Positive Selection by the Pre-TCR Yields Mature CD8+ T Cells

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It has been of much interest whether there is functional redundancy between the constitutively signaling pre-TCR (pre-TCR) and ligated TCRβ complexes, which independently operate the two distinct checkpoints during thymocyte development, i.e., the pre-TCR involved in β-selection at the CD4+CD8− double-negative stage and the TCRβ being crucial for positive/negative selection at the CD4+CD8+ double-positive stage. We found that the pre-TCR expressed on double-positive cells in TCRβ-deficient (TCRβ−/−) mice produced a small number of mature CD8+ T cells. Surprisingly, when pre-TCR was overexpressed, resulting in augmentation of pre-TCR expression, there was a striking increase of the CD8+ T cells. In addition, even in the absence of up-regulation of pre-TCR expression, a similar increase of CD8+ T cells was also observed in TCRα−/− mice overexpressing Egr-1, which lowers the threshold of signal strength required for positive selection. In sharp contrast, the CD8+ T cells drastically decreased in the absence of pre-TCR on a TCRα−/− background. Thus, the pre-TCR appears to functionally promote positive selection of CD8+ T cells. The biased production of CD8+ T cells via the pre-TCR might also support the potential involvement of signal strength in CD4/CD8 lineage commitment.


Materials and Methods

Generation of transgenic mice

A 1.3-kb fragment of pTα cDNA (kindly provided by H. J. Fehling, Ulm, Germany) was subcloned into the blunt-ended BamHI site of the expression vector controlled by the lck-proximal promoter (10). The complete plasmid (plek-pTα) was digested with NotI, and the transgene fragment no longer containing vector sequence was purified by GeneClean II kit (Bio 101, Vista, CA). DNA was microinjected into fertilized eggs of C57BL/6 mice. Resulting founders were screened for transgene by PCR and Southern blotting. Five independent founders carried the transgene. Two of the five mice that expressed transgene at relatively higher levels, as assessed by Northern blotting using thymus RNA, were backcrossed within TCRβ−/− background. All animals used in the experiments were cared in accordance with institutional guidelines.

RNA analysis

Total RNA was isolated from thymus and lymph nodes (LNs) by using RNAzol-B (Tel-Test, Friendswood, TX) from various mice. A quantity amounting to 15 μg total RNA was denatured, electrophoresed in a 1% agarose gel, blotted on a nylon membrane, and then hybridized with 32P-labeled either pTα or β-actin cDNA fragment.

Immunoprecipitation and Western blotting

Thymocytes from various mice (6.0 × 107) were lysed at a concentration of 1 × 108 cells/ml in a Triton X-100-containing lysis buffer (20 mM Tris-Cl, pH 7.6, 150 mM NaCl, 2.0 mM EDTA, and protease and phosphatase inhibitors). The TCRγ subunit was specifically immunoprecipitated with an anti-ζ mAb (6B10.2). The immunoprecipitates were separated on SDS-PAGE and transferred to Immobilon-P membranes (Millipore, Bedford, MA). Membranes were immunoblotted with anti-phosphotyrosine or anti-ζ mAb, as previously described (7).
FIGURE 1. Positive selection of CD8<sup>+</sup> T cells by the pre-TCR. A. The pTα expression was markedly enhanced in the thymus (Thy) of pTα-TG/TCRα<sup>-/-</sup> mice, but not in the LN, as assessed by Northern blotting. B. Increased CD3ζ chain phosphorylation under the overexpression of pTα in TCRα<sup>-/-</sup> mouse thymocytes. Lysates of thymocytes from WT, TCRα<sup>-/-</sup>, and pTα-TG/TCRα<sup>-/-</sup> mouse thymi were immunoprecipitated with an anti-CD3ζ mAb and probed sequentially on Western blots with anti-phosphotyrosine mAb 4G10 (top panel) and anti-CD3ζ mAb 6B10.2 (bottom panel). C,
Antibodies
Abs used in this study: anti-mouse CD4 (GK1.5), anti-mouse CD8α (53-6.7), anti-mouse TCRβ chain (H57-597), anti-mouse TCRγ (GL-3), anti-mouse CD8β (H35-17.2), anti-pan NK cells (DX-5), anti-Thy-1.2 (30-H12), anti-mouse CD25 (3C7), anti-mouse CD69 (H1.2F3), anti-mouse heat-stable Ag (HISA) (30-F), anti-phosphotyrosine (4G10), and anti-CD3ε (6B10.2) were purchased from BD PharMingen (San Diego, CA). Intracellular staining was performed by using the Cytofix/Cytoperm kit (BD PharMingen).

T cell activation
For IL-2 production, LN cells (1.0 × 10^6 cells/well) either from wild-type (WT) or pTα-transgenic (TG)/TCRα−/− mice were cultured in the presence or absence of 10 ng/ml of PMA for 48 h. The cells were then stained for intracellular IL-2 and cell surface CD8. CD8− cells were analyzed by FACSCalibur cytometer (BD Biosciences, San Jose, CA). For expression of activation markers, LN cells (1.0 × 10^6 cells/well) either from WT or pTα-TG/TCRα−/− mice were cultured in the presence or absence of 1 μg/ml of immobilized anti-CD3ε mAb or 1 μg/ml Con A in a 96-well plate for 16 h. The anti-CD3ε mAb was immobilized by preincubation of wells at 4°C overnight. The cells were then stained for intracellular TCRαβ (int), TCRαγ (int), and either for CD25 or CD69, and then analyzed by cytometer. CD8− γδ intβε cells were analyzed for expression of either CD25 or CD69.

Results
Positive selection of mature CD8+ T cells by the pre-TCR
First, we analyzed a set of mice expressing either low or high levels of the pre-TCR in the absence of TCRα chain, i.e., TCRα−/− (TCRα−/−) mice (11) or TCRα−/− mice expressing a transgenic α chain under the regulation of cek-proximal promoter. In thymocytes of TCRα−/− mice, the pre-TCR should be expressed at some level in DP thymocytes because most of the cells express the TCRβ chain due to β-selection, and still express the pTα chain that in this situation cannot be outcompeted by the TCRα chain (12). In the newly developed pTα-transgenic mice on a TCRα−/− background (pTα-TG/TCRα−/− mice), the pTα expression was much higher than in TCRα−/− mice in the thymus, although it was at undetectable level in the peripheral lymphoid organs by Northern blotting, which is compatible with the characteristic of the transgene promoter (10) (Fig. 1A). To assess whether the amount of signals mediated via the pre-TCR in each cell at the DP stage was actually strengthened by pTα overexpression in transgenic mice, we analyzed the tyrosine phosphorylation of CD3ζ chains in thymocytes, which are known to be functionally coupled to both the pre-TCR as well as the TCRαβ (7, 13–15). To this end, CD3ζ chains were immunoprecipitated from lysates of thymocytes from either pTα-TG/TCRα−/−, TCRα−/−, or WT mice, and their phosphorylation status was examined. In pTα-TG/TCRα−/− and TCRα−/− mice, CD3ζ chain should associate only with the pre-TCR, because of the lack of TCRαβ in the absence of α-chain. As illustrated in Fig. 1B, there was much more CD3ζ chain phosphorylation in thymocytes from pTα-TG/TCRα−/− mice compared with those in TCRα−/− (highlighted as ζ-PO4), whereas total amounts of precipitable ζ-chains were almost equal in both strains (bottom panel, CD3-ζ). Thus, pTα overexpression appeared to achieve efficiently increased signals through the pre-TCR in transgenic thymi.

Surprisingly, peripheral LNs of pTα-TG/TCRα−/− mice contained a large number (2.7 × 10^5 cells in all of collected mesenteric and inguinal LNs: 4.5% of total LN cells) of CD8+ cells that harbored intβε (Fig. 1C, right). A small, but clearly detectable number of the intβε CD8+ cells also did exist in LNs from TCRα−/− mice (1.2 × 10^5 cells in LNs, 0.2% of total LN cells; Fig. 1C, left). These intβε CD8+ cells were TCRγδ (Fig. 1D), Thy-1.2−, and CD8α−β−, and lacked expression of DX-5, a pan-NK cell marker (Fig. 1E), thus satisfying the criteria for αβ-lineage T lymphocytes despite the absence of TCRαβ complexes. These findings strongly imply that the pre-TCR harbors some potential of mediating signals required for positive selection of CD8+ SP cells. The presence of a small number of intβε CD8+ cells even in LNs from nontransgenic TCRα−/− mice is consistent with the very low, but significant amount of CD3ζ chain phosphorylation detectable in TCRα−/− thymocytes (Fig. 1B). Consistently, a far increased number of mature CD8+ SP thymocytes, displaying a low level of the HSA (CD24) expression, were detected in the thymus of pTα-TG/TCRα−/− mice than in the thymus of TCRα−/− mice (Table 1). In LNs from both TCRα−/− and pTα-TG/TCRα−/− mice, a small number of intβε CD4+ cells was also detected (Fig. 1C). Such intβε CD4+ cells had been previously reported as CD4+ TCRα−/− cells, and it was not clear whether they were derived from the thymus (16–18). In contrast to the intβε CD8+ cells, the number of these CD4+ cells was comparable in both types of mice (1.8 × 10^5 in LNs, 1.3 × 10^6 in the spleen in TCRα−/−, vs 1.6 × 10^6 in LNs, 1.1 × 10^6 in the spleen in pTα-TG/TCRα−/−), suggesting that the level of the pre-TCR complex in the thymus does not influence the development of the cells. Both strains also harbored intβε CD4+ CD8− cells, which consisted of γδ cells (3) and a proportion of DX-5−NK T-like cells (data not shown).

Egr-1 enhances the positive selection of CD8+ T cells by the pre-TCR
We further analyzed whether lowering the threshold for signaling required for positive selection by overexpressing of the Egr-1, a zinc-finger transcription factor, would increase the generation of intβε CD8+ T cells in TCRα−/− mice (19, 20). We previously reported that overexpression of Egr-1 in thymocytes markedly lowered the threshold of signal strength through the TCRαβ (or the avidity of TCR/MHC interaction) required for positive selection of mature SP thymocytes (19), and thus one might expect that Egr-1 could also lower the signal strength of the pre-TCR required for positive selection of intβε CD8+ T cells. The result illustrated in Fig. 2A shows that this is in fact true: the Egr-1 transgene expression on the TCRα−/− background (Egr-TG/TCRα−/−) resulted in a phenotype very similar to that of pTα-TG/TCRα−/− mice, in which a large (even larger than in pTα-TG/TCRα−/−) proportion (5.5 × 10^5 in LNs, 7.5% of total LN cells) of intβε CD8+ cells with a γδ−, Thy-1.2−, CD8α−β−, DX-5− phenotype was present in peripheral LNs. As shown in Fig. 2B, their
thymi also harbored a proportion of the HSA\textsuperscript{low} CD8\textsuperscript{+} mature SP thymocytes. Likewise, the absolute number of the HSA\textsuperscript{low} CD8\textsuperscript{+} mature SP cells in Egr-TG/TCR\textalpha\textsuperscript{−/−} mice was much smaller than in TCR\textalpha\textsuperscript{−/−} mice (compare Figs. 1C and 3). The precise reason for this difference is unclear. E\alpha might also influence the development of the intβ\textsuperscript{+} CD4\textsuperscript{+} CD8\textsuperscript{−} cells, although further study will be required. All facts considered, it is very likely that signals mediated by the pre-TCR and the ligated TCR\beta are functionally equivalent with regard to positive selection of CD8\textsuperscript{+} cells from DP precursors.

**CD8\textsuperscript{+} T cells selected by the pre-TCR are functional**

Having intβ\textsuperscript{+} CD8\textsuperscript{+} cells undergo positive selection by the pre-TCR, we then addressed whether these cells are functionally equivalent to the T cells from WT mice. First, we addressed the IL-2 production by the CD8\textsuperscript{+} cells in response to ionomycin + PMA. LN cells from either pT\alpha-TG/TCR\textalpha\textsuperscript{−/−} or WT mice were stimulated in the presence or absence of ionomycin + PMA for 16 h, then IL-2 production in the CD8\textsuperscript{+} cells were determined by intracellular staining analysis for IL-2. As shown in Fig. 4A, CD8\textsuperscript{+} cells from both pT\alpha-TG/TCR\textalpha\textsuperscript{−/−} and WT mice efficiently produced IL-2 upon stimulation. Second, the activation status of the CD8\textsuperscript{+} cells in response to either CD3\epsilon cross-linking or Con A...
stimulation was also studied. LN cells were stimulated by immobilized anti-CD3e Ab or Con A, then the expression levels of T cell activation markers, CD4, CD8, and HSA were analyzed by a cytometer. The absolute numbers of total thymocytes, whole CD8+ cells, and HSAlow mature CD8+ cells are presented. Numbers are the averages of five to seven mice analyzed. In TCRα−/− thymi, CD8+ cells were often undetectable. Numbers for CD8+ SP and HSAlow CD8+ SP in the TCRα−/− represent the maximum number observed for each population.

MHC molecules are not required for the positive selection events by the pre-TCR

Positive selection mediated by TCRαβ strictly requires interaction between the TCR and appropriate class I or class II MHC/Ag-peptide complex. We wondered whether positive selection of CD8+ cells operated by the pre-TCR is also dependent on MHC molecules. We investigated this question by analyzing the proportion of the intβ− CD8+ T cells in mice doubly deficient for class I MHC and TCRα, or for class II MHC and TCRα, which were generated by cross-breeding of TCRα−/− mice with either class I MHC−/− (K−/−, D−/−) (23, 24) or class II MHC−/− (Aβ−/−) (25) mice. No obvious difference in the size of the intβ− CD8+ T cell population was detected between the MHC/TCRα doubly deficient vs TCRα−/− mice (Table II), suggesting that the recognition of MHC

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**Table 1. Absolute numbers of mature CD8+ thymocytes in TCRα−/− and pTα-TG/TCRα−/− mice**

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<th>TCRα−/−</th>
<th>pTα-TG/TCRα−/−</th>
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<tr>
<td>Total thymocytes</td>
<td>1.1 × 10⁸</td>
<td>0.76 × 10⁸</td>
</tr>
<tr>
<td>CD8+ SP cells</td>
<td>&lt;1.9 × 10⁴</td>
<td>1.8 × 10⁴</td>
</tr>
<tr>
<td>HSAlow CD8+ SP cells</td>
<td>&lt;1.6 × 10⁴</td>
<td>1.4 × 10³</td>
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*Thymocytes of TCRα−/− or pTα-TG/TCRα−/− mice were stained for CD4, CD8, and HSA and analyzed by a cytometer. The absolute numbers of total thymocytes, whole CD8+ cells, and HSAlow mature CD8+ cells are presented. Numbers are the averages of five to seven mice analyzed. In TCRα−/− thymi, CD8+ cells were often undetectable. Numbers for CD8+ SP and HSAlow CD8+ SP in the TCRα−/− represent the maximum number observed for each population.*

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**FIGURE 3.** Peripheral CD8+ T cells were remarkably decreased in the absence of pTα. The presence of the CD8+ T cells was comparably analyzed in the LNs from TCRα enhancer-deficient (Eα−/−) mice and Eα, pTα doubly deficient (pTα−/−/Eα−/−) mice by staining, as in the previous experiments (see legends for Figs. 1 and 2). The intβ− CD8+ cells were drastically decreased in pTα−/−/Eα−/− mice.
Ags by the pre-TCR is not required for the selection event. We also studied β₂-microglobulin (β₂m−/−TCRa−/−) and TAP−/−TCRa−/− mice (26) to test possible involvement of nonclassical MHC molecules (most of which are dependent on β₂m and/or TAP for their cell surface expression). Again, a comparable fraction of the intβ⁺CD8⁻ T cells was observed in β₂m−/−TCRa−/− and TAP−/−TCRa−/− when compared with TCRα−/−mice (Table II). In addition, we also analyzed β₂m−/−Aβ⁻/−TCRa−/− mice, which lack both class I and class II MHC as well as most of nonclassical MHC molecules. Likewise, the number of intβ⁺CD8⁻ T cells in these mice was equivalent to that in TCRα−/− mice (Table II). Thus, recognition of MHC molecules (either classical or nonclassical) is not necessary for the pre-TCR to mediate positive selection. At the transition stage from the double-negative to the DP (β-selection), it has been shown that pre-TCR localizes cell autonomously to membrane rafts, where it appears to signal in a constitutive and ligand-independent manner (4, 27, 28).

Likewise, the recognition of an extracellular ligand might not be required for positive selection by the pre-TCR, while we cannot mutually exclude an involvement of non-MHC cell surface ligands.

### Discussion

In this study, we demonstrated that the pre-TCR can promote positive selection of CD8⁺ T cells in the thymus, by comparatively analyzing various strains of mice. Our observation strongly implies that the pre-TCR signals bear functional resemblance to those transduced by the TCRββ following ligand engagement.

One may wonder why the pre-TCR appeared to select predominantly CD8⁻ cells. One possible explanation might be insufficient signal strength to induce CD4⁺ cells in TCRα−/− mice, and even in pTα-TG/TCRa−/− or Egr-TG/TCRa−/− mice. Accumulating evidence suggests that the strength and/or duration of TCR signaling appear to influence CD4/CD8 lineage commitment (29–33). Indeed, positive selection of CD8⁻ cells by weak TCR signals has been demonstrated in various experiments (34, 35). The lower level of CD3ζ chain phosphorylation in thymocytes from pTα-TG/TCRa⁻/− (selecting predominantly CD8⁻ cells) as compared with WT animals (selecting both CD4⁺ and CD8⁻ cells) as shown in Fig. 1B might be sufficient for positive selection of CD8⁻ cells, but not for CD4⁺ cells. Recently, a new perspective, referred to as kinetic signaling model, was proposed based on a precise in vivo analysis of DP cells undergoing TCR signals (36–38). This model postulates that: DP cells terminate CD8 transcription and convert into CD4⁺CD8low⁺ intermediate cells in response to TCR (+co-receptor) signals; these CD4⁺CD8low⁺ intermediate cells are not yet lineage committed and retain the potential to differentiate into either CD4 or CD8 mature SP cells; sustained signaling in these cells results in their differentiation into CD4 SP cells, whereas cessation of signaling results in coreceptor reversal and differentiation into CD8 SP cells. In TCRα−/− as well as Egr-TG/TCRa−/− mice, the biased CD8⁺ cell production might fit with the model, because endogenous pTα expression is rapidly downregulated during the transition from the DP to the SP stage (lower in late DP cells than in early DP cells, then almost undetectable in mature SP thymocytes) (39), perhaps resulting in a decreased expression level of the pre-TCR in CD4⁺CD8low⁺ intermediate as compared in DP cells. However, the potential difference of the pTα transgene expression in DP and CD4⁺CD8low⁺ intermediate cells will need to be determined to extend this explanation to the priority of CD8⁺ cell production in pTα-TG/TCRa−/− mice as well. The alternative view of course is that pre-TCR signals never have the strength required for CD4⁺ T cell production.

In summary, our observations provide new insights on thymocyte development, two of which may be worth re-emphasizing. First, the fact that the pre-TCR can promote positive selection of CD8⁻ T cells further suggests the functional resemblance of the signaling pre-TCR and the ligated TCRββ. Second, the biased production of CD8⁻ T cells by the pre-TCR signals may support the propriety of hypothesis for the involvement of signal strength in CD4/CD8 lineage commitment.

### Acknowledgments

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### References


