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/TCRβ (pre-TCR) and ligated TCRαβ 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 TCRαβ 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 TCRαβ-deficient (TCRαβ−/−) 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 TCRαβ−/− 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 TCRαβ−/− 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 Iκκ-proximal promoter (10). The complete plasmid (pck-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 TCRαβ−/− background.

RNA analysis

Total RNA was isolated from thymus and lymph nodes (LN) by using RNAzolB (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⁺ T cells by the pre-TCR. A, The pTα expression was markedly enhanced in the thymus (Thy) of pTα-TG/TCRα⁻/⁻ mice, but not in the LN, as assessed by Northern blotting. B, Increased CD3ζ chain phosphorylation under the overexpression of pTα in TCRα⁻/⁻ mouse thymocytes. Lysates of thymocytes from WT, TCRα⁻/⁻, and pTα-TG/TCRα⁻/⁻ 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,
T cell activation

For IL-2 production, LN cells (1.0 x 10^6 cells/well) either from wild-type (WT) or pTa-Tg transgenic (TG)/TcRα−/− mice were cultured in the presence or absence of 10 ng/ml of PMA (5 x 10−7 M of ionomycin (Sigma-Aldrich, St. Louis, MO) in a 96-well plate for 16 h. The anti-CD3 mAb was immobilized by preincubation of wells at 4°C overnight. The cells were then stained for intracellular IL-2 and cell surface CD8. CD8+ cells were analyzed by FACSCalibur cytometer (BD Biosciences). CD4/CD8 double-positive thymocytes at the DP stage was actually strengthened by pTα expression at some level in DP thymocytes because most of the TCRβ chains were almost equal in both strains (D), and for CD8+ Thy-1.2, and DX-5 (E) by using specific mAbs. For expression of CD8α, Thy-1.2, or DX-5 presented in E, the intβ CD8+ cells gated on y6-negative population were analyzed. Dashed lines present positive or negative controls: y6 populations within CD4−CD8− cell population (for D), B220 (B cell marker)-positive cells within LN cells stained for CD8β or Thy-1.2, and DX-5-positive NK cell population within CD4−CD8− cell population (for E).
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\textsuperscript{α/−} thymi was larger (~2.5 times) than that in pT\textsuperscript{α/−}CD4/CD8\textsuperscript{+} mice (see Table I and Fig. 2B).

Direct evidence for the involvement of the pT\textalpha chain
Further evidence for the involvement of the pre-TCR in positive selection of int\textbeta CD8\textsuperscript{+} T cells was observed in TCR\textalpha enhancer-deficient (E\textalpha\textsuperscript{−/−}) mice (21): They likewise possessed a comparable (or even a slightly larger) proportion of int\textbeta CD8\textsuperscript{+} cells in the LNs as TCR\textalpha\textsuperscript{−/−} mice (1.9 \times 10\textsuperscript{4} in LNs, 0.4\% of total LN cells; Fig. 3, left panel). Moreover, in mice doubly deficient for E\textalpha and pT\textalpha (22) (pT\textalpha\textsuperscript{−/−}/E\textalpha\textsuperscript{−/−} mice), the number of the CD8\textsuperscript{+} cells drastically decreased (<5 \times 10\textsuperscript{3} in LNs; Fig. 3, right panel), confirming the indispensable requirement of the pT\textalpha chain for the selection event. Again, the presence of a similar proportion of the int\textbeta CD4\textsuperscript{+} cells in E\textalpha\textsuperscript{−/−} and pT\textalpha\textsuperscript{−/−}/E\textalpha\textsuperscript{−/−} mice (3.6 \times 10\textsuperscript{4} in LNs of E\textalpha\textsuperscript{−/−}, vs 3.4 \times 10\textsuperscript{4} in LNs of pT\textalpha\textsuperscript{−/−}/E\textalpha\textsuperscript{−/−}) supports the notion that the development of the int\textbeta CD4\textsuperscript{+} cells in the absence of TCR\textalpha is pre-TCR independent. Interestingly, the number of the int\textbeta CD4\textsuperscript{+} cells was larger in E\textalpha\textsuperscript{−/−} mice than in TCR\textalpha\textsuperscript{−/−} mice (see Fig. 1C). In addition, the proportion of the int\textbeta CD4\textsuperscript{+}CD8\textsuperscript{−} cells in E\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\textalpha might also influence the development of the int\textbeta 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\textalpha\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 int\textbeta 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\textalpha-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\textalpha-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\textepsilon cross-linking or Con A.

![FIGURE 2](http://www.jimmunol.org/)

**FIGURE 2.** Peripheral CD8\textsuperscript{+} T cells were also increased under the Egr-1 overexpression in TCR\textalpha\textsuperscript{−/−} mice. A, LN (mesenteric and inguinal) cells from Egr-1 transgenic mice on a TCR\textalpha\textsuperscript{−/−} background (Egr-TG/TCR\textalpha\textsuperscript{−/−} mice) were analyzed for the presence of int\textbeta CD8\textsuperscript{+} T cells by staining, as described in the legend for Fig. 1, C–E. B, Thymocytes from WT or Egr-TG/TCR\textalpha\textsuperscript{−/−} mice were stained for CD4, CD8, and HSA, then analyzed by cytometer. Demonstrated are the CD4/CD8 profiles of mature thymocytes, which were pregated on HSA\textsuperscript{low} populations.
stimulation was also studied. LN cells were stimulated by immobilized anti-CD3ε Ab or Con A, then the expression levels of T cell activation markers, CD25 and CD69, on the CD8⁺ cells were analyzed. As illustrated in Fig. 4B, CD8⁺ cells from pTα-TG/TCRα⁻/⁻ mice functionally responded to either stimulation, demonstrating up-regulation of these markers. Probably due to the less amount of CD3ε on the cell surface, as demonstrated in Fig. 4C, CD69 and CD25 expression levels after stimulation were lower in pTα-TG/TCRα⁻/⁻ than in WT. Together, the CD8⁺ cells selected by the pre-TCR were functionally competent.

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

Positive selection mediated by TCRβ 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**

<table>
<thead>
<tr>
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<th>TCRα⁻/⁻</th>
<th>pTα-TG/TCRα⁻/⁻</th>
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<tbody>
<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 × 10⁵</td>
</tr>
<tr>
<td>HSAlo CD8⁺ SP cells</td>
<td>&lt;1.6 × 10⁴</td>
<td>1.4 × 10⁵</td>
</tr>
</tbody>
</table>

*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 HSAlo mature CD8⁺ cells are presented. Numbers are the averages of five to seven mice analyzed for each population.

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**FIGURE 4.** CD8⁺ T cells selected by the pre-TCR are functionally competent. A, LN cells either from WT or pTα-TG/TCRα⁻/⁻ mice were cultured in the presence or absence of 10 ng/ml of PMA + 5 × 10⁻⁷ M of ionomycin. The cells were then stained for intracellular IL-2 and CD8. Intracellular IL-2 levels in CD8⁺ cells are present. The solid lines represent the level of intracellular IL-2 when incubated with stimulation, whereas the dashed lines represent those on cells incubated without stimulation. B, LN cells either from WT or pTα-TG/TCRα⁻/⁻ mice were cultured in the presence or absence of 1 μg/well of immobilized anti-CD3ε mAb or 1 μg/ml of Con A. The cells were then stained for CD8, γδ, intβ, and either for CD25 or CD69, and then analyzed by cytometer. CD25 and CD69 expression on CD8⁺γδ⁺ intβ⁻ cells are presented. The solid lines demonstrate the expression of CD25 or CD69 when incubated with stimulation, whereas the dashed lines represent those on cells incubated without stimulation. C, Lower expression of CD3ε on CD8⁺ cells from pTα-TG/TCRα⁻/⁻ mice. LN cells either from WT or pTα-TG/TCRα⁻/⁻ mice were stained for CD3ε, CD8, γδ, and intβ⁻, and analyzed by cytometer. CD3ε expression on CD8⁺γδ⁺ intβ⁻ cells is presented. Bold line, WT; solid line, pTα-TG/TCRα⁻/⁻; dashed line, negative control (CD3ε expression on B220⁺ LN B cells).
pre-TCR-mediated positive selection

Table II. Absolute numbers of intβ⁺ CD8⁺ cells in the LNs of various mice

<table>
<thead>
<tr>
<th>TCRα⁻/⁻</th>
<th>K⁻/⁻ D⁻/⁻ TCRα⁻/⁻</th>
<th>Aβ⁻/⁻ TCRα⁻/⁻</th>
<th>βm⁻/⁻ TCRα⁻/⁻</th>
<th>TAP⁻/⁻ TCRα⁻/⁻</th>
<th>βm⁻/⁻ Aβ⁻/⁻ TCRα⁻/⁻</th>
</tr>
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<tbody>
<tr>
<td>intβ⁺CD8⁺ (×10⁴)</td>
<td>1.2 ± 0.2</td>
<td>1.5 ± 0.2</td>
<td>1.2 ± 0.3</td>
<td>1.6 ± 0.4</td>
<td>1.6 ± 0.3</td>
</tr>
</tbody>
</table>

*LN s of the six different strains of mice were analyzed for the number of intβ⁺ CD8⁺ cells. Three to four mice for each strain were analyzed. All of the strains of mice harbored comparable number of the intβ⁺ CD8⁺ cells.

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 TCRαβ 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/TCRα⁻/⁻ or Egr-TG/TCRα⁻/⁻ mice. Accumulating evidence suggests that the strength and/or duration of TCR signaling appear to influence CD4/CD8 lineage commitment (29–33).

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 TCRαβ. 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