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The influence of the thymic environment on the CD4-versus-CD8 T lineage decision

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T cell receptor signaling is an essential factor regulating thymocyte selection, but the function of the thymic environment in this process is not clear. In mice transgenic for major histocompatibility complex class II–restricted T cell receptors, every thymocyte is potentially selectable for maturation in the CD4 lineage. To address whether selection frequency affects positive selection, we created hematopoietic chimeras with mixtures of selectable and nonselectable precursors. With increased proportions of nonselectable thymocytes, positive selection of MHC class II–specific precursors was enhanced, generating not only CD4 but also CD8 thymocytes. These results indicate that the CD4 versus CD8 fate of selectable precursors can be influenced by the selection potential of its neighbors.

During development in the thymus, T cell precursors expressing CD4 and CD8 coreceptors (double-positive, or DP, cells) are positively selected to mature based on the ability of the T cell receptor (TCR) to interact with peptide–major histocompatibility complex (MHC) complexes on thymic stromal cells. TCR specificity is intimately related to lineage commitment, as DP thymocytes expressing TCRs that recognize MHC class II mature as $CD4^+CD8^-$ (CD4 single-positive, SP) T cells, whereas those expressing a TCR specific for MHC class I mature as $CD4^-CD8^+$ (CD8 SP) T cells[1–4]. How TCR-MHC recognition is related to the CD4-versus-CD8 decision is an issue of considerable interest. Progress in understanding this relationship comes from experiments manipulating TCR signaling in developing thymocytes[5,6]. Mutations in the coreceptors[7–9], in lymphocyte protein tyrosine kinase (Lck)[10,11] and in mitogen-activated protein (MAP) kinase[12,13]; pharmacological inhibition of Lck or MAP kinases[14,15]; and antibody treatments that effect Lck function[16,17] all influence the CD4-versus-CD8 lineage decision. These studies have led to a general strength-of-signal model that suggests strong signals through the TCR promote a CD4 fate, whereas weaker signals favor a CD8 fate. In physiological conditions, Lck may be mainly responsible for these signaling differences because of its differential binding to the CD4 and CD8 coreceptors[8,18–20]. Accordingly, TCR recognition of MHC class II coengages CD4 and more Lck, producing strong intracellular signals, whereas TCR recognition of MHC class I coengages CD8 and less Lck, generating weaker signals[7,8,10,11,16,17,21,22].

Given that positive selection can be a prolonged process[23,24], the quantity and quality of intracellular signals could also be influenced by the number and duration of TCR-MHC interactions, integrated over time and space. In a two-step thymic organ culture, duration of signal was shown to be an essential factor, with longer signals favoring the CD4 and shorter signals favoring the CD8 lineage[25]. Other evidence supports a 'kinetic signaling' model in which persistence of TCR signals promotes CD4 development, whereas interrupted TCR signals result in CD8 development[26]. Indeed, there are common elements in all the models, and they need not be mutually exclusive.

Therefore, most studies of CD4-versus-CD8 lineage commitment have focused on intracellular signaling events during thymic selection with little attention given to the involvement of the microenvironment, localization, topography and migration. How and when thymocytes interact with each other and with thymic stromal cells during positive selection could have a large effect on cell fate[27]. In normal mice, relatively few precursors are selected for differentiation and migration from the cortex to the inner medullary region, where newly generated SP thymocytes appear. In fact, the proportion of precursors that undergo selection may be crucial for normal thymic architecture, as the thymuses of TCR transgenic mice have more medullary regions than those of normal mice[28,29]. This phenomenon is most likely because of the enhanced positive selection that occurs in these thymuses[30].

To examine the development of thymocytes bearing transgenic TCR in a more normal thymic environment, we generated mixed hematopoietic stem cell chimeras to limit the number of thymocytes with selectable TCRs. In agreement with previous studies[30,31], we found that decreasing the frequency of selectable precursors generally improved positive selection. Unexpectedly, however, decreasing the ratio of selectable to nonselectable precursors also affected lineage commitment. Whereas precursors with a MHC class II–selectable TCR usually generated CD4 SP thymocytes in $H\text{-}2^b$ hosts, dilution of selectable precursors with precursors unable to undergo positive selection caused a fraction of MHC class II–restricted thymocytes to mature as CD8 SP thymocytes. Examination of chimeric thymuses by confocal microscopy showed that CD4 and CD8 SP cells were segregated into distinct medullary regions. CD8 SP thymocytes predominated in medullas in which surrounding cortical areas were high in nonselectable precursors. These results indicated that a thymic microenvironment high in nonselectable precursors favors CD8 development, irrespective of MHC class specificity.

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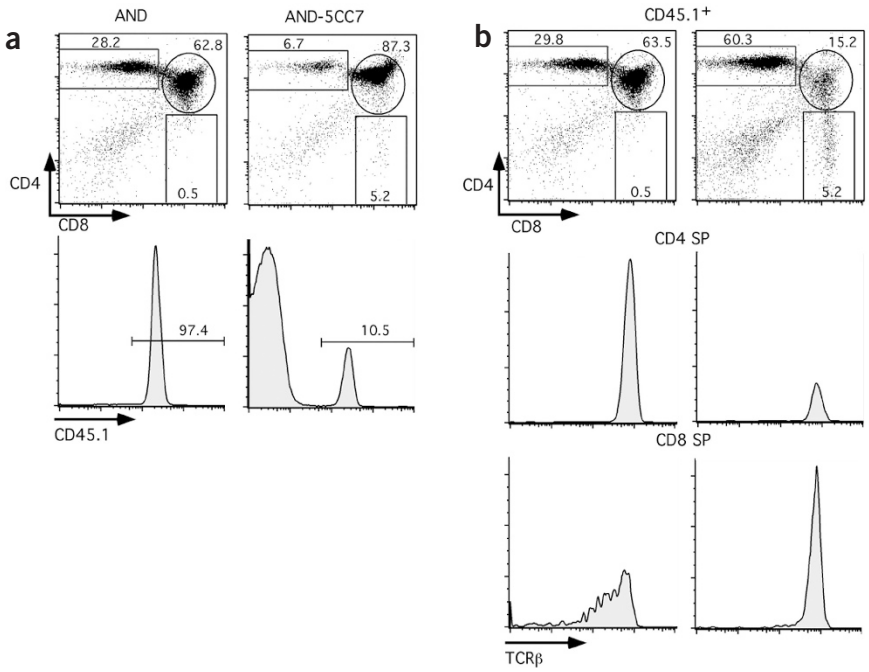

Figure 1 AND precursors generated CD8-lineage as well as CD4-lineage thymocytes in the presence of nonselectable 5CC7 thymocytes. Irradiated H-2^b RAG2-deficient hosts were reconstituted with T cell–depleted bone marrow cells from H-2^b RAG2-deficient AND (CD45.1) and H-2^b RAG2-deficient 5CC7 (CD45.2) mice, mixed at different ratios. At 5 weeks after transfer, thymocytes were collected, stained with fluorescence-labeled antibodies and analyzed by flow cytometry for expression of CD45.1, CD4, CD8 and TCRβ. (**a**) Coexpression of CD4, CD8 and CD45.1 for total thymocytes of AND control chimera (left) and AND-5CC7 mixed chimera (right). Percentages of total thymocytes subsets are indicated. (**b**) Top, coexpression of CD4 and CD8 for CD45.1$^+$ (AND-derived) thymocytes, gated as in **a**. Percentages of CD45.1$^+$ thymocyte subsets are indicated. Below, expression of TCRβ for gated CD45.1$^+$CD4 SP cells (middle) and CD45.1$^+$CD8 SP cells (bottom). Data are representative of three independent experiments.

RESULTS

Environmental effects on MHC class II–specific thymocytes

The fraction of selectable CD4$^+$CD8$^+$ cells is much higher in TCRαβ transgenic mice than in wild-type mice[30,32]. To study positive selection in more normal conditions, we designed an experimental model to reduce the number of selectable precursors. In MHC class II–specific, AND ($V_\alpha 11V_\beta 3$) TCR transgenic mice, DP thymocytes select on I-A^b, generating a large fraction of CD4 SP thymocytes[33]. Another MHC class II–specific TCR transgenic line, 5CC7 ($V_\alpha 11V_\beta 3$), selects on I-E^k to produce CD4 SP thymocytes[34]. This receptor cannot be selected in H-2^b mice, however, resulting in arrested development at the DP stage. Therefore, we used mixtures of donor bone marrow from H-2^b recombination activation gene 2 (RAG2)-deficient AND and 5CC7 transgenic mice to repopulate sublethally irradiated, H-2^b RAG2-deficient hosts. At certain dilutions of selectable AND precursors with nonselectable 5CC7 precursors, we expected a more normal thymus with fewer DP thymocytes undergoing positive selection, generating fewer mature SP thymocytes. In addition, the model would allow a comparison of marked, selectable (CD45.1) and nonselectable precursors (CD45.2) developing within the same thymus.

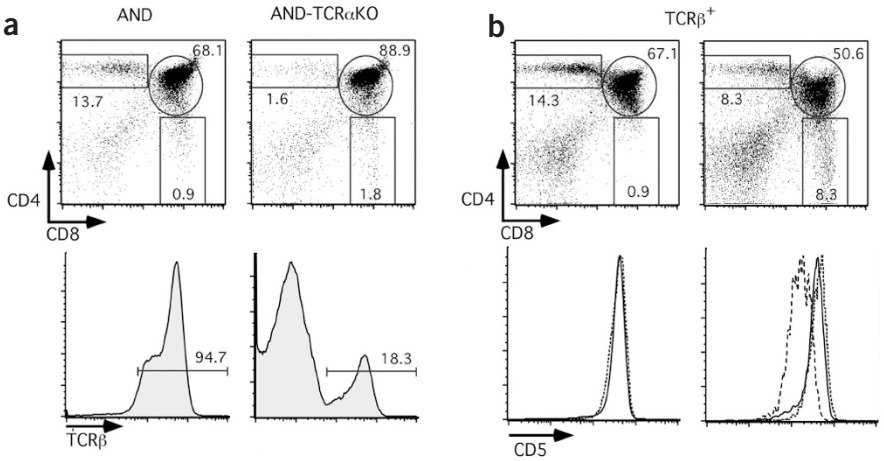

Figure 2 AND precursors generated CD8-lineage as well as CD4-lineage thymocytes in the presence of TCRα-deficient precursors. Irradiated H-2^b RAG2-deficient hosts were reconstituted with T cell–depleted bone marrow cells from H-2^b RAG2-deficient AND and H-2^b TCRαKO mice, mixed at different ratios. At 5 weeks after transfer, thymocytes were collected, stained with fluorescence-labeled antibodies and analyzed by flow cytometry. (**a**) Coexpression of CD4, CD8 and TCRβ for total thymocytes (top and middle) or gated for TCRβ$^+$ (AND-derived) thymocytes (bottom) of AND control chimera (left) or AND-TCRαKO mixed chimera (right). Percentages of total thymocyte subsets are indicated. (**b**) Thymocytes stained for CD4, CD8, TCRβ and CD5 were analyzed for CD5 expression on thymocyte subsets gated for TCRβ$^+$ (AND-derived), CD4 and/or CD8. Left, CD5 expression for TCRβ$^+$ DP (solid line) or TCRβ$^+$ CD4 SP (dotted line) thymocytes of AND control chimera; right, CD5 expression for TCRβ$^+$ DP (solid line), TCRβ$^+$ CD4 SP (dotted line) or TCRβ$^+$ CD8 SP (dashed line) thymocytes of AND-TCRαKO mixed chimera. Data are representative of three independent experiments.

At 5 weeks after transfer, we collected thymocytes from chimeric mice and analyzed them by flow cytometry. As expected, a higher proportion of DP and fewer CD4 SP thymocytes were present in AND-5CC7 mixed chimeras than in unmixed AND control chimeras (**Fig. 1a**). A population of CD8 SP thymocytes was also evident in AND-5CC7 mixed chimeras that was lacking in AND controls (**Fig. 1a**). Gating for CD45.1$^+$ AND-derived thymocytes showed that the proportion of CD8 SP thymocytes in AND-5CC7 chimeric mice was substantially increased (up to tenfold) compared with that of AND control mice (**Fig. 1b**). These AND-derived CD8 SP thymocytes expressed large amounts of TCRβ (**Fig. 1b**), an indication that they were mature. As the 5CC7 TCR is not selected by H-2^b, we did not find 5CC7-derived SP thymocytes in mixed chimeras (gating for CD45.1$^-$; data not shown).

Table 1 Analysis of AND thymocytes for positive selection and lineage development in hematopoietic chimeras

Chimeric mice	% AND-derived	AND-derived % $TCR^{hi}CD4$ SP[a]	AND-derived % $TCR^{hi}CD8$ SP[a]	AND-derived TCR^{hi} SP/DP[b]
AND	95.0	14.3	0.9	0.2
AND	93.0	11.0	1.1	0.2
AND	92.0	20.1	1.4	0.3
AND-TCRαKO	60.0	14.1	3.6	0.3
AND-TCRαKO	34.0	19.0	5.1	0.5
AND-TCRαKO	32.0	15.1	7.1	0.5
AND-TCRαKO	27.0	19.7	7.9	0.6
AND-TCRαKO	25.0	25.1	6.5	0.6
AND-TCRαKO	23.0	10.7	4.6	0.3
AND-TCRαKO	18.0	8.3	8.3	0.3
AND	91.8	24.0	0.6	0.4
AND	91.0	15.4	0.5	0.2
AND	90.7	15.0	0.6	0.2
AND-5CC7	33.3	28.7	4.0	0.7
AND-5CC7	30.7	31.2	4.4	0.8
AND-5CC7	23.5	19.4	3.9	0.4
AND-5CC7	21.2	53.9	6.9	2.4
AND-5CC7	18.5	51.6	6.9	2.6
AND-5CC7	13.3	28.4	7.1	1.0
AND-5CC7	7.2	11.2	7.7	0.5
AND-5CC7	1.7	0.6	6.9	0.3
AND-5CC7	1.3	5.2	3.2	0.3
AND-5CC7	0.6	0.0	3.7	0.2
AND	97.7	23.9	0.7	0.4
AND	96.0	35.4	0.5	0.6
AND	95.8	25.4	0.5	0.3
AND-B6	60.0	49.8	1.3	1.4
AND-B6	39.8	66.4	2.7	3.3
AND-B6	36.6	44.9	6.3	2.6
AND-B6	24.4	54.7	2.8	2.3
AND-B6	19.5	62.3	2.1	2.6
AND-B6	14.7	42.4	8.6	5.9
AND-B6	14.1	56.6	8.7	7.6

Percentages of thymocytes in individual chimeric mice were determined by flow cytometry. For calculation of the percentage of AND-derived for the AND control or AND (CD45.2)-TCRαKO (CD45.2) chimeras, total thymocytes were gated for $TCR\beta^+$; for the AND control or AND (CD45.2)-5CC7 (CD45.1) chimeras, total thymocytes were gated for $TCR\beta^+ CD45.1^-$; for the AND control or AND (CD45.1)-B6 (CD45.2) chimeras, total thymocytes were gated for $V_\alpha 11^+CD45.1^+$. The percent of AND-derived thymocytes in the AND control chimeras was less than 100%, as not all AND thymocytes expressed the transgenic TCR. Data are representative of two to three experiments. [a]Percent $TCR^{hi}CD4^+CD8^-$ or percent $TCR^{hi}CD4^-CD8^+$ of AND-derived thymocytes, as done in Fig. 2. [b]For TCR^{hi}SP/DP, the percent of AND-derived $TCR^{hi}CD4^+CD8^-$ + $TCR^{hi}CD4^-CD8^+$ was divided by the percent of AND-derived $CD4^+CD8^+$ thymocytes.

We assessed AND development in AND-5CC7 mixed chimeras made with increasing proportions of nonselectable precursors (**Table 1**). We calculated the percentages of AND-derived CD4 SP and CD8 SP ($CD45.1^+TCR\beta^+$) cells and ordered them by decreasing percentages of total AND-derived thymocytes in individual chimeras. AND-derived CD4 and CD8 SP thymocytes were both generated in AND-5CC7 mixed chimeras, but the percent of CD4 SP cells decreased substantially when the frequency of nonselectable precursors was higher than 95% (**Table 1**). Increasing the frequency of nonselectable precursors in the chimeras affected not only lineage development but also the overall efficiency of positive selection. Using the ratio of SP to DP as an estimate of the efficiency of positive selection[30], we found that selection of AND-derived thymocytes was improved in AND-5CC7 chimeras containing less than 80% of nonselectable precursors, compared with that of AND control chimeras (**Table 1**). However, this ratio dropped to control values when the frequency of nonselectable precursors was higher than 80%. The results were reminiscent of the improved positive selection found in mixed chimeras generated with precursors of transgenic TCR and wild-type mice[30,31]. As suggested before, improved selection on AND TCRs is most likely a consequence of the decreased competition among AND precursors for MHC ligand.

We considered whether CD8 development in mixed chimeras was because of attenuated TCR signaling in AND precursors. For example, 5CC7 TCRs might compete with or antagonize the interactions of AND TCRs with $I\text{-}A^b$. To eliminate any possibility of TCR-MHC competition from another TCR, we generated chimeras using mixtures of donor bone marrow from AND and TCRα-deficient (*Tcra*$^{-/-}$, referred to here as TCRαKO) mice. As with the AND-5CC7 chimera, analyses of total thymocytes from AND-TCRαKO chimeras showed an increase in the proportion of CD8 SP thymocytes compared with that of the AND control chimeras (**Fig. 2a**, top). Because the AND transgene is highly expressed at early stages of thymic development[35], TCRβ (gated to include both intermediate and high peaks) could be used to detect most AND-derived thymocytes (**Fig. 2a**, middle). Parallel analysis of the TCRαKO control chimera confirmed that TCRαKO-derived thymocytes did not fall in this gate (data not shown). A gated analysis for AND-derived thymocytes (**Fig. 2a**, bottom) showed a substantial increase in the percentage of CD8 SP thymocytes (up to tenfold) in the AND-TCRαKO chimera, compared with that of the AND control chimera. Similar results were obtained using a $V_\alpha 11$ antibody. The redirected MHC class II–specific CD8 SP thymocytes expressed large amounts of TCR and low amounts of heat-stable antigen; coexpressed CD8α and CD8β (data not shown); and responded to pigeon cytochrome c peptide presented by $I\text{-}E^k$-bearing antigen-presenting cells (**Supplementary Fig. 1** online). These properties are typical of mature CD8 T cells. The data clearly indicate that the alteration in AND lineage development cannot be due to MHC competition from another MHC class II–restricted TCR.

We assessed AND development in AND-TCRαKO mixed chimeras made with increasing proportions of nonselectable precursors (**Table 1**). Although AND precursors developed only as CD4 T cells in AND control chimeras, in all AND-TCRαKO mixed chimeras, a large fraction of these MHC class II–restricted thymocytes developed in the CD8 lineage. In mixed chimeras with the highest fraction of non

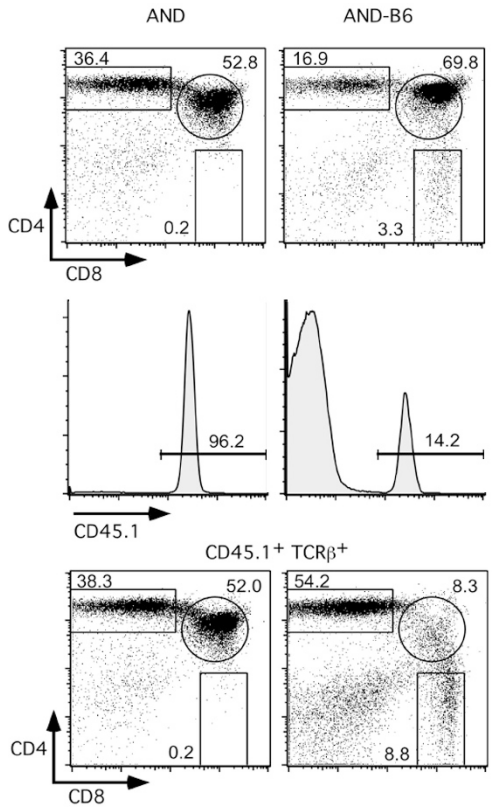

Figure 3 AND precursors generated CD8-lineage as well as CD4-lineage thymocytes in the presence of wild-type precursors. Irradiated H-2^b RAG2-deficient hosts were reconstituted with T cell–depleted bone marrow cells from H-2^b RAG2-deficient AND (CD45.1) and H-2^b C57BL/6 (B6.CD45.2) mice, mixed at different ratios. At 5 weeks after transfer, thymocytes were collected, stained with fluorescence-labeled antibodies and analyzed by flow cytometry. Coexpression of CD4, CD8 and CD45.1 by total thymocytes (top and middle) or gated for CD45.1$^+$ and TCRβ (AND-derived) thymocytes (bottom) of AND control chimera (left) or AND-B6 mixed chimera (right). Percentages of thymocyte subsets are indicated. Data are representative of three independent experiments.

selectable precursors (more than 80%), generation of AND-derived CD4 SP but not CD8 SP thymocytes decreased somewhat. The efficiency of positive selection (estimated by the SP/DP ratio) of AND-derived thymocytes was also generally improved in AND-TCRαKO mixed chimeras at ranges of nonselectable precursors of 40–80%, compared with that of AND control chimeras (**Table 1**). Nevertheless, the SP/DP ratio fell to control values in mixed chimeras with the highest proportion of nonselectable cells (more than 80%). As discussed below, the last phenomenon could be related to limited stromal cell access and/or abnormal trafficking in a thymus in which the proportion of nonselectable cells is abnormally high.

In many studies, attenuation of TCR signaling redirects MHC class II–specific thymocytes to the CD8 lineage. In AND-TCRαKO mixed chimeras, MHC class II–specific CD8 SP thymocytes were generated in the absence of obvious TCR signal attenuation. Because CD5 expression positively correlates with TCR signaling and thymic selection[36,37], we examined thymocyte subsets for CD5. Compared with CD5 expression on DP cells of AND control chimeras, CD5 expression on DP thymocytes of TCRαKO control chimeras was low (data not shown). CD5 expression was relatively high on AND-derived DP and SP thymocytes of both mixed and control chimeras; nevertheless, CD5 expression was higher on AND-derived CD4 SP than on CD8 SP thymocytes of AND-TCRαKO chimeric mice (**Fig. 2b**, lower panel). Previous studies have suggested that CD5 expression is higher on CD4 than on CD8 thymocytes of wild-type mice[36]. Our results showed, however, that CD4 and CD8 subsets differed in CD5 expression even when the same TCR was used to generate both cell types. Reduced TCR signaling in AND-derived CD8 SP thymocytes could be responsible for the higher ratio of CD4 to CD8 T cells found in lymph nodes compared with that of thymuses in mixed chimeric mice (data not shown). With the loss of CD4 for MHC class II recognition, TCR signals in some AND CD8 SP thymocytes may be insufficient to sustain survival in the thymic medulla and/or the periphery.

Results from both AND-5CC7 and AND-TCRαKO mixed chimeras indicated that some MHC class II–restricted thymocytes develop as CD8 single positive in the presence of nonselectable thymocytes. These findings raised the question of whether inappropriate lineage development would occur if AND precursors were mixed with wild-type precursors that have a diverse TCR repertoire. To test this, we created chimeras using mixtures of donor bone marrow from the AND and wild-type C57BL/6 (B6) mice. Even in the AND-B6 chimeras, we found a population of AND-derived CD8 SP thymocytes that was absent in AND controls (**Fig. 3**). We assessed AND development in AND-B6 mixed chimeras made with increasing proportions of wild-type precursors (**Table 1**). Similar to the other mixed chimeras, a fraction of AND thymocytes developed in the CD8 lineage in AND-B6 mixed chimeras. The production of AND-derived CD4 SP thymocytes was also increased in the mixed chimeras and was maintained even at the highest frequencies of wild-type thymocytes, as was the general efficiency of positive selection (as indicated by the SP/DP ratio). In contrast to these data, the efficiency of positive selection decreased at the highest proportions of nonselectable precursors in AND-TCRαKO and AND-5CC7 mixed chimeras. These results were especially intriguing, as the frequency of selectable thymocytes in mice with a diverse repertoire is presumed to be very low. The implication is that a small fraction of MHC class II–restricted thymocytes will commit to the CD8 lineage in the wild-type mouse. These findings emphasize an unappreciated function for the microenvironment, in that the extent of thymocyte selection can influence the cell fate and lineage decisions of MHC class II–specific thymocytes.

Environmental effects on MHC class I–specific thymocytes

In the presence of a large population of nonselectable precursors, some MHC class II–selectable DP precursors were able to commit to the CD8 lineage (**Table 1**). We sought to investigate the effect on lineage development of decreasing the proportion of selectable precursors in MHC class I–specific TCR transgenic mice. Therefore, we generated hematopoietic chimeras by reconstituting H-2^b RAG2-deficient recipients with mixtures of donor bone marrow from MHC class I–restricted P14 TCR ($V_\alpha2/V_\beta8.1$) transgenic[38] and TCRαKO mice. At 5 weeks after transfer, we collected thymocytes and analyzed them by flow cytometry (**Fig. 4**). P14-derived thymocytes could be distinguished from TCRαKO-derived thymocytes of mixed chimeras using a $V_\alpha2$ antibody (**Fig. 4**, middle). Nonselectable precursors had no obvious effect on lineage commitment, as P14-derived precursors generated only CD8 SP thymocytes in P14-TCRαKO mixed chimeras (**Fig. 4**, bottom).

As an indication of positive selection efficiency, we determined the ratio of SP to DP for P14-derived thymocytes in P14-TCRαKO mixed chimeras (**Table 2**). Similar to results from chimeras generated with MHC class I–specific HY TCR transgenic and wild-type precursors[30], these analyses showed that positive selection of P14 precursors was improved with increasing proportions of nonselectable TCRαKO precursor thymocytes. In contrast to the results obtained with a MHC

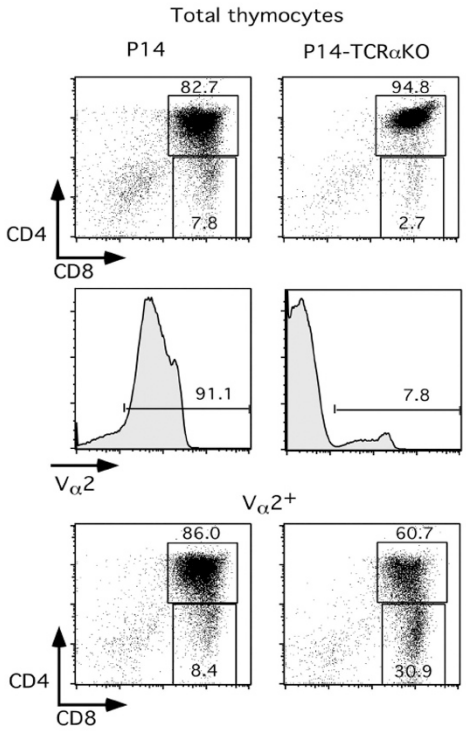

Figure 4 Increased efficiency of positive selection of P14 precursors in the presence of TCRα-deficient precursors. Irradiated H-2^b RAG2-deficient hosts were reconstituted with T cell–depleted bone marrow cells from H-2^b RAG2-deficient P14 and H-2^b TCRαKO mice mixed at different ratios. At 5 weeks after transfer, thymocytes were collected, stained with fluorescence-labeled antibodies and analyzed by flow cytometry. Coexpression of CD4, CD8 and $V_\alpha2$ by total thymocytes (top and middle) or gated for $V_\alpha2^+$ (P14-derived) thymocytes (bottom) of P14 control chimera (left) or P14-TCRαKO mixed chimera (right). Percentages of thymocyte subsets are indicated. Data are representative of three independent experiments.

Table 2 Analysis of P14 thymocytes for positive selection and lineage development in hematopoietic chimeras

	P14-derived		
Chimeric mice	% P14-derived	% TCR^{hi} CD8 SP[a]	% TCR^{hi} SP/DP[b]
P14	91.0	8.3	0.1
P14	87.0	11.4	0.1
P14-TCRαKO	30.0	20.3	0.3
P14-TCRαKO	12.0	24.1	0.3
P14-TCRαKO	10.0	23.9	0.4
P14-TCRαKO	9.0	23.6	0.4
P14-TCRαKO	8.0	30.6	0.5
P14-TCRαKO	6.0	26.7	0.5

Percentages of thymocytes in individual chimeric mice were determined by flow cytometry. For calculation of the percentage of P14-derived cells from the P14 control or P14 (CD45.2)-TCRαKO (CD45.2) chimeras, total thymocytes were gated for TCRβ$^+$. The percent of P14-derived thymocytes in the P14 control chimeras was less than 100%, as not all P14 thymocytes expressed the transgenic TCR. Data are representative of two to three experiments. [a]Percent TCR^{hi}CD4$^-$CD8$^+$ of P14-derived thymocytes. [b]For TCR^{hi}SP/DP, the percent of P14-derived TCR^{hi}CD4$^-$CD8$^+$ was divided by the percent of P14-derived CD4$^+$CD8$^+$ thymocytes.

class II–restricted TCR, improved selection of P14 precursors was evident even when the fraction of TCRαKO precursors was very large (>90%). Thus, the efficiency of positive selection of MHC class I–selectable precursors was enhanced by increasing the fraction of nonselectable precursors, but at no point was there any unpredicted effect on lineage development.

Effects of selection frequency on thymic organization

The findings described above indicated that the presence of a large cohort of nonselectable precursors can influence positive selection and the CD4 versus CD8 cell fate of selectable precursors. These results could be related to changes in thymocyte localization and/or in the organization of the microenvironment. The thymic architecture of TCRαβ transgenic mice is altered, with fragmented medullary tissue that is difficult to distinguish from the cortex[28]. As this phenomena could be related to the high efficiency of selection[30,32], we tested whether reducing the number of selectable precursors would restore normal cortical-medullary organization. We used confocal microscopy to examine mixed chimeras made by reconstituting H-2^b RAG2-deficient hosts with a mixture of bone marrow from AND and 5CC7 mice (**Fig. 5**). We used staining of CD45.1 to mark 5CC7-derived thymocytes, as shown by staining of control chimeras reconstituted with 5CC7 or AND bone marrow alone (**Fig. 5b**). CD45.1 staining of the AND-5CC7 mixed chimeras showed that nonselectable precursors were abundant in some areas of the thymus, whereas in other areas they were relatively rare. We selected three representative areas of the same thymus containing different densities of nonselectable precursors for examination based on the intensity of CD45.1 staining (**Fig. 5a**, A–C). Cytokeratin staining showed two morphologically distinct areas. Low-magnification images of thymic sections showed little staining for cytokeratin in the medulla relative to the cortex, causing the medulla to appear as black areas (**Fig. 5a**, D–F). We stained sequential sections for coordinate expression of CD4 and CD8. Cortical areas of AND-5CC7 chimeras high in nonselectable precursors showed CD4 and CD8 staining (**Fig. 5a**, G,J,M) similar to that of the 5CC7 control chimera (**Fig. 5b**, E,H,K). In comparison, cortical areas low in nonselectable precursors showed lower amounts of CD4 and CD8 (**Fig. 5a**, I,L,O), similar to AND control chimeras (**Fig. 5b**, F,I,L). This effect was also evident by flow cytometry, as CD4 and CD8 surface expression was lower on DP cells of AND control chimeras than on DP cells from AND-5CC7 mixed chimeras (**Fig. 1a**). Low CD4 and CD8 expression was probably a result of enhanced TCR signaling in thymocytes undergoing selection, as proposed before[39]. Accordingly, CD4 and CD8 expression was higher in the cortex of the wild-type thymus than in that of AND controls (**Fig. 5b**, G,J,M and F,I,L).

We were also able to visualize medullary areas by CD4 and CD8 staining, as well as by cytokeratin staining. As with other TCR transgenic mice[28], the thymuses of AND control chimeras contained multiple medullas, compactly filled with CD4 SP thymocytes (**Fig. 5b**, D,F). Areas of mixed chimeras with a low density of nonselectable precursors contained multiple medullas, but these were smaller and fewer than those of AND control chimeras (**Fig. 5a**, F,I, and **b**, D,F). The number of medullas decreased as the density of nonselectable precursors increased, and these medullas expressed less CD4 (**Fig. 5a**, D–I). When the density of nonselectable precursors was very high, thymic structure more closely resembled that of 5CC7 control chimeras (**Fig. 5a**, G, and **b**, E). The medullary areas in the 5CC7 control chimeras that could not generate SP thymocytes were notable (**Fig. 5b**, C,E). Under high magnification, these areas contained low numbers of thymocytes that did not stain for CD4 or CD8 (data not shown). However, mature T cell subsets can be found in thymuses with nonselectable, transgenic TCR (γδ T cells, TCRαβ$^+$

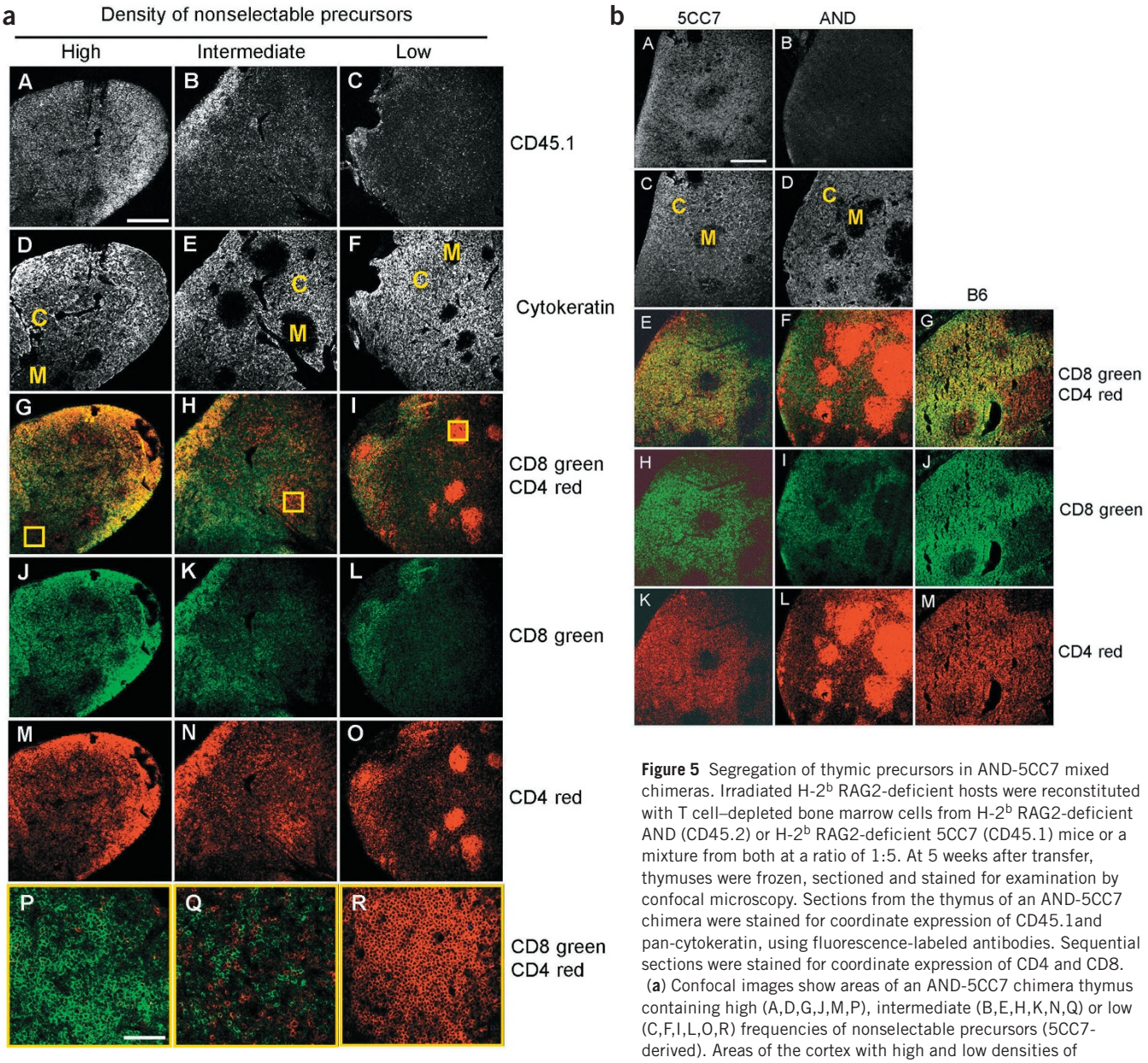

Figure 5 Segregation of thymic precursors in AND-5CC7 mixed chimeras. Irradiated H-2^b RAG2-deficient hosts were reconstituted with T cell–depleted bone marrow cells from H-2^b RAG2-deficient AND (CD45.2) or H-2^b RAG2-deficient 5CC7 (CD45.1) mice or a mixture from both at a ratio of 1:5. At 5 weeks after transfer, thymuses were frozen, sectioned and stained for examination by confocal microscopy. Sections from the thymus of an AND-5CC7 chimera were stained for coordinate expression of CD45.1and pan-cytokeratin, using fluorescence-labeled antibodies. Sequential sections were stained for coordinate expression of CD4 and CD8. (**a**) Confocal images show areas of an AND-5CC7 chimera thymus containing high (A,D,G,J,M,P), intermediate (B,E,H,K,N,Q) or low (C,F,I,L,O,R) frequencies of nonselectable precursors (5CC7-derived). Areas of the cortex with high and low densities of nonselectable precursors were determined by quantifying total CD45.1 pixel intensities in at least three adjacent areas of identical size (means, 65,263 ± 9,199 and 25,728 ± 2,102, respectively). P–R, High-magnification images (original magnification, 63) of medulla sections outlined in yellow in G–I (green, CD8; red, CD4). (**b**) Confocal images of sections from the thymus of 5CC7 (A,C,E,H,K) or AND (B,D,F,I,L) control chimeras or B6 (G,J,M) mice. **a**,**b**, Low-magnification images (original magnification, 10) show CD45.1 (**a**, A–C; **b**, A,B), pan-cytokeratin (**a**, D–F; **b**, C,D), CD8 in green and CD4 in red simultaneously (**a**, G–I; **b**, E–G), CD8 in green (**a**, J–L; **b**, H–J) or CD4 in red (**a**; M–O and **b**, K–M). In yellow (**a**, D–F; **b**, C,D): C, cortex, M, medulla. Scale bars represent 375 m (**a**, A–O, **b**, A–M) and 60 m (**a**, P–R). Data are representative of three independent experiments.

CD4$^-$CD8$^-$ cells)[35]. It is unknown whether these cells could be involved in medulla formation.

Morphological examination of cortical areas showed that 5CC7- and AND-derived precursors tended to segregate in AND-5CC7 mixed chimeras. We considered the possibility that the distribution of nonselectable precursors in different areas of the thymus may affect the CD4-versus-CD8 cell fate decision of AND-derived thymocytes. This seems to be the case, as medullas adjacent to cortical areas with a low density of nonselectable precursors contained mainly densely packed CD4 SP cells (resembling those of the AND controls), whereas medullas adjacent to cortex with a higher density of nonselectable precursors contained more CD8 SP thymocytes that were less densely packed (**Fig. 5a**, G,I,P,R). Accordingly, cortical areas with intermediate densities of nonselectable precursors generated medullas with both CD4 and CD8 SP thymocytes (**Fig. 5a**, H,Q). We obtained similar results with AND-TCRαKO mixed chimeras (data not shown). Thus, lowering the density of selectable precursors reduced the overall number of medullary areas, as expected. Very unpredicted, however, was the finding that some medullas contained mainly AND-derived CD8 SP thymocytes. These data indicated that an environment containing mainly nonselectable thymocytes can promote an inappropriate lineage choice in selectable class II–restricted thymocytes.

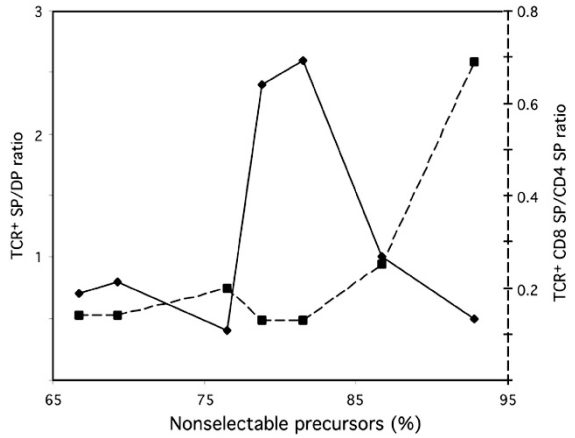

Figure 6 Differential effects of increasing the number of nonselectable precursors on the efficiency of positive selection and on the CD8/CD4 SP ratio. Irradiated H-2^b RAG2-deficient hosts were reconstituted with T cell–depleted bone marrow cells from H-2^b RAG2-deficient AND (CD45.1) and H-2^b RAG2-deficient 5CC7 (CD45.2) mice, mixed at different ratios, as in **Figure 1**. The TCR$^+$ SP/DP ratio (calculated as in **Table 1**) and TCR$^+$ CD8 SP/CD4 SP ratio for AND-5CC7 chimeras are represented by straight and dotted lines, respectively, with percentages of nonselectable precursors ranging between 65 and 95%

DISCUSSION

Experiments with TCRαβ transgenic mice have shown that TCR specificity for MHC class I or class II determines thymic selection and whether developing thymocytes will mature as CD4 or CD8 T cells. Although these mice have provided key information on thymic selection, they have some abnormal features. A most unusual characteristic is that every thymocyte expresses the same, and potentially selectable, TCR. This is in contrast to the highly diverse TCR repertoire of wild-type mice, in which relatively few thymocytes are selected to mature. As a consequence, thymic architecture is unusual in TCR transgenic mice. In contrast to the condensed, central medulla of wild-type mice, the thymuses of TCR transgenic mice have many medullary regions scattered throughout the cortex[29]. These observations raise questions about the nature of thymic selection in these mice and how accurately it reflects the process that occurs in wild-type mice.

In the studies reported here, we addressed these issues by creating a model in which the number of selectable precursor thymocytes was reduced by dilution with nonselectable precursors. We generated hematopoietic chimeras using mixtures of donor bone marrow from H-2^b RAG2-deficient MHC class II–specific AND TCR transgenic mice with H-2^b RAG2-deficient 5CC7 TCR transgenic mice. In the presence of nonselectable 5CC7 precursors, the efficiency of positive selection of AND-derived thymocytes was improved. Not only was the conversion from DP to SP thymocytes enhanced but also CD8 as well as CD4 SP thymocytes were generated. This was in contrast to control chimeras generated with AND bone marrow alone that yielded only CD4 SP thymocytes (as expected for MHC class II–restricted TCR). Histological examination of mixed chimeric thymuses provided another unexpected result. The CD4 and CD8 SP thymocytes were sometimes present in separate medullas. Medullas of mainly CD8 SP cells occurred in areas high in nonselectable precursors, whereas medullas containing mainly CD4 SP cells predominated in areas low in nonselectable precursors. These findings raise the question of why the CD4 versus CD8 fate should be influenced by the selection potential of neighboring thymocytes.

Given the growing body of evidence for a quantitative model for lineage commitment[5,40], we considered the possibility that some AND precursor thymocytes choose the CD8 lineage because they receive weaker TCR signals. AND precursors could receive weaker and/or fewer TCR signals if they competed with 5CC7 precursors for MHC. TCR competition between AND and 5CC7 is unlikely to cause this phenomenon, as chimeras generated with mixtures of AND and TCRαKO bone marrow also generated AND-derived CD8 SP cells. Nevertheless, the selectable thymocytes themselves must compete for MHC, as dilution of selectable AND precursors with nonselectable precursors increased AND positive selection efficiency. An improvement in positive selection has likewise been reported for mixed hematopoietic chimeras made with other TCR transgenic mice[30,31]. However, selection of AND precursors (as estimated by the SP/DP ratio) increased over a limited range of nonselectable precursors (75–90%). At the highest frequencies of nonselectable precursors, at which competition for ligand should be least, there was a negative effect on CD4-positive selection, whereas CD8 SP thymocytes were most efficiently generated at these points. It is easy to understand why positive selection could be compromised by large numbers of nonselected, dying cells that limit MHC and stromal cell access. It is less obvious why the CD8/CD4 ratio was altered when the proportion of nonselectable precursors was very high.

We have considered two explanations for the generation of AND-derived CD8 SP thymocytes in mixed chimeras. By a strength-of-signal model, DP precursors of TCR transgenic that received weaker and/or fewer TCR signals (perhaps because they stochastically express less TCR, coreceptors and adhesion molecules) would commit to the CD8 lineage. In fact, a few CD8 T cells were found in the periphery of all RAG1- or RAG2-deficient, MHC class II–restricted TCR transgenic mice that we have analyzed (refs. 9,41, B.J.F. and unpublished observations). In intact or control chimeric AND mice, specific MHCs can be limiting because of the large number of selectable precursors. In competition for limiting MHC, precursors with weaker avidity for MHC ligand would be out-competed by the many precursors with higher avidity that produce stronger TCR signals and commit to the CD4 lineage. In mixed chimeras, the number of AND precursors and competition for MHC ligand is reduced. Therefore, more of the less competitive precursors could be selected for development in the CD8 lineage.

Although the model described above relies on the idea that competition for MHC alters the efficiency of generating CD8-committed thymocytes, another (not mutually exclusive) possibility is that competition itself quantitatively alters the nature of positive selection signals. The development of AND-derived CD8 SP thymocytes was less compromised than that of CD4 SP thymocytes, causing a sharp increase in the CD8/CD4 ratio when the proportion of nonselectable precursors was very high. Furthermore, histology showed medullas containing mainly CD8 SP thymocytes in areas of the chimeric thymus where nonselectable thymocytes predominated. Thus, fewer CD4 and more CD8 SP thymocytes were generated in mixed chimeras when competition for MHC ligand should have been at its lowest and stromal cell access, at its worst. The strength-of-signal model is based on manipulations of TCR signals that influence the CD4-versus-CD8 fate decision, but it is uncertain whether commitment is related to the quantity or duration of TCR signals, or to the integration of multiple TCR signals over time and space. If the latter is important, fewer MHC 'hits' because of limited stromal cell access could favor a CD8 over a CD4 lineage choice. Thus, both the quantity and the nature of the selecting signals may affect CD4 versus CD8 lineage development. The frequency of nonselectable precursors can be high in local areas of a mixed chimeric thymus when the overall frequency of nonselectable precursors is low. This observation could explain why the relationship

between the frequency of nonselectable precursors and the generation of CD8 SP cells is only loosely correlated.

As decreasing competition for MHC ligand increases the efficiency of positive selection, it might be expected in P14-TCRαKO mixed chimeras that some MHC class I–restricted P14 precursors might receive stronger, longer and/or more TCR signals and commit to the CD4 lineage. We never found this outcome. As MHC class II–specific TCRs coengage CD4 and MHC, these receptors recruit more Lck to the receptor complex than do MHC class I–restricted receptors, which coengage CD8. Therefore, MHC class I–specific TCRs may never generate as much intracellular signal on thymic epithelium as MHC class II–specific TCRs. Alternatively, CD4 maturation may require additional interactions with MHC at later stages that are not required for CD8 maturation.

These discussions raise the question of whether lower or fewer TCR signals were indeed responsible for the AND-derived CD8 SP cells in the mixed chimeras. The fact that CD5 expression was lower on AND-derived CD8 than on CD4 SP thymocytes may be an indication that AND precursors, receiving less TCR signal, commit to the CD8 lineage. Alternatively, CD5 expression on CD4 versus CD8 SP thymocytes may be a consequence of lineage commitment. Nevertheless, CD8 SP thymocytes are generated in MHC class II–restricted TCR transgenic mice when TCR signals are attenuated in AND[9,10,12,14], A18 (ref. 15), DO11 (ref. 9), and HA[9] TCR transgenic mice. Moreover, there is no reason to believe that increasing the frequency of nonselectable precursor thymocytes would change MHC specificity or induce MHC class I cross-reactivity in the mixed chimeras. The CD8 development that occurs in CD4-deficient AND mice is MHC class II–dependent[9].

The alterations in positive selection and lineage commitment in the different chimeras are noteworthy. The efficiency of AND positive selection was greater and the ratio of CD8 to CD4 SP cells was lower in mixtures with wild-type precursors than in mixtures with 5CC7 or TCRαKO precursors. Clearly, there would be more active selection and less interference from dying cells in mixed chimeras of AND with wild-type than with 5CC7 or TCRαKO precursors, because little or no TCR signaling occurs in the last two. These results indicated that MHC engagement may be more prevalent in wild-type mice than is indicated by the frequency of fully mature thymocytes. Studies showing that peptide-MHC recognition is less specific for thymic selection than for mature T cell activation also contribute to this idea[42–45].

The precision of lineage development in MHC class I–deficient mice is difficult to reconcile with these and previous results showing that TCR signal is important in CD4-versus-CD8 lineage commitment. Previous studies of thymocytes with MHC class II–restricted transgenic TCR (developing in conditions that promote a CD8 lineage decision) have shown that maturation of CD8 SP cells is not only MHC class II–dependent but also dependent on noncognate MHC class I interactions (presumably for adhesion)[9,41]. The last observation could explain why no MHC class II–restricted CD8 T cells are generated in MHC class I–deficient mice. Studies in which a transgenic coreceptor was used to rescue developing thymocytes with mismatched TCR and coreceptor (for example, class I–specific DP thymocytes that down-regulate CD8) are also relevant, as they show that inappropriate lineage commitment occurs in mice with a normal TCR repertoire. Inappropriate commitment is relatively inefficient and is thought to be corrected by the requirement for continuous signaling and selection as thymocytes differentiate and mature[2]. This hypothesis could explain why in AND-TCRαKO mixed chimeras, the ratio of AND-derived CD4 to CD8 T cells increased in the periphery compared with that in the thymus, as DP precursors that commit to the CD8 lineage would lose MHC class II avidity by down-regulating CD4. Even though both genetics and the microenvironment can influence TCR signaling, resulting in the wrong lineage choice, these effects seem to be minimized over time and space. The requirement for continuous TCR-MHC signaling in the medulla and in the periphery not only serves to facilitate survival but also provides additional checkpoints to ensure that MHC specificity and effector functions are appropriately linked.

METHODS

Mice. B6.TCRα-deficient (*Tcra*$^{-/-}$) mice[46] backcrossed 12 times; congenic B6.SJL CD45a (Ly5a) (B6.CD45.1); C57BL/10 (B10).RAG2-deficient (*Rag2*$^{-/-}$)[47] backcrossed 20 times; and RAG2-deficient TCRαβ transgenic mice, B10.AND[33] backcrossed 14 times, B10.5CC7[34] backcrossed 12 times, and B6.P14[38] backcrossed 12 times were obtained from a National Institutes of Allergy and Infectious Diseases (NIAID) breeding contract with Taconic Farms. B10.RAG2-deficient AND or 5CC7 mice were crossed to RAG2-deficient B6.CD45.1 (Model 000461-M) mice[48] from Taconic Farms. B10.RAG2-deficient mice were used as recipients for bone marrow transfer. C57BL/6 (B6) mice were obtained from the Division of Cancer Treatment at the National Cancer Institute. Mice were bred and/or maintained in a NIAID Research Animal Facility according to American Association of Accreditation of Laboratory Animal Care specifications. All protocols for animal studies were approved by the NIAID Animal Care and Use Committee.

Hematopoietic bone marrow chimeras. Bone marrow chimeras were made as described before by reconstitution of sublethally irradiated RAG2-deficient recipients (650 rads, cesium source) with T cell–depleted bone marrow cells 2–12 h after irradiation[9]. Bone marrow suspensions were depleted of T cells by the use of antibodies to Thy1.2 (J1J)[49] and Ly1.2 (C3PO)[50] with low-toxicity rabbit complement (Cedarlane). Bone marrow cells were mixed at a ratio of 1:5, 1:10 or 1:50 in AND-5CC7 transgenic TCR chimeras to obtain an output of 5–60% AND thymocytes. In mixed chimeras, in which one donor expressed a transgenic TCR and the other did not, the TCR transgenic precursors were 'out-competed' at the transition from double negative to double positive, as reported before[51,52]. In these cases, the nontransgenic bone marrow cells were diluted with transgenic cells at a ratio of 1:5 or 1:10 to achieve the desired output (between 15 and 60% transgenic thymocytes). Mixtures of genetically marked, congenic donor bone marrow yielding unequal thymic reconstitution have been described before[53]. Recipients were injected intravenously with 2 10^7 bone marrow cells. Chimeric mice were killed and analyzed 5–6 weeks after bone marrow transfer. For lymph nodes analyses, mice were killed 8 weeks after bone marrow transfer. As is typical for hematopoietic chimeras, there was some variation in the absolute number of thymocytes recovered, but this variation did not relate to the ratio of selectable to nonselectable precursors.

***In vitro* peptide stimulation.** For cell purification, cells were incubated with antibody to heat-stable antigen (J11d)[49] and low-toxicity rabbit complement (Cedarlane), and were centrifuged over Ficoll (Pharmacia) gradients for removal of dead cells. After being washed, thymocytes were incubated at 37 °C with B10.A or B6 spleen cells previously irradiated at 3,000 rads and pulsed for 2 h with 10 M pigeon cytochrome c peptide (amino acids 81–104) or lymphocytic choriomeningitis virus peptide (amino acids 33–41). Cells were collected, centrifuged over Ficoll to exclude spleen cells and analyzed by flow cytometry after 16 h for CD69 expression. For interferon-γ expression, Golgi Stop (BD Pharmingen) was added to the culture after 30 h and cells were collected after another 16 h.

Antibodies. For flow cytometry, phycoerythrin-conjugated antibodies to CD4 (clone RM4-5), CD69 and interferon-γ, and fluorescein isothiocyanate–conjugated antibodies to CD5 (clone 53-7.3), CD45.1 (clone A20), V_α2 (clone B20.1) and V_α11 (RR8-1) were obtained from BD Pharmingen; Quantum Red–labeled antibody to CD8 (clone UCHT-4) was from Sigma; and allophycocyanin-conjugated antibody to TCRβ (clone H57-597) was from Caltag. Intracellular staining for flow cytometry was done using the Cytofix/Cytoperm kit (BD Pharmingen). For microscopy, purified antibodies to CD45.1, CD4 and CD8 (clone 53-6.7) from BD Pharmingen were labeled with Alexa-555, Alexa-488 or Alexa-647 dyes using labeling kits (Molecular Probes). Fluorescein isothiocyanate–conjugated antibody to pan-cytokeratin (clone C11) was obtained from Sigma.

Flow cytometry. Thymocytes were stained with antibodies and assessed by four-color cytometric analysis using a Becton Dickinson FACSCalibur flow cytometer with standard laser and optical filter configuration. For the four-color flow cytometric analysis, 100,000–200,000 events were collected.

Immunofluorescence. Thymus glands were fixed with a 4% paraformaldehyde solution in PBS, then washed with PBS and treated with a 20% sucrose solution in PBS. After a second wash, the thymus glands were immersed in optimum cutting temperature compound (Tissue Tek) for 1 h and 'snap frozen' using a mixture of 2-methylbutane (Sigma) and dry ice. Afterwards, section 10 m in thickness were obtained using a cryostat. The sections were fixed with 4% paraformaldehyde in PBS and stained for 5–12 h with antibodies in a solution containing 10% normal goat serum and 3% BSA. Sections were mounted with ProLong Antifade (Molecular Probes). The sections were analyzed using a Leica confocal microscope.

Note: Supplementary information is available on the Nature Immunology website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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