Stochastic Coreceptor Shut-off Is Restricted to the CD4 Lineage Maturation Pathway

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Summary

Kinetics of mature T cell generation in the thymus of normal or major histocompatibility complex (MHC) class I- or II-deficient mice were studied by the bromodeoxyuridine pulse labeling method. As previously described, the early activation and final maturation phases were found to be synchronous for the two T cell lineages, but CD4+8- cells were generated faster than CD4-8+ cells in MHC class I- and II-deficient mice, respectively. CD8 downregulation started on day 2 after cell proliferation even in the absence of MHC class II expression. CD8 downregulation thus appears to be stochastic at its beginning. By contrast, CD4 shut-off was found totally instructive, as the generation of CD4+8+ cells with a high TCR density was not observed in class I-deficient mice. The analysis of the Vβ14 TCR frequencies in CD4/8 subsets in normal and MHC-deficient mice confirmed that CD4 and CD8 generation pathways are not symmetrical. These findings show that commitment towards the CD4+8- or CD4-8+ phenotype is controlled at the CD81~ step for the former and at the CD4+8+ double-positive stage for the latter.

T cell selection in the thymus is controlled by the avidity of the TCR–peptide–MHC interaction, and therefore appears to be a globally instructive process (1–3). It is now well established that there is a high correlation between MHC recognition and single-positive cell generation. Indeed, CD4+8- cells are absent in MHC class II-deficient mice (4), whereas thymuses of mice that do not express β2-microglobulin (β2m) protein and therefore MHC class I proteins, contain very few mature CD4+8+ thymocytes (5–7). The mechanism by which CD4+8+ double-positive precursors are directed towards one of the two single-positive cell lineages is currently unclear. Two models have been proposed to explain this crucial step of thymic differentiation. The instructive model postulates that downregulation of one or the other coreceptors is strictly dependent on MHC restriction (8). The stochastic model proposes that coreceptor shut-off occurs stochastically on CD4+8+ thymocytes (5, 9). Experiments with TCR transgenic mice have been in favor of a strict instructive model for a long time (10). In contrast, the discovery of CD4+8+ and CD4+8+ thymocytes expressing high level of TCR-α/β in MHC class I- or II-deficient mice, respectively, supported the stochastic model predictions (4–7). Additional arguments concerning this problem came from experiments based on the forced expression of CD4 transgenes in class I-deficient mice (9, 11) or in mice bearing a MHC class II-restricted TCR (9). Symmetrically, class II-deficient mice (12) or MHC class I-restricted TCR transgenic mice (13–17) were rendered CD8 transgenic. If the stochastic predictions were true, in these mice, such coreceptor constitutive expression should allow the rescue of intermediate “stochastic” subsets that have downregulated the wrong coreceptor. Results obtained with this strategy were unclear: in some experiments no rescue was observed (13–15), and when it was obtained the rescue efficiency was low unless the coreceptor transgene was highly overexpressed (10, 12).

In all these approaches, kinetic experiments were missing. Furthermore, we and others (18–20) have previously observed in normal mice a 2-d lag between CD4+8- and CD4-8+ cell generation that is difficult to explain if coreceptor downregulation is totally stochastic. Other studies also predicted different postselection steps for the two lineage differentiation (21, 22). Using 5-bromo-2'-deoxyuridine (BrdUrd) as a kinetic marker (23), we investigated thymocyte lineage commitment in normal and MHC-deficient mice. We found that CD8 downregulation was at least partially stochastic whereas CD4 shut-off was totally instructive. Analysis of Vβ repertoire in normal mice compared to class I- and II-deficient mice confirmed these results.

Materials and Methods

Mice. Littermates as well as class I- (β2m−/−) (24) or II- (Δα+/+) (4) deficient mice were initially obtained from CSEAL (Orleans, France) and then bred in our own animal facilities. They were studied between 6 and 8 wk old.

BrdUrd Administration. Mice were pulsed on day 0 with two

Abbreviations used in this paper: BrdUrd, 5-bromo-2'-deoxyuridine; FSC, forward scatter; HSA, heat-stable antigen.
intraperitoneal injections of BrdUrd (1 mg each, 4 h apart). The thymus was taken at various times (from 1 to 7 d) after the second injection. Thymocytes were immediately suspended in ice-cold PBS containing 4% FCS and 0.2% sodium azide. In all mouse strains, the average total thymocyte number was 150 × 10^6. Thymuses containing fewer than 80 × 10^6 thymocytes were eliminated.

**Cell Surface Staining and BrdUrd Detection.** Thymocytes were distributed in 96-well round bottom microplates (10^6 cells/well; Greiner, Frickenhausen, Germany) and incubated first with 10 μl of biotinylated antibodies (anti-CD8, clone 53-6.7 [25]; anti-heat-stable antigen (HSA), clone J11D [26]; anti-CD69, clone H1.2F3 [27]; anti-Vα6, clone 44-22.1 [28]; anti-Vβ6.2, clone F23.2 [29]; anti-Vα14, clone 14-2 [30]) at optimal dilutions for 30 min at 4°C. The cells were washed and incubated with streptavidin conjugated to Tricolor (TC; CalTAG, San Francisco, CA) and with PE-conjugated antibodies (anti-CD4, clone GK1.5 [31] [Becton Dickinson Microbiology Sys., Cockeysville, MD] or anti-CD3, clone 500A2 [32] [PharMingen, San Diego, CA]). Double-stained cells were fixed in 200 μl of 1% paraformaldehyde containing 0.01% Tween 20 (PFAT; Sigma Chemical Co., St. Louis, MO) for 24–48 h at 4°C in the dark. Cells were washed in PBS and then in 40 mM Tris-HCl, pH 8.0, containing 10 mM NaCl and 6 mM MgCl₂ and incubated for 1 h at 37°C in the same buffer containing 50 Kunitz units of bovine pancreatic deoxyribonuclease I (Pharmacia, Uppsala, Sweden).

After a new wash in PBS, thymocytes were incubated in PBS containing 0.5% Tween 20 and anti-BrdUrd mAb (ascites fluid, clone 76/7 [33], a gift of T. Ternynck, Institut Pasteur, Paris) and with FITC-conjugated anti-mouse IgG1 (Southern Biotechnologies, Birmingham, NC). Both incubations were for 30 min at room temperature. A detailed evaluation of this technique has been published (23).

**Flow Cytometry.** Triple labeled cells were transferred into tubes containing PBS. Cells from three wells initially containing 10^6 cells stained in triplicate were often mixed to allow acquisition of very minor subsets. Acquisition of a minimum of 10,000 cells was done using a FACScan® flow cytometer (Becton Dickinson) appropriately set up for three-color fluorometry. Debris and aggregates were eliminated on the basis of forward and side cell scatter, with particular attention paid to preservation of thymoblasts. Surface stainings and cell scatter were virtually unmodified by the PFAT treatment.

**Results**

**Generation of CD4/8 Thymocyte Subsets in Normal and MHC-deficient Mice.** Thymocytes harvested at different times after BrdUrd injections were surface stained with PE-anti-CD8 and biotinylated anti-CD4 revealed by TC-streptavidin.

On the basis of CD4/CD8 fluorescence intensity, we defined six subsets (Fig. 1; top): CD4⁺8⁻, CD4⁺8ᵇ, CD4⁺8⁺, CD4ᵇ8⁺, CD4⁻8⁺, and CD4⁻8⁻ thymocytes.

Table 1 presents the absolute numbers of these subpopulations in MHC class I- and II-deficient mice compared to normal mice. In both strains of deficient mice, the thymus size was normal (mean = 150 × 10^6 thymocytes). In class II-deficient mice, the number of CD4⁺8⁻ cells represented <7% of the number found in littermates whereas CD4⁺8⁺ thymocytes represented >90% of the normal subset (Table 1).

In class I-deficient mice, the absolute number of CD4⁺8⁻ thymocytes was comparable to littermates (10.58 × 10^6 vs. 12.75 × 10^6 cells) whereas the absolute number of CD4⁺8⁺ subset was 40% lower. Furthermore, in these mice, very few CD4⁻8⁻ were found and the number of CD4ᵇ8⁺ was also diminished. Triple labeling of anti-TCR-α/β with anti-CD4 and anti-CD8 allowed us to calculate the absolute numbers of these subpopulations expressing intermediate or high level of the TCR-α/β, excluding immature CD4⁻8⁻ cells. It was found that CD4ᵇ8⁺ TCRhi thymocytes in class I-deficient mice were diminished by 93% in absolute number when compared to normal mice. On the contrary, in class II-deficient mice this subset was augmented and corresponded to 175% of that found in normal mice. In these mice, absolute number of CD4⁺8⁻ was also increased.

Using BrdUrd as a tracer of postcycling thymocytes in all three mouse strains, we investigated the CD4⁺8⁻ and CD4⁺8⁻ cell generation with a particular emphasis on CD4⁺8ᵇ and CD4ᵇ8⁺ intermediate subsets. On day 1 after BrdUrd injections, no significant difference could be noted in normal vs. MHC class I- or II-deficient mice: CD4⁺8⁻ thymocytes and immature CD4⁻8⁻ cells represented the great majority of BrdUrd⁺ cells (Fig. 1). On day 3, independently of the presence of MHC class II proteins, we observed the generation of a significant proportion of CD4⁺8ᵇ cells (Fig. 1, day 3). On day 5, in class I-deficient mice, as in littermates, >35% of labeled cells had a CD4⁺8⁻ phenotype whereas in class II-deficient mice, no or very few CD4⁺8⁻ thymocytes were detected. In class II-deficient mice as in normal mice, we observed CD4ᵇ8⁺ and CD4⁻8⁻ cell production. On the contrary, in class I-deficient mice very few cells with these phenotypes were detected (Fig. 1, day 5). On day 7, the majority of labeled cells corresponded to mature thymocytes expressing one or the other of the coreceptor CD4 or CD8. In MHC-deficient mice, only one of the two mature subsets was detected: CD4⁺8⁻ cells when MHC class II proteins were present and CD4⁻8⁻

**Table 1. Absolute Numbers of CD4/8 Thymic Subsets**

| CD4⁺8⁻ | CD4⁺8ᵇ | CD4⁺8⁺ | CD4ᵇ8⁺ | CD4⁻8⁻ | CD4⁻8⁺ |
|--------|--------|--------|--------|--------|--------|
| Littermate | 12.75 ± 0.74⁺ | 2.91 ± 0.14 | 119.85 ± 1.5 | 3.01 ± 0.28 | 5.86 ± 0.34 | 3.45 ± 0.3 |
| α⁺ | 0.94 ± 0.07 | 2.72 ± 0.28 | 128.07 ± 2.64 | 3.17 ± 0.38 | 8.40 ± 1.23 | 2.90 ± 0.24 |
| β⁻/⁻ | 10.58 ± 0.58 | 1.66 ± 0.10 | 129.80 ± 0.87 | 1.5 ± 0.09 | 0.69 ± 0.07 | 4.00 ± 0.24 |

⁺ Cell number: × 10⁻⁶ per thymus.
cells in the other case, confirming that the final result of thymocyte differentiation followed an instructive law. Furthermore, at this time, the majority of cells with an intermediate phenotype had disappeared.

To investigate more precisely the different steps of T cell lineage commitment, we calculated absolute numbers of the different CD4/8 subsets to study their generation and disappearance with time.

**Kinetics of CD4 Lineage Maturation Steps.** In all strains of mice, CD4⁺8⁺ thymocyte generation was observed from day 2 and this production reached a maximum on day 3 (Fig. 2). As on day 1 and 2, almost all BrdUrd⁺ thymocytes were CD4⁺8⁺ cells, CD4⁺8⁺ cells had to derive from CD4⁺8⁺ thymocytes by CD8 downregulation. Furthermore, these cells were absent (5) and their generation not observed using the BrdUrd kinetic technique in double MHC-deficient mice (data not shown) demonstrating that a TCR–MHC interaction was involved in their generation. Nevertheless, CD4⁺8⁺ cell generation was independent of the nature (class I or II) of MHC proteins present, suggesting that this intermediate cell production did not result from an instructive signal. Furthermore, at the peak on day 3 and compared to littermates, CD4⁺8⁺ thymocyte generation was slightly decreased in class II-deficient mice (0.98 × 10⁶ vs. 1.16 × 10⁶) and this decrease was strikingly more pronounced in class I-deficient mice (0.52 × 10⁶ vs. 1.16 × 10⁶).

In normal and class 1-deficient mice, absolute number of CD4⁺8⁻ thymocytes increased from day 2, reached a maximum on day 5 (around 1.9 × 10⁶), and then decreased (Fig. 2). In both strains, CD4⁺8⁻ cell production was quantitatively and kinetically comparable. Therefore, the reduced production of CD4⁺8⁻ cells in class 1-deficient mice did not result in a reduction of CD4⁺8⁻ cell production. Furthermore, the CD4⁺8⁻ intermediate subset was almost normally
produced in the absence of MHC class II proteins suggesting that the difference observed between normal and class I-deficient mice reflected a MHC class I-dependent production of CD4+8^b thymocytes in littersmates. In class II-deficient mice very few CD4+8^- thymocytes were produced; most of these cells in fact expressed low amounts of the CD8 protein (Fig. 1; days 3, 5, and 7) and disappeared at the same time as CD4+8^b cells, i.e., before the peak of CD4+8^- cell production in normal and class I-deficient mice (Fig. 2). These results suggest that the class I-restricted CD4+8^b thymocytes do not give rise to CD4+8^- cells. It is also noteworthy that CD4+8^b and CD4+8^- cell production were in fact simultaneous, without any real lag (Fig. 2). This observation suggests that CD4+8^b thymocytes with MHC class II-restricted TCRs very rapidly became CD4+8^- and represented in fact a transit cell type in continuous phenotypic transition.

The kinetic study of the CD4 lineage showed that CD8 downregulation started independently of TCR restriction whereas CD4+8^- cell generation was MHC class I dependent.

**CD8 Lineage Kinetics.** The differentiation of thymocyte precursors (CD4^-8^- CD3^-) into CD4+8^- CD3^b immature cells is achieved through sequential acquisition of CD8 and then CD4 (18). CD4+8^- and CD4+8^b cells therefore contain many immature transitional subsets. To exclude these immature cells, the analysis of BrdUrd+ cells was restricted to CD3^int/hi cells by staining with PE-anti-CD3 and either biotinylated anti-CD4 or CD8 or CD4 with CD8 and then by setting the gate for CD3^int/hi cells at the inflection point of three CD3 fluorescence intensity histograms (19). The percentage of each CD4/8 subset among CD3^int/hi thymocytes was determined by subtraction considering that CD4^b CD3^int/hi corresponded to the intermediate subset between CD4+8^- and mature CD4+8^- cells. Such a method applied to all CD3^int/hi cells gave results similar to those obtained by triple labeling using anti-TCR, anti-CD4, and anti-CD8 with a gate on TCR^int/hi cells. In addition, this method allowed the determination of the absolute number of CD4+8^- CD3^int/hi cells.

In class II-deficient mice as well as in littersmates, CD4+8+ CD3^int/hi cells were generated from day 3 to 5 after DNA synthesis and the absolute number of this intermediate subset rapidly decreased thereafter (Fig. 3 B). CD4+8^b CD3^int/hi thymocytes appeared after day 4 and their absolute number reached a maximum on day 6. So, comparing CD4+8^- cell generation in class I-deficient mice and CD4+8^- cell production in Class II-deficient mice, we verified the 2-d delay between CD4+8^- and CD4+8^- thymocyte generation already observed in normal mice (18, 19). On the contrary, in class I-deficient mice, no significant production of the CD4+8^- or the CD4+8^b subpopulations was observed. These results suggested that in opposition to CD8 downregulation, CD4 shut-off was completely instructive (MHC class I dependent). Furthermore, in class II-deficient mice, CD4+8^- and CD4+8^- cell production was increased compared to littersmates. This high positive selection of CD8 lineage cells in class II-deficient mice might be due to CD4, CD8, and TCR overexpression in these mice (5, 22).

Absolute number of BrdUrd+ CD4+8^- CD3^int/hi cells continually decreased from day 1 to 7 in all strains of mice (Fig. 3 A). The absolute numbers obtained in class II-deficient mice were greater than those obtained in littersmates that were again greater than those obtained in class I-deficient mice. Furthermore, in class I-deficient mice, 5.8 x 10^5 BrdUrd+ CD4+8^- CD3^int/hi thymocytes were found on day 3 whereas CD4+8^- cell generation seemed to be finished (Fig. 2) and in the absence of CD4^-/8^- cell production. These CD4+8^- cells therefore appear to be class II-restricted cells which have upregulated TCR while keeping a high CD8 expression and probably represent a dead-end subset without progeny. In fact, these class II-restricted cells seemed to be equivalent to class I-restricted CD4+8^- thymocytes observed in class II-deficient mice and certainly present in normal mice. These double-positive thymocytes were most probably present in normal mice but masked by CD4+8^- class I-restricted cells, precursors of the CD8 lineage.

**Early Activation and Final Maturation in Normal and MHC-deficient Mice.** By using the BrdUrd kinetic technique, we have previously shown that in normal mice the maturation
Figure 3. Absolute numbers of CD4*8*, CD4*8+, and CD4*8* CD3int/hi thymocytes in the progeny of cycling cells. Thymocytes were harvested on days 1–7 after BrdUrd injections and stained with PE-anti-CD3 and with biotinylated anti-CD4 or anti-CD8 or anti-CD4*CD8. Biotinylated Abs were revealed with TC-streptavidin. Analysis was restricted to BrdUrd* CD3int/hi cells and percentages of the different CD8 subsets determined by subtraction. Absolute numbers were then calculated by multiplying the percentage of each subset among CD3int/hi cells by the percentage of BrdUrd+ cells in CD3int/hi thymocytes and by the number of total CD3int/hi cells per thymus.

of newly produced TCRint/hi proceeded in three phases (19); cell activation, coreceptor downregulation, and then final maturation with downregulation of HSA correlated with the high expression of Qa-2, H2-K, and CD45RB. As opposed to single-positive cell generation that showed a delay between CD4*8* and CD4*8* subsets, cell activation and final maturation appeared synchronous for all CD3int/hi cells. It was interesting to compare these data with those obtained in MHC-deficient mice.

Thymocytes from normal and MHC-deficient mice were harvested at different times after BrdUrd injections and were surface stained with PE-anti-CD3 and biotinylated anti-HSA (Fig. 4, A and B) or anti-CD69 (Fig. 4 C). The analysis was restricted to CD3int/hi BrdUrd+ thymocytes by setting the gate for CD3int/hi cells at the inflection point of the CD3 fluorescence intensity histogram (19).

Blast-like cells were found in HSA+ cells on day 1 (Fig. 4 A) and the kinetics of CD69 expression were similar in all three mouse strains (Fig. 4 C). These results suggest that the early activation step preceding double-positive/single-positive cell transition was common to CD4 and CD8 single-positive cell lineage. Furthermore, in MHC double-deficient mice the few CD3int/hi cells observed among BrdUrd+ thymocytes on day 1 or 2 after BrdUrd incorporation were all CD69+ (data not shown), demonstrating that a MHC–TCR interaction was required for CD69 overexpression.

To investigate final maturation, we studied the loss of surface HSA in all strains of mice. In all cases, it started after day 4 and was more pronounced between days 5 and 6 (Fig. 4 B). Although we have demonstrated that there was a 2-d lag between CD4 and CD8 thymocyte generation, final maturation was synchronous for the two lineages and occurred at least 2 d after CD4 cell production and approximately at the same time as CD8 cell generation.

Analysis of Vb Repertoire as a Function of CD4/8 Expression in Normal and MHC-deficient Mice. Thymocytes were triple-labeled with PE-anti-CD4, FITC-anti-CD8, and biotinylated anti-TCR or biotinylated anti-Vb14. Vb6 and Vb8.2
frequencies were also estimated. The CD4/8 subsets were defined as shown in Fig. 1. In each subpopulation, the representation of each Vβ was estimated by dividing the percentage of specific Vβ-expressing cells by the percentage of TCRhi thymocytes.

As shown in Table 2, the Vβ6 frequency was the same in all four CD4/8 analyzed subsets. The most interesting results were obtained for Vβ14 frequencies. In normal mice, we noted a strong difference between CD4+8- vs. CD4+8+ thymocytes (8.4 ± 0.3 vs. 2.3 ± 0.1) and in CD4+8- cells, Vβ14+ thymocytes were half as frequent as in the CD4+8+ subpopulation (4.9 ± 0.4 vs. 8.4 ± 0.3). In class I-deficient mice, Vβ14 frequencies in CD4+8- and CD4+8+ thymocytes were identical (8.4 vs. 8.7), suggesting that, in normal mice,

Table 2. Analysis of Thymocyte Vβ Repertoire as a Function of CD4/8 Expression

| Vβ       | CD4+8- | CD4+8+ | CD4+8- | CD4+8+ |
|----------|--------|--------|--------|--------|
| Littermate | 11.0 ± 0.5 | 12.8 ± 0.8 | 12.8 ± 0.4 | 12.8 ± 0.4 |
| Aβ6+/-   | -      | 13.2 ± 0.4 | 15.6 ± 0.3 | 13.4 ± 0.5 |
| β2m--/-  | 12.2   | 14.6    | 10.15   | -      |
| Vβ8.2    |        |         |         |        |
| Littermate | 12.0 ± 0.4 | 9.8 ± 0.2 | 8.7 ± 0.5 | 8.4 ± 0.2 |
| Aβ8+/-   | -      | 8.7 ± 0.2 | 9.3 ± 0.2 | 8.6 ± 0.2 |
| β2m--/-  | 12.6   | 13.3    | 10.2    | -      |
| Vβ14     |        |         |         |        |
| Littermate | 8.4 ± 0.3 | 4.9 ± 0.4 | 2.5 ± 0.3 | 2.3 ± 0.1 |
| Aβ14+/-  | -      | 3.3 ± 0.2 | 3.3 ± 0.3 | 3.4 ± 0.1 |
| β2m--/-  | 8.7 ± 0.4 | 8.4 ± 1.2 | 9.1 ± 0.2 | -      |

Figure 4. Cell size and phenotypic evolution of recent CD3int/hi thymocytes. Thymocytes were harvested on days 1–7 after BrdUrd injections and stained with biotinylated anti-HSA (A or C), or anti-CD69 (B). Biotinylated Abs were revealed with TC-streptavidin. Surface-stained cells were then submitted to BrdUrd detection. Analysis of the markers listed above was restricted to BrdUrd+ CD3int/hi cells. (A) FSC/HSA fluorescence dot plots of day 1 BrdUrd+ CD3int/hi. (B) Evolution of CD69 (B) and HSA (C) expression by BrdUrd+ CD3int/hi thymocytes in normal and MHC-deficient mice.
the low $V_{\beta}14$ percentage in CD4$^+$$8^+$ subset was due to the presence of class I-restricted thymocytes. Indeed, in class II-deficient mice, CD4$^+$$8^+$ class I-restricted cells had the same low $V_{\beta}14$ representation as CD4$^+$$8^-$ cells (3.3 ± 0.3). The results obtained with $V_{\beta}8.2$ were similar but less significant because the frequency difference between CD4$^+$$8^-$ and CD4$^+$$8^+$ subsets was too small.

In contrast, in normal mice as well as in class II-deficient mice, CD4$^+$$8^-$ and CD4$^+$$8^+$ subpopulations presented identical $V_{\beta}14$ frequency (2.5 ± 0.3 vs. 2.3 ± 0.1 and 3.3 ± 0.3 vs. 3.4 ± 0.1). These results suggested that none or very few class II-restricted cells were present in the CD4$^+$$8^+$ subset of normal mice. Nevertheless, such cells existed and we have shown their existence in class I-deficient mice: they represented <7% of the absolute number of these cells obtained in normal mice and their $V_{\beta}14$ frequency was similar to that found in CD4$^+$$8^-$ cells (9.1 ± 0.2 vs. 8.7 ± 0.4). In fact, class II-restricted CD4$^+$$8^+$ thymocytes were too few to be detected in class I-deficient mice and did not alter the $V_{\beta}$ repertoire in normal mice. Their production could be compared to that of CD4$^+$$8^-$ thymocytes in class II-deficient mice but certainly not with class I-restricted CD4$^+$$8^+$ cells in normal and class II-deficient mice.

Analysis of thymocyte $V_{\beta}$ repertoire as a function of CD4/8 expression confirms results obtained with the BrdUrd kinetic technique: in normal mice, a large proportion of CD4$^+$$8^+$ cells were class I restricted whereas a nonsignificant percentage of CD4$^+$$8^+$ thymocytes were class II restricted confirming the idea that only CD8 downregulation followed a stochastic law.

Discussion

Using BrdUrd as a kinetic marker in normal and MHC-deficient mice has allowed a study of thymocyte commitment with a particular emphasis on cell subsets in transitions between CD4$^+$$8^-$ immature mature cells and mature single-positive thymocytes. We found that CD4$^+$$8^+$ cells were produced in comparable numbers and with the same kinetics in normal and class II-deficient mice. These cells, absent in cycling thymocytes and 24 h after DNA synthesis appeared on day 2, i.e., after CD4$^+$$8^+$ cells from which they are undeniably derived. A part of CD4$^+$$8^+$ cells were class I restricted, because their number was lower in class I-deficient mice, in which they also showed the same generation kinetics. Analysis of the $V_{\beta}$ repertoire confirmed these results: in the absence of MHC class I proteins, $V_{\beta}14$ frequency was identical in CD4$^+$$8^-$ and CD4$^+$$8^+$ subsets (∼8.5%) whereas in MHC class II-deficient mice, we found the same percentage of $V_{\beta}14$ among CD4$^+$$8^+$ and CD4$^+$$8^-$ subpopulations (∼3%). In normal mice $V_{\beta}14$ frequency in CD4$^+$$8^+$ was intermediate (4.9% ± 0.4). These results suggest that in normal mice the CD4$^+$$8^+$ subpopulation is a mixture of cells with class I- and II-restricted TCRs. In the present paper, we have shown that CD4$^+$$8^+$ thymocytes underwent CD8 downregulation, stochastically, i.e., independently of TCR–MHC class II interaction. Nevertheless, very few CD4$^+$$8^-$ cells were detected in class II-deficient mice. Moreover, $V_{\beta}14$ frequency among the CD4$^+$$8^-$ subset was found to be similar in normal and class I-deficient mice. Thus, the completion of CD8 shut-off seems to be restricted to class II-reactive cells and therefore is instructive. Nevertheless, a few class I-restricted CD4$^+$$8^+$ thymocytes were present in class II-deficient and normal mice (Fig. 1; Table 1) (5, 21, 34, 35); these cells were 20-fold less frequent than class II-restricted CD4$^+$$8^+$ cells. Chan et al. (5) have predicted that a second TCR–MHC engagement of CD4$^+$$8^+$ cells is necessary for CD4$^+$$8^-$ cell generation and is responsible for their class II restriction. The existence of two well defined successive steps is not supported by our kinetic data. CD4$^+$$8^-$ and CD4$^+$$8^+$ cell generation started simultaneously on day 2, suggesting that class II-restricted CD4$^+$$8^+$ thymocytes very rapidly finished downregulating CD8 to generate CD4$^+$$8^-$ cells and thus did not represent a stable stage of cells waiting for a second signal. A prolonged encounter with MHC rather than two separate signals is necessary to explain our kinetic results.

We can estimate the relative representation of class II- and I-restricted cells in the CD4$^+$$8^+$ subset. From $V_{\beta}14$ frequencies (4.9 in CD4$^+$$8^+$ vs. 8.4 in CD4$^+$$8^-$ and 2.3 in CD4$^+$$8^-$), we calculate 45% class II and 55% class I-restricted cells. These percentages are close to those obtained when we calculate the ratio of CD4$^+$$8^+$ cells produced in normal and in class I-deficient mice. There are two difficulties, however. First, it clearly appears on kinetic curves (Fig. 2) that class II-restricted cells do not stop at the CD4$^+$$8^+$ stage as opposed to class I-restricted ones, giving an overrepresentation of the class I-restricted CD4$^+$$8^+$ cells compared to their real production. Second, TCR, CD4, and CD8 densities are artificially augmented in class II-deficient mice, which also show an overproduction of CD4$^+$$8^+$ cells and so certainly of CD4$^+$$8^+$ thymocytes (4, 21).

What is the fate of the class I-restricted CD4$^+$$8^+$ subset? The most likely hypothesis proposes that these cells represent a dead-end subpopulation without progeny. Indeed, these cells seemed to be engaged to the CD4 lineage pathway as in class II-deficient mice, it was possible to rescue them adding a constitutive CD8 coreceptor expression (12) or after TCR ligation (36). We propose that CD8 shut-off in class I-restricted CD4$^+$$8^+$ thymocytes destabilized TCR–MHC class I interaction and resulted in the developmental arrest of these cells that died at the CD4$^+$$8^+$ stage. Nevertheless, the possibility that a small number of the class I-restricted CD4$^+$$8^+$ thymocytes could return to the CD4$^+$$8^+$ stage and then give rise to CD4$^+$$8^-$ cells can not be formally excluded.

In contrast to CD8 shut-off, CD4 downregulation seemed to be totally instructive i.e., dependent on a TCR–MHC class I interaction. Indeed, in class I-deficient mice, no significant CD4$^+$$8^-$ and CD4$^+$$8^+$ cell generation was observed. Class II-restricted CD4$^+$$8^+$ TCR$^+$ thymocytes have been described (5, 6, 21), but if such cells were present in a normal thymus, their proportion did not exceed that of class I-restricted cells among the CD4$^+$$8^-$ subpopulation (<7%). Their proportions in normal mice were too low to significantly change the $V_{\beta}14$ frequency among CD4$^+$$8^+$ cells in normal versus class II-deficient mice. Van Meerwijk et al. (6) as well
as Crump et al. (7) described a CD4$^{b8^+}$ TCR$^{int}$ subset in normal and class I-deficient mice. Postulating that these cells were the precursors of CD4$^{-8^+}$ mature thymocytes, they concluded that CD4 shut-off was stochastic. Another possibility is that these cells are not engaged in the CD8 maturation lineage pathway. Indeed, in our previous paper, we showed that CD4 shut-off only occurred on CD4$^{+8^+}$ cells that already expressed maximal TCR density (19). Marodon et al. (22) also observed that the upregulation of TCR preceded the downregulation of the CD4. Furthermore, Petrie et al. (21) have shown that purified CD4$^{+8^+}$ TCR$^{hi}$ thymocytes gave rise in vitro to CD4$^{-8^+}$ TCR$^{hi}$ cells only. Swat et al. (37) found similar results. Lundberg and Shortman (38) postulated that the CD4$^{-8^+}$ lineage cells spent several days in transit as CD4$^{+8^+}$ 3$^{bi}$ intermediates before losing CD4. All these results suggest that only CD4$^{b8^+}$ TCR$^{hi}$ cells, prog-
enemy of CD4+8+ TCRhi thymocytes are the precursors of CD4+8+ TCRhi mature cells. Experiments are currently under way in our laboratory to characterize the CD4+8hi TCRint thymocytes with more details (Lucas, B., G. Marodon, and C. Penit, manuscript in preparation). Nevertheless, the CD8-committed, class II-restricted cells equivalent to the class I-restricted CD4+8§ TCRhi thymocytes are the precursors of class II-restricted CD4+8+ TCRint mature cells. These double-positive thymocytes seemed to be arrested and to disappear at this stage. Furthermore, we and others (18-20) have previously shown a 2-3 day lag between CD4+8hi and CD4+8§ cell generation. CD4+8hi cell production also preceded CD4+8§ cell generation by 2 d. Then, kinetically, CD4+8§ TCRhi rather than CD4+8b cells were the developmental equivalent of CD4+8hi thymocytes. Furthermore, this conclusion is also supported by the recent report of Linette et al. (39) who showed that Bcl-2 overexpression at the CD4+8+ stage allowed the rescue of CD4+8hi thymocytes in class I-deficient mice. Therefore, it was logical to postulate that in class I-deficient mice CD4+8§ TCRhi cells observed after day 3 represented class II-restricted CD8-committed thymocytes, the stochastic step in CD8 lineage commitment occurring before CD4 downregulation.

It remains to be explained why, in class I-deficient mice, these cell II-restricted CD4+8§ TCRhi cells do not enter the CD4 lineage. Baron et al. (11) as well as Davis et al. (9) have shown that class II-restricted CD4+8hi cells rescued in class I-deficient mice had a cytolytic activity. More recently, Corbella et al. (17) demonstrated that class I-restricted CD4+8hi TCRint cell differentiation towards CD4+8+ TCRhi cells occurred after day 3 represented class II-restricted CD8-committed thymocytes, the stochastic step in CD8 lineage commitment occurring before CD4 downregulation.

From the data presented in this and in previous papers (18, 19) we propose that T cell lineage commitment proceeds in three steps, as depicted in Fig. 5: phase I: MHC-TCR interaction-dependent activation phase preceding stochastic CD4+8hi TCRint cell differentiation towards CD4+8hi or CD4+8§ TCRbhi thymocytes (this step is accompanied by CD69 overexpression and final TCR upregulation); phase II: instructive CD4+8hi/CD4+8§ or CD4+8hi/CD4+8hi transitions, giving rise to single-positive cells, still in an immature state (HSAhi); and phase III: final maturation, with downregulation of HSA and maturational marker upregulations. This model represents the main pathways of single-positive cell generation deduced from the data presented. It can not be excluded that some class I-restricted CD4+8hi cells or class II-restricted TCRhi CD4+8hi thymocytes could mature to CD4+8hi or CD4+8§ single-positive cells, respectively.

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