Evidence for down-regulation of highly expressed TCR by CD4 and CD45 on non-selected CD4\(^+\)CD8\(^+\) thymocytes

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Abstract

Immature CD4\(^+\)CD8\(^+\) double-positive (DP) thymocytes are positively selected for further development if they express TCR reacting with thymic ligands of low affinity. However, the majority of DP thymocytes express low TCR levels. This low level of TCR may be insufficient to recognize thymic ligands. To understand the basis for the low expression of TCR on DP thymocytes, we determined the density of TCR expression at various stages of their development using TCR transgenic (TCR-Tg) mice. We found that TCR expression was high in the thymocytes that had recently transited into the DP stage but then gradually decreased on DP cells if they were not selected by TCR interaction with MHC molecules. However, such TCR suppression was not observed in positively selected DP cells and in the non-selected DP cells obtained from CD45 deficient mice or from mice receiving anti-CD4 mAb. These findings suggest that the once highly expressed TCR at the DP stage is suppressed by CD45 and/or CD4 on non-selected thymocytes. Furthermore, TCR suppression is prevented by TCR-mediated signals. The maintenance of high TCR levels on positively selected DP thymocytes may facilitate their selection.

Introduction

The genes encoding \(\alpha\beta\) TCR undergo rearrangement and are expressed during T cell development within the thymus. However, because of the stochastic nature of TCR gene rearrangement, the T cell repertoire has to be selectively shaped in a given individual: immature thymocytes bearing TCR which react with self components are eliminated, whereas those able to recognize nominal antigens presented by self MHC are selected for further development. The latter process is called positive selection, whereas the former is called negative selection (1,2).

This phenomenon has become clear from studies using TCR transgenic (TCR-Tg) mice (3-6), but it is still controversial how developing thymocytes are either positively or negatively selected when their TCR recognizes thymic ligands composed of peptide plus MHC on stromal cells. A favorite hypothesis has been proposed by several reports: positive and negative selection of a given T cell clone is determined by the avidity of interaction between TCR and its ligand, peptide plus MHC on the thymic stromal cells (7-9). According to this avidity model, immature thymocytes develop into CD4\(^+\) or CD8\(^+\) single-positive (SP) cells if TCR engages with thymic ligand weakly and the thymocytes die if the engagement is strong. In fact, these studies showed that CD4\(^+\)CD8\(^+\) double-positive (DP) thymocytes can recognize ligands with low affinity and low density in positive selection.

In normal mice, DP thymocytes vary in their TCR expression from low to a high level, but the majority is low. TCR-Tg mouse studies showed that TCR on DP thymocytes is expressed at a higher level in positive selecting mice than that in non-selecting mice, implying that the TCR expression level is
Fig. 1. TCR expression is suppressed specifically on non-selected DP cells. TCR-Tg mice OVA23-3, which express MHC class II (I-A<sup>d</sup>)-restricted, OVA<sub>323-339</sub>-specific TCR, were backcrossed with C57BL/6 and BALB/c. The latter offspring were designated Tg-Posi and the former Tg-Neut. Eight weeks after birth, thymocytes of Tg-Neut and Tg-Posi were stained with PE-anti-CD4, FITC-anti-CD8, biotin-anti-TCR β or biotin-anti-TCR V<sub>a</sub>3 and Red670-streptavidin, and analyzed by FACScan. (Left panel) CD4 and CD8 expression on total thymocytes of Tg-Neut (upper) and Tg-Posi (lower); DP (R1) and DN (R2) cells of each mouse are indicated; (middle panel) TCR β expression on DN (shaded histograms) and DP (open histograms) cells; (right panel) transgene derived TCR V<sub>a</sub>3 expression on DN (shaded histograms) and DP (open histograms) cells.

increased by the positive selection process but remains low in non-selecting mice (10,11).

However, the observations and concepts described above have raised the question of how DP cells with fewer TCR than mature T cells can still recognize appropriate thymic ligands despite their lower density and affinity (12). In this paper we addressed this seeming paradox utilizing fetal thymocytes of TCR-Tg mice and showed that TCR once highly expressed is suppressed on DP cells without interaction with thymic ligands through TCR, but it is not suppressed on DP cells reacting with the ligands. It was also shown that the TCR suppression on non-selected DP cells is mediated by CD4 and CD45. These data provide insights into the mechanism of positive selection and the destination of non-selected DP cells.

**Methods**

**Mice**

I-A<sup>d</sup> plus OVA<sub>323-339</sub>-reactive TCR transgenic mice, OVA23-3, were produced as previously reported (13). These mice were backcrossed with BALB/c and C57BL/6. Their founders were designated Tg-Posi and Tg-Neut respectively. Tg-Posi and Tg-Neut mice were also backcrossed with CD45 exon-6-deficient mice (14).

**mAb and flow cytometry analysis**

Anti-mouse TCR V<sub>a</sub>3 mAb, 1H9, was produced in our laboratory by cell fusion between P3X63-Ag8.653 and spleen cells of Armenian hamster immunized with Tg-Posi spleen cells. Purified antibody obtained from hybridoma culture supernatants was shown to stain thymic and spleen T cells of OVA23-3 and 2C TCR-Tg mice, both TCR-Tg have TCR V<sub>a</sub>3 (Y. Kametani et al., submitted). Phycoerythrin (PE)-anti-mouse CD4 was purchased from Caltag (San Francisco, CA).

**Anti-CD4 mAb treatment of Tg-Neut**

Tg-Neut mice at 7 days after birth were i.p injected 200 µg/day for 3 days. As a control, equal volumes (100 µl) of PBS were injected daily. One day after the final injection, thymocytes were stained and analyzed.

**Results**

**DP cells of TCR-Tg mice with non-selecting MHC express TCR at a lower level than DN cells**

We have produced TCR-Tg mice, OVA23-3, the reactivity of which is MHC class II (I-A<sup>d</sup>) restricted, OVA<sub>323-339</sub> specific, and the mice were backcrossed with C57BL/6 (H-2<sup>b</sup>) and BALB/c (H-2<sup>d</sup>) (13). As was theoretically expected, positively selected CD4 SP cells were prominent in the latter offspring, but not in the former (Fig. 1, left panel). These TCR-Tg mice were designated as Tg-Posi (positive background) and Tg-Neut (neutral background), in which T cells develop in the thymus with positive selecting MHC and with non-selecting MHC respectively.

In Tg-Neut mice, expression levels of TCR β and transgene TCR V<sub>a</sub>3 were almost the same between double-negative (DN) (shaded histogram) and DP (open histogram) cells (Fig. 1, lower middle and right panels). In Tg-Neut, however, the expression of both TCR β and TCR V<sub>a</sub>3 was lower in DP cells than in DN cells (Fig. 1, upper middle and right panels).
indicates that this lower TCR expression is specific for DP cells of Tg-Neut. This characteristic of our TCR-Tg mice is not different from that of the previously reported class I- and class II-restricted TCR-Tg mice (3–6,15,16).

TCR expression of DP cells decreases following the course of gestation in TCR-Tg mice with non-selecting MHC

To determine whether the decrease of TCR expression occurs at the DP stage in Tg-Neut, fetal thymocytes in Tg-Neut and Tg-Posi were analyzed. As the expression of TCR β and TCR Vβ3 on DN and DP cells changed in a similar way in Tg-Neut and Tg-Posi, we examined TCR β expression as representing TCR αβ in this study if it was not otherwise mentioned. At gestation day (g.d.) 17 when DP cells clearly emerge, TCR expression on DP cells was at the same high level as that on DN cells in both Tg-Neut and Tg-Posi (Fig. 2A, upper row). One day later, however, at g.d. 18, DP cells expressing lower TCR were found in Tg-Neut but not in Tg-Posi (Fig. 2A, lower row). The TCR expression level on DN cells was not changed during these gestation days. As shown in Fig. 3, the mean values of the relative expression intensity of TCR β (Fig. 3A) and TCR Vβ3 (Fig. 3B) decreased in DP cells of Tg-Neut from g.d. 16 to 2 weeks after birth. During the fetal developing days, CD4 and CD8 expression did not change (data not shown). TCR expression of CD8 single-positive (SP) cells in Tg-Neut was also examined at g.d. 17–18 and showed a higher level (Fig. 2B). These CD8 SP cells are at an intermediate stage between DN and DP (designated as int 8SP) for the following reasons: (i) they had been isolated and then made the transition to DP cells during a 24 h cell suspension culture (data not shown), and (ii) because of MHC class II-restricted TCR specificity of these TCR-Tg, DP cells are not considered to develop into mature CD8 SP cells. These observations indicate that the decrease of TCR expression, which is initially high, occurs after thymocytes have transited into the DP stage if they are not positively selected.

At g.d. 16 and 17 when DP cells appeared in Tg-Posi and Tg-Neut, the cell number of DP cells per mouse was not different between Tg-Posi and Tg-Neut. After that, however, the increase in DP cell number was remarkably greater in Tg-Neut than Tg-Posi (Fig. 4). This suggests that pre-selected DP cells immediately after the transition remain inevitably at the DP stage without being selected for SP cells in Tg-Neut. On the contrary, DP cells in Tg-Posi are rapidly selected for SP cells without staying long at the DP stage. During the accumulating periods at the DP stage in Tg-Neut, highly expressed TCR may be down-regulated, resulting in an increased proportion of TCR low DP cells.

TCR suppression by engagement of CD45 and CD4 is found only on non-selected DP cells

It has been previously reported that the CD4-mediated negative signal suppresses the TCR expression on DP cells in non-transgenic mice (17–20). However, those studies did not determine whether TCR suppression upon CD4-mediated negative signal occurs on all DP cells or only on particular DP cells such as pre-selected or non-selected cells. Our TCR-Tg thymocytes are capable of clarifying this issue, because DP cells of Tg-Neut are dominantly composed of non-selected cells. In Tg-Neut neonates receiving i.p. injection of anti-CD4 mAb, TCR expression on DP cells was up-regulated to the same level as that on DN cells, whereas the injection of control antibody did not induce any change (Fig. 5). Such remarkable change of TCR was not observed in Tg-Posi DP cells and this treatment had no effect on the cell recovery (data not shown). This observation suggests that TCR suppression on non-selected DP cells in Tg-Neut is mediated by the interaction of CD4 with MHC class II on thymic stromal cells without TCR. In fact, in overnight cell suspension culture which dissociates such interactions, TCR expression on Tg-Neut DP cells increased (data not shown).

To further investigate the suppressive mechanism of TCR on DP cells, we produced double mutant mice by mating our TCR-Tg mice with CD45 exon 6-deficient (CD45−/−) mice and examined TCR expression on their thymocytes, because CD45 is reported to be involved in activating Ick which is associated with CD4 (21–24). TCR expression on DP cells was much higher in CD45−/− Tg-Neut than CD45+/+ Tg-Neut, but such a difference was not found in DP cells between CD45+/+ Tg-Posi and CD45−/− Tg-Posi (Fig. 6). The TCR expression was also higher in CD45−/− non-TCR-Tg than CD45+/+ non-TCR-Tg, but the increase was not so remarkable because DP cells of CD45+/+ non-TCR-Tg contain
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Fig. 3. TCR expression on non-selected DP cells decreases following the course of gestation. Mean fluorescence intensity of DP cells stained with anti-TCR β (A) and anti-TCR Vα3 (B). Thymocytes of fetus and neonates (NB) at indicated age of Tg-Neut were stained and analyzed separately. Mean fluorescence intensity was calculated by FACScan and the mean values of mean fluorescence intensity (± SD) are expressed as a log scale.

Discussion

Using fetal thymocytes of MHC class II-restricted TCR-Tg mice, we have shown that the highly expressed TCR on DP cells decreased its expression level in the absence of a positive selecting signal through TCR, and that this down-regulation without any TCR signal is mediated by CD4 and CD45.

As far as was examined in adult TCR-Tg, TCR expression was lower in non-selected DP cells than in selected ones and lower than in DN cells (Fig. 1). Similar findings have been reported in TCR-Tg mice by several groups (15,16), but there was not sufficient discussion about the mechanism of this phenomenon. In the present study analyzing fetal thymocytes of TCR-Tg, we demonstrated that Tg-Neut DP thymocytes at g.d. 17, which are supposed to have recently transited into DP stage, expressed TCR at a high level, the same as DN cells and Tg-Posi DP cells, but the TCR expression decreased at g.d. 18 only in Tg-Neut DP cells (Fig. 2A). This finding provides the first evidence that low TCR in non-selected DP cells is a result of the suppression of high TCR. Russell et al. speculated that a high expression of TCR on DN cells of their adult TCR-Tg was due to their maturity because they had some characteristics of mature T cells (16). However, fetal DN thymocytes were mostly composed of immature DP precursors even if they expressed high TCR in our TCR-Tg. Furthermore, the TCR expression level was also high in Tg-Neut CD8 SP cells which are at an intermediate stage between DN and DP (int 8SP) (Fig. 2B). Thus, it is concluded that DP cells develop through the int 8SP stage from DN cells with high TCR and decrease their TCR expression if they are not positively selected.

Previous reports using H-Y TCR-Tg mice showed that DP cells of larger cell size express higher TCR, and this was observed in both selecting and non-selecting MHC background mice (25,26). It is also reported that the large DP cells are cycling and express high TCR in non-TCR-Tg mice (27–29) (Hozumi, unpublished data). Penit et al. also...
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Fig. 5. TCR suppression on non-selected DP cells is mediated by surface molecules including CD4, TCR β (left) and TCR Vα3 (right) expression on anti-CD4-treated mice (open histograms) and control mice daily injected with equal amounts of rat Ig (shaded histograms) Tg-Neut mice at 7 days after birth were i.p. injected with purified anti-CD4 (GK1.5) at 200 μg/day for 3 days. One day after the final injection, thymocytes were obtained and first incubated with anti-CD4 (GK1.5), followed by FITC-anti-rat Ig, PE-anti-CD8, biotin-anti-TCR β and Red670-streptavidin, and analyzed as in Fig 1. Three mice for each group were analyzed separately and typical data are illustrated.

Fig. 6. TCR suppression on non-selected DP cells requires CD45 expression. (A) CD4 and CD8 expression on CD45 positive (CD45+/+) and CD45 exon 6-deficient (CD45-/-) TCR-Tg mice. (B) TCR β expression on DP cells of Tg-Posi (left) and Tg-Neut (right) mice. CD45+/+ mice of Tg-Posi and Tg-Neut are illustrated as shaded histograms, and CD45-/- mice are illustrated as open histograms.

demonstrated that only a small proportion of the large DP cells becomes SP cells (27). Taken together with the observations of H-Y TCR-Tg and our TCR-Tg, it is speculated that the large DP cells are mostly at the pre-selected stage, from which selected cells develop into SP cells and non-selected cells become resting DP cells. This speculation is supported by our other data showing that the cell number of DP cells in Tg-Neut was greater than that in Tg-Posi. At g.d. 16–17, the
DP cell number was similar between Tg-Neut and Tg-Posi but then became less in Tg-Posi with age. Furthermore, when Tg-Posi was backcrossed with RAG2-deficient (RAG2-/-) mice (30), DP cells were fewer in Tg-Posi RAG2-/- than in Tg-Posi RAG2+/- (data not shown). These results imply that positively selected DP cells develop into the SP stage rapidly in Tg-Posi but non-selected cells remain in the DP stage for a certain period. This interpretation is applicable to normal mice in which DP cells contain a significant number of resting cells with low TCR. However, the thymocyte cell number in Tg-Neut was slightly smaller than in normal mice (data not shown). It is probably because TCR expression on the DN stage may provide any effect on transition from the DN to DP stage.

It has been reported that DP cells can detect ligands with very low affinity and low densities for developing into SP cells (7-9,31). This observation has raised the question of how DP cells with greatly fewer TCR than mature T cells can recognize appropriate ligands, because the majority of DP cells express TCR at a lower level (DP TCR+) in normal mice (12). In addition, these DP cells with fewer TCR show reduced Ca2+ influx response following TCR cross-linking (19,32,33). Now, our study is able to provide a new interpretation for this issue. DP cells immediately after the TCR gene rearrangement transiently express TCR at a higher level and have the potential for interacting with MHC on thymic stromal cells with low avidity. Then DP cells reacting to appropriate ligands maintain a higher expression of TCR, whereas DP cells without such recognition decrease their TCR expression.

This novel concept seems to be inconsistent with some previous reports. Huesmann et al. reported that BrdUrd labeling was delayed in DP TCR+ cells as compared to DP TCR+ cells, suggesting that DP TCR+ cells originate from DP TCR+ cells (34). In vivo transfer studies showed that DP TCR+ cells contain precursors of mature T cells (35-37). However, as discussed above, DP cells in normal mice contain pre-selected immature DP cells which have begun to express TCR at a low level and non-selected DP cells. Such pre-selected DP cells with low TCR at the beginning have the potential to undergo positive selection for further development after their TCR expression has increased.

In this study, we clarified that CD45- and/or CD4-mediated suppression for TCR occurs only on non-selected DP cells. According to the model of Nakayama et al., CD4 bind with MHC class II on stromal cells mediates the negative signal, leading to TCR suppression, and dissociation of this interaction results in higher TCR expression (19,20). However, the physiological significance and the relationship to the selection process in this event has remained unclear, since it is impossible in normal mice to clarify whether DP cells which acquired high TCR expression by anti-CD4 mAb are at the pre-stage for positive selection or belong to non-selected cells (38). In the present report, we have shown directly that TCR which has been initially highly expressed is suppressed specifically in DP cells that have not been positively selected and that positively selected DP cells overcome this suppression. Furthermore, the present data have made clear that this suppression is mediated by surface molecules including CD45 and CD4. Nakayama et al. suggested that p56ck plays a role in the CD4-mediated suppressive mechanism (20,39).

Indeed, in p56ck-deficient mice, as well as CD45-deficient mice as shown in this report, the TCR suppression on DP cells is not observed (40). Since it is reported that CD45 can activate p56ck by dephosphorylation of C-terminal tyrosine (21-23), p56ck may play a crucial role in TCR suppression on non-selected DP cells (41).

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Abbreviations

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<th>Description</th>
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<tr>
<td>DN</td>
<td>double negative</td>
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<td>DP</td>
<td>double positive</td>
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<td>g.d.</td>
<td>gestational day</td>
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<td>PE</td>
<td>phycoerythrin</td>
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<td>SP</td>
<td>single positive</td>
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<td>Tg</td>
<td>transgenic</td>
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