

## The Cytoplasmic Domain of CD4 Promotes the Development of CD4 Lineage T Cells

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### Summary

Thymocytes must bind major histocompatibility complex (MHC) proteins on thymic epithelial cells in order to mature into either CD8<sup>+</sup> cytotoxic T cells or CD4<sup>+</sup> helper T cells. Thymic precursors express both CD8 and CD4, and it has been suggested that the intracellular signals generated by CD8 or CD4 binding to class I or II MHC, respectively, might influence the fate of uncommitted cells. Here we test the notion that intracellular signaling by CD4 directs the development of thymocytes to a CD4 lineage. A hybrid protein consisting of the CD8 extracellular and transmembrane domains and the cytoplasmic domain of CD4 (CD884) should bind class I MHC but deliver a CD4 intracellular signal. We find that expression of a hybrid CD884 protein in thymocytes of transgenic mice leads to the development of large numbers of class I MHC-specific, CD4 lineage T cells. We discuss these results in terms of current models for CD4 and CD8 lineage commitment.

The differentiation of immature thymocytes into either CD4<sup>+</sup> or CD8<sup>+</sup> mature T cells is linked to the specificity of the TCRs expressed on developing thymocytes. Recognition of class I MHC by developing thymocytes requires the coordinate binding of both a class I-specific TCR and a CD8 coreceptor, and can give rise to mature CD8<sup>+</sup> T cells. In contrast, recognition of class II MHC requires corecognition by a class II-specific TCR and the CD4 coreceptor and can give rise to mature CD4<sup>+</sup> T cells. Although immature thymocytes express both CD4 and CD8, as thymocytes mature, they downregulate expression of one of the coreceptors, giving rise to mature CD4<sup>+</sup>CD8<sup>-</sup> or CD4<sup>-</sup>CD8<sup>+</sup> T cells. Thus lineage commitment in the thymus is linked to both the specificity of the TCR and the expression of the CD4 and CD8 coreceptors.

The observation that the specificity of TCR for class I or II MHC influences the choice between CD4 and CD8 lineages is consistent with a model in which binding of class I MHC by a CD4<sup>+</sup>CD8<sup>+</sup> thymocyte leads to the downregulation of CD4 expression and commitment to a CD8 lineage. Likewise, binding of class II MHC may lead to the downregulation of CD8 expression and commitment to a CD4 lineage (1). This model fits well with observations from TCR transgenic mice, in which expression of a class I-specific TCR generally leads to an increase in the production of mature CD8 cells, and expression of a class II-specific TCR generally leads to an increase in the production of mature CD4 cells (2–5).

The analysis of putative transitional intermediates in MHC-deficient mice has been interpreted as evidence against an in-

structive model. For example, a population of CD4<sup>+</sup>CD8<sup>low</sup> thymocytes is observed in mice mutant for class II MHC, but is missing in mice mutant for both class I and II MHC (6, 7). It has been proposed (7) that this population represents class I-selected transitional cells that are committed to a CD4 lineage, a population that would not be predicted by an instructive model. There is, however, no direct evidence that the CD4<sup>+</sup>CD8<sup>low</sup> cells are the precursors of CD4 lineage cells. Indeed, recent experiments indicate that at least some CD4<sup>+</sup>CD8<sup>low</sup> thymocytes give rise to CD8 lineage cells (8), raising questions about the implications of this population for the mechanism of CD4/CD8 lineage commitment.

Another line of evidence that lineage commitment need not correlate with MHC specificity comes from mice expressing constitutive CD8 or CD4 transgenes (9–15). Although these experiments provide evidence for a stochastic component to lineage commitment, it is striking that the generation of T cells of the “wrong” lineage (i.e., CD4<sup>+</sup> with class I-specific TCRs or CD8<sup>+</sup> with class II-specific TCRs) is invariably inefficient. For example, class II MHC mutant, CD8 transgenic mice have only ~3% of the mature CD4<sup>+</sup> thymocytes that are found in mice that are wild type for class II MHC (10). Moreover, another class I-specific TCR, anti-HY, failed to give rise to any detectable CD4 lineage cells when coexpressed with a CD8 transgene (16–18), implying that not all class I-specific TCRs can permit the development of CD4 lineage cells.

In light of these considerations, we decided to examine more directly the question of whether intracellular signals generated upon MHC recognition might influence the fate

of uncommitted thymocytes. Here we directly test the hypothesis that CD4 intracellular signals promote the development of CD4 lineage T cells. A hybrid molecule consisting of the extracellular domain of CD8 and the cytoplasmic domain of CD4 would be expected to bind class I MHC, yet deliver a CD4 intracellular signal. If CD4 signals direct thymic precursors to choose the CD4 lineage, then the recognition of class II MHC by thymocytes expressing such a hybrid molecule would direct development to a CD4 instead of a CD8 lineage. Indeed, we find that coexpression of a hybrid CD8/4 molecule with the F5 TCR leads to a dramatic increase in mature CD4 cells, and a decrease in mature CD8 cells. These results indicate that the cytoplasmic domain of CD4 delivers a signal that favors the development of CD4 lineage cells.

## Materials and Methods

**Generation of Transgenic Mice Expressing CD884 Hybrid Molecules.** A NaeI site was introduced into the CD8.1 cDNA (16) at amino acid 196 by site-directed mutagenesis. The sequence of the mutagenic oligonucleotide was 5'-aac acg ctg ccg gct cct gtg-3'. This NaeI site was ligated to the existing NaeI site at amino acid 400 of the murine CD4 gene. The resulting hybrid cDNA encodes a fusion protein whose extracellular and transmembrane domains derive from the CD8 gene (through amino acid Ser 195 [19]), and whose cytoplasmic domain derives from CD4 (from amino acid Arg 400 [20]). The hybrid cDNA was inserted into the EcoRI site of the human CD2 expression cassette, to produce a transgenic construct that is analogous to the "T11-8" construct that was used to generate CD8.1 transgenic mice (16). This construct was coinjected along with a CD8 $\beta$ .1 genomic clone (21) into C57Bl/10 embryos. One founder which expresses transgenic CD8.1 and CD8 $\beta$ .1 at levels comparable to the endogenous CD8 genes was chosen for further analysis. Hematopoietic stem cell chimeras were generated by injecting mixtures of T cell-depleted bone marrow cells from F5 TCR and F5 TCR/CD884 transgenic mice ( $2 \times 10^7$  cell/recipient) into unirradiated Rag1 mutant mice (22).

**Analysis of Lck Association.** Thymocytes ( $10^7$  cells/sample) were lysed in 1 ml of lysis buffer (50 mM Tris, pH 7.5, 0.1% NP-40, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 10 mM NaF, 10 mM Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>, 1 mM PMSF, 10  $\mu$ g/ml aprotinin, and 10  $\mu$ g/ml leupeptin). Lysates were centrifuged for 1 min at 14,000 rpm and the supernatants were incubated with 25  $\mu$ l protein G beads (GammaBind G Sepharose; Pharmacia, Piscataway, NJ) that had been precoated with either anti-CD4 (GK1.5) or anti-CD8 (53-6.72). After 3 h at 4°C, beads were washed twice with lysis buffer without detergent. Nonreducing SDS loading buffer was added to the samples, which were heated for 5 min at 95°C. Samples were run on an SDS polyacrylamide gel and transferred to nitrocellulose (Hybond-ECL; Amersham Corp., Arlington Heights, IL). Immunoblotting was performed using the enhanced chemiluminescence system (ECL; Amersham Corp.) according to the manufacturer's instructions. Anti-Lck antibody was kindly provided by Dr. Joe Bolen (Bristol-Myers Squibb, Princeton, NJ) and was visualized using horseradish peroxidase goat anti-mouse Ig (Southern Biotechnology Associates, Birmingham, AL).

**Analysis of Lck Activation.** CD8 and CD884 constructs were cloned into a SV40 expression cassette (23) and introduced by electroporation into Jurkat cells along with a selectable plasmid.

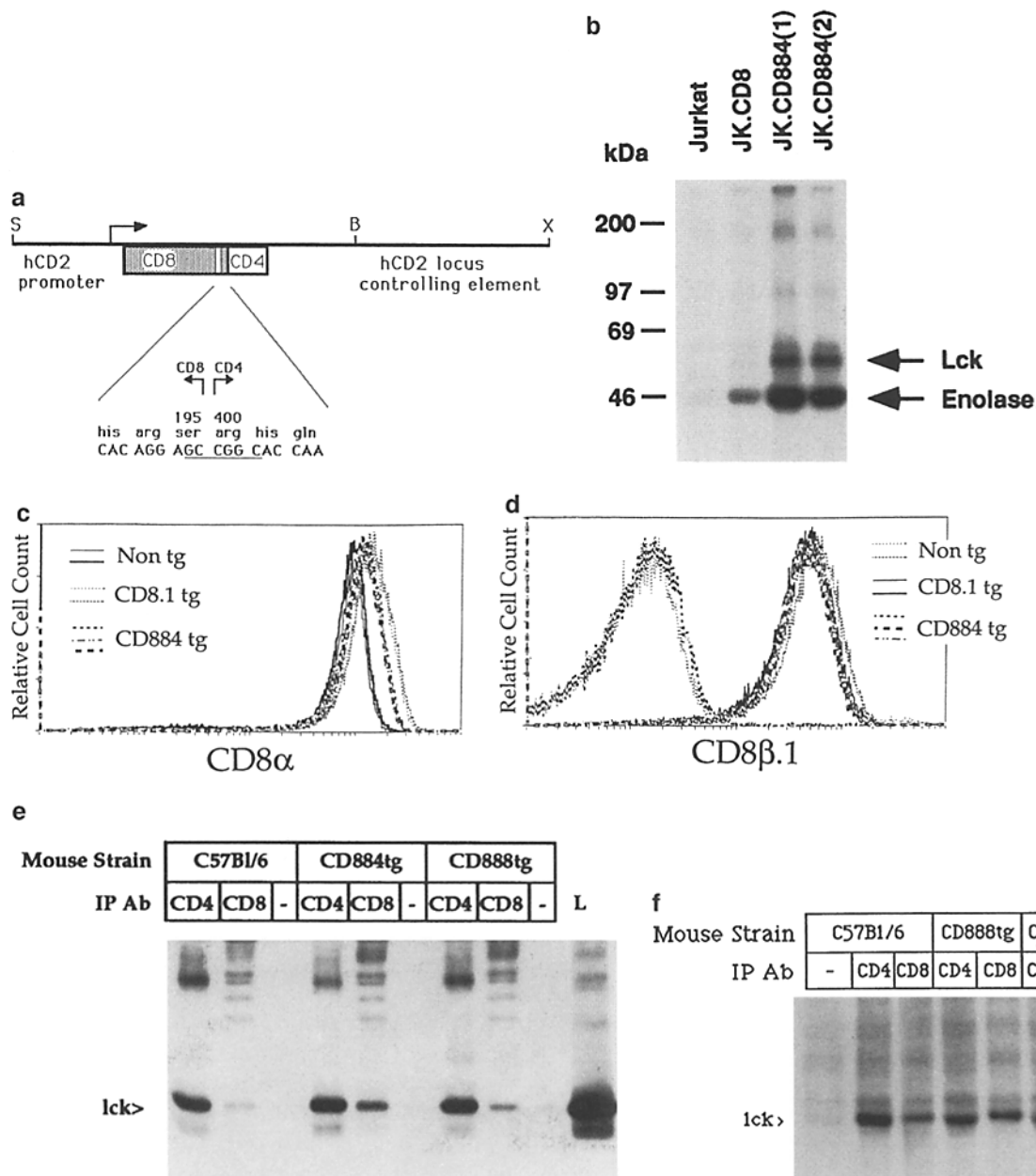
Individual hygromycin B resistant clones expressing comparable cell surface levels of CD8 were selected for cross-linking experiments. Cross-linking was performed as follows.  $10^7$  cells were incubated with 20  $\mu$ g rat anti-murine CD8 IgG (clone 53-6.72) for 15 min on ice, then with 60  $\mu$ g goat anti-rat IgG (Cappel Laboratories, Durham, NC) for 15 min on ice. Cells were then incubated for 10 min at 37°C, rinsed with cold PBS containing 1 mM Na<sub>3</sub>VO<sub>4</sub> and 10 mM NaF, and lysed for 20 min in lysis buffer on ice. Lysates were cleared by centrifugation for 10 min at 10,000 g and incubated with 25  $\mu$ l protein G beads for 6 h at 4°C. Beads were then washed four times with lysis buffer (without Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub> and PMSF), once with kinase buffer (50 mM HEPES, pH 7.5, 100 mM NaCl, 5 mM MgCl<sub>2</sub>, and 5 mM MnCl<sub>2</sub>), and resuspended in 30  $\mu$ l kinase buffer containing 10  $\mu$ Ci  $\gamma$ -[<sup>32</sup>P]ATP and 10  $\mu$ g acid-denatured enolase (Sigma Chemical Co., St. Louis, MO). Reactions were run for 10 min at 30°C, quenched with 15  $\mu$ l 3 $\times$  sample buffer (150 mM Tris, pH 6.8, 30% glycerol, 6% SDS, 3% 2-ME and 0.05% bromophenol blue), and reaction products were analyzed by SDS-PAGE. The gel was then fixed and dried for autoradiography. Quantification of radioactive bands was performed using the PhosphorImager/ImageQuant system (Molecular Dynamics, Sunnyvale, CA). For the in vitro kinase assay on thymocytes, immunoprecipitations were performed as described above for the Western blot. Immunoprecipitates were resuspended in kinase buffer and assays were performed as described above.

**Analysis of T Cell Populations.** Class II MHC mutant, anti-HY and F5 TCR transgenic mice have been previously described (3, 24, 25). All mice were homozygous for H-2<sup>b</sup>. Cell suspensions of thymocytes and lymph node T cells were prepared and labeled with fluorescent antibodies as previously described (11). For analysis of mature thymocytes, thymocytes were treated with anti-heat stable antigen (HSA)<sup>1</sup> and complement as previously described (10). Antibodies used were T3.70 (culture supernatant), PE-labeled goat anti-mouse IgG1 (Caltag Laboratories, South San Francisco, CA), PE-labeled CD4 (Beckton Dickinson & Co., Mountain View, CA), anti-CD8.2 $\alpha$  FITC (2.43), Tricolor<sup>TM</sup>-labeled streptavidin (Caltag), and rat gamma globulin (Calbiochem-Novabiochem Corp., San Diego, CA), anti-CD8.1 (49-31.1; Cedarlane Laboratories, Westbury, NY), FITC-labeled goat anti-mouse IgG3 (Caltag), biotinylated anti-CD4 (YTS 191.1; Caltag), biotinylated anti-V $\alpha$ 2 (PharMingen, San Diego, CA), and biotinylated anti-V $\beta$ 11 (KT11). Data (50,000 events) were collected and analyzed using a FACScan<sup>®</sup> flow cytometer (Becton Dickinson & Co.) or X-cell flow cytometer (Coulter Corp., Hialeah, FL). Dead cells were excluded on the basis of forward and side scatter.

## Results

**Transgenic Mice Expressing a Hybrid CD8/CD4 Transgene.** To redirect a CD4 intracellular signal, we constructed a hybrid cDNA consisting of the extracellular and transmembrane domains of CD8 $\alpha$  and the cytoplasmic domain of CD4 (CD884). The CD8 $\alpha$  cDNA used encodes the CD8.1 allele, which can be distinguished from the endogenous CD8 allele (CD8.2) using monoclonal antibodies. The hybrid cDNA (Fig. 1 a) was inserted into an expression cassette containing the human CD2 promoter, minigene, and 3' locus controlling region, to generate a construct analogous

<sup>1</sup>Abbreviations used in this paper: HSA, heat stable antigen; NP, nucleoprotein.



**Figure 1.** Redirecting a CD4 intracellular signal with a hybrid CD8/CD4 coreceptor. (a) Construct used in the generation of CD884 transgenic mice. (*White*) Transmembrane domain. (*Arrow*) Direction of transcription; the sequence surrounding the fusion is depicted below. (*Underlined*) *NaeI* site. The map is not drawn to scale. Restriction endonuclease sites: *SalI* (*S*), *BamHI* (*B*), and *XbaI* (*X*). The CD884 transgene was coinjected with a 15-kb *BamHI* genomic fragment encoding CD8 $\beta$ .1 (21) into C57Bl/10 embryos. (b) CD884 protein is more effective than CD8 in activating Lck. The CD884 and CD8 cDNAs were cloned into a SV40 expression vector and used to transfect the human T cell line Jurkat. Anti-CD8 immunoprecipitates from stable transfectants expressing comparable levels of murine CD8 cell were analyzed using an in vitro kinase assay as described in Materials and Methods. Lck activity was quantitated by integrating signals of enolase bands. (*Arrows*) Migration positions of Lck and enolase. (*Left*) Migration positions of molecular weight (*kDa*) standards. (c) CD8 $\alpha$  expression on CD884 and CD8.1 transgenic (*tg*) mice. Thymocytes were stained with anti-CD8 $\alpha$ , which recognized both endogenous and transgenic CD8. (d) CD8 $\beta$ .1 expression on CD884 and CD8.1 transgenic mice. Thymocytes were stained with anti-CD8 $\beta$ .1, which recognizes transgenic, but not endogenous CD8 $\beta$ . (e) Lck associated with CD4 and CD8 in thymocytes of CD884 and CD8.1 transgenic mice. Thymocytes from the indicated mice were subjected to immunoprecipitation with the indicated antibodies. Immunoprecipitates were resolved by PAGE and analyzed by Western blot analysis using anti-Lck antibodies. The experiment was performed twice with equivalent results. (f) Active Lck associated with CD4 and CD8 in thymocytes of CD884 and CD8.1 transgenic mice. Thymocytes from the indicated mice were subjected to immunoprecipitation with the indicated antibodies. Immunoprecipitates were then subjected to in vitro kinase assays as described in Materials and Methods. The experiment was performed twice with equivalent results.

**Table 1.** *Thymocytes and Lymph Node T Cells from CD884 Transgenic Mice*

Genotype	No. of cells/thymus × 10 <sup>6</sup>	Percent total thymocytes		Percent HSA-Thymocytes		<i>n</i>
		CD4 <sup>+</sup> 8.2 <sup>-</sup>	CD4 <sup>-</sup> 8.2 <sup>+</sup>	TCR <sup>+</sup> CD4 <sup>+</sup> 8.2 <sup>-</sup>	TCR <sup>+</sup> CD4 <sup>-</sup> 8.2 <sup>+</sup>	
		%		%		
Nontransgenic	225 (126)	7.1 (1.7)	2.4 (0.9)	—	—	5
CD884 transgenic	171 (66)	10 (2.6)	2.3 (0.6)	—	—	8
Class II <sup>-</sup>	230 (33)	1.3 (0.3)	2.9 (0.4)	2.4 (0.6)	68 (5.6)	3
Class II <sup>-</sup> /CD884 transgenic	193 (61)	5.1 (0.6)	2.2 (0.2)	26 (7.5)	49 (3.3)	3
		Percent lymph node T cells				
		CD4 <sup>+</sup> 8.2 <sup>-</sup>	CD4 <sup>-</sup> 8.2 <sup>+</sup>	4:8 ratio		<i>n</i>
		%				
Nontransgenic	56 (3.4)		38 (3.2)	1.5		9
CD884 transgenic	63 (5.2)		24 (2.5)	2.6		8
Class II <sup>-</sup>	3.4 (0.2)		88 (4.2)	0.04		3
Class II <sup>-</sup> /CD884 transgenic	33 (2.2)		58 (1.6)	0.6		3

Total thymocytes, mature thymocytes (HSA depleted), or lymph node T cells (B cell depleted) were stained with antibodies against TCR, CD4, and CD8.2 (endogenous CD8), as described in Materials and Methods. CD884 transgenic mice were either heterozygous or homozygous for the CD884 transgene. Average values are given with standard deviations in parentheses, and *n* is the number of mice analyzed of each genotype.

to one previously used to direct constitutive expression of a wild-type CD8 cDNA (16, 26).

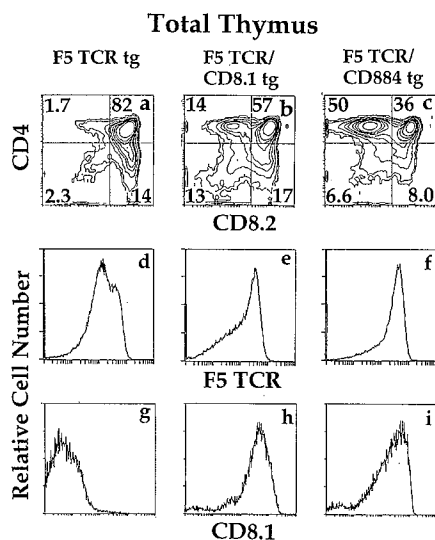
To test whether the CD4 cytoplasmic domain is functional in the context of the CD884 protein, we examined the ability of the CD884 molecule to activate the tyrosine kinase p56<sup>lck</sup> (Lck). Although the cytoplasmic domains of both CD4 and CD8 interact with Lck, cross-linking of CD4 activates Lck more strongly than does cross-linking of CD8 (27–29). We introduced both the CD884 and the wild type-CD8 constructs into the human T cell line Jurkat, and isolated stable transfectants expressing comparable surface levels of either CD884 or wild-type CD8. Transfectants were then subjected to cross-linking using antimurine CD8 antibodies, and *in vitro* kinase assays were performed on anti-CD8 immunoprecipitates. We find that cross-linking of CD884 molecules leads to approximately eightfold higher activation of Lck than does cross-linking of wild-type CD8 (Fig. 1 *b*). This indicates that the cytoplasmic domain of CD4, when fused to the extracellular and transmembrane domain of CD8, activates Lck more strongly than does the cytoplasmic domain of CD8.

Although CD8 $\alpha$  can be expressed as a homodimer, it is generally found as a heterodimer paired with a  $\beta$  chain, and recent evidence indicates that the  $\beta$  chain of CD8 plays an important role in thymic development (30–32). To ensure that CD8 $\beta$  is not limiting, and to mimic more closely the original CD8 transgenic mice, we coinjected a genomic clone encoding CD8 $\beta$  (21) along with the CD884 transgenic construct. A line that expresses CD884 and CD8 $\beta$  on virtually all thymic and peripheral T cells at levels compar-

able to endogenous CD8 was chosen for further analysis. The cell surface expression of CD8 $\alpha$  on thymocytes of CD884 transgenic mice is comparable to endogenous CD8 expression and is very similar to the levels on previously described CD8.1 transgenic mice (Fig. 1 *c*). Both CD884 and CD8.1 transgenic mice also express comparable surface levels of CD8 $\beta$ .1 (Fig. 1 *d*).

Because the cytoplasmic domain of CD4 associates strongly with Lck, we suspected that thymocytes from CD884 transgenic mice would have more CD8-associated Lck than thymocytes from nontransgenic or CD8.1 transgenic mice. To investigate this question, we prepared anti-CD8 and anti-CD4 immunoprecipitates from thymocytes of nontransgenic, CD884 transgenic, and CD8.1 transgenic mice, and performed both *in vitro* kinase assays and anti-Lck. Western blot analysis (Fig. 1, *e* and *f*). As expected, we found an increase in both Lck immunoreactivity (Fig. 1 *e*) and kinase activity (Fig. 1 *f*) in anti-CD8 immunoprecipitates from CD884 transgenic mice relative to nontransgenic or CD8 transgenic mice. It is also interesting to note that anti-CD8 immunoprecipitates from thymocytes of CD8 transgenic mice have an increased association with Lck relative to nontransgenic mice. This may reflect the fact that the level of CD8 expression in thymocytes of CD8 transgenic mice is slightly elevated relative to nontransgenic, and/or the fact that the CD8 transgene does not encode CD8 $\alpha'$ , an alternative splice version of CD8 that cannot associate with Lck (33).

*Class I-specific, CD4 Lineage T Cells Develop in CD884 Transgenic Mice.* In our initial analysis of CD884 transgenic mice, we examined thymocytes and lymph node T cells for



**Figure 2.** Thymic subsets in mice coexpressing the F5 TCR and the CD884 transgenes. Expression of CD4 and CD8.2 (endogenous CD8) (a-c), V $\beta$ 11 (d-f), or CD8.1 (transgenic CD8) (g-i) in thymocytes from F5 TCR (a, d, and g), F5 TCR/CD8.1 (b, e, and h) or F5 TCR/CD884 (c, f, and i) transgenic mice. Thymocytes were analyzed with fluorescent antibodies as described in Materials and Methods. The numbers inside the quadrants represent the percentage of cells in each population.

expression of TCR, CD4, and endogenous CD8 (CD8.2) by flow cytometry (Table 1). It is interesting to note that we observed a slight increase in the ratio of mature CD4 to CD8 T cells in the lymph node and thymus of CD884 transgenic mice. This increase in the ratio of CD4 to CD8 cells is consistent with the possibility that both class I- and II-specific T cells are present in the mature CD4 population in CD884 transgenic mice.

If mature CD4<sup>+</sup> T cells in CD884 transgenic mice are selected on class I MHC, they should not be dependent on class II MHC for their development. To examine this question, we backcrossed the CD884 transgene to mice

that are deficient for class II MHC because of a targeted disruption of the I-A  $\beta$  gene (24). Whereas class II MHC mutant mice have very few peripheral CD4 cells (24, 34), the ratio of peripheral CD4 to CD8 cells in CD884 transgenic, class II mutant mice is 0.6, compared to 1.5 in mice that are wild type for class II MHC (Table 1).

There is also a substantial population of mature CD4 cells (TCR<sup>high</sup>CD4<sup>+</sup>CD8.2<sup>-</sup>) in the thymus of CD884 transgenic, class II mutant mice (Table 1). To more accurately determine the ratio of mature CD4 to CD8 thymocytes, we depleted thymocytes of immature cells by treating them with anti-HSA and complement. The resulting mature thymic population was then analyzed for expression of TCR, CD4, and CD8.2. When analyzed in this manner, non-transgenic mice have a ratio of mature CD4 to CD8 cells of approximately 2, whereas class II-deficient mice show a ratio of <0.05 [10]. In contrast, expression of the CD884 transgene in a class II mutant background results in a thymic CD4 to CD8 ratio of 0.5. As previously shown, expression of a wild-type CD8 transgene in class II MHC mutant mice also restores the development of some mature CD4 thymocytes, however the CD4 to CD8 ratio is only 0.08 in CD8 transgenic, class II mutant mice (10). This is in spite of the fact that the surface expression of the wild-type CD8 transgene is slightly higher than the CD884 transgene (Fig. 1 c and Fig. 2, h and i). Thus the CD884 molecule is much more efficient than wild-type CD8 at permitting the development of class I-selected CD4 lineage T cells.

*The Effect of the CD884 Transgene on Selection of the F5 TCR.* We also examined the effect of the CD884 transgene expression on the selection of two individual class I-specific TCRs, F5 and anti-HY. The F5 TCR recognizes a nucleoprotein peptide bound to the class I MHC protein, H-2D<sup>b</sup>, and mice expressing an F5 TCR transgene have greatly increased numbers of mature CD8 thymocytes and very few mature CD4 thymocytes (25; Table 2 and Fig. 2 a). It is striking that in mice expressing both the F5 TCR

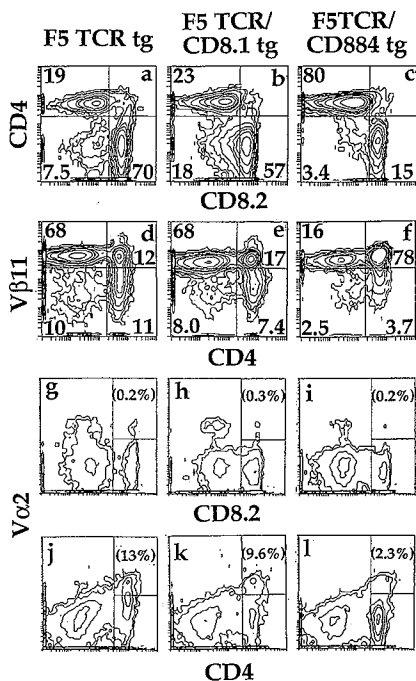
**Table 2.** Thymocytes and Lymph Node T Cells from Mice Coexpressing the F5 TCR and CD884 Transgenes

Genotype	No. of cells/thymus $\times 10^6$	Percent total thymocytes			n
		CD4 <sup>+</sup> 8.2 <sup>-</sup>	CD4 <sup>-</sup> 8.2 <sup>+</sup>	4:8 ratio	
F5 TCR transgenic	198 (97)	2.4 (0.9)	15 (4.6)	0.2	6
F5 TCR/cd884 transgenic	114 (20)	50 (8.9)	7.4 (1.6)	6.8	6
		Percent lymph node T cells			
		CD4 <sup>+</sup> 8.2 <sup>-</sup>	CD4 <sup>-</sup> 8.2 <sup>+</sup>	4:8 ratio	n
F5 TCR transgenic	7.6 (1.2)	87 (1.7)		0.1	6
F5 TCR/CD884 transgenic	56 (15)	32 (11)		1.8	8

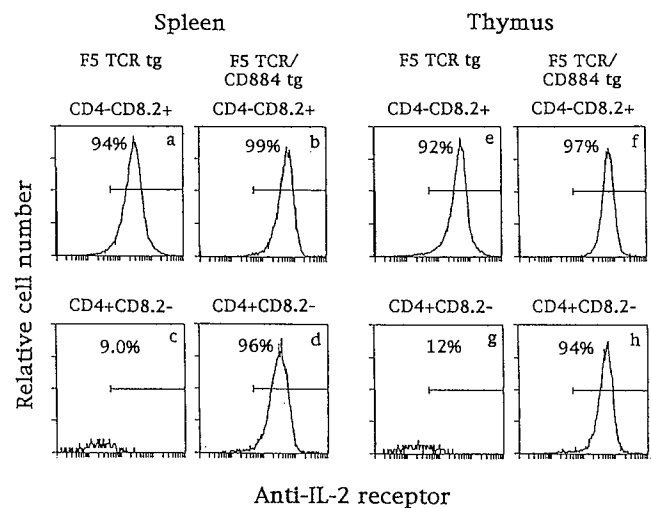
Thymocytes or lymph node T cells (B cell-depleted lymph node cells) were stained with antibodies against CD4 and CD8.2 (endogenous CD8) as described in Materials and Methods. Representative data are shown in Figs. 2 and 3. Average values are given with standard deviations in parentheses, and n is number of mice of each genotype analyzed.

and the CD884 transgenes, this pattern is reversed (Fig. 2 *c* and Table 2). In the thymus of F5 TCR/CD884 transgenic mice, the mature CD4 subset represents ~50% of thymocytes and the mature CD8 subset is reduced approximately twofold compared to F5 TCR transgenic mice. Expression of a wild-type CD8 transgene also leads to an increased number of mature CD4 cells (11, and Fig. 2 *b*); however, this effect is much less dramatic than that observed with the CD884 transgene, in spite of the higher level of expression of the CD8 transgene (Fig. 1, *c* and *d* and Fig. 2, *h* and *i*). Moreover, in F5 TCR/CD884 transgenic mice, the increase in the CD4 subset is accompanied by a decrease in the mature CD8 subset.

A similar trend is observed in the lymph node of F5 TCR/CD884 transgenic mice (Fig. 3 and Table 2). The average ratio of CD4 to CD8 cells is 1.8 in F5 TCR/CD884 transgenic mice, compared to 0.1 in F5 TCR and 0.3 in F5 TCR/CD8.1 transgenic mice (11). The majority of the CD4 cells in the lymph node of F5 TCR/CD884 transgenic mice also express V $\beta$ 11 (Fig. 3 *f*), consistent with the notion that they express the F5 TCR. Because some rearrangements of the endogenous TCR genes can occur in TCR transgenic mice, however, it is important to confirm that the CD4<sup>+</sup> T cells express the F5 TCR rather than endogenous TCRs. Because there is no anticonotypic antibody



**Figure 3.** Lymph node T cells in mice coexpressing the F5 TCR and the CD884 transgenes. Expression of CD4, CD8.2 (endogenous CD8), V $\beta$ 11 (transgenic TCR $\beta$ ), and V $\alpha$ 2 (endogenous V $\alpha$ ) in lymph node T cells from F5 TCR (*a*, *d*, *g*, and *j*), F5 TCR/CD8.1 (*b*, *e*, *h*, and *k*), or F5/CD884 (*c*, *f*, *i*, and *l*) transgenic mice. The numbers inside the quadrants represent the percentage of cells in each population. Numbers in parentheses represent the frequency of V $\alpha$ 2<sup>+</sup> cells as a percentage of CD8.2<sup>+</sup> T cells (*g*–*i*) or as a percentage of CD4<sup>+</sup> T cells (*j*–*l*). T cells (B-depleted lymph node cells) were analyzed with fluorescent antibodies as described in Materials and Methods.



**Figure 4.** Expression of IL-2 receptor after *in vitro* stimulation with NP peptide. Splenocytes (*a*–*d*) or thymocytes (*e*–*h*) from F5 TCR or F5 TCR/CD884 transgenic mice were incubated with NP peptide and H-2<sup>b</sup> splenocytes for 48 h and analyzed for expression of CD4, endogenous CD8 (CD8.2), and IL-2 receptor as described in Materials and Methods. IL-2 receptor levels on gated CD4<sup>+</sup>CD8.2<sup>-</sup> and CD4<sup>-</sup>CD8.2<sup>+</sup> populations are shown.

available to the F5 TCR, we took two alternative approaches to address this question. First, we examined expression of an endogenous V $\alpha$ , V $\alpha$ 2 (Fig. 3, *g*–*l*). Whereas V $\alpha$ 2 is normally expressed on 8–15% of mature T cells from normal mice (35, and data not shown), <0.5% of the CD8<sup>+</sup> T cells from F5 TCR, F5 TCR/CD8.1, or F5 TCR/CD884 transgenic mice express V $\alpha$ 2 (11, and Fig. 3, *g*–*i*), consistent with the notion that the F5 TCR  $\alpha$  and  $\beta$  chains are expressed on these cells. In contrast, 13% of the CD4<sup>+</sup> T cells from F5 TCR transgenic mice express V $\alpha$ 2 (Fig. 3 *j*), consistent with the notion that these cells were selected using endogenous TCRs. It is striking that only 2% of the CD4<sup>+</sup> T cells in F5 TCR/CD884 transgenic mice express V $\alpha$ 2 (Fig. 3 *l*), implying they, like the CD8<sup>+</sup> T cells, express the F5 TCR.

As an additional confirmation that CD4<sup>+</sup> T cells in F5 TCR/CD884 transgenic mice express the F5 TCR, we also examined the response of T cells from mice to nucleoprotein (NP) peptide as measured by IL-2 receptor induction. Although >90% of CD8<sup>+</sup> T cells from both F5 TCR and F5 TCR/CD884 transgenic mice expression IL-2 receptor upon stimulation with NP peptide (Fig. 4, *a*–*f*), only ~10% of CD4<sup>+</sup> T cells from F5 TCR are IL-2 receptor positive after stimulation (11, and Fig. 4, *c* and *g*). In contrast, >90% of CD4<sup>+</sup> T cells from F5 TCR/CD884 transgenic mice express IL-2 receptor in response to NP peptide (Fig. 4, *d* and *h*), confirming that they express the F5 TCR.

*Competition for Limiting “Niches” Does Not Account for the Reduction in CD8 Lineage Cells in F5 TCR Transgenic Mice.* The decrease in the mature CD8 thymocytes in F5 TCR/CD884 transgenic mice (Table 2) is consistent with the notion that the CD884 transgene is causing uncommitted thymocytes to switch fates and choose the CD4 instead of

the CD8 lineage. We also considered an alternative explanation for the decrease in the mature CD8 population; that the large number of mature CD4 cell thymocytes in F5 TCR/CD884 transgenic mice are preventing CD8 lineage cells from being selected because of competition between CD4 and CD8 lineage cells for limiting niches in the thymus (36). If cellular competition for limiting niches is the explanation for the reduction in the CD8 population, we could expect the proportion of CD8 lineage cells within the F5 TCR/CD884 transgenic cells to increase as the proportion of F5 TCR<sup>+</sup>CD884<sup>+</sup> thymocytes decreases. On the other hand, if the CD884 transgene is diverting thymocytes from the CD8 into the CD4 lineage, we would expect the ratio of mature CD4 to CD8 lineage cells to remain constant as the proportion of F5 TCR/CD884 transgenic thymocytes decreases. To investigate this question, we constructed mixed hematopoietic stem cell chimeras from bone marrow of F5 TCR and F5 TCR/CD884 transgenic mice. 3 wk after reconstitution, we analyzed thymocytes for expression of CD4, endogenous CD8 (CD8.2), and transgenic CD8 (CD8.1). As shown in Table 3, there is no correlation between the proportion of thymocytes that are derived from F5 TCR/CD884 transgenic bone marrow and the relative proportion of mature CD4 and CD8 lineage cells within this population. These data indicate that cellular competition for limiting niches does not account

**Table 3.** Ratio of Mature CD4 to CD8 Lineage Thymocytes in Mixed Hematopoietic Stem Cell Chimeras from F5 TCR/CD884 Transgenic and F5 TCR Transgenic Mice

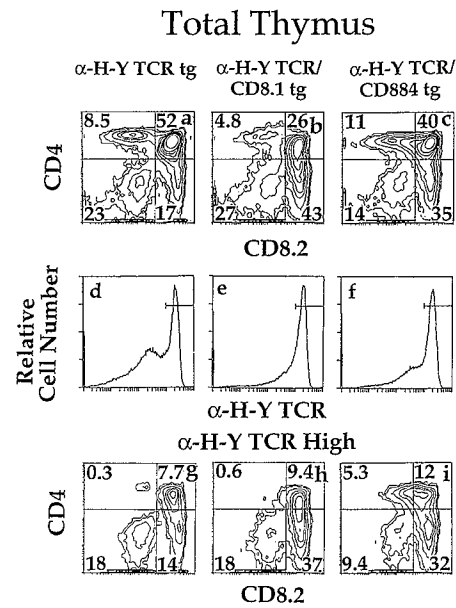
Percent thymocytes derived from F5 TCR/CD884 transgenic donor (CD8.1 <sup>+</sup> )	Ratio of CD4 <sup>+</sup> CD8.2 <sup>-</sup> /CD4 <sup>-</sup> CD8.2 <sup>+</sup> thymocytes within the F5 TCR/CD884-derived subset (CD8.1 <sup>+</sup> )
3.4	3.6
20	2.8
20	12
23	3.8
32	6.3
54	4.2
81	3.7
87	4.8
89	3.6

Mixed hematopoietic stem cell chimeras were generated by reconstituting the immune systems of Rag1 mutant mice with mixtures of bone marrow from F5 TCR/CD884 and F5 TCR transgenic mice. After 3–4 wk to allow reconstitution, thymocytes were analyzed by flow cytometry for expression of CD8.1 (as a marker for F5 TCR/CD884 transgene-derived cells), CD4, and endogenous CD8 (CD8.2). Thymocytes that were CD4<sup>-</sup> and CD8.2<sup>-</sup> (double negative) were excluded from the analysis because their genotype could not be assessed. The percentage of remaining thymocytes that expressed the CD884 transgene (CD8.1<sup>+</sup>) is indicated. Each line represents the data from an individual chimera.

for the reduction in the mature CD8 population in F5 TCR/CD884 transgenic mice.

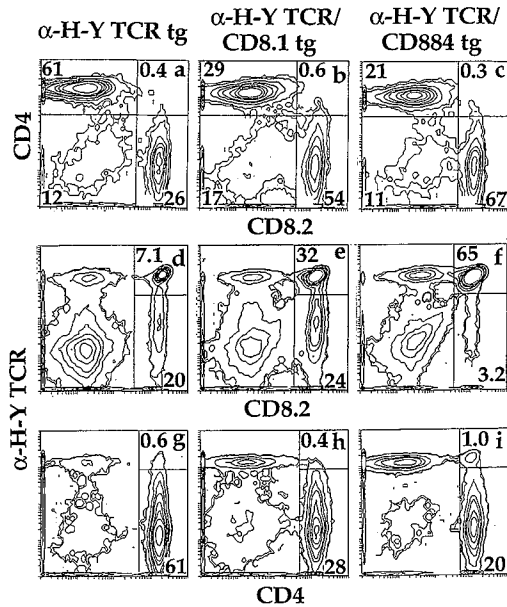
**The Effect of the CD884 Transgene on Selection of the anti-HY-TCR.** We also examined the effect of the CD884 transgene on selection of the anti-HY TCR. This TCR recognizes a male antigen bound to the class I molecule, H-2D<sup>b</sup>. In the thymus of female anti-HY TCR transgenic mice, there is a substantial population of CD4<sup>-</sup>CD8<sup>+</sup> cells that express high levels of the anti-HY TCR, but very few anti-HY TCR<sup>+</sup>CD4<sup>+</sup>CD8<sup>-</sup> cells (37, and Fig. 5 *g*). As previously observed, coexpression of a constitutive CD8.1 transgene leads to an increased number of anti-HY TCR<sup>+</sup>CD4<sup>-</sup>CD8.2<sup>+</sup> thymocytes, but does not permit the development of CD4<sup>+</sup>CD8.2<sup>-</sup> cells bearing the anti-HY TCR (16, and Fig. 5 *h*). In contrast, coexpression of the hybrid CD884 transgene leads to the appearance of a significant population of anti-HY TCR<sup>+</sup>CD4<sup>+</sup>CD8.2<sup>-</sup> cells (Fig. 5 *i*). On close examination, however, it is apparent that this population expresses low levels of endogenous CD8, suggesting that they may not be fully mature.

If CD4<sup>+</sup> thymocytes expressing the anti-HY TCR are fully mature, they should emigrate from the thymus and populate the periphery. To examine this question, we analyzed peripheral T cells of anti-HY TCR/CD884 mice. Expression of the anti-HY TCR in peripheral T cells of female H-2<sup>b</sup> mice is generally low due to extensive rearrangements of endogenous TCR  $\alpha$  genes (37) and peripheral selection for cells that do not express the anti-HY



**Figure 5.** Thymic subsets in female mice coexpressing the anti-HY TCR and the CD884 transgenes. Expression of CD4, CD8.2 (endogenous CD8), and anti-HY TCR (T3.70) in thymocytes from anti-HY TCR (*a*, *d*, and *g*), anti-HY TCR/CD8.1 (*b*, *e*, and *h*), or anti-HY TCR/CD884 (*c*, *f*, and *i*) transgenic mice. Thymocytes were analyzed by three-parameter flow cytometry using fluorescent antibodies as described in Materials and Methods. The numbers inside the quadrants represent the percentage of cells in each population. Total thymocytes (*a–f*) or thymocytes expressing high levels of anti-HY TCR (*g–i*) are shown. The gate for discriminating anti-HY TCR high thymocytes is indicated (*d–f*).

## Lymph Node T Cells



**Figure 6.** Lymph node T cells in female mice coexpressing the anti-HY TCR and the CD884 transgenes. Expression of CD4, CD8.2 (endogenous CD8), and anti-HY TCR in lymph node T cells from anti-HY TCR (*a*, *d*, and *g*), anti-HY TCR/CD8.1 (*b*, *e*, and *h*), or anti-HY TCR/CD884 (*c*, *f*, and *i*) transgenic mice. The numbers inside the quadrants represent the percentage of cells in each population. Four mice of each genotype were analyzed and representative data are shown. T cells (B-depleted lymph node cells) were analyzed by three-parameter flow cytometry using fluorescent antibodies as described in Materials and Methods.

TCR (38). There is, however, a distinct population of CD8 cells expressing high levels of the anti-HY TCR (37, and Fig. 6, *a* and *d*). Coexpression of a constitutive CD8.1 transgene leads to an increase in anti-HY TCR<sup>+</sup>CD8.2<sup>+</sup> lymph node T cells, but does not increase the anti-HY TCR<sup>+</sup>CD4<sup>+</sup> population (16, and Fig. 6, *b*, *e*, and *h*). In mice expressing both the CD884 and the anti-HY TCR transgene, there is a further increase in the anti-HY TCR<sup>+</sup>CD8<sup>+</sup> population (Fig. 6, *c* and *f*). It is important to note that there is also a small (1%) but distinct population of CD4 cells expressing high levels of the anti-HY TCR (Fig. 6 *i*). Taken together, these data indicate that expression of the CD884 transgene permits the development of some anti-HY TCR<sup>+</sup> CD4 lineage cells. However, unlike the F5 TCR, for which coexpression of the CD884 transgene leads to a predominance of CD4 cells over CD8 cells, coexpression of the CD884 protein with the anti-HY TCR produces predominately CD8 lineage T cells and only a small number of mature CD4 lineage cells.

## Discussion

In this paper we show that expression of a hybrid molecule consisting of the extracellular and transmembrane portions of CD8 and the cytoplasmic portion of CD4 leads to the development of large numbers of mature class I-specific, CD4<sup>+</sup> T cells. In contrast, expression of a wild-type

CD8 transgene at comparable levels allows only a small number of class I-specific, CD4 lineage cells to develop. These results indicate that the cytoplasmic tail of CD4 delivers an intracellular signal that favors the development of CD4 lineage T cells. This observation provides a molecular basis for differential signaling in response to class I or II MHC recognition during thymic development which could serve to direct the CD4/CD8 lineage decision.

One feature of our data is most compatible with instructive models for lineage commitment. We find that in F5 TCR/CD884 transgenic mice, the increase in CD4 lineage cells is accompanied by a decrease in the mature CD8 population. This decrease in the mature CD8 compartment is seen even when the F5 TCR/CD884-bearing thymocytes represent a minority of total thymocytes, indicating that the effect is not due to cellular competition. Whereas an instructive model predicts that mature CD4 cells might appear at the expense of CD8 cells, if CD884 expression “rescues” already committed CD4 lineage cells, it is not obvious why the development of CD8 lineage cells should be impaired. Because this feature of the data is not readily explained by a stochastic model, we favor the explanation that the CD884 hybrid molecule acts at an uncommitted stage of T cell development to instruct cells to choose the CD4 lineage.

The different abilities of the CD884 hybrid coreceptor and wild-type CD8 to generate class I-specific, CD4 lineage T cells focuses attention on the CD4 cytoplasmic domain. What feature of these 31 amino acids favors the development of CD4 lineage cells? The CD4 cytoplasmic domain interacts with the tyrosine kinase Lck, raising the possibility that Lck is ultimately responsible for the effects of the CD4 cytoplasmic domain on T cell development. If this is the case, this effect is likely to be quantitative, because the cytoplasmic domain of CD8 $\alpha$  also interacts with Lck, albeit more weakly (27–29). It is also possible that there are qualitative differences between the CD4 and CD8 intracellular signals and that as yet unidentified signaling molecules are associated with CD4 or CD8. Experiments to identify more precisely the region of the CD4 tail that is responsible for these effects are in progress.

The quantitative difference in the ability of CD4 and CD8 to associate with and activate Lck suggests a possible explanation for the effect of the CD4 cytoplasmic tail on T cell fate. Signal transduction through the TCR occurs via a series of tyrosine phosphorylations and involves the sequential interaction of at least two tyrosine kinases, Lck and ZAP-70 (for a review see reference 39). We suggest that the quantity of Lck associated with the TCR complex during MHC recognition might impose a bias on lineage commitment, such that MHC engagement that produces weak Lck activation would be more likely to result in CD8 lineage cells, whereas strong Lck activation would be more likely to result in CD4 lineage cells. Because CD8 binds Lck more weakly than CD4, class I MHC recognition would tend to produce CD8 lineage cells, whereas class II MHC recognition would be more likely to produce CD4 lineage cells. Although differential recruitment of Lck by CD4 or CD8



would have a strong impact on lineage commitment, the level of Lck associated with the TCR could be affected by other factors as well. It has been shown that phosphorylation of the  $\zeta$  chain of the TCR by Lck and recruitment of ZAP-70 can occur in the absence of CD4 and CD8, implying that there are multiple pathways for activating Lck (for a review see reference 39). Although other means of recruiting Lck to the TCR complex have not yet been characterized, it is possible that additional factors, such as the extent of TCR cross-linking by MHC, could affect the level of Lck in the TCR complex and influence the CD4/CD8 lineage decision independently of coreceptor engagement.

Obviously, quantitative considerations alone cannot explain the strict correlation between TCR specificity and CD4 or CD8 lineage commitment that is normally observed. However, indications that positive selection may require prolonged TCR engagement (40, 41), along with the observation that constitutive coreceptor expression can

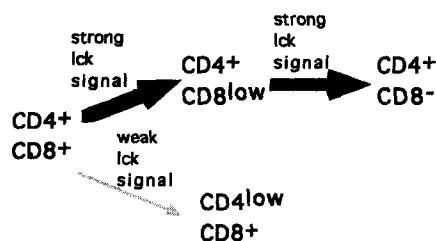
sometime “rescue” cells of the wrong lineage (9–15), suggest that there is a “confirmation step” after the initial MHC encounter. Based on these observations, as well as our data, we favor the model depicted in Fig. 7. In this model, the initial encounter with MHC leads to a bias in lineage commitment such that most thymocytes expressing class II-specific TCRs receive a strong signal and begin to downregulate CD8. However, some thymocytes bearing class II-specific TCRs (perhaps those with weaker avidity for peptide/MHC on thymic epithelial cells) turn off CD4 instead. Because CD4 would be required for continued class II-specific TCRs that retain expression of CD4 could survive, whereas those that have downregulated CD4 would die. Likewise, most cells expressing class I-specific TCRs would receive a weaker Lck signal and would downregulate CD4. Cells that continue to receive a weak signal could complete maturation to a CD8 lineage. Thymocytes with class I-specific TCRs that receive a stronger TCR signal might downregulate CD8 instead of CD4, however those cells would not complete maturation to a CD4 lineage because of the continued requirement for class I recognition throughout maturation.

It is interesting to consider the possibility that the confirmation step might also be instructive, i.e., that it may affect the lineage decision of an uncommitted cell. Although it is often assumed that CD4<sup>+</sup>CD8<sup>low</sup> thymocytes are committed to a CD4 lineage, there is little evidence supporting this assumption. Indeed, intrathymic transfer of CD4<sup>+</sup>CD8<sup>low</sup> thymocytes indicates that some of these cells can differentiate into CD8 lineage cells (8). If CD4<sup>+</sup>CD8<sup>low</sup> thymocytes are uncommitted, then the signal that they receive upon MHC recognition might be instructive. A strong signal would lead them to become CD4 cells, whereas a weak signal would divert them to the CD8 lineage.

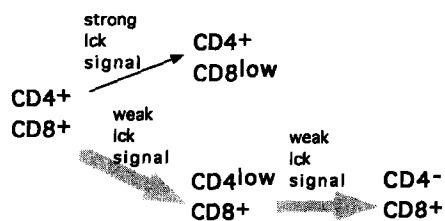
This quantitative model may provide an explanation for the different effects of the CD884 transgene on two individual class I-specific TCRs. If the F5 TCR is relatively effective at generating an intracellular signal (due to the high avidity of the F5 TCR for peptide/MHC or due to a high density of the positive-selecting ligand on thymic epithelial cells), then the Lck associated with the CD884 protein might be sufficient to divert the majority of cells to the CD4 lineage. On the other hand, thymocytes expressing the anti-HY TCR might experience a relatively weak intracellular signal during positive selection. If that were the case, then increasing the activation of Lck by engaging the CD884 protein might not be sufficient to complete maturation to the CD4 lineage. It is interesting to note that the thymus of anti-HY TCR/CD884 transgenic mice contains a very prominent CD4<sup>+</sup>CD8<sup>low</sup> population. This may reflect the fact that thymocytes expressing both the anti-HY TCR and CD884 transgenes receive a stronger intracellular signal that may be sufficient to downregulate CD8, but not to permit complete maturation to the CD4 lineage.

Earlier studies (42) indicated that CD4 intracellular signals are dispensable for the development of some CD4 lineage cells. At first glance, these results appear to contradict the role for the CD4 cytoplasmic tail indicated here. How-

#### THYMOCYTES WITH CLASS II SPECIFIC TCRs



#### THYMOCYTES WITH CLASS I SPECIFIC TCRs



**Figure 7.** A quantitative, instructive model for the CD4/CD8 lineage decision. This model is based on the assumption that positive selection is not a “single-hit” event, and that MHC engagement influences the lineage choice of an uncommitted thymocyte. The width of the arrow denotes the proportion of thymocytes that take a particular pathway and the darkness of the arrow indicates the extent of Lck activation. According to this model, MHC recognition that leads to weak activation of Lck biases development to the CD8 lineage, whereas MHC recognition that leads to strong Lck activation biases toward the CD4 lineage. Most thymocytes bearing class II-specific TCRs would receive a strong Lck signal (due to Lck activation by CD4) and would begin to downregulate CD8. These thymocytes would continue to receive a strong intracellular signal through the TCR and CD4, and would therefore continue to mature. Most cells bearing class I-specific TCRs would receive a weaker signal (due to weak Lck activation by CD8) and would downregulate CD4. A continued weak signal through TCR and CD8 would permit their final maturation. The requirement for continued MHC recognition after the downregulation of one of the coreceptors serves as a “confirmation” step to insure that cells that downregulate the wrong coreceptor do not mature. Note that if cells that have begun CD8 downregulation are not yet lineage committed (8), then the confirmation step could also influence the lineage choice of uncommitted cells.

ever, it is possible that the CD4 cytoplasmic tail could promote the development of CD4 lineage cells and still not be essential for the development of all CD4 cells. The CD4 cells that develop in the absence of a CD4 cytoplasmic tail may bear high avidity TCRs that would generate strong intracellular signals. Such cells might not require the Lck associated with CD4 in order to develop into CD4 lineage T cells. The fact that the substitution of physiological levels of tail-less CD4 for endogenous CD4 restores only a fraction of the normal numbers of mature CD4 cells is consistent with this explanation.

An earlier report describing a hybrid CD8/CD4 transgene (18) differs from our results in a number of important respects. In the previous report, a population of peripheral T cells expressing both CD4 and endogenous CD8 was observed. We see no evidence for such a population in our studies. Moreover, in a preliminary study, expression of the previously described CD8/CD4 hybrid transgene in class II mutant mice did not produce an increase in the mature CD4 population (Jane Parnes, personal communication).

The failure to generate class I-specific CD4 cells using this particular hybrid coreceptor could be due to the fact that CD8 $\beta$  was not coinjected with the CD8/CD4 hybrid transgene or due to the level or timing of expression of the transgene. It may also be relevant that our CD8/CD4 hybrid transgene uses the transmembrane segment of CD8, whereas the one described in the earlier study uses the transmembrane segment of CD4.

A number of recent studies have emphasized the stochastic nature of the process of CD4/CD8 lineage commitment and the lack of an absolute requirement for CD4 and CD8 intracellular domains in thymic development. In spite of this, the critical issue of whether intracellular signals generated upon MHC recognition influence the fate of uncommitted thymocytes remains unresolved. The data in our paper clearly demonstrate that the CD4 intracellular domain does play an important role in the CD4/CD8 lineage decision. Moreover, our data are most compatible with the notion that CD4 intracellular signals act on uncommitted thymocytes to influence their lineage decision.

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We thank Mimi Mong for technical assistance, Peter Schow for assistance with flow cytometry, Paul Gottlieb (University of Texas, Austin, TX) for providing the CD8 $\beta$ .1 genomic clone, David Baltimore (MIT, Boston, MA) for providing Rag1 mutant mice, Tak Mak (Amgen, Toronto, Canada) for providing CD8 $\alpha$  mutant mice, Laurie Glimcher (Harvard University, Boston, MA) for providing class II MHC mutant mice, Harald von Boehmer (Basel Institute, Switzerland) for providing anti-HY TCR transgenic mice, Joe Bolen (Bristol-Myers Squibb, Princeton, NJ) for providing anti-Lck antisera, and R. Axel (Columbia University, New York), F. Ramsdell (Darwin Molecular, Seattle, WA), D. Raulet (University of California, Berkeley, CA), and members of the Robey laboratory for comments on the manuscript.

This work was supported by National Institutes of Health (NIH) grant AI-32985 to E. Robey. R. Salmon was supported by Fondation pour la Recherche Médicale. The initial phases of this work were carried out in Richard Axel's laboratory (Columbia University, New York), and were supported by the NIH.

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Received for publication 25 August 1995 and in revised form 2 November 1995.

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