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*J Immunol* 1999; 162:5764-5774; ;
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Limiting TCR Expression Leads to Quantitative But Not Qualitative Changes in Thymic Selection

Vibhuti P. Dave, David Allman, David L. Wiest, and Dietmar J. Kappes

Thymic selection is controlled in part by the avidity of the interaction between thymocytes and APCs. In agreement, the selective outcome can be modulated by altering the expression levels of selecting ligands on APCs. Here we test the converse proposition, i.e., whether changing TCR levels on thymocytes can alter the selective outcome. To this end, we have generated mice in which all thymocytes express two transgenic TCRs simultaneously (dual TCR-expressing (DTE) mice), the class I-restricted HY TCR and the class II-restricted AND TCR. Due to mutual dilution, surface expression levels of the two individual transgenic TCRs are diminished in DTE relative to single TCR-expressing mice. We find that thymic selection is highly sensitive to these reductions in TCR surface expression. Positive selection mediated by the AND and HY TCRs is severely impaired or abolished, respectively. Negative selection of the HY TCR in male DTE mice is also partly blocked, leading to the appearance of significant numbers of double positive thymocytes. Also, in the periphery of male, but not female, DTE mice, substantial numbers of single positive CD8<sup>bright</sup> cells accumulate, which are positively selected in the thymus but by a highly inefficient hemopoietic cell-dependent process. Overall our results favor the interpretation that the outcome of thymic selection is not determined solely by avidity and the resulting signal intensity, but is also constrained by other factors such as the nature of the ligand and/or its presentation by different subsets of APCs.


Received for publication June 30, 1998. Accepted for publication February 23, 1999.

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1 This work was supported by National Institutes of Health Grants CA74620 and AI34472, Institutional Grant CA6927 from the National Institutes of Health, and also by an appropriation from the Commonwealth of Pennsylvania.

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3 Abbreviations used in this paper: DP, double positive; SP, single positive; DTE, dual TCR-expressing; RAG, recombinase-activating gene; β<sub>2</sub>m, β<sub>2</sub>-microglobulin; B6, C57BL/6; HSA, heat stable antigen; CD62L, CD62 ligand; STE, single TCR-expressing.
by switching the cytoplasmic tail of CD8α for that of CD4, which is presumed to enhance TCR/coreceptor-mediated signaling due to the higher efficiency of lck binding to CD4 (33). Other models of lineage commitment postulate either that commitment requires qualitatively distinct signals for both lineages (“traditional” instructive model (34)) or only the CD8 lineage (asymmetric model (35)) or is independent of TCR/coreceptor-mediated signaling altogether (stochastic model (36, 37)).

While the functional importance of ligand density on thymic development has been examined in several studies, the converse situation involving modulation of TCR density has not yet been explored. The approach we have employed here is to cross two different TCR transgenic lines to generate dual TCR-expressing (DTE) mice, under the assumption that total TCR surface levels will remain constant while the levels of particular transgenic TCR heterodimers will be relatively diminished. Consistent with this expectation, we find that in DTE mice expressing the AND and hydrophobic region HY TCRs the expression levels of both TCRs are reduced, albeit to different extents. We have examined the consequences of this reduced expression on the ability of transgenic TCRs to support positive and negative selection in the presence of one or two selecting ligands. We find that lowering TCR expression levels reduces the efficiency of both processes, but cannot redirect thymocytes to a qualitatively different developmental fate.

Materials and Methods
Animals
Recombinase-activating gene 1-deficient (RAG1−/−), β−/−microglobulin-deficient (β−/−), B10.A-H2b(2R)/Sgnd (K1A1E1D1), and AND TCR transgenic mice were obtained from The Jackson Laboratory (Bar Harbor, ME); MHC class II-deficient (I-Ab−/−) mice were obtained from Taconic (Germantown, NY), and C57BL/6 (B6) mice were obtained from the Fox Chase Cancer Center Laboratory Animal Facility (Philadelphia, PA). HY TCR transgenic mice were obtained from S. Tonegawa from H. von Boehmmer (38). Double TCR (HY ‘AND’) expressing (DTE) mice were generated as follows: AND and HY TCR transgenic lines maintained on a B6 background were intercrossed to generate DTE RAG−/− mice. These DTE mice were then bred for another four to six generations to RAG1−/− mice of the B6 background obtained from The Jackson Laboratory (designated C57BL/6-rag1tm1 Mom; previously backcrossed for 10 generations to B6 of the B6 background obtained from The Jackson Laboratory (designated C57Bl6J-tgHY1.1Ab10.3/1Ab10.4, previously backcrossed for 10 generations to B6 according to the supplier). The resulting DTE RAG1−/− mice possess an essentially pure B6 background. DTE RAG1−/− mice were further crossed to β−/−, class II−/−, or B10.A(2R) mice. Typing for TCR transgene expression and expression of MHC molecules was conducted by flow cytometric analysis of PBLs using specific fluorescently labeled Abs. Because the RAG and β−/−m loci are both located on mouse chromosome 2, generation of doubly deficient RAG−/−β−/−m−/− mice required more extensive breeding. RAG−/−β−/−m−/− F1 animals were backcrossed to RAG−/− to permit the occurrence of crossovers. F2 progeny, which typed as RAG−/− by FACS analysis of PBLs, were screened by PCR for the β−/−m knockout allele. A few RAG−/−β−/−m−/− mice were identified that were then intercrossed to generate doubly deficient RAG−/−β−/−m−/− mice.

Cell preparation and flow cytometry
PBLs were obtained by retro-orbital bleeding and were purified by density gradient centrifugation over Lympholyte M (Cedarlane, Hornby, Ontario, Canada). Thymocytes, lymph node cells, and splenocytes were obtained by grinding up the respective organs through a metal sieve. Then, 105 cells were incubated with the relevant combinations of fluorescently labeled Abs for 15 min at 4°C and analyzed using FACStarplus or FACScan (Becton Dickinson, San Jose, CA). Fluorescently labeled Abs against CD4, CD8, TCRβ, I-Aa, H-2Kk, H-2Kb, V88, Vβ3, Vα11, CD5, CD69, heat stable antigen (HSA), and CD62 ligand (CD62L) were obtained from PharMingen (San Diego, CA). Anti-TCR-HYα (T3.70) was purified from ascites. Fluorescent staining for HYα was conducted in four steps to maximize the signal-to-background ratio, i.e., sequentially with purified T3.70, anti-mouse IgG1 (of the mouse IgG1 isotype), biotin-labeled anti-mouse IgG2b, and streptavidin-cychrome.

Radiation chimeras
Bone marrow was isolated from the rear leg bones of DTE mice and depleted of β−/− TCR+ cells by FACS. Then, 5–10 × 106 T cell-depleted bone marrow cells in 0.2 ml of RPMI 1640 were injected i.v. into RAG−/− or nude recipients of the H-2b haplotype that had been lethally irradiated (900 rad) 24 h previously. In some experiments, 1 × 106 SP CD8 male DTE splenocytes were transferred into nude or RAG−/− recipients. Peripheral blood and thymus samples were obtained 5–6 wk following bone marrow transfer.

Results
Positive selection is severely impaired in DTE H-2b mice
HY+ thymocytes are positively selected by the D b class I molecule, while AND+ thymocytes can be positively selected either by the I-Aα or I-Eα class II molecules (39, 40). I-Eα acts as a stronger selecting ligand with respect to the AND TCR than I-Aα, as evidenced by the generation of greater numbers of SP CD4 thymocytes in mice expressing I-Eα (32, 40–42). To test the effect of dual TCR expression on thymic development, we first generated DTE mice of the H-2b background, i.e., expressing both D b and I-Aα molecules. All mice analyzed were females. Importantly, DTE H-2b mice are of the B6 strain, which lacks Vβ3- or Vβ8-specific superantigens that could distort the outcome of thymic selection. Comparison of DTE H-2b mice with single TCR-expressing (STE) H-2b controls revealed a marked reduction in the proportions and absolute numbers of both SP CD4 and CD8 thymocytes (Fig. 1a, top left panels). In principle, this reduction could reflect either an impairment of positive selection or an enhancement of negative selection. To evaluate these possibilities, we introduced the I-Eα ligand into DTE mice by crossing to the B10.A(2R)-H-2h2 strain to generate DTE H-2h2b mice (like B6 mice, the B10.A(2R) strain lacks Vβ3- or Vβ8-specific superantigens). As mentioned above, I-Eα is known to act as a higher-affinity ligand for the AND TCR than I-Aα (32, 40, 41). Hence, expression of I-Eα should increase the overall avidity of thymocytes for thymic APCs. This would be expected to restore SP CD4 development in DTE mice, if low numbers of SP CD4 cells resulted from weakened positive selection. In contrast, if SP CD4 cells are being deleted by negative selection, the addition of a higher-affinity ligand for the AND TCR should not rescue them. We found that in DTE H-2h2b mice, which express the I-Eα ligand in addition to the I-Aα ligand, the proportion of SP CD4 thymocytes is substantially restored with respect to DTE H-2b mice (Fig. 1a, right panels). Because these experiments were conducted with RAG−/− mice, it was important to show that SP CD4 cells rescued in H-2h2b mice in fact expressed the transgenic rather than endogenous TCRα-chains. Cotaining with ANDα- and HYα-specific Abs clearly demonstrated that H-2h2b DTE RAG+ mice exclusively express the transgenic TCRα-chains, in contrast to SP CD4 T cells from H-2b DTE RAG+ mice, many of which express endogenous TCRα-chains (Fig. 1b). The restoration of positive selection of SP CD4 thymocytes in DTE mice by a stronger ligand for the AND TCR strongly supports the interpretation that low SP numbers in DTE mice are caused by impaired positive selection rather than enhanced negative selection.

Expression levels of transgenic TCRs are reduced in DTE mice
The most likely explanation for the impairment of positive selection observed in DTE H-2b mice is that TCR signaling is diminished due to a reduction in TCR surface expression levels. To determine surface expression levels of transgenic TCRs on thymocytes of DTE and STE mice, we employed both flow cytometry and biochemistry. We first conducted staining of thymocytes and peripheral T cells from DTE H-2b mice (backcrossed to a
RAG1\textsuperscript{−/−} background to exclude endogenous TCR rearrangement) with Abs specific for TCR constant and variable domains used by the transgenic TCRs. The AND and HY TCRs consist of Vβ3/Nα11 and Vβ8/Vα3 heterodimers, respectively (39, 43). Anti-TCRβ staining revealed identical expression patterns for STE and DTE mice, indicating that total TCR levels are not changed by expression of multiple TCR chains (data not shown). Staining with Vβ-specific Abs showed that both transgenic TCRβ-chains were down-modulated 2-fold on cells from DTE mice (Fig. 2, a and b).

Staining with Vα-specific Abs showed that surface levels of the AND TCRα-chain were not markedly altered in DTE mice, while those of the HY TCRα-chain were down-modulated significantly by about 10-fold (Fig. 2, a and b). This indicates that HY TCRα competes poorly with AND TCRα for association with one or both TCRβ-chains. To precisely define the associations of transgenic TCR chains on DTE thymocytes, we employed partial endo F treatment to modify different TCR chains so that they could be distinguished electrophoretically (44). Surface TCR complexes on
FIGURE 2. Surface expression of AND and HY TCR heterodimers is diminished in DTE mice. Total thymocytes (a) or gated SP peripheral T cells (b) from STE and DTE RAG<sup>−/−</sup> mice, as indicated, were stained with fluorescently labeled Abs specific for TCRα and β subunits of the HY and AND TCR heterodimers and analyzed by flow cytometry. Biphasic staining patterns in a reflect different staining intensities of SP (TCR<sup>Bright</sup>) and DP (TCR<sup>low-med</sup>) subsets, respectively. b, VβAND and VαAND stainings were conducted on SP CD4 T cells from female mice, while VβHY and VαHY stainings were conducted on SP CD8 T cells from male mice (to avoid comparisons of SP CD4 and CD8 T cells, which express different total surface TCR levels). c, Total thymocytes from the indicated STE or DTE mice were subjected to surface biotinylation, lysis, and immunoprecipitation with Abs specific for Vβ-AND (Vβ<sub>3</sub>) or Vβ-HY (Vβ<sub>8</sub>). Immunoprecipitates were subjected to two-dimensional nonequilibrium pH-gradient gel electrophoresis/SDS-PAGE analysis and visualized with avidin-HRP. Identification of specific TCRβ- and α-chains was accomplished by overlaying autoradiographs of STE and DTE samples, using the invariant CD3 chains as internal guides.
total thymocytes were biotinylated, immunoprecipitated with Vβ-specific Abs, endo F treated, and separated by two-dimensional nonequilibrium pH-gradient gel electrophoresis/SDS-PAGE. A comparison of immunoprecipitates from DTE and STE mice shows that all four transgenic TCR chains can be distinguished by this method, consistent with previously published analyses (Fig. 2c) (44). By determining the ratios of band intensities between TCRα- and β-chains within a given immunoprecipitate, it is possible to estimate changes in association of TCR chains between STE and DTE mice. We find that both transgenic TCRβ-chains associate predominantly with the ANDα-chain (Vα11). The Vβ: Vα3 (HYβ:HYα) ratio in anti-Vβ8 immunoprecipitates of DTE thymocytes is reduced dramatically, on the order of 10-fold, with respect to HY STE thymocytes, indicating that 90% of HYβ-chains are associated with ANDα. Similarly, we estimate that at least 80% of ANDβ-chains are associated with ANDα. Taking all the data into consideration, we estimate that the AND TCR is reduced by 20- to 2-fold with respect to HY STE mice, while the HY TCR is reduced by 10- to 20-fold with respect to HY STE mice.

The AND TCR is responsible for inefficient positive selection in DTE H-2b RAG−/− mice

Although, as shown above, positive selection is severely impeded in DTE RAG−/− H-2b mice, some SP cells still arise and populate the periphery (Fig. 1). The development of these cells can reflect an inefficient positive selection using the transgenic TCRs and/or rearrangement of endogenous TCRs, giving rise to novel selectable TCR specificities on a small fraction of developing thymocytes. To determine whether the transgenic TCRs could, indeed, support positive selection, we analyzed female DTE H-2b mice on a RAG−/− background, in which endogenous TCR rearrangement is precluded. HY STE and AND STE RAG−/− littermates gave rise exclusively to SP CD8+ and CD4+ thymic and peripheral T cell subsets, respectively, as expected (Fig. 3a and Table I). Significantly, DTE H-2b RAG−/− mice generated SP CD4+ thymocytes and peripheral T cells in equivalent numbers to those seen in RAG−/− DTE mice, indicating that the AND TCR remains capable of supporting positive selection in these mice, albeit less efficiently so than CD4+ T cells. A comparison of absolute SP CD4 thymocyte and peripheral T cell numbers between DTE and AND STE RAG−/− mice indicates a 4-fold reduction in both subsets (Table I). In contrast, absolute numbers of SP CD8+ cells in DTE RAG−/− mice are reduced to background values, i.e., by >10-fold in the thymus (the precise degree of reduction cannot be determined due to contamination with immature SP CD8 cells, i.e., DP precursors (45)) and >20-fold in the periphery with respect to HY STE mice (Table I). To gauge the activation status of DP thymocytes in DTE mice, we examined the surface expression of CD5, which is known to increase on DP thymocytes in response to TCR-mediated activation both during positive selection and in response to Ab-mediated TCR engagement in vitro (46). In control AND+ and HY+ STE mice, CD5 levels are markedly elevated on all DP thymocytes on positively selecting H-2b backgrounds, but not on nonselecting H-2b backgrounds (Fig. 3b and data not shown), in agreement with previous reports (47). Significantly, CD5 levels on DP thymocytes from DTE H-2b mice are also elevated, indicative of a substantial degree of activation, although evidently not always sufficient for complete positive selection.

To definitively test the capacities of the AND and HY TCRs in DTE mice to function independently of one another, we backcrossed DTE mice to doubly deficient RAG−/− ββm−/− and RAG−/− class II−/− backgrounds. RAG−/− ββm−/− DTE mice generate similar numbers of SP CD4 thymocytes as β2m−/− DTE mice, while RAG−/− class II−/− DTE mice generate none, demonstrating that engagement of the AND TCR alone is necessary and sufficient for the development of SP CD4 cells in DTE mice (Fig. 3c). Conversely, no detectable SP CD8 thymocytes are generated in either background, demonstrating that the HY TCR is functionally inert due to its greatly diminished surface expression (Fig. 3c). This analysis also specifically excludes the possibility that signaling through the AND TCR is responsible for blocking HY-mediated development to the CD8 lineage in DTE mice. CD5 levels are elevated on DP thymocytes from DTE ββm−/− mice, but not DTE class II−/− females (Fig. 4d), providing further evidence that only the AND TCR supports meaningful functional interactions in DTE mice. In summary, transgenic TCRs support development exclusively to the CD4 lineage in DTE mice. This is dependent on class II but not class I expression, consistent with the exclusive involvement of the AND TCR. In contrast, the HY TCR is down-modulated so severely as to be functionally inert.

HY-mediated negative selection is impaired in DTE mice

While the HY TCR induces positive selection in female H-2b mice, in male animals it induces massive negative selection, presumably reflecting the presence of higher-affinity male-specific ligand(s) (38). Here, we test whether the reduced surface density of the HY TCR in DTE mice affects this male-specific negative selection and, in particular, whether it converts it to positive selection, as would be predicted by the differential avidity model. Negative selection in HY STE mice is characterized by the virtual absence of DP thymocytes and a consequent reduction in thymic cellularity to about 5% of normal. Nevertheless the periphery contains significant numbers of both double negative and SP CD8 cells expressing the transgenic TCR. The SP CD8 cells express characteristically low levels of surface CD8 (Ref. 38 and Fig. 4a). In male DTE RAG−/− mice, thymic cellularity is substantially reduced (30% of normal), indicating that deletion of DP thymocytes is still occurring, albeit with distinctly diminished efficiency (Fig. 4a). Absolute numbers of SP CD4 thymocytes are 2-fold lower in male than female DTE mice (Table I). Significantly, however, the absolute number of DP thymocytes (total thymocytes minus SP thymocytes) is similarly reduced, so that the percentage of SP CD4 thymocytes in fact remains very similar between male and female mice. This suggests that numbers of SP CD4 thymocytes in males are limited by the number of DP precursors. The absolute number of peripheral SP CD4 T cells in male DTE mice is comparably reduced to that of SP CD4 thymocytes. The absolute number of SP CD8 thymocytes in male DTE mice is not significantly increased with respect to female DTE mice. Furthermore, the majority of these cells are actually immature, as determined by their HSA+ phenotype (Fig. 4a, top right panels). However, a distinct population of mature HSA+ CD62L+ SP CD8 thymocytes is also detectable. Interestingly, SP CD8 T cells are found abundantly in the periphery, bearing normal high levels of the CD8 coreceptor in contrast to SP CD8+ cells from HY+ STE mice, which bear 5- to 7-fold lower levels (Fig. 4d). Given that these SP CD8bright cells arise only in male DTE mice, it is clear that their generation requires engagement of HY TCRs by APCs presenting male Ag. To explore whether the engagement of HY TCR is sufficient for the generation of these cells, we examined their development on a male RAG−/− background in which the AND TCR would not be engaged. Abundant peripheral SP CD8bright cells still arise, demonstrating that the AND TCR plays no role in their development. In the thymus, CD5 levels are notably up-regulated on DP thymocytes from male DTE class II−/− mice, in contrast to female DTE class II−/− mice, confirming that they are undergoing...
stimulation via their HY TCRs. In summary, the HY TCR can mediate a functional interaction in male DTE mice leading to the generation of SP CD8 cells, despite its significantly reduced level of surface expression. This developmental pathway does not interfere with the simultaneous generation of SP CD4 thymocytes using the AND TCR.

**FIGURE 3.** SP CD4⁺ thymocytes in DTE mice do not require endogenous TCR rearrangement or class I expression for their maturation. Cells of the indicated origin were stained with fluorescently labeled Abs specific for CD4, CD8, and CD5 and analyzed by flow cytometry. Data in a and b are from mice of the RAG⁻/⁻ background. c, mice are of the B₂m⁻/⁻ RAG⁻/⁻ or I-A β⁻/⁻ RAG⁻/⁻ backgrounds, as indicated.
HY-mediated generation of SP CD8<sup>+</sup> T cells is thymus dependent but does not require presentation of male Ag by epithelial cells

The disproportion between thymic and peripheral CD4:CD8 ratios in male DTE mice raises the issue of whether peripheral SP CD8 T cells in these mice are in fact thymus derived. We have addressed this question by generating radiation chimeras in which bone marrow from male DTE mice is transferred into male nude (B6) recipients. As shown in Fig. 5 (bottom panel), this results in essentially no peripheral SP CD8 T cells being generated, demonstrating that SP CD8 cells in male DTE mice are thymus derived. We further used radiation chimeras to determine the thymic cell types required for the generation of these SP CD8 cells. Previous experiments have shown that HY-mediated negative selection in males is mediated by hemopoietic cells (48), while HY-mediated positive selection in females is mediated by radioreistant epithelial cells (49). The generation of SP CD8<sup>low</sup> cells in HY males is, however, strongly outcompetes HY<sup>b</sup>-chains (50). Certain combinations of TCR<sub>α</sub>- and β-chains are incapable of association (51). Further, where two TCR<sub>α</sub>-chains are coexpressed with a single TCRβ-chain, there can be strong competition for association with this TCRβ product leading to predominant expression of a single TCRαβ combination (50, 52). This turns out to be the case for DTE mice, in which AND<sub>α</sub> strongly outcompetes HY<sub>α</sub> for association with transgenic TCRβ-chains. Interestingly, however, AND<sub>α</sub> does not show any detectable preference for a particular one of the transgenic TCRβ-chains. As a consequence of this intracellular competition for association, there is a 2-fold reduction in surface expression of the AND TCR and a 10- to 20-fold reduction for the HY TCR. In addition, mismatched HYβ/AND<sub>α</sub> heterodimers are expressed at substantial levels.

Mismatched HYβ/AND<sub>α</sub> heterodimers are extremely unlikely to influence thymic selection in DTE mice for the following reasons. First, a high proportion of T cells from normal mice and humans express two functionally rearranged TCRα alleles (53–55). Because positive selection terminates the rearrangement process, the high frequency of such cells strongly indicates that most random TCRαβ combinations do not generate a selectable TCR specificity. Second, the generation of SP CD4 thymocytes is substantially restored in DTE mice by the expression of a stronger AND-selecting ligand. If mixed TCR heterodimers in DTE mice contribute to thymic selection, they must act to enhance negative not positive selection, because SP thymocytes are reduced in DTE mice. In this case, SP thymocyte numbers should not be restored using a novel dual TCR expression system. Specifically, we sought evidence that lowering TCR density could 1) convert negative into positive selection, as suggested by the differential affinity model of thymic selection, and 2) redirect class II-restricted thymocytes from the CD4 to the CD8 lineage, as suggested by the quantitative model of lineage commitment. We find that reductions in TCR expression lower the efficiency of both processes, but fail to induce substantial numbers of thymocytes to undergo a qualitatively different developmental fate.

In our dual TCR expression model, the transgenic AND and HY TCRs are coexpressed on the same developing thymocytes. Because the total level of TCR surface expression is tightly regulated during development, we anticipated that levels of individual transgenic TCRs would be diminished in DTE mice due to mutual dilution. This was indeed confirmed to be the case. Less predictable was the possibility of further diminishment of transgenic TCRs due to the formation of mismatched heterodimers. It has been shown that there are wide differences in the efficiency of heterodimer formation for different combinations of TCRα- and β-chains (50). Certain combinations of TCR<sub>α</sub>- and β-chains are incapable of association (51). Further, where two TCR<sub>α</sub>-chains are coexpressed with a single TCRβ-chain, there can be strong competition for association with this TCRβ product leading to predominant expression of a single TCRαβ combination (50, 52). This turns out to be the case for DTE mice, in which AND<sub>α</sub> strongly outcompetes HY<sub>α</sub> for association with transgenic TCRβ-chains. Interestingly, however, AND<sub>α</sub> does not show any detectable preference for a particular one of the transgenic TCRβ-chains. As a consequence of this intracellular competition for association, there is a 2-fold reduction in surface expression of the AND TCR and a 10- to 20-fold reduction for the HY TCR. In addition, mismatched HYβ/AND<sub>α</sub> heterodimers are expressed at substantial levels.

Table 1. Thymic and peripheral T cell subsets

<table>
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<tr>
<th>Strain (n = 4–6)</th>
<th>Total (×10&lt;sup&gt;6&lt;/sup&gt;)</th>
<th>CD8&lt;sup&gt;+&lt;/sup&gt; (×10&lt;sup&gt;6&lt;/sup&gt;)</th>
<th>CD8&lt;sup&gt;+&lt;/sup&gt; (%)</th>
<th>CD4&lt;sup&gt;+&lt;/sup&gt; (×10&lt;sup&gt;6&lt;/sup&gt;)</th>
<th>CD4&lt;sup&gt;+&lt;/sup&gt; (%)</th>
<th>CD8&lt;sup&gt;-&lt;/sup&gt; (×10&lt;sup&gt;6&lt;/sup&gt;)</th>
<th>CD8&lt;sup&gt;-&lt;/sup&gt; (%)</th>
<th>CD4&lt;sup&gt;-&lt;/sup&gt; (×10&lt;sup&gt;6&lt;/sup&gt;)</th>
<th>CD4&lt;sup&gt;-&lt;/sup&gt; (%)</th>
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<tr>
<td>HY&lt;sup&gt;−&lt;/sup&gt;</td>
<td>6.2 ± 1.3</td>
<td>9.7 ± 2.9</td>
<td>1.6 ± 0.5</td>
<td>&lt;2</td>
<td>&lt;0.2</td>
<td>27.4 ± 10.8</td>
<td>104 ± 30</td>
<td>39.6 ± 7.7</td>
<td>&lt;2</td>
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<td>DTE &lt;sup&gt;+&lt;/sup&gt;</td>
<td>51.3 ± 10.2</td>
<td>50.3 ± 13.0</td>
<td>1.0 ± 0.3</td>
<td>426 ± 96</td>
<td>8.4 ± 1.5</td>
<td>19.6 ± 9.7</td>
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<td>132 ± 21</td>
<td>791 ± 243</td>
<td>6.3 ± 2.3</td>
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<td>144 ± 9</td>
<td>&lt;30</td>
<td>&lt;0.2</td>
<td>3955 ± 467</td>
<td>27.7 ± 4.4</td>
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<td>&lt;2</td>
<td>&lt;0.3</td>
<td>80.6 ± 22.7</td>
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<sup>a</sup> The severe reduction in splenic cellularity in RAG<sup>−/−</sup> mice is due primarily to the absence of B cells. Numbers of splenocytes expressing transgene-encoded TCRs are considerably higher in equivalent RAG<sup>+</sup> mice.

<sup>b</sup> WT, wild type.

Discussion

The present study addresses the influence of quantitative differences in TCR-mediated interactions on thymic selection in vivo.
by increasing the avidity of the interaction between DTE thymocytes and thymic APCs, but rather should be subject to increased deletion. Contrary to this expectation, expression of the higher-affinity I-Ek ligand for the AND TCR substantially restores SP CD4 development in DTE mice. This argues strongly that the reduction of SP thymocytes in DTE mice is due to an impairment of positive selection. The appearance of a minor population of SP CD8 T cells in DTE H-2b/b mice expressing transgenic TCR specificities (Fig. 1b) deserves some comment, as it would not be predicted based on the known specificity of the HY TCR. Significantly, this population also arises in AND+ STE H-2b/b mice, but not H-2b/b mice (Ref. 32 and data not shown), and so is unrelated to the expression of mixed TCR heterodimers in DTE mice. This unusual minor maturation pathway mediated by the AND TCR requires both MHC class I and II expression (32). Last, limiting TCR expression by means that do not involve the expression of multiple surface TCRs reduces the positive selection of AND+ thymocytes to the same extent as in DTE mice. We have generated

FIGURE 4. Large numbers of SP CD8+ cells develop in male DTE mice. Cells from mice of the indicated genotype were stained with fluorescently labeled Abs specific for CD4, CD8, CD5, HSA, and CD62L and analyzed by flow cytometry. Histograms represent total thymocyte and lymph node samples, unless otherwise indicated.
a line of mice in which AND TCR surface expression is limited by impeding the intracellular assembly of the CD3 complex (V.P.D. et al., manuscript in preparation). When expression of the AND TCR is reduced 2-fold below normal by this means, the same degree of reduction in SP CD4 thymocytes is observed as in DTE mice (data not shown). This confirms that 2-fold down-modulation of the AND TCR is by itself sufficient to cause impaired positive selection leading to a severe reduction in SP CD4 thymocytes, without any contribution by mismatched heterodimers.

Based on the above considerations, it is highly improbable that mismatched heterodimers play a significant role in thymic selection in DTE mice. While logically compelling, our arguments are necessarily indirect. A direct demonstration must await generation of transgenics that express only the mismatched TCR combinations in question.

There is strong evidence from studies of T cell clones that the extent of TCR engagement correlates quantitatively with the cell’s biological responsiveness, as measured, for example, by the amount of cytokine release (56). In these studies, the extent of TCR engagement was modulated by stimulating T cells with APCs that bore different densities of relevant MHC/peptide ligands. In DTE mice, we have applied a conceptually similar approach to thymic development by reducing specific TCR density on DP thymocytes, while leaving the density and repertoire of MHC/peptide ligands unaltered. If TCR density and by extension TCR signaling are limiting for a given thymic selection process, this approach should elicit detectable changes in selective outcomes. Consistent with this expectation, we observe clear changes in both positive and negative selection in DTE mice.

We have used female DTE mice to analyze the effect of TCR down-modulation on positive selection mediated by the HY and AND TCRs. On an H-2b background, the ability of the AND TCR to mediate positive selection and development to the CD4 lineage is reduced by 4-fold. It has been reported that there are precise thresholds for T cell activation in terms of the number of TCRs that must be engaged (57). Assuming that similar thresholds apply to the positive selection of thymocytes, it is apparent that the level of AND expression on DTE thymocytes must be within the range required to support positive selection, as otherwise no thymocytes would be positively selected. The fact that some but not other thymocytes are selected presumably reflects differences in the proportion of TCRs that are actually engaged on individual thymocytes, which may vary due to microheterogeneity of the thymic environment. The sensitivity of AND-mediated positive selection in the H-2b background to a modest 2-fold decrease in TCR expression suggests that the interaction of AND with I-A\(^b\) ligands must fall close to the borderline between positive selection and death by neglect. In contrast positive selection by the I-E\(^k\) ligand is relatively insensitive to this degree of TCR down-modulation, consistent with a higher affinity of interaction. SP CD8 thymocytes and peripheral T cells are undetectable in female DTE H-2b RAG\(^{-/-}\) mice, indicating that a 10- to 20-fold down-modulation reduces the number of HY TCRs below a critical threshold for positive selection.

The effect of TCR down-modulation on the development of class II-restricted thymocytes in H-2b DTE mice does not support a purely quantitative model of lineage commitment. Specifically, we observe a diminution in the proportion of SP CD4 thymocytes, but no corresponding increase in SP CD8 thymocytes. This indicates that those thymocytes receiving a signal that is too weak for development to the CD4 lineage instead undergo death by neglect rather than alternate development to the CD8 lineage, as the quantitative model would predict. The quantitative model derives largely from experiments in which coreceptor expression is manipulated either by switching the cytoplasmic domains of coreceptors or eliminating one of the coreceptors altogether (32, 33). It is postulated that the resultant changes in thymocyte fate reflect the quantitative contribution of coreceptors to TCR-mediated signaling, as determined by their relative affinities for p56\(^{ck}\). However, these data are also consistent with an alternate model, whereby
coreceptors mediate qualitatively different signals. It is noteworthy in this regard that development of class II-restricted thymocytes to the CD8 lineage in CD4-decient mice requires the presence of class I MHC, consistent with a requirement for CD8 engagement and a specific CD8-mediated signal (32). Using male DTE mice, we have examined the effect of reduced TCR density and signaling on negative selection. We provide compelling evidence that T cells in DTE mice are receiving and responding to HY-mediated signals. First, there is a substantial reduction in thymic cellularity in male but not female DTE mice, consistent with continued negative selection even at these diminished levels of HY TCR expression. However, it is less efficient than in male HY + STE mice, as demonstrated by the retention of substantial numbers of DP thymocytes, which are essentially absent in HY + STE mice. Second, CD5 levels are up-regulated on all DP thymocytes from male but not female DTE class II −/− mice. Last, male but not female DTE mice accumulate substantial numbers of peripheral thymus-derived SP CD8 T cells, which can only be explained by a specific HY-mediated differentiation and/or survival signal. The presence of substantial numbers of DP thymocytes in male DTE mice allowed us to test whether negatively selecting ligands present in the male background could support HY-mediated positive selection of DP thymocytes to the CD8 lineage. Indeed, a significant number of SP CD8bright T cells were present specifically in the periphery of male but not female DTE mice, indicating that HY TCR-mediated positive selection could occur. However, the generation of these SP CD8 cells is atypical in two important respects. First, the frequency of SP CD8 relative to SP CD4 cells is much lower in the thymus than in the periphery of male DTE mice. Indeed, SP CD8 thymocytes are quite difficult to detect at all. This suggests that peripheral SP CD8 T cells accumulate in these mice either because they are longer lived than SP CD4+ T cells or undergo expansion in the periphery. Consistent with the latter hypothesis, SP CD8 spleen cells from male DTE mice showed at least a 5-fold expansion following transfer into nude or Rag−/− recipients after 6 wk (data not shown). Second, the generation of SP CD8 T cells in male DTE mice does not require T-cell expression of male Ag on the thymic epithelium. In both these respects, SP CD8bright T cells from DTE males resemble the SP CD8low T cells found in male HY + STE mice (38, 48). It has been proposed that down-modulation of CD8 on SP CD8low cells in HY STE mice represents a necessary adaptation to avoid harmful autoreactivity in the periphery (58). In DTE mice, the down-modulation of HY TCR may already obviate this problem, allowing CD8 levels to remain high. The inefficiency of SP CD8 generation in male DTE mice is quite consistent with the fact that it depends on hemopoietic rather than epithelial cells. Thus, it has been previously shown that positive selection of SP CD8 T cells by hemopoietic cells is extremely inefficient (6).

Previous efforts to gauge the influence of avidity on thymic selection have used fetal thymic organ cultures supplemented with different types and concentrations of antigenic peptides. Two different results were obtained depending on the experimental system employed, i.e., the same agonist peptides could induce either negative or positive selection, depending on the concentration of specific peptide employed (24–26), or negative and positive selection were induced by different agonist and antagonist peptides (31). Our inability to efficiently convert negative to positive selection of SP CD8 thymocytes in male DTE mice may be explained in the context of the above observations in several ways. First, the specific peptide responsible for HY TCR-mediated negative selection, which remains undefined, may be intrinsically incapable of supporting positive selection, as shown for a subset of strong agonist peptides in fetal thymic organ cultures (31). Second, the presence of the relevant male Ag peptide responsible for negative selection may not be equivalent on all types of thymic APCs, in particular those required for efficient positive selection, because different cell types may process and present intracellular Ags differently (59). Last, the HY TCR may be down-modulated too severely in DTE mice for optimal positive selection, i.e., signal intensity may be reduced too much. Future experiments will seek to distinguish these possibilities. In particular, we will determine how HY-mediated thymic selection is affected over a range of TCR surface expression levels using a series of transgenic lines in which surface TCR levels are progressively reduced in (2-fold) increments.

Acknowledgments

We thank R. Hardy for critical reading of the manuscript.

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