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The Role of CD8α' in the CD4 Versus CD8 Lineage Choice[1]

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During thymic development the recognition of MHC proteins by developing thymocytes influences their lineage commitment, such that recognition of class I MHC leads to CD8 T cell development, whereas recognition of class II MHC leads to CD4 T cell development. The coreceptors CD8 and CD4 may contribute to these different outcomes through interactions with class I and class II MHC, respectively, and through interactions with the tyrosine kinase p56^{lck} (Lck) via their cytoplasmic domains. In this paper we provide evidence that an alternatively spliced form of CD8 that cannot interact with Lck (CD8α') can influence the CD4 vs CD8 lineage decision. Constitutive expression of a CD8 minigene transgene that encodes both CD8α and CD8α' restores CD8 T cell development in CD8α mutant mice, but fails to permit the development of mismatched CD4 T cells bearing class I-specific TCRs. These results indicate that CD8α' favors the development of CD8-lineage T cells, perhaps by reducing Lck activity upon class I MHC recognition in the thymus. *The Journal of Immunology,* **1999, 163: 5312–5318.**

During T cell maturation in the thymus, precursors that coexpress CD4 and CD8 down-regulate either CD8 or CD4 to give rise to mature CD4 or CD8 lineage T cells. This process is influenced by MHC recognition, such that thymocytes bearing TCRs specific for class I MHC molecules give rise to mature CD8 lineage T cells, and thymocytes bearing TCRs specific for class II MHC give rise to mature CD4 lineage T cells (reviewed in Refs. 1 and 2). During this process, the coreceptors CD8 and CD4 cooperate with the TCR by stabilizing interactions with MHC class I and class II, respectively, and by participating in signal transduction through their interactions with the cytoplasmic tyrosine kinase, p56^{lck} (Lck) (reviewed in Refs. 3 and 4). To what extent the coreceptors generate intracellular signals that influence CD4 vs CD8 lineage commitment remains a subject of some controversy.

Based on the observation that CD4 is more effective than CD8 in recruiting Lck, it has been suggested that differential recruitment of Lck during class I or class II MHC recognition could influence the CD4 vs CD8 lineage choice (5, 6). CD8 engagement during class I MHC recognition would weakly activate Lck, promoting the CD8 cell fate, whereas CD4 engagement during class II MHC recognition would strongly activate Lck, promoting the CD4 cell fate. Several lines of evidence support this "quantitative Lck" hypothesis. Hybrid coreceptors consisting of the extracellular domain of CD8 and the cytoplasmic domain of CD4, which strongly activate Lck, were found to be effective at promoting the development of CD4 T cells bearing class I-specific TCRs (5, 7). In addition, while the expression of class II-specific TCR transgenes generally leads to the development of CD4 lineage T cells, the expression of class II-specific TCR transgenes in CD4 mutant mice leads to the development of CD8 lineage T cells (6). Since the absence of CD4 during class II MHC recognition would lead to reduced Lck activity, these results also support the quantitative Lck hypothesis.

Manipulations that quantitatively alter the TCR signal can also influence CD4 vs CD8 cell development. For example, enhancing signaling through the MAP kinase pathway by expression of an activated form of ERK favors the CD4 cell fate, while inhibiting the MAP kinase pathway using a MEK inhibitor favors CD8 cell development (8). Finally, in studies using Ab treatments to induce T cell development in thymic organ cultures, forms of anti-CD3 Ab that are agonists for mature T cells and that strongly activate Lck induce CD4 T cell development, whereas a form of anti-CD3 that is antagonistic for mature T cells and that weakly activates Lck induces CD8 cell development (9, 10). Thus, several lines of evidence support the idea that differential activation of Lck could influence the CD4 vs CD8 lineage decision.

The differential activation of Lck by CD4 vs CD8 can be attributed in part to the fact that the cytoplasmic domain of CD4 interacts more strongly with Lck than does the cytoplasmic domain of CD8 (11–14). This differential Lck activation could be further enhanced by the presence of an alternatively spliced form of CD8 called CD8α' that lacks the cytoplasmic domain and is incapable of interacting with Lck (15, 16). Interestingly, while mature T cells express a CD8 heterodimer composed of full-length CD8α paired with CD8β (CD8$\alpha\beta$), approximately half the CD8 on the surface of murine thymocytes consists of CD8$\alpha'\beta$ heterodimers (15, 16). This suggests that CD8α' may act as a naturally occurring dominant negative form of CD8 that serves to further dampen Lck activation during recognition of class I MHC in the thymus and may thereby contribute to promoting the CD8 cell fate.

Despite evidence that MHC recognition can influence lineage commitment though the engagement of CD8 or CD4, there are also indications that lineage commitment does not always correlate with MHC class I or II recognition. This together with indications that continuous or prolonged MHC recognition may be required for T cell maturation (17, 18) suggest that the continued requirement for coreceptor recognition of MHC as thymocytes are turning off CD4 or CD8 expression could serve as a check to ensure that only thymocytes that retain expression of the correct coreceptor finally mature. Thus, CD4 committed thymocytes bearing class I-specific TCRs would be generated transiently but would fail to mature because, as they lose CD8 expression, they would also lose the ability to recognize class I MHC. This idea is supported by

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experiments showing that constitutive expression of CD8 can sometimes lead to the development of mismatched CD4 lineage T cells bearing class I-specific TCRs (19–25). These data together with similar evidence from CD4 transgenic mice (26–28) suggest that a late requirement for coreceptor engagement contributes to the strict correlation between CD8 or CD4 expression and MHC specificity that is normally observed.

The idea that CD8α' might play a role in CD8 lineage commitment by reducing Lck activation upon class I MHC recognition suggests a potential problem with the interpretation of experiments using CD8 transgenic mice. All the previously described CD8 transgenes are derived from CD8α cDNA that do not encode CD8α'. Thus, these CD8 transgenes may not accurately reflect the normal function of CD8 in the thymus. Indeed, in our previous analysis of CD8 transgenic mice, we noted that transgene-encoded CD8 has a higher level of associated Lck activity compared with endogenous CD8 (5). This raises the possibility that some of the class I-specific CD4 lineage T cells found in CD8 transgenic mice could result from abnormally high Lck activation rather than constitutive CD8 expression per se.

To explore this question, we have constructed a new constitutive CD8 transgene that can encode both the α and α' forms of CD8. The CD8 encoded by this transgene has associated levels of Lck activity very similar to those of endogenous CD8. Compared with previously described constitutive CD8 transgenes, the CD8 minigene (CD8mg)[4] transgene is ineffective at promoting the development of mismatched CD4 lineage T cells bearing class I-specific TCRs. Moreover, expression of the CD8mg transgene inhibits the ability of a constitutive CD8α transgene to promote the development of class I-specific CD4 lineage T cells. These data suggest that expression of CD8α' in thymocytes serves to dampen Lck activation upon class I MHC recognition and provide additional support for a model in which weak activation of Lck during positive selection favors the CD8 T cell fate.

Materials and Methods

Generation of transgenic mice

The CD8mg construct was generated by modifying the previously described CD8.1 transgenic construct (T11-8) (19) by replacing the sequences containing the 3′ half of the CD8α cDNA and the human CD2 minigene with CD8α genomic sequences. In brief, a 3-kb *Eco*RI-*Bam*HI fragment from T11-8 was replaced with a 1.3-kb *Eco*RI-*Bam*HI genomic fragment of CD8α (29) (provided by Paul Gottlieb) that extends from the *Eco*RI site within the CD8α-coding region to a *Bam*HI site in the third intron of the CD8α gene. The resulting plasmid was then linearized at the unique *Bam*HI site, and a 3.3-kb *Bam*HI fragment containing exons 4 and 5 (encoding IC1 and IC2) of the CD8α genomic clone was inserted in the correct orientation with respect to CD8 transcription. Plasmid sequences were removed by digestion with *Sal*I, and the fragment was coinjected into $(B6 \times CBA/J)F_2$ embryos along with a 17-kb *Bam*HI genomic DNA clone containing the entire CD8β-coding region (30).

Analysis of Lck activation

Cells (20×10^6) were incubated with saturating amount of rat anti-murine CD8 IgM (3.155) for 15 min on ice, then with 100 μl of goat anti-rat IgM (Cappel, Durham, NC) for 15 min on ice. Cells were then washed with 500 μl of cold DMEM (Life Technologies, Grand Island, NY) with 0.5% bovine albumin fraction V (Sigma, St. Louis, MO) and lysed in 500 μl of ice-cold lysis buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl, 1 mM Na_3VO_4, 10 mM NaF, 10 mM $Na_2P_2O_8$, 1% Brij97, 1 mM PMSF, and 10 μg/ml each of pepstatin A, aprotinin, and leupeptin for 15 min on ice. Lysates were cleared by centrifugation for 10 min at $10,000 \times g$ and were incubated with 50 μl of protein G-Sepharose beads for 2 h at 4°C. Beads were then washed three times with ice-cold wash buffer (50 mM Tris (pH 7.5), 150 mM NaCl, 1 mM Na_3VO_4, 10 mM NaF, 0.1% Brij97, and 10 μg/ml each of pepstatin A, aprotinin, and leupeptin) and once with kinase reaction buffer (50 mM Tris (pH 7.5), 150 mM NaCl, and 10 mM $MnCl_2$), and resuspended in 20 μl of kinase reaction buffer with 10 μCi of [γ-^{32}P]ATP and 10 μg of acid-denatured enolase (Sigma). Kinase assays were performed for 3 min at 30°C and quenched with 20 μl 3× sample buffer (150 mM Tris (pH 6.8), 30% glycerol, 6% SDS, 3% 2-ME, and 0.05% bromophenol blue). The samples were boiled for 5 min, and the reaction products were analyzed by 10% PAGE-SDS. The gel was then soaked in a solution of 10% AcOH and 40% MeOH for 15 min, dried, and visualized by autoradiography. Quantification of radioactive bands was performed using the PhosphorImager/ImageQuant system (Molecular Dynamics, Sunnyvale, CA).

Analysis of T cell populations

F5 TCR (31), A-β mutant (32), CD8.1 (19), and CD884 transgenic (5) mice have been previously described. Transgenic offspring were identified by Southern blot and PCR typing. For analysis by flow cytometry, thymus and lymph nodes (cervical, axillary, brachia, and mesenteric) were teased apart in cold medium 199 (Life Technologies) supplemented with 2% FBS, and the cells were filtered through nylon mesh. For analysis of mature thymocytes, thymocytes were treated with anti-HSA (Jll.D) and complement. Cells (10^6) were incubated with 10 μl of Ab on ice for 20 min, then washed twice with staining buffer containing 1× HBSS (Fisher, Fairlawn, NJ), 0.2% sodium azide, and 0.2% bovine albumin (Sigma). Data (50,000 events) were collected and analyzed using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA) or EPICS XL-MCL flow cytometer (Coulter, Hialeah, FL). Dead cells were excluded on the basis of forward and side scatter. Dot-plot images were produced with the aid of WinMDI version 2.1.2 by Joseph Trotter (Scripps Research Institute, La Jolla, CA).

The Abs used were T3.70 (culture supernatant), FITC-labeled anti-CD8α (53-6.7; PharMingen, San Diego, CA), FITC-labeled anti-CD8β.2 (53-5.8; PharMingen), anti-CD8β.1 (5034-29.5; Serotec, Oxford, U.K.), FITC-labeled goat anti-mouse IgG1 (Caltag, South San Francisco, CA), anti-vβ11 (KT11), RED613-labeled goat anti-rat IgG (Life Technologies), rat γ-globulin (Calbiochem-Novabiochem, San Diego, CA), PE-labeled anti-CD4 (GK1.5; Becton Dickinson), anti-CD8.1 (49-31.1; Cedarlane, Westbury, NY), FITC-labeled goat anti-mouse IgG3 (Caltag), HO-3.1 (culture supernatant) (33), FITC-labeled goat anti-mouse IgM (Caltag), FITC-labeled anti-mouse I-A^b (AF6-120.1; PharMingen), RED613-labeled anti-CD4 (H129.19; Life Technologies), FITC-labeled anti-CD8.2α (2.43; rat anti-mouse IgG2b affinity purified), and PE-labeled anti-TCR-$\alpha\beta$ (H57–597; PharMingen).

Results

A constitutive CD8 transgene that encodes both CD8 α and α'

A previously described constitutively expressed CD8.1 transgene consists of CD8.1α cDNA under the control of the human CD2 promoter and locus control region (19). To generate a new CD8mg construct that encodes both CD8α and α', we modified the original CD8.1 transgenic construct by replacing the 3′ half of the CD8α cDNA and human CD2 minigene with CD8α genomic sequences (Fig. 1). We then generated transgenic mice by coinjecting the CD8mg construct with a CD8β.1 genomic clone (30). We chose the founder line with levels of CD8 expression that were closest to endogenous CD8 for further analysis. Flow cytometric analysis of total CD8α surface expression on thymocytes of transgenic and nontransgenic mice is shown in Fig. 2*A*. Thymocytes from CD8mg transgenic mice express several times more surface CD8α than thymocytes from nontransgenic mice and slightly higher levels than the previously described CD8.1 and CD884 transgenic mice. Flow cytometric analysis using a transgene-specific Ab (CD8.1; Fig. 2*B*) likewise shows comparable, but slightly higher, CD8.1α expression on thymocytes of CD8mg transgenic mice compared with CD8.1 and CD884 transgenic mice. Although the CD8mg transgene, like the CD8.1 and CD884 transgenes, is cointegrated with a CD8β genomic clone (data not shown), surface expression of transgenic CD8β (CD8β.1) on thymocytes from CD8mg transgenic mice is low and variable (Fig. 2, *C* and *D*). The reason for this difference in CD8β expression between the two CD8 transgenes is unknown.

[4] Abbreviations used in this paper: CD8mg, CD8 minigene; HSA, heat-stable Ag; ITAM, immunoreceptor tyrosine-based activation motif.

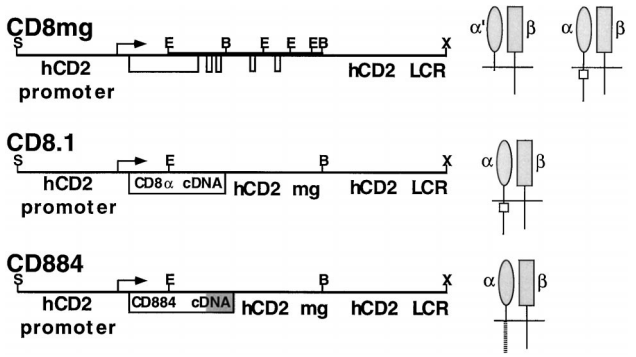

FIGURE 1. Structure of the CD8mg transgene. Schematic structures of the transmembrane CD8 receptors expressed from the CD8 transgenes discussed in this paper. For the CD8mg transgene, alternative splicing generates both CD8α (full-length) and CD8α' (tailless) isoforms. Open squares represent the Lck binding domain of CD8α. Both CD8α and CD8α' can associate with CD8β to form $\alpha\beta$ or $\alpha'\beta$ heterodimers. The CD8.1 transgene (19) contains the CD8.1 cDNA, which encodes only the α form of CD8; thus, only $\alpha\beta$ heterodimers will be generated. For the CD884 transgene, a hybrid cDNA consisting of the extracellular and transmembrane domains of CD8α and the cytoplasmic domain of CD4 encodes a fusion protein as described previously (5). The shaded area indicates the region derived from the CD4 cDNA. Restriction sites are indicated: E, *Eco*RI; B, *Bam*HI; X, *Xba*I; and S, *Sal*I.

Because the CD8mg transgene, like the endogenous CD8 gene, can encode a form of CD8 that does not interact with Lck, we expected that thymocytes expressing the CD8mg transgene might have wild-type levels of CD8 associated Lck activity. To examine this question, we performed in vitro kinase assays on CD8 immunoprecipitates from thymocytes of CD8mg transgenic and nontransgenic mice (Fig. 3). CD8.1 and CD884 transgenic mice were included for comparison. As previously shown, CD8 immunoprecipitates from CD884 transgenic mice had ~6-fold higher Lck activity compared with CD8 immunoprecipitates from nontransgenic mice (5). This was consistent with observations that the cytoplasmic domain of CD4 associates more strongly with Lck than does the cytoplasmic domain of CD8 (11–14). In contrast, CD8 immunoprecipitates from thymocytes of CD8mg transgenic mice had levels of CD8associated Lck comparable to those in nontransgenic mice. Although we have not directly measured the ratio of

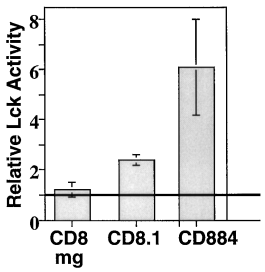

FIGURE 3. Lck kinase activity associated with transgenic forms of CD8. Thymocyte extracts from the indicated mice were subjected to immunoprecipitation with anti-CD8 Abs. Immunoprecipitates were then subjected to an in vitro kinase assay as described in *Materials and Methods*. Data are compiled from 10 experiments. Lck activities were normalized to the values in the nontransgenic mice indicated by the horizontal bar across the graph (relative activity = 1), for each independent experiment. The bars represent the mean, and the error bars indicate the SD.

CD8α to CD8α' produced by the CD8 transgene, the observation that thymocytes from CD8mg transgenic mice have lower levels of CD8associated Lck activity than the CD8.1 transgene despite the higher level of CD8 expression is consistent with the idea that CD8α' encoded by the CD8mg transgene acts to reduce the Lck activity associated with CD8. These data indicate that the CD8mg transgene may be more similar to endogenous CD8 than the previously described CD8 transgene.

To confirm that the CD8mg transgene is functional, we asked whether it could substitute for the presence of endogenous CD8 to permit the development of CD8 lineage T cells. We crossed mice bearing the CD8mg transgene with mice bearing a targeted deletion of the CD8α locus (34) and examined lymph nodes for the presence of $\alpha\beta$TCR$^+$CD4$^-$ cells (Table I). For comparison, we also examined the ability of previously described CD8.1 and CD884 transgenes to substitute for endogenous CD8. We found that all three CD8 transgenes can restore the development of CD8 lineage T cells to levels comparable to those found in wild-type mice.

Effect of the CD8mg transgene on thymic selection

To determine whether the CD8mg transgene can permit the development of CD4 lineage T cells bearing class I-specific TCRs, we

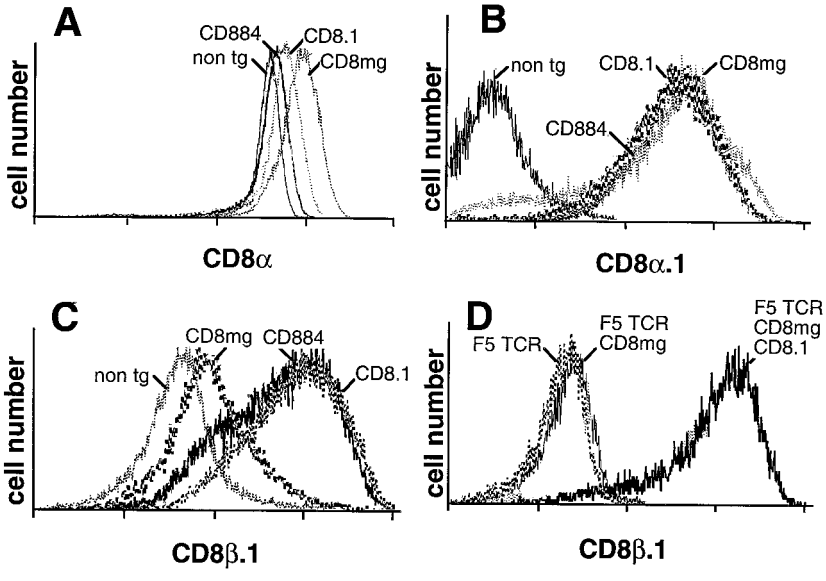

FIGURE 2. Expression of the CD8mg transgene on thymocytes. *A*, Levels of CD8α (endogenous and transgenic) on thymocytes of nontransgenic and CD8 transgenic mice. Levels of transgenic CD8α (CD8.1; *B*) and CD8β (CD8β.1; *C* and *D*) on thymocytes are shown. Total thymocytes from mice of the indicated genotypes were analyzed by flow cytometry as described in *Materials and Methods*. The *x*-axis is in arbitrary fluorescent units (4-decade log scale).

Table I. *Lymph node T cells from CD8 knockout mice mice expressing CD8 transgenes*[a]

	Percent Lymph Node T Cells			
	TCR^+ $CD4^+$	TCR^+ $CD4^-$	$CD4^+$: $CD4^-$ Ratio	*n*
$CD8\alpha^{+/-}$	42 ± 3.1	17 ± 2.2	2.5 ± 0.2	7
$CD8\alpha^{-/-}$	55 ± 5.6	1.7 ± 0.5	40 ± 21	16
$CD8\alpha^{+/-}$CD8mg transgenic	39 ± 4.5	18 ± 4.1	2.3 ± 0.7	12
$CD8\alpha^{-/-}$CD8mg transgenic	42 ± 1.6	15 ± 5.7	3.1 ± 0.9	4
$CD8\alpha^{-/-}$CD8.1 transgenic	49	20	2.5	1
$CD8\alpha^{-/-}$CD884 transgenic	48	27	1.8	2

[a] Lymph node T cells were stained with Abs against CD4, CD8.2 (endogenous CD8), and TCR as described in *Materials and Methods.* Average values are given with SDs in parentheses, and *n* is number of mice of each genotype analyzed.

crossed the CD8mg transgenic mice with Aβ knockout mice (32). Because Aβ mutant mice are deficient for class II MHC, the appearance of a large population of CD4 lineage T cells in these mice is likely to reflect the selection of T cells bearing class I-specific TCRs into the CD4 lineage. As previously shown (32, 35), the lymph nodes of class II-deficient mice contain a very small population of $\alpha\beta TCR^+CD4^+CD8^-$ cells, resulting in a CD4:CD8 ratio of 0.03 compared with ~2.0 in wild-type mice (Table II). Expression of the CD8.1 transgene in class II-deficient mice leads to significant increase in CD4 lineage lymph node T cells (CD4:CD8 ratio of 0.19) (22), whereas expression of the CD884 transgene results in an even further increase in lymph node CD4 lineage T cells (CD4:CD8 ratio of 0.6) (5). In contrast, in CD8mg transgenic, class II-deficient mice there is only a small increase in $\alpha\beta TCR^+CD4^+CD8.2^-$ lymph node cells (CD4:CD8 ratio of 0.05) over that seen in class II-deficient mice without a CD8 transgene. These results indicate that the CD8mg transgene is relatively ineffective at permitting the development of CD4 lineage T cells bearing class I-specific TCRs.

We also examined thymocytes of class II MHC-deficient CD8mg transgenic mice for the presence of mature CD4 lineage T cells. In the thymus, quantitation of the mature CD4 lineage T cells is complicated by the presence of $CD4^+CD8.2^{int}HSA^{int}$ cells that may represent developmental intermediates (36, 37) as well as a $CD4^+CD8.2^-TCR^{int}$ population that appears to represent a distinct T cell lineage (38–41). To exclude these populations, we first enriched for mature cells by depleting thymocytes of HSA-expressing cells. We then analyzed the remaining mature thymocytes for expression of CD4, CD8.2, and $\alpha\beta$TCR. When analyzed in this manner, mature thymocytes from class II-deficient mice display a CD4 to CD8 ratio of <0.1 (5, 22) (Table II), indicating that very few mature CD4 lineage thymocytes are present. Previous analysis indicates that expression of the CD8.1 transgene in class II-deficient mice leads to only a small increase in the CD4 to CD8 ratio of mature thymocytes, whereas expression of the CD884 transgene leads to a CD4 to CD8 ratio that approaches normal levels (0.53 compared with 3.3) (5, 22). Consistent with the analysis of lymph node cells, we found only a small increase in mature CD4 lineage thymocytes in class II-deficient mice bearing the CD8mg transgene. Together these data indicate that the CD8mg transgene is ineffective in permitting the development of CD4 lineage T cells bearing class I-specific TCRs.

We also examined the ability of the CD8mg transgene to permit the development of class I-specific CD4 lineage T cells using a rearranged TCR transgene encoding the class I-specific F5 TCR (31). Mice expressing the F5 TCR transgene have increased numbers of mature CD8 lineage T cells and decreased numbers of CD4 lineage T cells compared with nontransgenic mice, reflecting the selection of the majority of thymocytes on class I MHC (31) (Fig. 4). As previously shown, coexpression of the CD8.1 transgene with the F5 TCR transgene leads to increased numbers of CD4 lineage T cells bearing the F5 TCR. These CD4 lineage cells are selected using the F5 TCR rather than endogenous TCRs, as indicated by the reduced frequency of endogenous Vα2 on these cells (21) as well as the fact that the number of CD4 lineage T cells in CD8.1 transgenic, F5 TCR transgenic mice remains high in a recombinase-activating gene-2 mutant background (24). Coexpression of the CD884 transgene leads to an even further increase in CD4 lineage T cells as well as a decrease in CD8 lineage T cells (5) (Fig. 4). In contrast, coexpression of the CD8mg transgene with the F5 TCR transgene leads to a very modest increase in CD4 lineage thymocytes, and no detectable increase in CD4 lineage lymph node cells compared with those in F5 TCR transgene only mice. These data are in line with the results from class II-deficient mice and indicate that the CD8mg transgene does not permit the development of CD4 lineage thymocytes bearing class I-specific TCRs.

Table II. *Lymph node T cells and thymocytes from CD8mg transgenic, class II MHC-deficient mice*[a]

		Percent Lymph Node T Cells		Percent Total Thymocytes		Percent HSA- Thymocytes			
Genotype	No. of Cells/ Thymus ($\times 10^6$)	$CD4^+8.2^-$ TCR^+	$CD4^-8.2^+$ TCR^+	TCR^+ $CD4^+8.2^-$	TCR^+ $CD4^-8.2^+$	TCR^+ $CD4^+8.2^-$	TCR^+ $CD4^-8.2^-$	4:8 Ratio	*n*
Nontransgenic		35 ± 2.0	21 ± 1.1					1.7	8
CD8mg transgenic		40 ± 1.5	20 ± 0.6					2.0	9
Class II^-		1.6 ± 0.1	50 ± 1.8					0.033	10
Class II^-/CD8mg transgenic		2.8 ± 0.2	50 ± 1.2					0.046	7
Nontransgenic	224 ± 16			7.0 ± 0.2	5.2 ± 0.3	66 ± 0.5	20 ± 1.0	3.3	8
CD8mg transgenic	174 ± 11			8.0 ± 0.5	6.2 ± 0.5	61 ± 0.9	25 ± 0.9	2.4	9
Class II^-	197 ± 15			1.1 ± 0.2	6.8 ± 0.3	5.0 ± 0.2	71 ± 2.0	0.068	10
Class II^-/CD8mg transgenic	209 ± 18			1.6 ± 0.1	7.0 ± 0.6	7.1 ± 0.4	74 ± 1.5	0.096	7

[a] Total thymocytes, mature thymocytes (HSA depleted), or lymph node T cells were stained with Abs against TCR, CD4, and CD8.2 (endogenous CD8) as described in *Materials and Methods.* Average values and SDs are given, and *n* is the number of mice of each genotype analyzed.

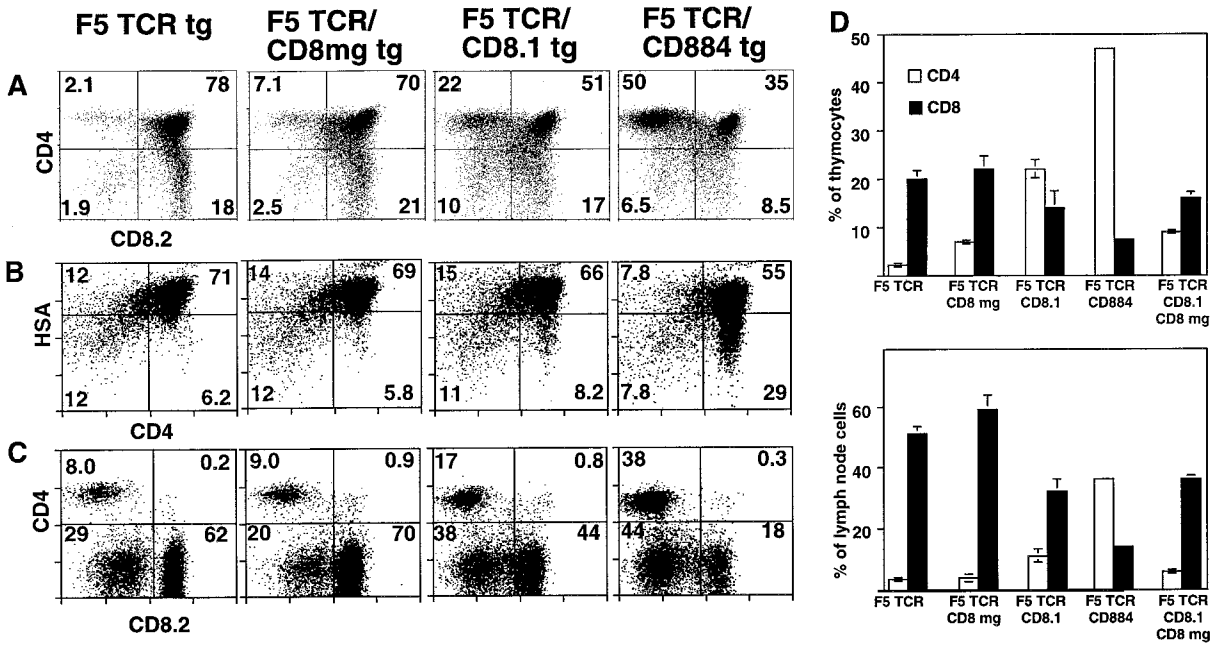

FIGURE 4. Thymic subsets and lymph node cells in mice coexpressing the F5 TCR and CD8 transgenes. Representative flow cytometric analysis of thymocytes and lymph node cells from F5 TCR transgenic mice crossed with CD8 transgenic mice are shown. *A*, CD4 and endogenous CD8 expression on thymocytes. *B*, Expression of HSA and CD4 on thymocytes. *C*, Expression of CD4 and endogenous CD8 in lymph node cells. Thymocytes and lymph node cells were analyzed with fluorescent Abs as described in *Materials and Methods*. The anti-CD8.2 Ab recognizes endogenous, but not transgenic, CD8. The numbers inside the quadrants represent the percentage of cells in each population. The *x*- and *y*-axis are in arbitrary fluorescent units (4-decade log scale). *D*, Compiled flow cytometric data for F5 TCR, F5 TCR/CD8mg transgenic, F5 TCR/CD8.1 transgenic, F5 TCR/CD884 transgenic, and F5 TCR/CD8.1/CD8mg transgenic mice. For thymocytes, the percentage of $CD4^+CD8.2^-$ or $CD4^-CD8.2^+$ thymocytes is displayed. For lymph node cells, the percentage of $V\beta11^+CD4^+CD8.2^-$ or $V\beta11^+CD4^-CD8.2^+$ cells is displayed. Bars represent the mean, and error bars indicate the SD. The numbers of mice of each genotype analyzed were: F5 TCR, 14; F5 TCR/CD8mg, 6; F5 TCR/CD8.1 transgenic, 3; F5 TCR/CD884 transgenic, 2; and F5 TCR/CD8mg/CD8.1, 3.

The CD8mg transgene acts dominantly to prevent the development of class I-specific CD4 lineage T cells

The absence of class I-specific CD4 lineage T cells in CD8mg transgenic mice could be due to the presence of transgene-encoded CD8α′. However, CD8mg transgenic mice express low and variable levels of transgenic CD8β compared with the CD8.1 transgenic mice (Fig. 2, *C* and *D*). Because CD8β is known to participate in class I recognition during thymic development (42–44), we considered the possibility that the failure to develop class I-specific CD4 lineage T cells in these mice could be due to the absence of sufficient levels of CD8β expression. To distinguish between these possibilities, we generated mice bearing the F5 TCR transgene, the CD8mg transgene, and the CD8.1 transgene. If CD8α′ is preventing the development of CD4 lineage cells, we would expect that F5 TCR mice bearing both CD8 transgenes would have reduced numbers of CD4 lineage T cells compared with F5 TCR transgenic mice expressing only the CD8.1 transgene. On the other hand, if the lack of CD8β expression from the CD8mg transgene is responsible, F5 TCR transgenic mice expressing both CD8 transgenes should resemble F5 TCR/CD8,1 transgenic mice. The results of flow cytometric analysis of thymocytes and lymph node cells from F5 TCR/CD8mg/CD8.1 transgenic mice are presented in Figs. 2*D* and 4*D*. As expected, expression of transgene-encoded CD8β is high in F5 TCR/CD8mg/CD8.1 transgenic mice due to the contribution of CD8β expression from the CD8.1 transgene (Fig. 2*D*). Interestingly, the number of mature CD4 T cells in both the thymus and the lymph node is reduced 2-fold in triple transgenic mice compared with that in mice expressing only the F5 TCR and CD8.1 transgenes. The observation that the CD8mg transgene is dominant over the CD8.1 transgene in reducing the development of class I-specific CD4 lineage T cells implies that the difference between the CD8mg and the CD8.1 transgene is due to the presence of CD8α′ in the former, rather than the lack of transgenic CD8β expression.

Discussion

Although both CD4 and CD8 interact with Lck via their cytoplasmic domains, CD4 is more effective than CD8 at recruiting Lck. This difference between CD4 and CD8 may be accentuated by the presence of CD8α′, an alternatively spliced form of CD8 that cannot interact with Lck and that constitutes half the surface CD8 on murine thymocytes. Here we describe a constitutively expressed CD8mg transgene (CD8mg) that encodes both CD8α and α′, and we compare this new CD8 transgene to a previously described CD8 transgene that does not encode CD8α′. We find that while CD8 from CD8α only transgenic thymocytes has abnormally high Lck activity, CD8 from CD8mg transgenic thymocytes has levels of associated Lck activity comparable to those of endogenous CD8. Both CD8 transgenes can restore CD8 T cell development in CD8α mutant mice. However, while the CD8α-only transgene can permit the development of mismatched CD4 lineage T cells bearing class I-specific TCRs, the CD8mg transgene is ineffective at promoting the development of such mismatched CD4 T cells. Finally, we show that the CD8mg transgene inhibits the production of class I-specific, CD4 lineage T cells when coexpressed with the CD8α only transgene. These results support the hypothesis that CD8α′ acts to reduce Lck recruitment upon class I MHC recognition in the thymus, and that reduced Lck recruitment may, in turn, favor the CD8 lineage over the CD4 lineage.

In light of our results, it is worth re-examining the interpretation of earlier experiments using constitutive CD8 transgenic mice. In those experiments, the appearance of mismatched CD4 T cells bearing class I-specific TCRs was generally interpreted in terms of a stochastic/selection model for CD4 vs CD8 lineage commitment (21–25). In this model, thymocytes bearing class I-specific TCRs randomly choose either the CD4 or CD8 lineage. Thymocytes that choose the CD4 lineage and turn off CD8 expression would normally die because they can no longer bind class I MHC, but are rescued by the presence of the constitutive CD8 transgene. However, in light of our results, the appearance of CD4 T cells bearing class I-specific TCRs could be attributed in part to the abnormally high Lck activity associated with transgene-encoded CD8 that does not contain CD8α'.

To what extent can the effect of constitutive CD8 or CD4 expression be attributed to the rescue of lineage-committed thymocytes with mismatches between TCR specificity and endogenous coreceptor expression? The observation that CD8mg transgenic mice do show a slight increase in CD4 lineage T cells bearing class I-specific TCRs over that seen without a CD8 transgene suggests that constitutive CD8 expression per se may also allow some mismatched T cells to develop. In addition, constitutive expression of CD4 leads to the appearance of a small number of mismatched thymocytes (CD8 lineage bearing class II-specific TCRs) (26–28). Thus, we favor a model in which the initial encounter with MHC imposes a bias on lineage commitment, such that the majority of thymocytes that recognize class I MHC develop along the CD8 pathway, and the majority of thymocytes that recognize class II MHC develop along the CD4 pathway. The continued requirement for MHC recognition following CD4 or CD8 down-regulation then serves as a reinforcement step to ensure that no mismatched T cells emerge.

Our data indicate that in mouse thymocytes, CD8α' serves to reduce Lck signaling and favor the CD8 cell fate. Interestingly, the human CD8α gene also undergoes alternative splicing to produce a truncated form, but in contrast to mouse CD8α', which lacks the cytoplasmic domain, alternatively spliced human CD8 encodes a secreted form that lacks the transmembrane domain (45, 46). Human CD8 can be found in association with CD1 (47, 48). In addition, multiple forms of CD8β have been described in the human (49). It is possible that some of these modifications of CD8 serve a function equivalent to that proposed for mouse CD8α'. Alternative forms of CD8 have not been detected in rat thymocytes (50). Interestingly, rat thymocytes also differ from mouse thymocytes in that strong TCR signals induce CD8 cell development, and it has been suggested that the lack of CD8α' in the rat may underlie this difference in lineage commitment between the mouse and the rat (50). These observations suggest that alternative structures of CD8 are rapidly evolving, and that different species have found different solutions to the problem of how to discriminate between class I and class II MHC recognition during thymic development.

How might a quantitative difference in Lck recognition influence the CD4 vs CD8 lineage decision? Lck is thought to phosphorylate the ITAMs in the CD3 and ζ-chains of the TCR complex, and the Lck recruited to the TCR complex by CD4 or CD8 may thus contribute to ITAM phosphorylation (3). Greater Lck recruitment by CD4 engagement might lead to more extensive phosphorylation of ITAMs, and this may, in turn, favor the CD4 fate over the CD8 fate. It is also possible that the Lck recruited by CD4 or CD8 acts on other substrates (51–53). Finally, in addition to its catalytic function, Lck may serve an adapter function, increasing the association between coreceptor and the TCR complex and thereby enhancing the adhesive function of CD4 and CD8 (54, 55). According to this idea, CD4 might make a greater adhesive contribution than CD8, since it would be expected to spend more time associated with the TCR complex by virtue of its stronger association with Lck. This improved binding to MHC could, in turn, influence the CD4 vs CD8 lineage decision by increasing the overall strength of the TCR signal.

In addition to the effects of MHC recognition, Notch activity has been shown to influence CD4 vs CD8 lineage commitment (56). An activated form of Notch causes thymocytes bearing class II-specific TCRs that would normally develop as CD4 lineage T cells to choose the CD8 lineage instead. Although alternative interpretations of the data are possible (57), we favor the view that Notch activity is normally regulated by MHC class I or II recognition. Thus, it is tempting to speculate that class I MHC recognition would lead to weak Lck activation, which would, in turn, up-regulate Notch signaling, thus favoring the CD8 cell fate. Class II MHC recognition would lead to strong Lck activation, which would, in turn, down-regulate Notch signaling, thus favoring the CD4 cell fate. A more complete understanding of the function and regulation of both Lck and Notch during thymic development is essential to dissecting the molecular events underlying the CD4 vs CD8 lineage decision.

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