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Coreceptor Signal Strength Regulates Positive Selection but Does Not Determine CD4/CD8 Lineage Choice in a Physiologic In Vivo Model

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TCR signals drive thymocyte development, but it remains controversial what impact, if any, the intensity of those signals have on T cell differentiation in the thymus. In this study, we assess the impact of CD8 coreceptor signal strength on positive selection and CD4/CD8 lineage choice using novel gene knockin mice in which the endogenous CD8α gene has been re-engineered to encode the stronger signaling cytoplasmic tail of CD8, with the re-engineered CD8α gene referred to as CD8.4. We found that stronger signaling CD8.4 coreceptors specifically improved the efficiency of CD8-dependent positive selection and quantitatively increased the number of MHC class I (MHC-I)-specific thymocytes signaled to differentiate into CD8+ T cells, even for thymocytes expressing a single, transgenic TCR. Importantly, however, stronger signaling CD8.4 coreceptors did not alter the CD8 lineage choice of any MHC-I-specific thymocytes, even MHC-I-specific thymocytes expressing the high-affinity F5 transgenic TCR. This study documents in a physiologic in vivo model that coreceptor signal strength alters TCR-signaling thresholds for positive selection and so is a major determinant of the CD4:CD8 ratio, but it does not influence CD4/CD8 lineage choice.

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in which to examine coreceptor signal strength without the potential artifacts induced by aberrant CD8 coreceptor expression that plagued CD8 coreceptor transgenic mice. Indeed, the results obtained with CD8.4 knockin mice are remarkably unequivocal: increased CD8 coreceptor signal strength significantly increased the efficiency of MHC-I-specific positive selection and the number of CD8+ T cells generated in the thymus, but it had no effect on CD4/CD8 lineage choice. Thus, coreceptor signaling intensity affects TCR signaling thresholds for positive selection and so is a key determinant of the CD4/CD8 ratio, but it does not influence CD4/CD8 lineage choice.

Materials and Methods

**Mice**

C57BL/6 (B6) were purchased from The Jackson Laboratory; F5 TCR transgenic mice (27), CD8α+ mice (28), CD8β+ mice (29), MHC-II+ mice (30), and Ella-Cre transgenic mice (31) were bred in our own animal colony. All mice used in this study were cared for in accordance with National Institutes of Health guidelines.

**Generation of CD8.4 knockin mice**

The CD8.4 knockin locus was constructed as depicted in Fig. 1. A targeting construct containing 5′ and 3′ flanking regions homologous to the endogenous CD8α locus was constructed in a pKO917 plasmid (Stratagene) backbone that contains a 2-kb thymidine kinase (TK) selection cassette derived from pKO-Select-TK (Stratagene) and a 1.65 kb floxed neomycin (NEO) selection cassette from the pGKneoLoxP plasmid (32). A 3.9-kb region of the CD8α locus that includes exons 1–3 was PCR amplified to generate the 5′ flank. This fragment was digested with BglII and XhoI and ligated into the corresponding sites of pKO917, between the TK and NEO selection cassettes. One part of the 3′ flank was generated by PCR amplifying CD4 cytoplasmic tail sequences from a plasmid containing a murine CD4 cDNA using a hybrid forward primer containing bases from CD8α exon 3 (RR8-1), TCR-Vα1 (blunted with Klenow) and HindIII and ligated into the Smal and HindIII sites of the plasmid pBluescript II (Stratagene). The remainder of the 3′ flank was amplified from CD8α genomic DNA (using primers with the appropriate restriction sites and inserted into the Cil and SalI sites of pBluescript II containing the CD8α/4 hybrid sequence). A SacII-HindII fragment of the resulting plasmid comprised the full 3′ flank and was inserted into the corresponding sites of pKO917 already containing the 5′ flank. All PCR were performed with 129 R1 embryonic stem (ES) cell DNA unless otherwise noted.

The resulting targeting construct encodes a chimeric gene with CD8α exons 1–3 provided by the 5′ flank, and a chimeric exon 4 provided by the 3′ flank. The amino acid sequence at the modified junction is as follows: IITLICYH/R CRHQQ.... and bold sequences are from CD4: H57-597; anti-CD4 (GK1.5) and anti-CD8α (53-7.3), TCRβ (H57-597), TCR-Var2 (B20.1), TCR-Var3 (RR3-16), TCR-Var8 (B21.14), TCR Var11 (RR5-1), CD4 (GK1.5), and Qa2 (1-1-2) were obtained from BD Pharmingen; mAbs specific for CD8α (CT-CD8α) were obtained from Caltag Laboratories; and mAbs specific for granzyme B (clone 16G6) and CD154 (CD40L) were obtained from eBioscience.

**Cell lysis and immunoprecipitations**

DP thymocytes were lysed for 30 min at 4°C in buffer containing 1% Nonidet P-40 (Pierce), 10 mM Tris-HCl (pH 7.2), 140 mM NaCl, 2 mM EDTA, 1 mM Na3VO4, 1 mM orthovanadate, and protease inhibitors. Insoluble material was removed by centrifugation for 10 min at 4°C. Lysates were incubated overnight with anti-CD8α mAbs coupled to protein G-Sepharose beads (Amersham Biosciences). Proteins were resolved by reducing SDS-PAGE and transferred to polyvinylidene difluoride membranes. Blots containing anti-CD8 immunoprecipitates and total cell lysates were incubated with anti-Lck (3A5 mAb; Santa Cruz Biotechnology) followed by HRP-conjugated protein A, with reactivity revealed by ECL (Pierce).

**Intracellular calcium mobilization**

Thymocytes were loaded with the calcium dye Indo-1 (Molecular Probes; 1.8 nM) at 37°C for 30 min. Cells were stained for 40 min on ice with the indicated concentrations of the following biotinylated mAbs: anti-TCRβ (H57-597); anti-CD4 (GK1.5) and anti-CD8α (53-6.72). DP thymocytes were detected using anti-CD4 and anti-CD8 mAbs that do not cross-react with the biotinylated Abs. For stimulation, cells were warmed for 5 min before being applied to the flow cytometer. Calcium mobilization was analyzed following avidin cross-linking (4 μg/ml) and acquisition was recorded for 4 min. Calcium concentrations were calculated using FlowJo software (Tree Star).

**BrdU staining**

For intravenous BrdU labeling, mice received daily i.p. injections of 100 μl of a 10 mg/ml solution of BrdU in PBS in addition to having BrdU in the drinking water at a final concentration of 0.8 mg/ml. Single-cell suspensions of thymocytes were obtained on time point and stained for TCRβ, CD4, and CD8α. Following surface staining, cells were fixed, stained for intracellular BrdU by using a FITC-BrdU Flow kit (BD Pharmingen), and analyzed by flow cytometry.

**Results**

CD8.4 mice expressing endogenously encoded chimeric CD8α/CD4 coreceptor proteins

To increase the signaling intensity of endogenously encoded CD8 coreceptors, we re-engineered the endogenous CD8α gene locus in two ways: we replaced the exon encoding the CD8α cytoplasmic tail with a chimeric exon containing the CD4 cytoplasmic tail, and we removed the alternative splice site in the CD8α locus that gives rise to the differentially tailless CD8α′ proteins (33). The molecular approach we used is schematized in Fig. 1A. Our targeting construct consisted of five components: a TK selection cassette, exons 1–3 of CD8α encoding the CD8α extracellular domain, a floxed NEO selection cassette, chimeric exon 4 encoding the CD8α transmembrane domain ligated to the CD4 cytoplasmic tail, and a truncated version of noncoding CD8α exon 5. The targeting construct was electroporated into ES cells so that homologous recombination with the endogenous CD8α locus would generate the CD8α+ locus (Fig. 1A), and an ES cell clone containing the CD8α+ locus was used to generate CD8α+ knockin mice. CD8α+ knockin male mice were bred to Ella-Cre transgenic female mice whose offspring deleted the floxed NEO selection cassette in their germline (31), giving rise to mice with the CD8.4 (Fig. 1A). We then bred the CD8.4 gene into CD8α-exon 5 mice to generate CD8.4/CD8α heterozygous mice expressing only chimeric CD8.4 coreceptor proteins, and these mice subsequently gave rise to CD8.4/CD8α homozygous mice. Both heterozygous and homozygous CD8.4 mice were used in this study.

Southern blotting of ES cell DNA confirmed that the selected ES cell clone contained the targeted CD8α′ allele (Fig. 1B, left panel), and PCR analyses of tail DNA from CD8.4 mice confirmed the presence of the CD8.4 allele (Fig. 1B, right panels). Only tail
DNA from CD8.4 mice amplified a PCR product from primers designated “3” and “4” (see Fig. 1A), which were specific for sequences in the targeting construct and for downstream sequences in the CD8α gene locus (Fig. 1B, top right panel). Tail DNA from both CD8.4 and WT CD8α mice amplified PCR products from primers designated “1” and “2” (see Fig. 1A) that were specific for sequences present in the CD8α gene and that flanked the NEO cassette in the targeting construct (Fig. 1B, bottom right panel). Notably, the PCR product obtained with primers 1 and 2 from CD8.4 tail DNA was ~50 bp larger than that from CD8α tail DNA, the size difference expected from a residual loxP site remaining in the CD8α locus after Cre-mediated deletion of the NEO cassette (which was ~2000 bp in length) from the CD8α locus (Fig. 1B, bottom right panel).

Biochemical characterization of proteins encoded by the CD8.4 gene locus confirmed that they had the structural characteristics...
proteins in DP thymocytes. CD8 coreceptors on DP thymocytes from B6 and CD8.4 mice were solubilized in Nonidet P-40 detergent, immunoprecipitated (Immpt) with anti-CD8α mAb, resolved by reducing SDS-PAGE, and blotted for Lck. The relative intensities of the Lck bands were determined by densitometry and compared with B6, which was set at 1.0. B, CD8.4 coreceptors increase in vivo signaling intensity in DP thymocytes. DP thymocytes from B6 and CD8.4 mice were analyzed by multicolor flow cytometry. Single parameter histograms of CD5 expression on electronically gated CD4+8+ DP thymocytes from B6 (dashed line) and CD8.4 (solid line) mice are shown. CD5 surface expression on both cell populations was quantified into linear fluorescence units, with CD5 expression on B6 DP thymocytes set equal to 100. C, CD8.4 coreceptors increase in vitro signaling intensity in DP thymocytes. Thymocytes from WT and CD8.4 mice were loaded with the calcium-sensitive dye Indo-1 (1.8 μM) and coated with biotinylated mAbs specific for TCRβ (5 μg/ml), CD4 (1 μg/ml), or CD8α (1 μg/ml). Signaling was induced by addition of streptavidin (1 μg) (indicated by the arrow) that cross-linked the stimulatory biotinylated mAbs. DP thymocytes were identified using anti-CD4 and anti-CD8 mAbs which do not cross-react with the biotinylated Abs used for stimulation. Intracellular calcium concentrations were determined by the ratio of Indo-1 fluorescence at 405 vs 510 nm.

FIGURE 2. Increased Lck binding and increased signaling intensity by CD8.4 coreceptors. A, More Lck is associated with CD8.4 than WT CD8α proteins in DP thymocytes. CD8.4 coreceptors on DP thymocytes from B6 and CD8.4 mice were solubilized in Nonidet P-40 detergent, immunoprecipitated (Immpt) with anti-CD8α mAb, resolved by reducing SDS-PAGE, and blotted for Lck. The relative intensities of the Lck bands were determined by densitometry and compared with B6, which was set at 1.0. B, CD8.4 coreceptors increase in vivo signaling intensity in DP thymocytes. DP thymocytes from B6 and CD8.4 mice were analyzed by multicolor flow cytometry. Single parameter histograms of CD5 expression on electronically gated CD4+8+ DP thymocytes from B6 (dashed line) and CD8.4 (solid line) mice are shown. CD5 surface expression on both cell populations was quantified into linear fluorescence units, with CD5 expression on B6 DP thymocytes set equal to 100. C, CD8.4 coreceptors increase in vitro signaling intensity in DP thymocytes. Thymocytes from WT and CD8.4 mice were loaded with the calcium-sensitive dye Indo-1 (1.8 μM) and coated with biotinylated mAbs specific for TCRβ (5 μg/ml), CD4 (1 μg/ml), or CD8α (1 μg/ml). Signaling was induced by addition of streptavidin (1 μg) (indicated by the arrow) that cross-linked the stimulatory biotinylated mAbs. DP thymocytes were identified using anti-CD4 and anti-CD8 mAbs which do not cross-react with the biotinylated Abs used for stimulation. Intracellular calcium concentrations were determined by the ratio of Indo-1 fluorescence at 405 vs 510 nm.

Quantitative effect of enhanced CD8 coreceptor signaling on positive selection of CD8+ T cells
To assess the impact of enhanced CD8.4 coreceptor signaling on positive selection of CD4+ and CD8+ T cells, we compared thymocytes and peripheral T cells from CD8.4 mice with WT littermate mice,
FIGURE 3. The CD4 tail on CD8.4 coreceptor molecules specifically increases CD8+ T cell number and reverses the CD4:CD8 ratio. A, Thymocytes from B6, WT littermate (WT LM), and CD8.4 mice were analyzed by multicolor flow cytometry. CD4 vs CD8 contour plots on various cell populations from these mice are shown (left panels). Within the contour plots, the numbers inside each box indicates the percentage of cells falling within that box. Numbers below the contour plots indicate the total number of cells obtained. The CD4:CD8 ratios (±SEM) in WT littermate (LM) and CD8.4 mice are shown (right panel). A total of seven mice from each strain was analyzed. SEM are indicated by the error bars. Values of $p$ were obtained by the two-tailed Student $t$ test. B, Effect of CD8.4 coreceptors on T cell subpopulations. The frequency (±SEM) and absolute number (±SEM) of CD4+ and CD8+ T cells in various lymphoid populations from WT LM (□) and CD8.4 (■) mice are shown. A total of seven mice from each strain were analyzed.
both of which contained one CD8α− null allele and so were heterozygous for either the CD8.4 or WT CD8α gene (Fig. 3A). Thymus cellularity and thymocyte profiles were similar in these two strains, except that CD8.4 mice contained a higher frequency of SP thymocytes (both CD4+ and CD8+) than WT littermate mice (Fig. 3A). Because positively selected DP thymocytes first appear as CD4+8low transitional cells whether they are differentiating into CD4+ or CD8+ T cells (37, 38), some thymocytes that fall into the CD4SP gate may actually be CD8 lineage cells in the process of differentiating into CD8+ T cells. Consequently, to accurately determine the frequencies of committed CD4 lineage and CD8 lineage thymocytes in each strain, we focused on Qa2+ thymocytes as these are terminally differentiated and functionally mature T cells (39) (Fig. 3A). Unlike WT littermate mice that contained a greater number of CD4SP than CD8SP cells among Qa2+ thymocytes, CD8.4 mice contained the reverse (i.e., a greater number of CD8SP than CD4SP cells among Qa2+ thymocytes).
We thought it likely that the increased number of mature CD8 T cells in CD8.4 mice (Fig. 3B) was due to increased positive selection of CD8 T cells while only 1% of B6 DP thymocytes were positively selected to differentiate into CD4+ T cells. Assess-ment of peripheral T cells in the spleen and lymph node (LN) of CD8.4 mice revealed precisely the same finding, namely that CD8.4 mice had more CD8+ T cells in the periphery and therefore also displayed a CD4:CD8 ratio of <1 (Fig. 3A).

Reversal of the normal CD4:CD8 ratio in every lymphoid compartment in CD8.4 mice might have resulted from reduced CD4+ T cell numbers, increased CD8+ T cell numbers, or both. Assessment of CD4+ and CD8+ T cell frequencies in CD8.4 mice revealed that, compared with WT littermates, CD4+ T cell frequencies were reduced and CD8+ T cell frequencies were increased (Fig. 3B, right). However, assessment of absolute T cell numbers revealed that CD8.4 coreceptors had no effect on CD4+ T cell numbers as absolute numbers of CD4+ T cells were equivalent in all lymphoid compartments in CD8.4 and WT littermate mice (Fig. 3B, left). In contrast, CD8.4 coreceptors specifically increased absolute numbers of CD8+ T cells in every lymphoid compartment of CD8.4 mice (Fig. 3B, left).

We thought it likely that the increased number of mature CD8+ T cells in CD8.4 mice was due to increased positive selection of CD8 lineage thymocytes by stronger signaling CD8+ coreceptors. Consequently, we wished to quantitate and compare positive selection in CD8.4 and B6 mice. We injected young adult mice daily with BrdU and also put BrdU in their drinking water to continuously label proliferating pre-DP thymocytes so that we could track their subsequent differentiation into DP and SP thymocytes (Fig. 4). BrdU+ cells appeared as DP thymocytes with equal rapidity in CD8.4 and B6 mice (Fig. 4A, top panel). Positive selection induced BrdU+ DP thymocytes to convert into BrdU+ CD4SP and BrdU+ CD8SP thymocytes, with their conversion rates indicated by the slopes of their linear regression lines (Fig. 4A, middle and bottom panels) (40). Note that CD4SP and CD8SP thymocytes in this experiment were also gated to be TCRβhigh cells. Although BrdU+ DP thymocytes converted into BrdU+ CD4SP (TCRβhigh) thymocytes at equivalent rates in CD8.4 and B6 mice (Fig. 4A, middle panel), BrdU+ DP thymocytes converted into BrdU+ CD8SP (TCRβhigh) thymocytes at significantly different rates in CD8.4 and B6 mice (Fig. 4A, bottom panel). As indicated by the slopes of their linear regression lines, the conversion rate of BrdU+ DP thymocytes into BrdU+ CD8SP thymocytes was 2.5-fold greater in CD8.4 mice than in B6 mice (Fig. 4A, bottom panel). Thus, increased CD8+ coreceptor signaling by the CD4 cytotoxic tail specifically increased positive selection of CD8+ T cells without affecting positive selection of CD4+ T cells.

The efficiency of positive selection is defined as the relative number of DP thymocytes signaled to undergo further differentiation and can be quantitated in BrdU-labeling experiments as conversion efficiency (40). In B6 mice, the conversion efficiency of DP thymocytes into CD4SP and CD8SP thymocytes was 3 and 1%, respectively, indicating that 3% of B6 DP thymocytes were positively selected to differentiate into CD4+ T cells while only 1% of B6 DP thymocytes were positively selected to differentiate into CD8+ T cells (Fig. 4B). However, in CD8.4 mice, the conversion efficiency of DP thymocytes into CD4SP and CD8SP thymocytes was ~3% for each T cell subset (Fig. 4B), revealing that increased CD8+ coreceptor signaling by the CD4 cytotoxic tail quantitatively increased the efficiency of CD8+ T cell positive selection to the point that it was equivalent to that of CD4+ T cell positive selection.

**Cellular function of CD8.4+ T cells**

CD8+ T cells function as T-killer (Tk) cells and CD4+ T cells function as Th cells in normal B6 mice. To determine whether stronger signaling CD8.4 coreceptors altered these cellular functions in CD8.4 mice, we measured CD8 Tk function by assessing intracellular expression of granzyme B and we measured CD4 Th function by assessing TCR induced up-regulation of CD40L expression (Fig. 4C). We found that expression of stronger signaling CD8.4 coreceptors had no effect on cellular function, as CD8 T cells from CD8.4 and B6 mice stained identically for granzyme B (Fig. 4C), and anti-TCR stimulation failed to induce CD8 T cells in either CD8.4 or B6 mice to up-regulate CD40L (CD154) expression, even though both T cell populations were stimulated by anti-TCR to up-regulate CD69 (Fig. 4C).

**Does enhanced CD8+ coreceptor signaling affect lineage choice?**

Finally, we wished to determine whether strengthened signaling by endogenously encoded CD8.4 coreceptors affected the lineage choice of MHC-I-specific thymocytes. Notably, the strength of signal hypothesis of lineage choice originated to explain why a co-receptor transgene encoding a chimeric CD8/CD4 coreceptor protein identical with the CD8.4 protein appeared to direct the differentiation of MHC-I-specific thymocytes into CD4+ T cells (20). To determine the effect of endogenously encoded CD8.4 coreceptors on the lineage choice of MHC-I-specific thymocytes, we bred the CD8.4 gene locus into Aβ1–7 mice deficient in MHC-II...
FIGURE 6. Effect of stronger signaling CD8.4 coreceptors on positive selection and lineage choice of thymocytes selected by transgenic F5 TCR. A, The F5 TCR transgene was bred into RAG<sup>0</sup> mice expressing either CD8<sub>α</sub> or CD8.4 coreceptors to generate F5(RAG<sup>0</sup>)CD8<sub>α</sub> and F5(RAG<sup>0</sup>)CD8.4 mice. Thymocytes (left panels) and lymph node cells (right panels) from these mice were analyzed by multicolor flow cytometry and analyzed for expression of CD4 vs CD8<sub>α</sub>. The numbers inside each box indicate the percentage of cells within that box. The numbers under each strain name indicate the total numbers of cell obtained. B, The F5 TCR transgene (TCR-Vα4Vβ11) was bred into RAG<sup>0</sup>/H<sub>11001</sub> mice expressing either CD8<sub>α</sub> or CD8.4 coreceptors to generate F5 CD8<sub>α</sub> and F5 CD8.4 RAG<sup>0</sup>/H<sub>11001</sub> mice. Thymocytes (left panels) and lymph node cells (right panels) from these mice were subjected to multicolor flow cytometry and analyzed for expression of CD4, CD8<sub>α</sub>, TCR-Vβ11, and TCR-Vα2<sup>3</sup>Vβ11. Note that mAb to TCR-Vβ11 detects the transgenic F5 TCR β-chain, but that the mixture of mAbs to
expression (MHC-II), because in these mice, only MHC-I-specific thymocytes are signaled to undergo positive selection and to differentiate into mature T cells. As expected, LN T cells from MHC-II mice expressing WT CD8α coreceptors were almost exclusively CD8+, with few CD4+ T cells (Fig. 5). But, in direct conflict with the strength-of-signaling hypothesis, LN T cells from MHC-II mice expressing CD8.4 coreceptors were also almost exclusively CD8+, with few CD4+ T cells (Fig. 5). In fact, CD8.4 coreceptors resulted in greater absolute numbers of MHC-I-specific CD8+ T cells than did WT CD8 coreceptors (Fig. 5, bottom panel). The few CD4+ T cells found in both CD8.4 and WT littermate MHC-II mice were presumably NKT cells with specificity for minor MHC-I determinants (41).

To assess the effect of CD8.4 coreceptors on lineage choice by thymocytes expressing an MHC-I-specific transgenic TCR, we introduced the F5 TCR transgene that encodes TCR-Vα4 chains for which there is no available mAb). Because positive selection is inherently inefficient, only a small minority of F5-expressing preselection DP thymocytes are signaled to undergo positive selection into intermediate thymocytes and to differentiate into CD8SP thymocytes. However, the number of F5-expressing DP thymocytes that can be signaled to undergo positive selection is influenced by the signaling intensity of CD8 coreceptors, with the effect that stronger signaling CD8.4 coreceptors increase the number of F5-expressing DP thymocytes that are signaled to undergo positive selection into intermediate thymocytes and to differentiate into CD8SP thymocytes.

FIGURE 7. Schematic representations of findings and the kinetic signaling model. Schematic representation that CD8.4 coreceptors improve the efficiency of positive selection of thymocytes expressing the F5 TCR transgene. All preselection DP thymocytes in F5(RAG) mice obligatorily express the MHC-I-specific transgenic F5 TCR. Because positive selection is inherently inefficient, only a small minority of F5-expressing preselection DP thymocytes are signaled to undergo positive selection into intermediate thymocytes and to differentiate into CD8SP thymocytes. However, the number of F5-expressing DP thymocytes that can be signaled to undergo positive selection is influenced by the signaling intensity of CD8 coreceptors, with the effect that stronger signaling CD8.4 coreceptors increase the number of F5-expressing DP thymocytes that are signaled to undergo positive selection into intermediate thymocytes and to differentiate into CD8SP thymocytes.
transgenic mice (Fig. 6C). These experiments revealed two important points. First, they confirmed that MHC-I-specific positive selection of DP into CD8SP thymocytes was 20-fold more efficient when DP thymocytes expressed the F5 TCR transgene than when they expressed a diversity of polyclonal TCR (compare Figs. 6C and 4B). Second, and more importantly, they revealed that positive selection of DP into CD8SP thymocytes signaled by F5 transgenic TCR was increased more than 2-fold by CD8.4 coreceptors compared with CD8α coreceptors (Fig. 6C). Remarkably, >60% of F5 DP thymocytes were positively selected in CD8.4 mice to differentiate into CD8+ T cells (Fig. 6C). Thus, stronger signaling CD8.4 coreceptors increased the efficiency of MHC-I-specific T cell positive selection even for DP thymocytes expressing a single TCR specificity.

We conclude that stronger signaling CD8.4 coreceptors do not effect CD8 lineage choice by MHC-I-specific thymocytes, but they do increase the efficiency of CD8+ T cell positive selection and promote the generation of greater numbers of CD8+ T cells in the thymus, even for thymocytes expressing a single transgenic TCR specificity (schematized in Fig. 7).

Discussion

This study documents that the signaling intensity of CD8 coreceptors quantitatively regulates the efficiency of CD8-dependent positive selection and thereby determines the number of CD8+ T cells generated in the thymus, but it does not influence CD4/CD8 lineage fate. The present results were remarkably clear cut and definitive because they were obtained in a physiologic in vivo model using gene knockin mice whose endogenous CD8α gene locus had been re-engineered to encode CD8 coreceptors expressing the stronger signaling tail of CD4. The in vivo expression pattern of CD8.4 coreceptors was identical with that of WT CD8α coreceptors and therefore avoided the expression artifacts that plagued CD8 coreceptor transgenic mice. Thus, this study reveals that coreceptor signal strength during positive selection is a quantitative regulator of positive selection and a major determinant of the CD4:CD8 ratio, but it does not determine CD4/CD8 lineage choice.

Regulating positive selection and the CD4:CD8 ratio

In this study, replacement of the CD8 cytosolic tail with the stronger signaling tail of CD4 increased the number of CD8− T cells generated in the thymus as a direct result of more efficient positive selection. We measured positive selection efficiency in BrdU continuous labeling experiments and determined that the efficiency of MHC-I-specific CD8+ T cell positive selection was improved 3-fold (3 vs 1%) by stronger signaling CD8.4 coreceptors compared with WT CD8α coreceptors. In fact, CD8.4 coreceptors improved positive selection of MHC-I-specific CD8+ T cells to the point that it occurred with equal efficiency as MHC-II-specific CD4+ T cells. It is likely that stronger signaling CD8.4 coreceptors induced thymocytes with lower affinity TCR to undergo positive selection than were induced by WT CD8α coreceptors. However, selection of thymocytes with lower affinity TCR does not fully explain the effect of CD8.4 coreceptors we observed on positive selection, as CD8.4 coreceptors also increased positive selection of F5 TCR transgenic thymocytes expressing a single, fixed TCR specificity. That is, CD8.4 coreceptors increased positive selection of MHC-I-specific CD8+ T cells to the spectrum of affinities and also increased positive selection of F5 thymocytes expressing only a single TCR specificity. So how might stronger CD8.4 coreceptors increase CD8+ T cell positive selection when DP thymocytes all express a single TCR specificity?

Our results indicate that stronger signaling CD8.4 coreceptors reduce the TCR signaling threshold required for positive selection, with the result that more DP thymocytes receive positive selection signals—even when all thymocytes express a single transgenic TCR (schematized in Fig. 7). Our reasoning is
based on the fact that positive selection is inherently inefficient (40, 42–44), because DP thymocytes express relatively few surface TCR, and these TCR transduce intracellular signals only when they engage intrathymic ligands with the appropriate affinity, and because TCR signal transduction requires the protein tyrosine kinase Lck that is present in limiting amounts and is associated with CD4 and CD8 coreceptors, making TCR signal transduction in DP thymocytes highly coreceptor dependent (14, 15). As a consequence, TCR signaling is frequently unsuccessful in DP thymocytes even when their TCR have appropriate specificity for intrathymic ligands, with the result that relatively few eligible DP thymocytes are successfully signaled to undergo positive selection. Because Lck preferentially associates with the CD4 tail rather than the CD8 tail, DP thymocytes are more likely to be successfully signaled to undergo positive selection signal when their TCR are coengaged with coreceptors bearing the CD4 tail than the CD8 tail, regardless of the diversity or specificity of their TCR (schematized in Fig. 7).

This study fulfills predictions that we previously based on experiments in CD8 coreceptor transgenic mice (24), namely that the CD4:CD8 ratio might reflect the relative signaling intensities of CD4 and CD8 coreceptors. Importantly, this study significantly extends our original hypothesis by documenting that CD8 coreceptor signal strength influences the number of CD8^+ T cells generated even when all thymocytes express only a single TCR specificity. Moreover, this study documents the mechanism by which this occurs, namely by improving the positive selection efficiency of MHC-I-specific thymocytes and thereby increasing the number of CD8^+ T cells generated. Because CD4 molecules normally transduce stronger coreceptor signals than CD8 molecules, coreceptor signal strength is a major reason why mammalian immune systems normally contain more MHC-II-specific CD4^+ T cells than MHC-I-specific CD8^+ T cells. Of course, other factors such as TCR specificity, quantitative differences in MHC-I and MHC-II expression, and peptide diversity may also contribute to the CD4:CD8 ratio (45). But we think that the CD4:CD8 ratio primarily reflects the relative signaling intensities of CD4 vs CD8 coreceptors during positive selection.

Because both CD8.4 and CD4 coreceptors expressed the CD4 cytosolic tail and both signaled with equal intensity, why was the CD4:CD8 ratio in CD8.4 mice less than 1 rather than equal to 1? DP thymocytes express 4-fold more CD8 than CD4 surface molecules (14) so that the total amount of Lck associated with CD8.4 coreceptors is greater than the total amount of Lck associated with CD4 coreceptors in each CD8.4 DP thymocyte, providing an explanation for why the CD4:CD8 ratio is <1 in CD8.4 mice.

CD4/CD8 lineage choice

This study refutes two classical models of CD4/CD8 lineage choice, the instructional model and the strength of signal model. The instructional model postulates that coreceptor signals transduced by the CD4 cytosolic tail specifically direct thymocytes to differentiate into CD4^+ T cells, whereas coreceptor signals transduced by the CD8 cytosolic tail specifically direct thymocytes to differentiate into CD8^+ T cells (34). The present demonstration that MHC-I-specific thymocytes differentiated into CD8 lineage T cells regardless of the identity of the cytosolic tail on CD8 coreceptors directly refutes the instructional model, a conclusion also drawn by others (25). Similarly, the strength-of-signal model postulates that strong coreceptor signals (such as those transduced by the CD4 cytosolic tail) direct thymocytes to differentiate into CD4^+ T cells, whereas weak coreceptor signals (such as those transduced by the CD8^+ cytosolic tail) direct thymocytes to differentiate into CD8^+ T cells (19–23). In direct contradiction to the strength-of-signal model, this study documents that strong CD8.4 coreceptor signals transduced by the CD4 cytosolic tail do not determine CD4/CD8 lineage choice.

Because the strength-of-signal model provides a widely accepted explanation for CD4/CD8 lineage choice that remains highly popular (18), it is worth discussing two issues related to the strength of signal model in greater detail (1). First, the strength-of-signal model was originally proposed to explain observations in mice expressing a coreceptor transgene that encoded a chimeric CD8/CD4 molecule in which the cytosolic tail of CD8α was replaced by the stronger signaling tail of CD4 (20). In these mice, transgenic CD8/CD4 chimeric coreceptors appeared to redirect the differentiation of MHC-I-specific thymocytes, especially those expressing the F5 transgenic TCR, into CD4^+ T cells. Indeed, the fundamental observation on which the strength of signal model was based was that increased signaling intensity by transgenically encoded CD8/CD4 chimeric coreceptors redirected F5 TCR transgenic thymocytes to differentiate into CD4^+ T cells (20). However, in contrast to these previous observations in coreceptor transgenic mice, our study demonstrates that when the identical CD8/CD4 chimeric coreceptor proteins are encoded by endogenous CD8α genes rather than extrinsic transgenes, F5 TCR transgenic thymocytes differentiate exclusively into CD8^+ T cells. The critical difference between endogenously encoded CD8 coreceptors and transgenically encoded CD8 coreceptors is that TCR-mediated positive selection signals are unable to terminate coreceptor transgene expression, leading to persistent expression of transgenic CD8 coreceptor proteins throughout positive selection that abnormally prolongs CD8-dependent TCR signaling (discussed in more detail below) (2). Second, the strength of signal model requires that strong signals transduced by the CD4 tail direct thymocytes to differentiate into CD4^+ T cells, regardless of the MHC specificity of their TCR. Conflicting with this requirement, CD8.4 coreceptors—which signaled via the CD4 tail and which signaled with the same intensity as CD4 coreceptors—failed to redirect MHC-I-specific thymocytes to differentiate into CD4^+ T cells. Thus, this study directly contradicts the original observation that prompted formulation of the strength-of-signal model and directly contradicts a necessary requirement of the strength-of-signal model.

The strength-of-signal model has recently been reformulated as a "duration-of-signal" model (23) in which signal strength is proposed to affect both the intensity and duration of positive selection signaling in DP thymocytes such that long/strong signals lead to CD4^+ T cell differentiation and short/weak signals lead to CD8^+ T cell differentiation. As described in this model (23), the duration/intensity of MHC-II-specific CD4/TCR signals is greater than that of MHC-I-specific CD8/TCR signals because of the greater association of intracellular Lck with CD4 than CD8 coreceptors in DP thymocytes. Importantly, this reformulation is also contradicted by this study because Lck association with CD4 coreceptors is not greater than with CD8.4 coreceptors in CD8.4 DP thymocytes. Likewise, MHC-I-specific CD8.4/TCR signals still only generated CD8^+ T cells and MHC-II-specific CD4/TCR signals still only generated CD4^+ T cells in the CD8.4 thymus. Thus, by demonstratively that CD4/CD8 lineage choice is not determined by the extent of Lck/coreceptor associations, this study directly contradicts the duration-of-signal model (23).

We think that CD4/CD8 lineage choice is best described by the kinetic signaling model which postulates that positive selection and lineage choice are developmentally distinct events (Fig. 8) (4, 46, 47). In the kinetic signaling model, TCR plus coreceptor mediated positive selection signals, regardless of their MHC specificity, terminate CD8 coreceptor gene expression causing signaled
DP thymocytes to transcriptionally convert into CD4^+8^- intermediate thymocytes; lineage direction is then determined by whether positive selection signaling persists or ceases in CD4^+8^- intermediate thymocytes. If TCR-mediated positive selection signaling persists despite absent CD8 coreceptor expression, intermediate thymocytes differentiate into CD4^+ T cells; if TCR-mediated positive selection signaling ceases, intermediate thymocytes re-initiate CD8^+ gene expression and differentiate into CD8^+ T cells. In this way, positively selected thymocytes determine lineage choice by assessing the CD8β dependence of their TCR. For example, CD8^-independent positive selection signals mediated by MHC-II-specific TCR would persist in CD4^-8^- intermediate thymocytes despite absent CD8^+ gene expression, resulting in differentiation into CD4^+ T cells, whereas CD8^-dependent positive selection signals mediated by MHC-I-specific TCR would cease in CD4^-8^- intermediate thymocytes because of absent CD8^+ gene expression, resulting in differentiation into CD8^+ T cells. Notably, the kinetic signaling model can explain why MHC-I-specific thymocytes were originally observed to differentiate into CD4^+ T cells in CD8^-co-receptor transgenic mice encoding chimeric CD8/CD4 co-receptor proteins (20): because CD8^-co-receptor transgene expression was regulated by heterologous transcriptional control elements (rather than endogenous CD8^α transcriptional control elements), transcription of transgenic CD8^-coreceptors was not terminated by TCR-mediated positive selection signals, causing continuous CD8^+ expression and persistent signaling by MHC-I-specific TCR which induced intermediate thymocytes to differentiate into CD4^+ T cells. Indeed, increasing evidence supports kinetic signaling as the mechanism of CD4/CD8 lineage determination in the thymus (24, 46, 48–50).

Conclusions
This study reveals that coreceptor signal strength is a key regulator of positive selection, determining positive selection efficiency and thereby the CD4^-CD8 ratio, but coreceptor signal strength does not determine CD4^-CD8^ lineage choice. That coreceptor signal strength influences positive selection without affecting lineage choice is precisely in accord with the kinetic signaling concept that positive selection and lineage choice are discrete and separable events during thymocyte differentiation.

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Disclosures
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References