed elevated levels of CD8α and was chosen, along with lines 151 and 140, for further characterization.

The CD8α.1 Transgene Is Expressed at Distinct and Elevated Levels on Thymocytes and Peripheral T Cells of Lines 151, 2761, and 140. Splenocytes and thymocytes from lines 151, 2761, and 140 were stained with antibodies to CD4 and CD8, and an example of the resulting two-color FACS® profiles is shown in Fig. 2. In lines 151 and 2761, 100% of the $CD4^+$ splenocytes coexpress CD8. Relative to endogenous levels of CD8 on control B6 splenocytes, the $CD4^+CD8^+$ splenocytes in Fig. 2 express 2.4- and 3.8-fold elevated levels of CD8 in, respectively, lines 151 and 2761. Averaging relative values of CD8 fluorescence from several independent transgenic mice, the CD8 levels on $CD4^+CD8^+$ splenocytes range from 1.5 to 3 times in line 151 and two to four times in line 2761. In line 140, the pNeZCD8α.1 transgene is expressed on ~83% of $CD4^+$ splenic cells, generating a $CD4^+CD8^+$ subset that represents 20% of the T cell–enriched splenocytes. The level of transgene expression on the double-positive cells is approximately sixfold higher than endogenous gene expression, as indicated by the difference in mean CD8 fluorescence between the $CD8^+$ single-positive cells in the control B6 mouse and the $CD4^+CD8^+$ double-positive cells in the line 140 mouse, 46 and 280, respectively. The data in Fig. 2, as well as additional two-color FACS® analyses of splenocytes from line 140 mice (see Fig. 6), reveal that there are two distinct populations of the $CD4^-CD8^+$ single-positive T cells in line 140 mice, $CD8^{normal}$ and $CD8^{hi}$; the latter contain ~60% of the $CD4^-CD8^+$ subset and stain with a mean CD8 fluorescence that is approximately sevenfold more intense than that on $CD8^{normal}$ cells (see Fig. 6). In contrast, only a single $CD4^-CD8^+$ population is observed in lines 151 and 2761, staining at levels approximately twofold higher than those on controls.

Fig. 2 also shows that lines 151, 2761, and 140 express CD8 on nearly 100% of all thymocytes. Line 151 thymocytes express CD8 at 2.2-fold increased levels, giving a mean CD8 fluorescence of 177 compared with a mean CD8 fluorescence of 81 on the $CD4^+CD8^+$ thymocytes in the control B6 mouse. Line 2761 thymocytes express CD8 at 2.8-fold elevated levels (a mean CD8 fluorescence of 226 vs. 81) and line 140 $CD4^+CD8^+$ thymocytes at 7.8-fold elevated levels (a mean CD8 fluorescence of 630 vs. 81). These elevated levels of CD8 have been consistently observed in numerous animals of various ages and thus are a stable phenotype of these transgenic lines.

To establish whether the elevated levels of CD8 expression in the CD8 transgenic lines are due entirely to the expression of the pNeZCD8α.1 transgene, splenic T cells from transgenic and nontransgenic B6 littermates were costained with anti-CD4 and either anti-CD8α.1 or anti-CD8α.2 antibodies. Fig. 3 *A* presents the staining profile of the purified splenic T cells from a line 140 mouse. The $CD4^+CD8^+$ subset reacts with the anti-CD8α.1 antibody but not the anti-CD8α.2 antibody, demonstrating that virtually all of the CD8 expression on the double-positive peripheral T cells represents transgene expression. Likewise, since the level of CD8α.2 staining on the CD8 single-positive subset does not vary between control B6 and line 140 mice (Fig. 3 *A*), the enhanced levels of CD8 staining on $CD4^-CD8^+$ subpopulation must result exclusively from transgene expression. Comparable results were also obtained from FACS® analyses examining CD8α.1 and CD8α.2 expression on splenocytes from line 151 and 2761 mice.

Fig. 3 *B* shows the two-color FACS® profile of thymo-

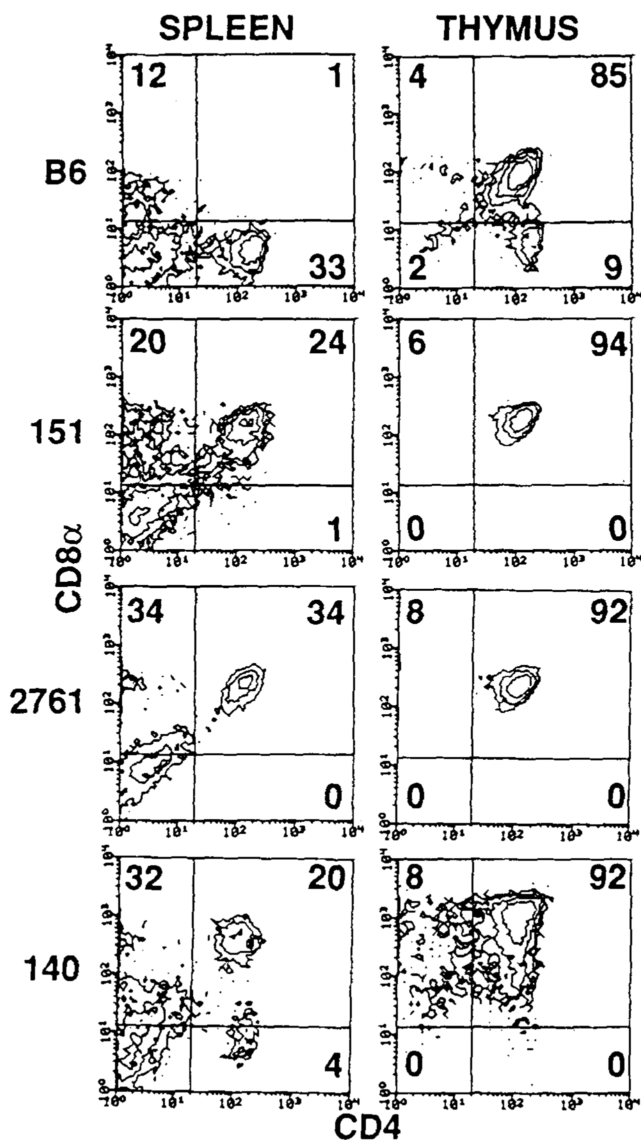

Figure 2. CD4 and CD8 surface phenotype of splenic T cells and thymocytes from normal and CD8α.1 transgenic mice. Flow cytometric analysis was performed by double-color staining with PE-conjugated anti-CD4 and FITC-conjugated anti-CD8. The percentages of total cells in each quadrant are indicated on each plot. Fluorescence intensities are indicated on a logarithmic scale. Greater than 10 mice from each transgenic line have been analyzed at ages between 3 wk and 8 mo. The data shown for nontransgenic C57BL/6J (B6) or transgenic lines 151, 2761, and 140 represent cells from a single mouse killed at 8 wk of age.

cytes from line 140 transgenic and nontransgenic littermates after staining with anti-CD4 and either anti-CD8α.1 or anti-CD8α.2 antibodies. As found with the splenic T cells, thymocytes from both line 140 and B6 control mice express comparable levels of the endogenous CD8α.2 gene, arguing that the approximately eightfold elevated levels of CD8 in the thymus result entirely from transgene expression. Similar FACS® analyses, using CD8α.1- and CD8α.2-specific antibodies to stain thymocytes from lines 151 and 2761, show that levels of the endogenous CD8α.2 gene product are unaffected and thus that the elevated CD8 levels in these transgenics also result from expression of CD8α.1 transgene. Consequently, in none of the CD8 lines has expression of the pNeZCD8α.1 transgene perturbed expression of the endogenous CD8α gene product on either immature or mature T cells.

Our data indicate that, on average, 80% and 60%, respectively, of the peripheral CD4$^+$ and CD8$^+$ T cell subsets express the pNeZCD8α.1 transgene in line 140 mice. In addition, based on staining with the CD8α.1 antibody, only 80% of thymocytes in line 140 mice express the CD8 transgene. The incomplete expression of the pNeZCD8α.1 transgene in line 140 mice most likely represents a chromosomal position effect, as lines 151 and 2761, as well as additional lines of mice carrying pNeZCD8α.1 or other CD3-δ-driven transgenes, express these transgenes on 100% of peripheral T cells and thymocytes (N.A. Lee, D. Grass, and E. Lacy, unpublished results). Such chromosomal position effects, leading to line-specific patterns of transgene expression, are commonly observed in transgenic mice (36–39).

pNeZCD8α.1 Transgene-expressing Cells Show Normal Levels of Other T Cell Surface Markers. To determine whether elevated levels of CD8 expression affect normal T cell development, we have examined the expression of CD8β, CD3, and the α/β TCR on thymocytes and splenic T cells in the CD8 transgenic mice. CD8 is expressed on the surface of most thymocytes and mature T cells as a disulfide-linked α/β heterodimer (40–42). In the absence of CD8β, however, CD8α can be expressed as a homodimer (43, 44). CD8β, on the other hand, requires CD8α for cell surface expression (44–46). We have investigated whether the high levels of CD8α expression in the line 140 mice can increase the surface levels of CD8β by performing three-color FACS® analyses using antibodies to CD8β, CD4, and CD8α (Fig. 4). Our results show that surface levels of CD8β are not enhanced in line 140 mice. Consequently, we conclude that a pool of excess CD8β chains does not normally exist in T cells and that the surface level of the CD8α/β heterodimer is not determined solely by limiting quantities of CD8α chain. In addition, no CD8β expression is detected on the CD4$^+$CD8$^+$ peripheral T cells expressing the CD8α.1 transgene. Together, these findings argue that the elevated levels of CD8 are expressed in the form of α/α homodimers on thymocytes and peripheral T cells of the CD8 transgenic mice.

When stained with an anti-CD3 antibody, thymocytes from both line 140 mice and nontransgenic littermates yielded comparable single-color FACS® profiles (data not shown). The CD8α and TCRα/β two-color staining profile (Fig. 5, *bottom*) shows that ~80% of the CD8 cells in lines 151, 2761, and 140 express the TCR-α/β. The non-TCR-α/β staining CD8 cells presumably result from expression of the CD3-δ enhancer–driven CD8 transgene in B lymphocytes. Although the CD3-δ enhancer functions preferentially in T lymphocytes, characterization of other transgenes driven by the CD3-δ enhancer indicates that it has some specificity for B cells as well (D. Grass and E. Lacy, unpublished data). In Fig. 5, *top* and *middle*, the CD8 fluorescence of total lymphocytes and

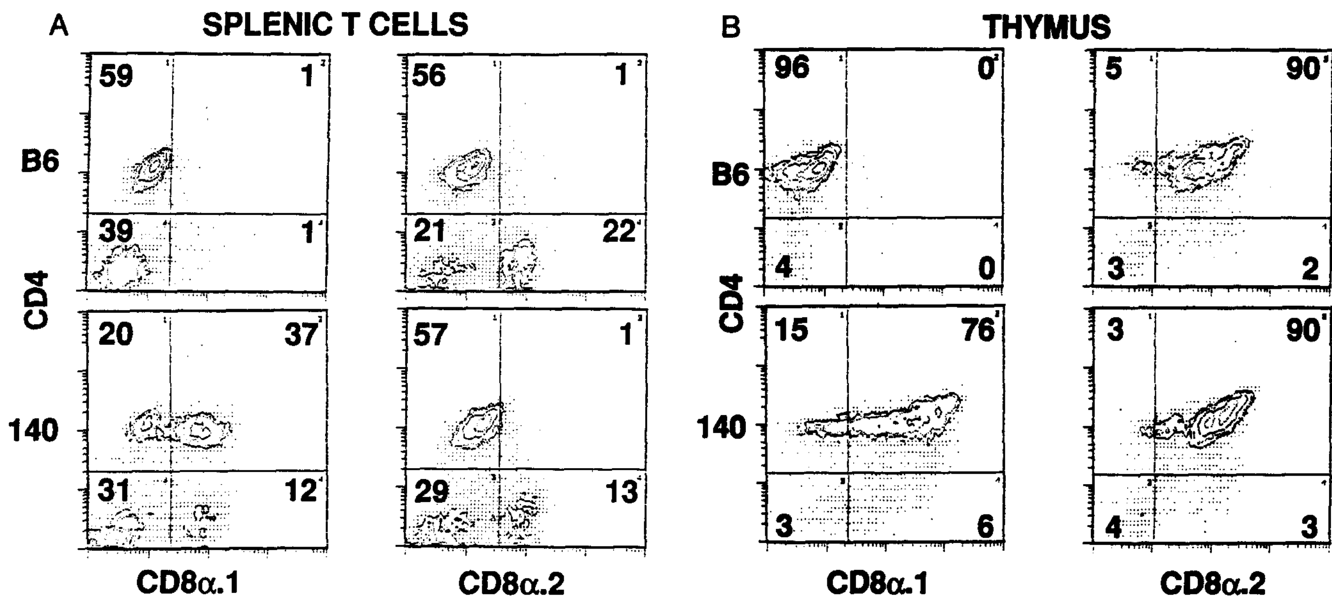

Figure 3. Surface phenotype of splenic T cells and thymocytes from normal and line 140 mice. Flow cytometric analysis was performed as described in Fig. 2, except that cells were stained with PE-conjugated anti-CD4 and either anti-CD8α.1 (clone 116-13.1) or anti-CD8α.2 (clone 19/178) followed by staining with FITC-conjugated goat anti-mouse Ig. This CD8α.1 antibody stains lymphocytes from line 140 with a mean channel value of CD8 fluorescence that is five- to sixfold greater than that of lymphocytes from CBA/J mice, which carry the endogenous CD8α.1 allele (data not shown).

the TCR-α/β^+ subset is plotted as a histogram. These data demonstrate directly that the vast majority of TCR-α/β^+ cells do express the elevated levels of CD8 characteristic of lines 151, 2761, and 140. All subsets in the transgenic mice ($CD4^+CD8^-$, $CD4^+CD8^{hi}$, $CD4^-CD8^{normal}$ [in line 140], and $CD4^-CD8^{hi}$) express levels of the TCR-α/β complex that are identical to those detected on splenic T cells from nontransgenic mice. This is shown for the $CD8^+$ cells in Fig. 5, *bottom*, in which the mean TCR-α/β fluorescence of the $CD8^+$TCR-α/β^+ splenic T cells is 104 for the B6 mouse, and 104, 107, and 96, respectively, for the line 151, 2761, and 140 mice. Consequently, on the basis of TCR-α/β expression, we have found no evidence that high levels of CD8 grossly perturb T cell maturation.

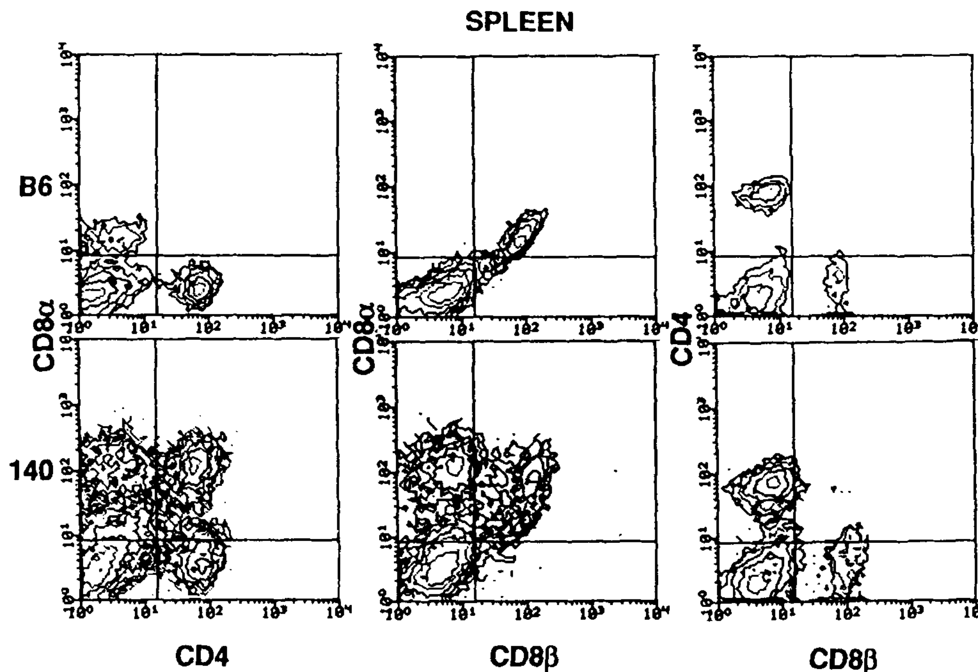

Figure 4. Expression of the endogenous CD8β gene is unaffected in transgenic line 140 mice. Three-color FACS® analysis was performed on splenocytes from nontransgenic C57BL/6J (B6) and transgenic line 140 mice. Splenocytes were stained with PE-conjugated anti-CD4, FITC-conjugated anti-CD8α, and biotinylated anti-CD8β, followed by staining with streptavidin red 613. Five mice of each genotype were analyzed; the data shown represent those of one individual. The mean CD8β fluorescence on splenic T cells is 85 in control B6 mouse and 77 in the line 140 mouse.

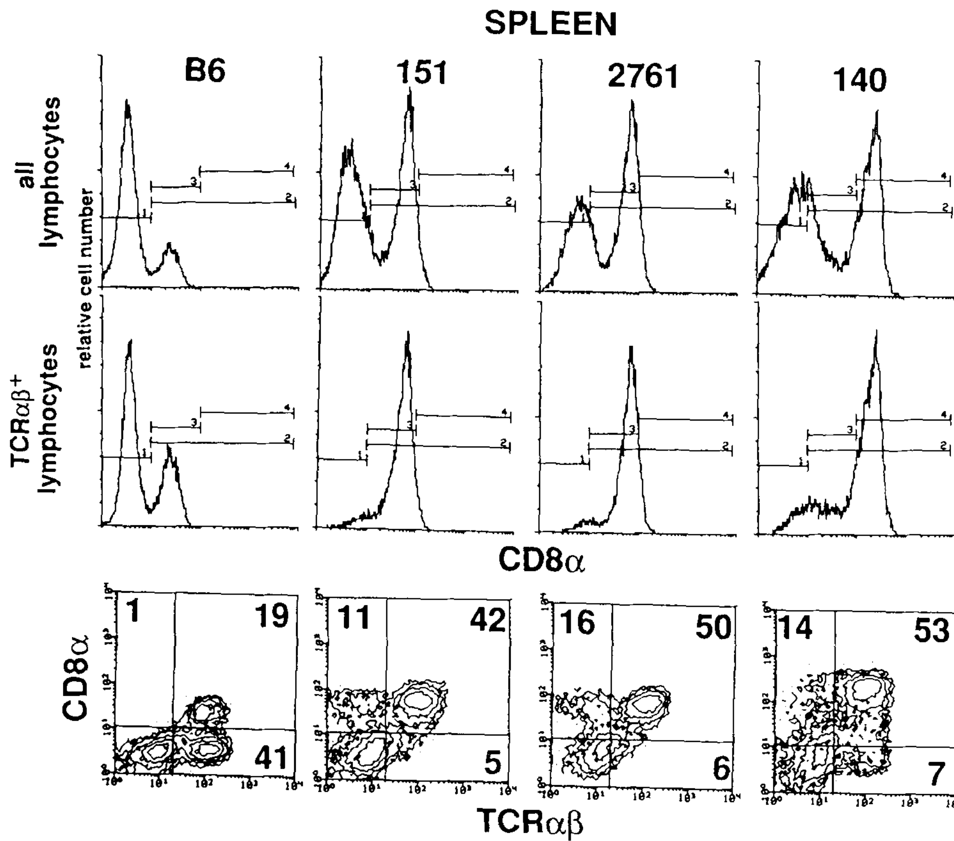

Figure 5. Cell surface expression of the TCR-α/β on splenic T cells in line 151, 2761, and 140 transgenics. Splenic T cells from nontransgenic C57BL/6J (B6) and transgenic line 151, 2761, and 140 mice were labeled with biotinylated anti-TCR-α/β, anti-CD8 (FITC), and anti-CD4 (PE), followed by staining with streptavidin red 613. Four mice of each genotype were analyzed; the data shown represent those of one individual. (*Top* and *middle*) Relative cell number versus CD8 fluorescence for, respectively, total splenic lymphocytes and TCR-α/β^+ splenic T cells. The histogram markers are numbered 1–4, from bottom to top. (Marker *2*) The left boundary of which is set at 10, distinguishes the CD8$^-$ cells from the CD8$^+$ cells; (marker *4*) the left boundary of which is set at 100, distinguishes the CD8^{normal} cells form the CD8^{hi} cells. (*Top*) The mean CD8 fluorescences of all lymphocytes staining for CD8 (marker *2*) are 26 (B6), 55 (151), 61 (2761), and 148 (140); the mean CD8 fluorescence for the CD8^{hi} cells in line 140 (marker *4*) is 228. (*Middle*) The mean CD8 fluorescences (marker *2*) for the TCR-α/β^+ lymphocytes are 26 (B6), 59 (151), 69 (2761), and 175 (140); the mean CD8 fluorescence of the CD8^{hi} TCR-α/β^+ cells in line 140 is 225. (*Bottom*) CD8α vs. TCR-α/β FACS® profile of T cell–enriched splenocytes in nontransgenic B6 mice and line 151, 2761, and 140 mice. The numbers in each corner of the contour plots represent the percentage of cells staining in each quadrant. The mean TCR-α/β fluorescences of the CD8$^+$ TCR-α/β^+ cells are 104 (B6), 104 (151), 107 (2761), 96 (140), and 103 (CD8^{hi}, 140).

CD8^{hi} Cells Are Deleted in 2C TCR Transgenic Mice. Line 151, 2761, and 140 mice were crossed to transgenic mice expressing the 2C class I–restricted TCR to determine whether elevated levels of CD8 expression could alter the developmental fate of a maturing thymocyte bearing a TCR of known specificity on its cell surface. This TCR transgenic line, which like the CD8 transgenics is on an H-2^b background, carries the rearranged α and β chain genes of the TCR expressed in the L^d-specific cytotoxic T cell clone, 2C (47–48). The 2C clone was originally derived from a BALB/b (H-2^b) anti-BALB/c (H-2^d) MLC (35). Previous studies of Sha et al. (27) have demonstrated that positive selection of the 2C TCR is mediated by the K^b class I molecule. On an H-2^b background, 55–75% of peripheral T cells express the 2C TCR

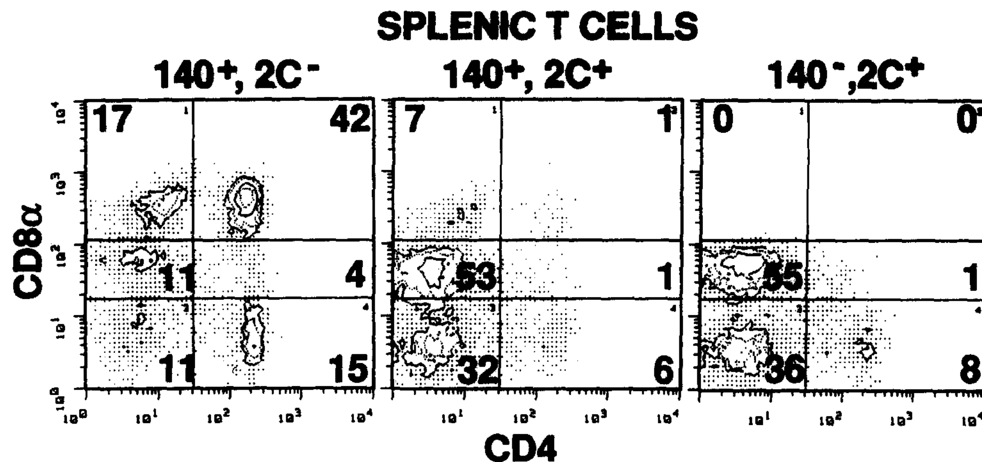

Figure 6. Splenic T cells expressing high levels of CD8 are absent in the line 140$^+$2C$^+$ double transgenics. Two-color FACS® analysis was performed on splenic T cells from line 140 or 2C single transgenics and 140$^+$2C$^+$ double transgenic mice stained with FITC-conjugated anti-CD8 and PE-conjugated anti-CD4. Plots were analyzed with two sets of quadrants and the numbers shown represent the percentages of total cells in each of the depicted six divisions. Representative data from one individual (of 10 analyzed at 8–12 wk of age) of each genotype are shown.

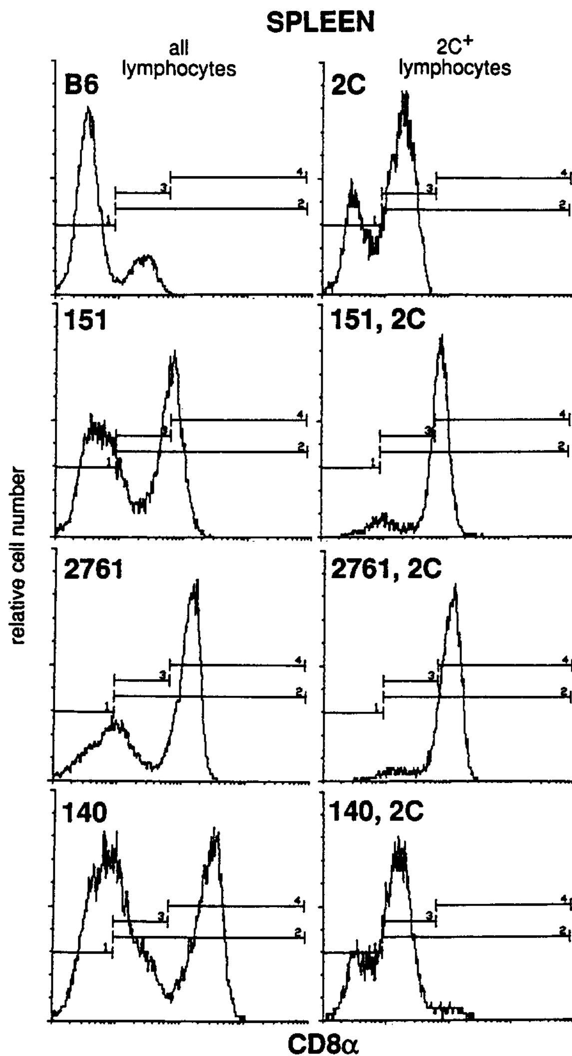

Figure 7. Three-color flow cytometry analysis of line 151^+2C^+, 2761^+2C^+ and 140^+2C^+ double transgenics. Splenocytes were stained with FITC-conjugated anti-CD8, PE-conjugated anti-CD4, and biotinylated anti-2C (1B2), followed by staining with streptavidin red 613. (*Left*) CD8 fluorescence plotted versus relative cell number for all splenocytes; (*right*) CD8 fluorescence plotted versus relative cell number for cells staining positive for the 2C TCR ($1B2^+$). Markers are numbered 1–4, from bottom to top. (Marker *2*) The left boundary is set at 10, and distinguishes the $CD8^-$ cells from the $CD8^+$ cells; (marker *4*) the left boundary is set at 66, and distinguishes the $CD8^{normal}$ from the $CD8^{hi}$ cells. The mean CD8 fluorescences of the $CD8^+$ cells are 25 (B6), 58 (151), 109 (2761), 171 (140), 285 ($CD8^{hi}$, 140), 21 (2C), 77 (151, 2C), 102 (2761, 2C), 25 (140, 2C), and 123 ($CD8^{hi}$; 140, 2C).

transgene and >90% of these are $CD4^-CD8^+$. The initial 2C × CD8 transgenic crosses used the line 140 mice, since these animals expressed the highest levels of the CD8α.1 transgene. Three classes of transgenic progeny were generated in the 2C × line 140 cross (140^+2C^-, 140^+2C^+, and 140^-2C^+) and 5–10 animals in each class were examined by two- and three-color FACS® analyses to monitor the fate of the $2C^+$ thymocytes and peripheral T cells. Samples of the data obtained are presented below.

Fig. 6 presents the CD4/CD8 profile of splenic T cells from the transgenic progeny produced in the 2C × line 140 cross. The percentage of $CD4^-CD8^+$ splenic T cells in the 140^+2C^+ mice (60%) is comparable with that in the 140^-2C^+ single transgenic (55%). However, whereas 59% of the 140^+2C^- splenic T cells stain brightly with anti-CD8 antibodies (including 17% that stain as $CD4^-CD8^+$), only 8% of splenic T cells stain at this level in the 140^+2C^+ double transgenic progeny. Specifically, the percentage of $CD4^-CD8^{hi}$ cells is decreased, from 17% in the 140^+2C^- transgenic to 7% in the 140^+2C^+ double transgenic. When comparing the relative levels of CD8 fluorescence among the three classes of transgenic progeny, we find that the mean CD8 fluorescence of the $CD4^-CD8^+$ population is similar in the 140^+2C^+ double and the 140^-2C^+ single transgenics: 66 and 50, respectively. In contrast, the mean CD8 fluorescence of the $CD4^-CD8^{hi}$ cells in the 140^+2C^- mice is 364, approximately sevenfold higher than the CD8 levels found on splenic T cells in the 140^-2C^+ single transgenics (Fig. 6). A small number of $CD4^-CD8^{hi}$ cells is observed in the double transgenic and these cells stain with a mean CD8 fluorescence of 241, a value that is reduced relative to the mean CD8 fluorescence (364) observed on $CD4^-CD8^{hi}$ cells in the 140^+2C^- transgenic mouse. The absence of peripheral $CD8^{hi}$ cells is also reflected by the decrease in the number of cells staining brightly for the CD8α.1 transgene. In line 140, ∼60–80% of peripheral $CD8^+$ cells stain brightly for CD8α.1, whereas, in the 140^+2C^+ double transgenic, <12% of $CD8^+$ cells stain brightly for CD8α.1 (data not shown; these numbers represent averages from four mice).

The data in Fig. 6 clearly show that there is a reduction in the percentage of $CD8^{hi}$ cells in the 140^+2C^+ double transgenic relative to the 140^+2C^- single transgenic. Furthermore, the level of CD8 staining on the $CD4^-CD8^{hi}$ cells appears to be somewhat lower in the double transgenic compared with the 140^+2C^- single transgenic. These data do not establish, however, whether the 2C TCR^+ cells in the 140^+2C^+ double transgenic express normal, moderate, or high levels of CD8. Therefore, we performed three-color FACS® analyses on splenocytes using the 1B2 antibody, a clonotypic antibody specific for the 2C TCR (35), and anti-CD4 and anti-CD8 antibodies. To determine whether the loss of $CD8^{hi}$ splenocytes in a CD8 × 2C double transgenic is dependent on the actual level of CD8 transgene expression, single and double transgenic progeny from the 2C × 151 and 2C × 2761 crosses were included in this analysis. The data are shown in Fig. 7, which plots CD8 fluorescence versus relative cell number (*left*) for total splenic lymphocytes from nontransgenic B6 and line 151, 2761, and 140 single transgenic mice, and (*right*) for 2C TCR^+ splenocytes from a $2C^+$ single transgenic mouse and 151^+2C^+, 2761^+2C^+, and 140^+2C^+ double transgenic mice. Splenocytes from the three CD8 transgenic lines show an increased level of CD8 expression relative to that seen in control nontransgenic B6 littermates: 2.3 times (n = 1) for 151, three times (n = 4) for 2761, and six times (n = 11) for 140. In the 2C TCR^+

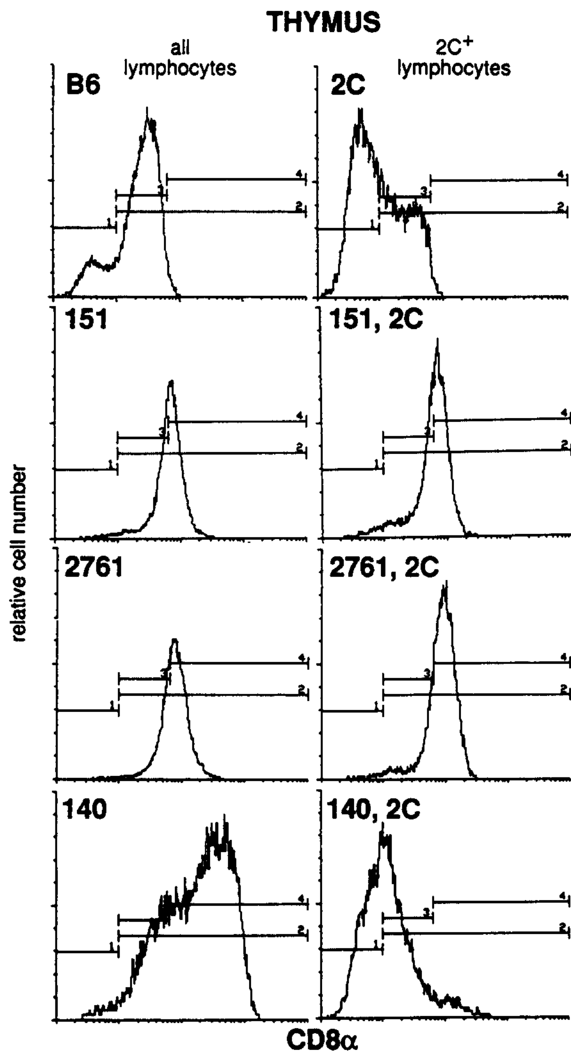

Figure 8. High cell surface levels of CD8 trigger deletion of 2C TCR$^+$ thymocytes. The CD8 fluorescence intensities of thymocytes from nontransgenic C57BL/6J (B6), line 151, 2761, 140, and 2C TCR single transgenics, and 151$^+$2C$^+$, 2761$^+$2C$^+$, and 140$^+$2C$^+$ double transgenics are shown. Thymocytes were stained with FITC-conjugated anti-CD8, PE-conjugated anti-CD4, and biotinylated anti-2C (1B2), followed by staining with streptavidin red 613. The histograms depict all cells analyzed (*left*) or all cells positive for the 2C TCR (*right*). Markers are shown as in Fig. 7. The mean CD8 fluorescences of the CD8$^+$ thymocytes are 32 (B6), 74 (151), 86 (2761), 295 (140), 474 (CD8^{hi}, 140), 29 (2C), 70 (151, 2C), 95 (2761, 2C), 37 (140, 2C), and 150 (CD8^{hi}; 140, 2C).

lymphocytes from the 151$^+$2C$^+$ and 2761$^+$2C$^+$ double transgenics, the relative level of CD8 fluorescence does not change significantly from that observed in the CD8 single transgenics, three times (n = 5) and four times (n = 2), respectively. In the 140$^+$2C$^+$ double transgenics, however, the mean CD8 fluorescence decreases fivefold from that measured in line 140 single transgenic mice: 28 (n = 10; 140$^+$2C$^+$) vs. 143 (n = 11; 140$^+$) in Fig. 7.

The 2C$^+$ single transgenic shows two peaks of fluorescence on splenocytes, CD8$^-$ and CD8^{normal}, denoted by markers 1 and 3, respectively, in Fig. 7. The 140$^+$2C$^+$ double transgenic contains three peaks of fluorescence in the splenocyte population. The first peak represents the CD8$^-$ cells, whereas the second peak represents CD8^{normal} cells, which express levels of CD8 that are similar to those detected on 2C$^+$ cells in the 140$^-$2C$^+$ single transgenic, a mean fluorescence of 19 vs. 21. The third peak includes cells staining with a CD8 fluorescence >70 and is designated by marker 4. The number of cells staining with a CD8 fluorescence >70 in the 140$^+$2C$^+$ double transgenic drops dramatically compared with that in the 140$^+$ single transgenics. In line 140, 32% of splenocytes fall within marker 4 (54% of the CD8$^+$ cells) and these stain with a mean CD8 fluorescence of 248. In contrast, in 140$^+$2C$^+$ double transgenic mice, <5% of the 2C TCR$^+$ cells fall within marker 4, and these cells stain with a mean CD8 fluorescence of 122. In the subsequent discussions of these data, we will refer to T cells expressing CD8 at levels >200 as the CD8^{hi} cells; we will refer to the 2C TCR$^+$ T cells expressing CD8 at levels >70 as the CD8^{mod} cells.

The data in Figs. 6 and 7 clearly demonstrate a specific loss of CD4$^-$CD8^{hi} splenic T cells relative to CD4$^-$CD8^{mod} and CD4$^-$CD8^{normal} splenic T cells in the 140$^+$2C$^+$ double transgenic. These results raise the question of whether this absence of CD8^{hi} cells results from thymocyte deletion or the absence of positive selection.

CD8^{hi} Cells Are Deleted in the Thymus of the 140$^+$2C$^+$ Double Transgenic. To determine if 2C$^+$CD8^{hi} cells are deleted in the thymus of the double transgenic, thymocytes from single and double transgenics were stained with anti-CD8, anti-CD4, and 1B2 antibodies. The relative levels of CD8 fluorescence among the three classes of transgenic progeny are plotted in Fig. 8. For comparison, progeny from the 151 × 2C and 2761 × 2C crosses were also included in this analysis. Thymocytes from 151 and 2761 single transgenic mice stain with a mean CD8 fluorescence that is, respectively, 2.3- and 2.7-fold elevated above that measured on control B6 thymocytes (74 for 151 and 86 for 2761, compared with 32 for B6). Similar to the analysis of splenocytes in Fig. 7, no significant change was observed in the CD8 profile of thymocytes from the 151$^+$2C$^+$ and 2761$^+$2C$^+$ double transgenics compared with that of the single transgenics. Thymocytes from the 151$^+$2C$^+$ and 2761$^+$2C$^+$ double transgenics stained with a relative mean CD8 fluorescence of two and three times (70 and 95, respectively). Thymocytes from the 140$^+$2C$^+$ double transgenics, however, exhibit very few CD8^{hi} cells compared with line 140 single transgenics. 98% of thymocytes stain with a mean CD8 fluorescence of 295 in line 140 single transgenics. In contrast, only 54% of 2C$^+$ thymocytes express CD8 and they do so with a mean fluorescence of 37, an eightfold reduced level relative to the line 140 single transgenics. Furthermore, whereas 74% of thymocytes in line 140 single transgenics stain at levels of fluorescence that are >70 in Fig. 8, only 6% of 2C$^+$ thymocytes in the 140$^+$2C$^+$ double transgenics stain at or above this level. Consequently, these data show that 2C$^+$CD8^{hi} cells, as well as most 2C$^+$CD8^{mod} cells, are deleted in the thymus of 140$^+$2C$^+$ double transgenics. The absence of thymic CD8^{hi} cells is also shown by the decrease in the number of cells staining for the CD8α.1 transgene. In the thymus of line 140 mice, ~80% of thymocytes stain as CD8α.1$^+$;

however, in the 140^+2C^+ double transgenic, only 8% of thymocytes stain as CD8α.1$^+$ (data not shown; these numbers represent averages from two to five mice). In contrast, no decrease in the percent of CD8α.1$^+$ thymocytes is observed in the 151^+2C^+ and 2761^+2C^+ double transgenics compared with the 151^+ and 2761^+ single transgenics (data not shown).

Discussion

To investigate the effect of increased levels of CD8α surface expression on the developmental fate of maturing thymocytes, we generated a T cell–specific expression cassette capable of promoting high level expression of heterologous genes in all T cell subsets in transgenic mice. This expression cassette, pNeZ, contains the promoter and enhancer elements of the murine CD3-δ gene, which is normally expressed on all TCR-α/β and -γ/δ-bearing thymocytes and peripheral T cells, and which is transcriptionally active in the thymus by embryonic day 15–16 (49). Transient expression experiments performed with cultured cells have demonstrated that while the CD3-δ promoter is not cell type specific, the CD3-δ enhancer functions preferentially in T cells (33).

As discussed in this paper, one transgenic line generated with the pNeZCD8α.1 construct, line 140, expressed the CD8 transgene on 75% of T cells at levels that are elevated 6–10-fold relative to endogenous CD8α gene expression. An additional seven lines have been produced that carry either human CD4 or murine and human CD8 transgenes inserted into the pNeZ cassette; all of these lines, including line 151 discussed in this paper, express the heterologous transgenes on virtually 100% of thymocytes and mature T cells (N. Lee, D. Grass, D. Smith, and E. Lacy, unpublished results). Furthermore, the surface levels of expression of the CD4 and CD8 transgenes are equivalent on immature and mature T cells, indicating that the activity of the CD3-δ regulatory sequences is not modulated during thymocyte maturation.

Previous experiments have suggested that the density of CD8 molecules on the surface of maturing thymocytes plays a crucial role in tolerance induction. For example, analysis of transgenic mice expressing a TCR specific for the HY male antigen has shown that T cells expressing this receptor, as well as low levels of CD8, can escape clonal deletion in males (50). Similarly, Sha et al. (27) observed deletion of $CD8^{hi}$, but not of $CD8^{lo}$, T cells in transgenic mice expressing the 2C TCR on a K^{bm11} or K^{bm3} mutant background. Transgenic lines expressing elevated but very distinct levels of CD8α (2–4 vs. 6–10 times) allowed us to test directly whether surface levels of CD8 can modulate the selection of the mature TCR repertoire. We found no effect on thymocyte maturation in the double transgenic progeny from crosses between a line expressing the class I–restricted 2C TCR and lines 151 and 2761, which express two- to fourfold increased levels of CD8. However, markedly different results were obtained in double transgenic progeny produced in the cross between the 2C mice and line 140 mice, which express 6–10-fold elevated levels of CD8. The numbers of peripheral T cells that are $CD8^{hi}$, and thus that are CD8α.1 transgene positive, are dramatically reduced in the 140^+2C^+ double transgenics. The percentage of 2C TCR–expressing splenic T cells, however, is comparable between the 140^+2C^+ double transgenics and the $2C^+$ single transgenics. Although peripheral 2C TCR^+ T cells that express elevated levels of CD8 are found in the double transgenics, their numbers are few and their surface density of CD8 is lower than the mean density of CD8 on splenic T cells in line 140. In addition, none of the 2C TCR^+ splenic T cells in the double transgenics express the highest levels of CD8 observed in the line 140 mice (Figs. 6 and 7). As no $CD8^{hi}2C^+$ cells are detected in the thymus of the 140^+2C^+ double transgenic, these cells must be deleted during thymocyte maturation. The loss of the peripheral double-positive cells in the double transgenic could reflect either deletion of most $CD4^+CD8^{hi}$ thymocytes expressing the 2C TCR and/or an instructional mechanism for thymocyte maturation (51, 52).

The overexpression of the CD8α transgene does not perturb endogenous CD8α or CD8β gene expression, and the excess CD8α molecules are likely to be expressed in the form of α/α homodimers rather than α/β heterodimers. Since the CD8β chain cannot bind *lck* (53), it is formally possible that the deletion of the $2C^+CD8^{hi}$ cells in the 140^+2C^+ double transgenics results from a perturbation in signaling due to limiting quantities of *lck* or an increase in the amount of *lck* bound per CD8 dimer. We think such a general, nonspecific mechanism for the deletion of the $2C^+CD8^{hi}$ cells is unlikely, as the overexpression of the CD8α transgene in line 140 mice is without apparent global effect on thymocyte maturation. Normal staining patterns are obtained with antibodies to other T cell surface markers, for example CD3 and TCR-α/β, in line 140 mice as well as in line 151 and 2761 mice (Fig. 5). In addition, the CD8 transgene–positive cells in line 140 show normal proliferative responses in mixed lymphocyte reactions to class I and class II alloantigens (N. A. Lee and E. Lacy, unpublished results). The line 140 mice have also been crossed with the transgenic line expressing the H-Y-specific TCR (54, 55) on the H-2^b positively selecting background. Interestingly, in the double transgenic female progeny the $CD8^{hi}$, H-Y TCR–expressing thymocytes are not deleted, but positively selected (N. A. Lee, H. von Boehmer, and E. Lacy, unpublished results). This finding indicates that the effect of high levels of CD8 on thymocyte maturation is determined directly by the specificity of the TCR-α/β. Thus, if the high levels of CD8 in line 140 mice are, in fact, globally altering signaling in maturing thymocytes, then the cellular response to these altered signals must be dictated by the specificity of TCR/self-Ag-MHC interactions.

Another interpretation of our data, and the one we favor, is that the deletion of the $2C^+CD8^{hi}$ cells reflects the outcome of a synergistic collaboration between the excess levels of CD8 and the 2C TCR, and that this collaboration acts to increase the avidity of the interaction between immature thymocytes and selecting MHC class I–positive stromal cells. T cell maturation requires positive and negative selection, both of which are mediated by interactions between a TCR on a maturing thymocyte and a MHC/self-peptide ligand on a selecting cell in the thymus (27–30; 54). Thus, to avoid

deleting or inactivating the positively selected TCR repertoire, either the thymocyte itself or the selecting stromal cell must be able to distinguish between positively and negatively selecting interactions between the TCR and MHC. The affinity model (29, 56) proposes that the choice between positive and negative selection is based on the affinity of the interaction between a TCR and a MHC/self-peptide ligand. Thymocytes expressing TCRs with high affinity for MHC/self-peptide ligands would be eliminated by the mechanisms for tolerance induction, whereas thymocytes expressing receptors with low or moderate affinities would be allowed to mature. A refinement of this model is that the levels of binding by CD8 and CD4 to class I and class II molecules, respectively, can contribute either to the affinity of the TCR complex for its selecting MHC molecule or to the overall avidity of a thymocyte for a selecting stromal cell.

In the 2C × 140 cross, as well as in the 2C × 151 and 2761 crosses, we have not altered the expression of the 2C TCR transgene nor of endogenous MHC/self-peptide complexes; thus, we believe our data demonstrate directly that CD8 levels can affect the developmental fate of a maturing thymocyte. Our data also argue in support of an affinity model for thymocyte selection in which the affinity of a TCR: MHC/self-peptide interaction is translated into a graded signal that is measured by the maturing thymocyte. A signal above a certain threshold would result in clonal deletion, whereas a signal below a certain level would result in positive selection; a signal in an intermediate range, on the other hand, might trigger clonal anergy. A distinct advantage of the 2C TCR transgenic system is that this interpretation of our findings can be confirmed and extended by crossing the 2C TCR and line 140 $CD8^{hi}$ transgenes onto different H-2 backgrounds. Sha et al. (27, 48) have shown that the selection of the 2C TCR varies dramatically on different H-2 backgrounds. On the $H-2^s$, $H-2^k$, and H-2 K^{bm8} backgrounds, the 2C TCR is weakly positively selected, whereas it is weakly negatively selected on the H-2 K^{bm3} and K^{bm11} backgrounds. If the affinity model is an accurate interpretation of the selection process, then these observations most likely reflect the different affinities of the 2C TCR for the different selecting MHC class I/self-peptide ligands. Furthermore, the affinity model argues that the line 140 $CD8^{hi}$ transgene should affect the selection of the 2C TCR in predictable ways on these different H-2 backgrounds. For example, K^{bm8} should become a strongly positively selecting background and K^{bm3} and K^{bm11} should become strongly negatively selecting backgrounds. Another question that can be addressed using this transgenic system is whether the $2C^+CD8^{mod}$ T cells in the 151^+2C^+ and 2761^+2C^+ double transgenics are functionally competent against the L^d antigen or whether they are anergic. The finding that the 2C TCR^+CD8^{mod} cells are anergic would support the proposal that the choice to clonally anergize a thymocyte is also determined by the affinity of a TCR:MHC/self-peptide interaction.

Although our experimental results support an affinity model for thymocyte selection, they do not address the mechanism by which CD8 could be contributing to the avidity of thymocyte/stromal cell interactions. Studies on CD8 function during alloreactive cytolytic responses indicate that CD8 participates in T cell activation largely as a coreceptor that engages the same MHC molecule as the TCR (9, 10). Numerous other studies (57–61) have demonstrated that CD8 can also contribute to T cell recognition and activation as an adhesion molecule that binds bystander MHC class I molecules not involved in antigen presentation to the responding TCR. Analysis of CD8-deficient mice has shown that CD8 is essential for the selection of class I–restricted TCR (62). It is not known, however, whether CD8 participates in thymocyte selection as a coreceptor and/or as an adhesion molecule. Thus, the elevated levels of CD8α could be affecting the avidity of the 2C TCR^+ thymocytes via one or both of these mechanisms.

If the excess levels of CD8α enhance the avidity of thymocyte/stromal cell interactions through a coreceptor mechanism, then the number of 2C TCRs that can coengage an MHC/self-peptide ligand with CD8 must be increased in the 2C TCR/140 double transgenics. This would imply that the elevated surface levels of CD8 may effectively increase the frequency with which both CD8 and the TCR simultaneously contact MHC/self-peptide. Alternatively, the increased expression of CD8 in line 140 may result in elevated levels of a particular form of CD8 (α/α, α' etc.) that alters the affinity of the TCR:MHC/self-peptide interaction. Another possibility is that the elevated levels of CD8α might increase the overall avidity of thymocyte/stromal cell interactions by binding class I molecules not engaged by the 2C TCR. Consistent with such a mechanism are the data from O'Rourke et al. (58) showing that CD8 binding to bystander class I molecules is increased by TCR engagement by an anti-TCR antibody. Through either a coreceptor or adhesion function, the increased levels of CD8α may enhance signals generated during thymocyte selection, such that they surpass the threshold required to trigger negative selection.

Another model to resolve the paradox of thymocyte selection is the altered ligand model. This model proposes that a distinct set of self-peptides, presented exclusively by the cortical epithelium, mediates positive selection, whereas another set of peptides, expressed both by thymic stromal cells and nonthymic tissues, mediates negative selection (21, 63). Our experiments do not address this model, because we do not know whether the same K^b self-peptide ligand that positively selects the 2C TCR in the single transgenic also negatively selects the 2C TCR in the double transgenic. For example, in the double transgenic, the 2C TCR may be positively selected by the same cortical K^b self-peptide ligand as in the single transgenic, but deleted by another MHC/self-peptide due to an enhanced affinity. However, even if distinct sets of self-peptides and antigen-presenting cells are used for positive and negative selection, our results would argue that affinity is still a component of the selection process. The availability of transgenic mice expressing different levels of CD8 will allow us to explore different facets of the role of affinity in thymocyte maturation.

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