Intermediate Steps in Positive Selection: Differentiation of CD4+8int TCRint Thymocytes into CD4-8+TCRhi Thymocytes

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Summary

The differentiation potential of putative intermediates between CD4+8+ thymocytes and mature T cells has been examined. Such intermediate populations were sorted, in parallel with CD4+8+ thymocytes, from three types of C57BL/6 mice: major histocompatibility complex (MHC) class II-deficient mice, mice transgenic for an α/β T cell receptor (TCR) restricted by class I MHC and normal mice. The sorted populations were then transferred into the thymus of nonirradiated C57BL/Ka mice differing in Thy 1 allotype, and the progeny of the transferred cells were analyzed 2 d later. Surprisingly, with all three types of donor mice, a major proportion of the CD4+8intTCRint-derived progeny were found to be CD4-8+ TCRhi cells, thus delineating a new alternative pathway for development of the CD8 lineage. In contrast, the transfer of CD4int8+ TCRint thymocytes produced CD4-8+ TCRhi cells but no significant proportion of CD4+8- TCRhi cells, suggesting that there is no equivalent alternative pathway for the CD4 lineage. The results negate some of the evidence for a stochastic/selective model of lineage commitment, and point to an asymmetry in the steps leading to CD4-8+ versus CD4+8- T cells.

Mature T lymphocytes can be divided into two subpopulations based on their expression of the coreceptor molecules CD4 and CD8. Both populations recognize antigens in the form of peptides presented by MHC molecules. In general, CD4+8+ T cells recognize antigens presented on class I MHC, whereas CD4+8- T cells recognize antigens presented on class II MHC. This recognition process involves both an antigen-specific interaction between the TCR and the peptide-MHC complex (1) and an interaction between the coreceptor, either CD4 or CD8, and a conserved region of the same MHC molecule, class II or class I, respectively (for a review see reference 2).

Both CD4+8+ and CD4+8- T cells develop within the thymus from a common CD4+8+TCRint cortical thymocyte population, which itself arises by a developmental process involving successive rearrangements of TCR α and β genes (for reviews see references 3 and 4). CD4+8+ TCRint thymocytes appear destined to die unless they receive a selective set of signals that involve interaction of the TCR, and either CD4 or CD8 with peptide-MHC complexes expressed by the thymic stroma. The overall result is the upregulation of TCR, the downregulation of either CD4 or CD8, and the rescue of the cell from programmed death. The entire process, which dictates the antigen specificity and the MHC restriction of the emerging T cell populations, is termed positive selection (5).

The precise sequence of events that occurs during positive selection has not been elucidated, but two different models, known as the “instructive” and the “stochastic/selective” models, have dominated the discussion about mechanisms of positive selection. In the instructive model, engagement of MHC class I gives the uncommitted CD4+8+ thymocyte a positive selection signal that at the same time also instructs the thymocyte to downregulate CD4 and become a CD8+ T cell. In the same way, MHC class II recognition instructs the uncommitted CD4+8- thymocyte to downregulate CD8 and become a CD4+ T cell. In the same way, MHC class II recognition instructs the uncommitted CD4+8+ T cell to downregulate CD4 and become a CD8+ T cell. In the same way, MHC class II recognition instructs the uncommitted CD4+8+ T cell to downregulate CD8 and become a CD8+ T cell. In the same way, MHC class II recognition instructs the uncommitted CD4+8+ T cell to downregulate CD8 and become a CD8+ T cell. In the same way, MHC class II recognition instructs the uncommitted CD4+8+ T cell to downregulate CD8 and become a CD8+ T cell. In the same way, MHC class II recognition instructs the uncommitted CD4+8+ T cell to downregulate CD8 and become a CD8+ T cell.
mice and a CD4<sup>int</sup>8<sup>−</sup>TCR<sup>int</sup> population in MHC class I-deficient mice (8, 10–12). Both populations have also been identified in the thymus of normal mice (13), but are absent in MHC class I and II double-deficient mice (8, 11). These intermediate populations have features in common with both immature CD4<sup>+</sup>8<sup>+</sup> and mature CD4<sup>+</sup>8<sup>−</sup> and CD4<sup>+</sup>8<sup>+</sup> single positive cells and it has been proposed that they represent a transitional differentiation step in the progression from an immature double positive to a mature single positive stage (8, 11, 14, 15). Thus, according to the stochastic/selective model, the CD4<sup>+</sup>8<sup>+</sup>TCR<sup>−</sup> population observed in MHC class II-deficient mice has been formed from CD4<sup>+</sup>8<sup>+</sup>TCR<sup>−</sup> cells that have interacted with class I MHC, received a first positive selection signal, and in a stochastic manner down-regulated CD8. It is assumed that these cells are doomed to die since the second survival signal fails to come, because of their low CD8 expression. The reciprocal population of CD4<sup>+</sup>8<sup>+</sup>TCR<sup>−</sup> cells, formed by random downregulation of CD4 after the same first signal, can on the other hand be positively selected and differentiate into mature CD4<sup>+</sup>8<sup>+</sup>T cells, since they have "matched" TCR and CD8 molecules. Following the same arguments, the CD4<sup>+</sup>8<sup>+</sup>TCR<sup>−</sup> thymocytes of normal mice should consist of a mixture of class I MHC-restricted, TCR-bearing cells doomed to die and class II MHC-restricted, TCR-bearing cells en route to CD4<sup>+</sup>8<sup>+</sup>mature T cells.

In this report we isolate transitional intermediates from three different types of mice: MHC class II-deficient, class I MHC–restricted TCR-transgenic (Tg), and normal mice, and examine their fate when transferred into the thymus of normal recipient mice. The CD4<sup>+</sup>8<sup>+</sup>TCR<sup>−</sup> population produced, as expected, only CD4<sup>+</sup>8<sup>+</sup> cells as mature progeny. Surprisingly, the CD4<sup>+</sup>8<sup>+</sup>TCR<sup>−</sup> population also produced a predominance of CD4<sup>+</sup>8<sup>+</sup> cells as well as some CD4<sup>+</sup>8<sup>−</sup> cells. Thus we provide evidence for a new pathway of T cell maturation.

Materials and Methods

Mice. MHC class II–deficient mice (12) and mice expressing a Tg α/β TCR specific for an OVA peptide in context of the MHC class I molecule K<sup>+</sup> (OVATCR-1) (16) were used, along with normal C57BL/6/ (Spy 1.2) and C57BL/Ka (Spy 1.1) mice. The MHC class II–deficient and OVATCR-1 Tg mice were on a C57BL/6 (Spy 1.2) background. Only female mice were used, at 5–8 wk of age, and they were bred and maintained under conventional conditions at the Walter and Eliza Hall Institute animal facility.

Cell Preparation. Mice were killed with CO2, the thymuses were removed and transferred into cold balanced salt solution (BSS) containing 2% FCS. Thymocyte suspensions were made by pressing the tissue through a wire mesh into BSS-2% FCS and washing once by centrifugation through an underlayer of FCS.

Purification of Thymocyte Subpopulations. The CD4<sup>+</sup>8<sup>+</sup>, CD4<sup>+</sup>8<sup>+</sup>, and CD4<sup>+</sup>8<sup>+</sup> populations were isolated by cell sorting. Thymocytes were stained with PE-conjugated anti-CD4 (GK1.5; Becton Dickinson, San Jose, CA) and FITC-conjugated anti-CD8 (53-6.7; Becton Dickinson) using procedures previously described (17). The cells were then single or double sorted in a modified FACStar II instrument (Becton Dickinson) using the gates indicated in Figs. 1, 3, and 4 (the gate used for CD4<sup>+</sup>8<sup>−</sup> cells is not shown). The fluorescence intensity was checked throughout the whole sort and sorting gates were adjusted if necessary. Dead cells were excluded on the basis of forward light scatter and propidium iodide (PI) staining. The purity of all sorted populations was checked by reanalysis and the values given in Results. The CD4<sup>+</sup>8<sup>−</sup> population was obtained by depletion. Cells bearing CD3, CD4, CD8, together with non-T lineage cells, were removed from thymocyte suspensions using a sequence of complement-mediated cytotoxicity followed by immunomagnetic bead depletion, as described in detail elsewhere (17, 18). The purity of this depleted population was >99.9% as determined by flow cytometric analysis after staining the cells for residual depletion mAb with PE-conjugated goat anti–rat IgG. In each purification experiment, a separate sample of the unsorted thymocytes was stained and analyzed for CD4, CD8, and CD3 expression using the following antibodies. PE-conjugated anti-CD4, FITC-conjugated anti-CD8, and biotinylated anti-CD3 (KT3-1.1; 19) revealed with Texas red-streptavidin (TR-av) (Amersham International plc, Amersham, UK).

Intrathymic Transfer. 6–8-wk old C57BL/Ka (Spy 1.1) recipient mice (not irradiated) were anesthetized and intrathymically injected as described previously (20). Various numbers of purified thymocytes were suspended in 10 μl of BSS and injected into a single lobe of the recipient thymus.

Analysis of Recipient Thymuses for Donor-derived Thymocytes. At 1, 2, or 3 d after transfer, cell suspensions were prepared from the injected thymus lobes. Before analysis for donor-derived cells, most of the host thymocytes and non-T lineage cells were removed. Depletion was performed by coating the cells with antibodies against host thymocytes, Thy 1.1 (19F12; 21), erythrocytes (TEK-119; provided by Dr. T. Kina, Department of Immunology, Chest Disease Research Institute, Kyoto University, Kyoto, Japan), granulocytes (RB6-8C5; 22), macrophages (M1/70.15; 23), B cells, B220 (RA3-68B2; 24) and dendritic cells, MHC class II, (M5/114; 25) and then removing the coated cells with anti-Ig–coated magnetic beads using a 4:1 ratio of beads to cells and a mixture of equal parts of anti-rat and anti-mouse Ig-coupled Dynabeads (Dynal Inc., Oslo, Norway). The depleted suspensions were stained in four fluorescent colors with FITC-anti-Thy 1.2 (26), PE-anti-CD4, APC-anti-CD8, and biotin-anti-CD3 followed by TR-av. The stained cells were then analyzed by flow cytometry, gating for Thy 1.2<sup>+</sup> (donor-derived) cells.

Results

Development of CD4<sup>+</sup>8<sup>+</sup> Thymocytes Isolated from MHC Class II-deficient Mice. To determine the maturation potential of CD4<sup>+</sup>8<sup>+</sup> thymocytes, these cells were purified in parallel with CD4<sup>+</sup>8<sup>+</sup> cells from MHC class II–deficient mice and injected intrathymically into nonirradiated C57BL/Ka mice. After 2 d, suspensions of the recipient thymus lobes were depleted of host cells and the donor-derived cells were analyzed.

The sorting gates used for isolating the CD4<sup>+</sup>8<sup>+</sup> and CD4<sup>+</sup>8<sup>+</sup> thymocytes are shown in Fig. 1. Reanalysis of the sorted populations after a single sort showed that the CD4<sup>+</sup>8<sup>+</sup> preparations were >99.7% pure in the two experi-
ments performed, the only contaminants being CD4-8- thymocytes. The purity in the CD4-8+ preparations was >99%, the contaminants being of CD4-8- type. The CD4-8int population expressed intermediate to high levels of CD3 and the cells were larger than CD4-8+ small thymocytes but smaller than CD4-8- and CD4-8+ single positive cells (data not shown), suggesting that the CD4-8int cells had undergone at least one stage of positive selection.

Surprisingly, after transfer, the CD4-8int population produced a high proportion (42-62%) of CD4-8+ cells amongst the recovered progeny, the remainder being some CD4-8- cells, some unchanged CD4-8int cells, and some of intermediate phenotype (Fig. 1). In contrast, the majority (92-95%) of the cells recovered after transfer of the CD4-8+ population remained CD4-8+, with only 1% being CD4-8- (Fig. 1). The total recovery of donor thymocytes was in the same range for the two populations (2-3%). However, because of the low numbers of CD4-8int cells obtainable by sorting, only 0.1-0.2 x 10^6 were injected, compared to 2.9-3.7 x 10^6 CD4-8+ cells, explaining the low number of cells recovered 2 d after the transfer of the CD4-8int fraction. Based on the absolute production of CD4-8+ progeny cells per precursor cell injected, the CD4-8int population produced over 40 times more CD4-8+ progeny than the CD4-8+ population (Table 1). Furthermore, the few CD4-8- cells derived from the CD4-8+ population might have represented CD4-8+ contaminants in the original sorted CD4-8+ preparation. Thus the generation of CD4-8+ progeny from the CD4-8int population could not be attributed to any overlap with, or equivalence to, the CD4-8+ population.

Development of CD4-8int and CD4-8+ Thymocytes Isolated from OVA-TCR-I Tg Mice. The population of CD4-8int cells in MHC class II-deficient mice, as well as in normal mice, is very small. To increase the number of CD4-8int cells and thereby increase the number of cells recovered after transfer, OVA-TCR-I Tg mice were used. These mice express a MHC class I-restricted Tg TCR, which causes heavy skewing towards production of CD4-8+ cells. In addition, the thymic CD4/CD8 phenotype is different from that of most other MHC class I-restricted Tg TCR mice, in that there is a high proportion of CD4-8int cells (Figs. 2 and 3). These cells are also prominent in OVA-TCR-I Tg mice with disrupted recombinase-activating gene (RAG)-1, indicating they are not the consequence of endogenous gene rearrangement (our unpublished results). These CD4-8int cells have a high expression of the Tg TCR (measured by Vα2 density) and are bigger than CD4-8+ cells. They have presumably developed as a result of a selection signal from MHC class I, since this CD4-8int population is very small in OVA-TCR-I Tg mice on a nonselecting, H-2bm1, background (Fig. 2).

Table 1. CD4-8+ Thymocyte Formation by CD4-8int and CD4-8+ Cells from Class II MHC-deficient, OVA-TCR-I Tg, and Normal Mice

<table>
<thead>
<tr>
<th>Mice</th>
<th>Population transferred</th>
<th>CD4-8+ progeny per 10^6 cell transferred</th>
</tr>
</thead>
<tbody>
<tr>
<td>Class II deficient</td>
<td>CD4-8int</td>
<td>9,700</td>
</tr>
<tr>
<td></td>
<td>CD4-8+</td>
<td>400 ± 40</td>
</tr>
<tr>
<td>OVA-TCR-I Tg</td>
<td>CD4-8int</td>
<td>1,800 ± 800</td>
</tr>
<tr>
<td></td>
<td>CD4-8+</td>
<td>2,700 ± 700</td>
</tr>
<tr>
<td>Normal C57BL/6</td>
<td>CD4-8int</td>
<td>4,000 ± 2,100</td>
</tr>
<tr>
<td></td>
<td>CD4-8+</td>
<td>300 ± 100</td>
</tr>
</tbody>
</table>

CD4-8int and CD4-8+ cells from class II MHC-deficient, OVA-TCR-I Tg, and normal mice were injected intrathymically into Thy1.1 recipient mice. 2 d after transfer the injected thymus lobes were removed and most host-thymocytes depleted before staining with anti-Thy1.2, anti-CD4, anti-CD8, and anti-CD3 antibodies and analysis of the donor-derived population. The numbers are the means ± SD. SD is given where three or more values contributed to the mean.
The sorting gates used for isolating the CD4^+8^- and CD4^+8^+ populations are indicated in Fig. 3. The purity of the CD4^+8^- preparations was always >87% and the contaminating cells were entirely of the CD4^-8^- type. The CD4^+8^+ preparations were always >99% pure. The percent total recovery of donor thymocytes from the CD4^+8^- and CD4^+8^+ populations 2 d after intrathymic transfer ranged from 0.1 to 0.3% and 1.0 to 3.6%, respectively. As in the previous experiments with cells from MHC class II-deficient mice, the majority of the donor thymocytes recovered after transfer of the CD4^+8^- population differentiated into CD4^-8^- cells (72-78%), a small proportion had differentiated into CD4^-8^- cells (8-14%), and a few cells remained CD4^-8^- or had an intermediate phenotype (Fig. 3). The CD4^-8^- population also produced CD4^-8^- cells (6-32%), in accordance with the Tg specificity, although the majority (56-60%) still remained double positive after 2 d, in agreement with our earlier published transfer data (18).

The transfer experiments also gave some clues to the origin of the CD4^+8^- population of OVA-TCR-I Tg mice. Transfer of the CD4^-8^- population produced, as well as CD4^-8^- cells, a proportion of CD4^-8^- cells, <1% of CD4^-8^- cells, with the majority of recovered cells remaining CD4^-8^- (data not shown). Both these results were generally as expected, in contrast to the behavior of the CD4^-8^- population.

The transfer experiments also gave some clues to the origin of the CD4^-8^- population of OVA-TCR-I Tg mice. Transfer of the CD4^-8^- population produced, as well as CD4^-8^- cells, a proportion of CD4^-8^- cells, <1% of CD4^-8^- cells, with the majority of recovered cells remaining CD4^-8^- (data not shown). Both these results were generally as expected, in contrast to the behavior of the CD4^-8^- population.
Development of CD4⁺8⁻ and CD4⁺8⁺ Thymocytes Isolated from Normal Mice. To determine if the transition of CD4⁺8⁻ cells into CD4⁻8⁺ cells was a peculiarity of cells from MHC class II-deficient and OVA-TCR-I Tg mice, or if this occurred also in normal mice, the experiments were repeated using cells from normal C57BL/6 mice. The CD4⁺8⁻, CD4⁺8⁺, and CD4⁺8⁺ populations were isolated, using the sorting gates indicated in Fig. 4. The purity of the CD4⁺8⁻ preparation was always >85%, the contaminants being only CD4⁺8⁻ cells. The purity of the CD4⁺8⁺ preparation was always >99.5%. The CD4⁺8⁺ population was found to be a heterogeneous group, including both putative late intermediate thymocytes and immature single positives, as considered below in Fig. 5. Of these sorted populations 0.2-0.3 × 10⁶ CD4⁺8⁻, 2 × 10⁶ CD4⁺8⁺, and 0.2 × 10⁶ CD4⁺8⁺ cells were separately transferred to C57BL/Ka mice. The total recovery of donor cells 2 d later was ~2% from all populations. The CD4⁺8⁻ population again produced a high proportion (27-40%) of CD4⁻8⁺ cells, but now along with CD4⁺8⁻ cells (34-51%), the remaining 22-30% of cells were of intermediate phenotype (Fig. 4). The CD4⁺8⁺ population also produced CD4⁻8⁺ and CD4⁺8⁻ cells, but in much smaller yields (1 and 2%, respectively) and in the proportions characteristic of the normal thymus. On an absolute production per injected cell basis, the CD4⁺8⁻ population produced eight times more CD4⁻8⁺ cells than did the CD4⁺8⁺ population (Table 1).

The CD4⁺8⁺ population produced mainly CD4⁻8⁺ cells (58%), CD4⁺8⁻ cells (40%), and a few CD4⁺8⁻ cells (2%) (Fig. 4).

Comparison of the Surface Phenotype of CD4⁺8⁻ and CD4⁺8⁺ Thymocytes in Normal Mice. All the preceding results suggested an asymmetry in the development of CD4⁺8⁻ and CD4⁺8⁺ T cells, with both CD4⁺8⁻ and CD4⁺8⁺ cells having an orientation to CD4⁻8⁺ T cell production. This contrasted with the assumptions of the stochastic/selective model that these intermediates would be equivalent developmental stages but with opposite lineage orientation. The CD4⁺8⁻ TCR⁻ population from both normal and MHC class I-deficient mice has been shown to exhibit a phenotype which suggests it has been positively selected (13) or at least has received a primary selection signal (8). To determine if the CD4⁺8⁻ population showed equivalent characteristics, the size and CD3 expression of these two intermediate populations were compared to those of immature CD4⁺8⁻ and mature CD4⁺8⁻ and CD4⁻8⁺ thymocytes (Fig. 5).

Cells in the CD4⁺8⁻ region were a reasonably homogeneous group, larger than CD4⁺8⁺ small thymocytes but smaller than CD4⁺8⁻ mature thymocytes. In addition, their level of surface CD3 was higher than CD4⁺8⁺ thymocytes, lower than most CD4⁺8⁻ thymocytes, but almost the same as that of CD4⁺8⁻ thymocytes. In contrast, the cells in the CD4⁺8⁺ region formed two populations with regard to
Figure 5. CD4, CD8, and CD3 expression and cell size of different thymocyte populations. Total thymocytes from normal C57BL/6 mice were triple labeled with anti-CD4-PE, anti-CD8-FITC, and anti-CD3-biotin antibodies followed by TR-Av. The upper dot plot shows the thymocyte populations studied: CD4⁺8⁻ (R1), CD4⁺8⁺ (R3), CD4⁻8⁺ (R4), and CD4⁻8⁻ (R5). Left histograms compare the cell size and right histograms compare the CD3 expression of all populations, R1-R5.

size and CD3 expression. One population consisted of blast-sized cells with low surface CD3, corresponding in characteristics to the "immature single positives" that are known to be preselection cells that mature into CD4⁺8⁺ cortical thymocytes (27). The other CD4⁺8⁻ population did have properties suggestive of a postselection intermediate. The cells of this population were larger than CD4⁺8⁺ ones but slightly smaller than CD4⁻8⁻ mature thymocytes. Furthermore, their CD3 expression was at mature thymocyte levels, higher than on CD4⁺8⁺ and CD4⁻8⁻ ones, suggesting they were at a later stage of differentiation than both these subpopulations.

Correlation of TCR Expression with CD4 and CD8 Expression in OVA-TCR-I Tg Mice. The progeny cells found after intrathymic transfer of intermediate subpopulations pointed to an unexpected developmental sequence leading to CD4⁺8⁻ T cells, from CD4⁺8⁺ to CD4⁺8⁺ to CD4⁻8⁻. To check if this was likely to be a significant pathway and not just a feature of the transfer system, the entire thymocyte population was analyzed, using increasing levels of TCR expression as a marker of postselection developmental stage. This was carried out on the OVA-TCR-I Tg mice and using mAb recognizing the Vα2 transgene marker, to focus attention on the pathway leading to CD4⁻8⁺ T cells. Five different levels of Tg expression were analyzed for CD4 and CD8 status (Fig. 6). Cells expressing the lowest levels were mainly CD4⁺8⁺. Cells expressing increasing Tg levels up to the mature values formed a "boomerang" linking the CD4⁺8⁺ population, the CD4⁺8⁺ population, and the CD4⁻8⁻ population. Although this analysis provided no direct evidence of directional flow, it is entirely compatible with the pathway deduced from the transfer studies, and with the preceding analysis of the intermediate populations in normal mice.

Discussion

The identification of a CD4⁺8⁻TCR⁻ population in MHC class II-deficient mice and a corresponding, but less prominent, CD4⁺8⁺TCR⁻ population in MHC class I-deficient mice (8, 10-12), has been used as evidence for the stochastic/selective model of positive selection. The evidence is based on the assumption that an initial downregulation of either CD8 or CD4 indicates the commitment to the CD4⁻8⁻ or CD4⁺8⁺ lineage, respectively. Although this seems a logical assumption, the lack of direct evidence for such lineage commitment in these intermediate populations

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has been a weak point in the arguments for a stochastic/selective model. By using an intrathymic transfer procedure, we now examined the differentiation potential of both CD4\(^{+}\)8\(^{int}\) and CD4\(^{+}\)8\(^{+}\) thymocytes from three different murine systems, and found in all three cases that CD4\(^{+}\)8\(^{int}\) thymocytes can differentiate into CD4\(^{-}\)8\(^{+}\) cells. This indicates that a reduced expression of CD8 does not necessarily mean a commitment to the CD4 lineage. Further, the existence of CD4\(^{+}\)8\(^{int}\) cells in class II-deficient mice, as well as in class I-restricted TCR Tg mice, need no longer be attributed to a stochastic process, since it is a simple consequence of these cells being on a class I-restricted CD4\(^{-}\)8\(^{-}\) lineage pathway. A reciprocal pathway for generation of CD4\(^{-}\)8\(^{-}\) T cells does not seem to exist, since the transfer of CD4\(^{+}\)8\(^{+}\) thymocytes did not give rise to any significant numbers of CD4\(^{-}\)8\(^{-}\) cells, but did produce CD4\(^{-}\)8\(^{+}\) progeny. The comparison of cell size and CD3 expression of the two intermediate populations agreed with the data of Marodon and Rocha (15), and suggested that most cells of the CD4\(^{+}\)8\(^{-}\) population are more mature than cells of the CD4\(^{+}\)8\(^{+}\) population. It therefore seems likely that CD4\(^{+}\)8\(^{int}\) cells (excluding the CD4\(^{+}\)8\(^{+}\)8\(^{+}\)) immature single positive) are all late intermediates committed to the CD4\(^{-}\)8\(^{-}\) lineage.

The surprising differentiation of CD4\(^{+}\)8\(^{int}\) thymocytes into CD4\(^{-}\)8\(^{-}\) cells could not be attributed to CD4\(^{-}\)8\(^{+}\) contaminants, since transfer of sorted CD4\(^{-}\)8\(^{+}\) thymocytes gave a different pattern of progeny. In addition, when different CD4\(^{+}\)8\(^{int}\) populations were sorted and transferred from the same mice, the frequency of CD4\(^{-}\)8\(^{+}\) progeny formation decreased with the advance of the sorting gate towards the cells, a matter we have discussed in detail previously (18). The comparison of cell size and CD3 expression of the two intermediate populations agreed with the data of Marodon and Rocha (15), and suggested that most cells of the CD4\(^{+}\)8\(^{-}\) population are more mature than cells of the CD4\(^{+}\)8\(^{+}\) population. It therefore seems likely that CD4\(^{+}\)8\(^{int}\) cells (excluding the CD4\(^{+}\)8\(^{-}\)8\(^{-}\)) immature single positive) are all late intermediates committed to the CD4\(^{-}\)8\(^{-}\) lineage.

Not all cells in the CD4\(^{+}\)8\(^{int}\)TCR\(^{int}\) population are committed to the CD8 lineage. In a previous study (18) we showed that CD4\(^{-}\)8\(^{-}\) progeny are formed within 1–2 d of transferring CD4\(^{+}\)8\(^{+}\) thymocytes, whereas it takes 3–4 d to form CD4\(^{-}\)8\(^{-}\) progeny. This implies that complete downregulation of CD8 proceeds much faster than complete downregulation of CD4, and suggests that despite the larger total number of CD4\(^{-}\)8\(^{-}\) lineage thymocytes, fewer of them will be found in the form of these transient intermediates, complicating analysis of their development from CD4\(^{-}\)8\(^{+}\) cortical thymocytes. Nevertheless our transfer studies on CD4\(^{+}\)8\(^{int}\) TCR\(^{int}\) cells from normal mice indicated a production of both CD4\(^{-}\)8\(^{+}\) and CD4\(^{-}\)8\(^{-}\) progeny. This is in accord with the conclusions of Marodon and Rocha (5) who suggested that downregulation of CD8 and upregulation of TCR occur together, producing CD4\(^{+}\)8\(^{int}\)TCR\(^{int}\) intermediates en route to CD4\(^{-}\)8\(^{-}\) T cells. However, their model must now be modified since not all CD4\(^{+}\)8\(^{int}\)TCR\(^{int}\) thymocytes are on a pathway leading to CD4\(^{-}\)8\(^{-}\) T cells. The concept that both types of mature thymocytes are normally produced from these intermediates also fits with experiments showing that the frequency of CD4\(^{+}\)8\(^{int}\)TCR\(^{int}\) cells in class II–deficient mice is reduced by <50% compared to normal mice, whereas the frequency of CD4\(^{-}\)8\(^{+}\)TCR\(^{hi}\) cells in class I–deficient mice is only ~1% of normal mice (15). Overall, these data support the concept that the CD4\(^{+}\)8\(^{int}\) population in the normal mouse thymus consists of both CD4 and CD8 lineage committed cells, whereas the CD4\(^{+}\)8\(^{+}\) population contains only CD8 lineage committed cells.

Consistent with the above model, the production of CD4\(^{-}\)8\(^{-}\) lineage cells from CD4\(^{+}\)8\(^{int}\) intermediates of
normal mice was about half that of CD4$^8$ int intermediates from class II MHC-deficient mice, which should not contain class II MHC-restricted intermediates. The fact that CD4$^8$ cells from normal and class II MHC-deficient mice produced similar numbers of CD4$^8$ + mature cells is consistent with this population containing predominantly unselected cells. It was, however, surprising that CD4$^8$ int cells from OVA-TCR-I Tg mice produced mature CD4$^8$ + cells at a frequency similar to that of normal mice, and below that of the class II MHC-deficient mice. No obvious explanation can be offered for this lower than expected frequency, but it may relate to a higher proportion of the Tg population being negatively selected, as indicated by the approximately 10-fold lower recovery from CD4$^8$ int cells of OVA-TCR-I Tg mice, compared with normal and class II MHC-deficient mice.

Although our results may negate one of the main lines of evidence offered in support of the stochastic/selective model, the low cell recoveries in the transfer system make it difficult to test the basic theoretical differences between the models in any definitive way. The results are consistent with a multistep process in the development of CD4$^8$ + cells, as in the stochastic/selective model, but each of the steps could involve instructive signals. The “rescue” of cells with presumed mismatched TCRs and coreceptors by the introduction of a constitutively expressed coreceptor transgene (9, 31, 32) cannot easily be explained by an instructive interpretation of our data. However, the number of cells rescued by this method have so far been very low. Thus, it remains to be determined if these cells represent the majority of developing thymocytes, or a minority with some of the cells normally expressing “mismatched” TCR coreceptors (33, 34).

The nature of the transient intermediates between CD4$^8$ + cortical thymocytes and mature CD4$^8$ - and CD4$^8$ medullary thymocytes clearly provides some insights into the mechanism of positive selection. In this study the insights go beyond the issue of instructive versus stochastic/selective models. Normally, thymocyte development after the CD4$^8$ + stage is pictured as a symmetrical process, regardless of the positive selection model espoused, the CD4$^8$ - or CD4$^8$ + lineages developing in a unidirectional manner as mirror images of each other. In contrast, this study points to a basic asymmetry. It suggests that there may be more than one route even for cells committed to the same lineage. It also demonstrates, in agreement with our own and other earlier studies (18, 14), that the differentiation pathways leading to CD4$^8$ - and CD4$^8$ + T cells differ in the nature of the steps involved, as well as in the end product.

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