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 $CD4^{lo}8^+$ cells with a high TCR density was not observed in class I–deficient mice. The analysis of the $V_\beta 14$ 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 $CD8^{lo}$ 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 β_2-microglobulin (β_2m) 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 $CD4^{lo}8^+$ and $CD4^+8^{lo}$ 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 "stochastive" 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)[1] 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 V_β 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– ($\beta_{2m}{}^{-/-}$) (24) or II– ($A_\beta{}^{o/o}$) (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

[1] *Abbreviations used in this paper:* BrdUrd, 5-bromo-2′-deoxyuridine; FSC, forward scatter; HSA, heat-stable antigen.

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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-$V_\beta6$, clone 44-22.1 [28]; anti-$V_\beta8.2$, clone F23.2 [29]; anti-$V_\beta14$, 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-$CD3_\epsilon$, 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 $MgCl_2$ 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 IgG_1 (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-CD4 and biotinylated anti-CD8 revealed by TC-streptavidin.

On the basis of CD4/CD8 fluorescence intensity, we defined six subsets (Fig. 1; *top*): $CD4^+8^-$, $CD4^+8^{lo}$, $CD4^+8^+$, $CD4^{lo}8^+$, $CD4^-8^+$, 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 $CD4^+8^{lo}$ 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 $CD4^+8^{lo}$ subset was 40% lower. Furthermore, in these mice, very few $CD4^-8^+$ were found and the number of $CD4^{lo}8^+$ 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 $CD4^{-/lo}8^+$ cells. It was found that $CD4^{lo}8^+$ TCR^{hi} 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 $CD4^+8^{lo}$ and $CD4^{lo}8^+$ 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 $CD4^{-/lo}8^+$ cells represented the great majority of $BrdUrd^+$ cells (Fig. 1) (18). On day 3, independently of the presence of MHC class II proteins, we observed the generation of a significant proportion of $CD4^+8^{lo}$ 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 $CD4^{lo}8^+$ 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*

	$CD4^+8^-$	$CD4^+8^{lo}$	$CD4^+8^+$	$CD4^{lo}8^+$	$CD4^-8^+$	$CD4^-8^-$
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
$A_\beta^{o/o}$	0.94 ± 0.07	2.72 ± 0.28	128.07 ± 2.64	3.17 ± 0.38	8.40 ± 1.23	2.90 ± 0.24
$\beta_{2m}^{-/-}$	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.

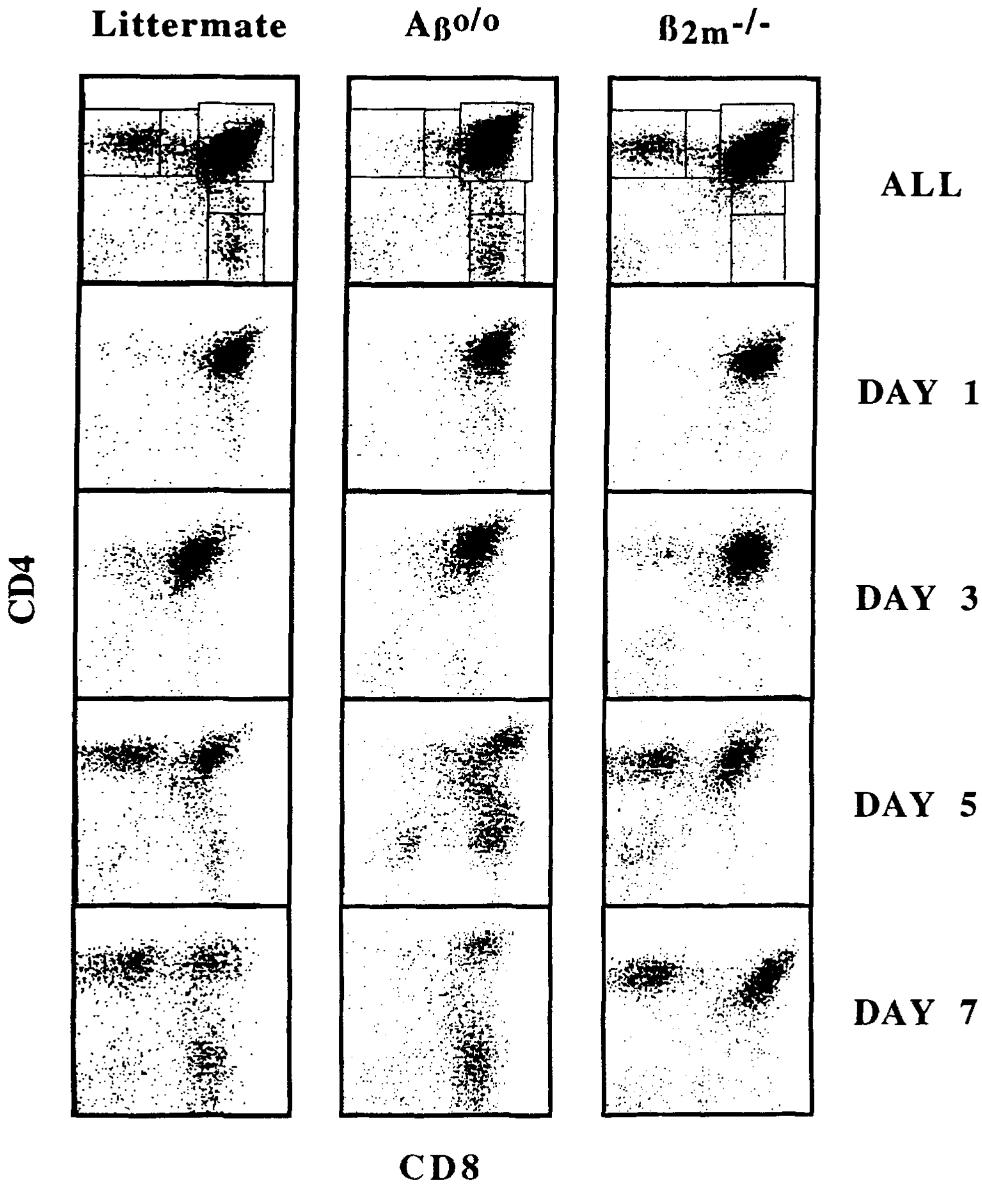

Figure 1. CD4/8 phenotypic evolution of recent (postcycling) thymocytes in normal and MHC-deficient mice. BrdUrd was injected twice on day 0 at a 4-h interval. Thymocytes were harvested on days 1–7, surface stained with PE–anti-CD4 and biotinylated anti-CD8 plus TC-streptavidin, and submitted to BrdUrd detection. CD4/8 fluorescence dot plots obtained for total (*top*) and for gated BrdUrd+ thymocytes (days 1, 3, 5, and 7) time points are presented. The five regions used for phenotype analysis are shown (*top*). The results expressed in absolute numbers are presented in Figs. 2 and 3.

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, $CD4^+8^{lo}$ 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, $CD4^+8^{lo}$ 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, $CD4^+8^{lo}$ 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, $CD4^+8^{lo}$ thymocyte generation was slightly decreased in class II–deficient mice (0.98×10^6 vs. 1.16×10^6) and this decrease was strikingly more pronounced in class I–deficient mice (0.52×10^6 vs. 1.16×10^6).

In normal and class I–deficient mice, absolute number of $CD4^+8^-$ thymocytes increased from day 2, reached a maximum on day 5 (around 1.9×10^6), and then decreased (Fig. 2). In both strains, $CD4^+8^-$ cell production was quantitatively and kinetically comparable. Therefore, the reduced production of $CD4^+8^{lo}$ cells in class I–deficient mice did not result in a reduction of $CD4^+8^-$ cell production. Furthermore, the $CD4^+8^{lo}$ intermediate subset was almost normally

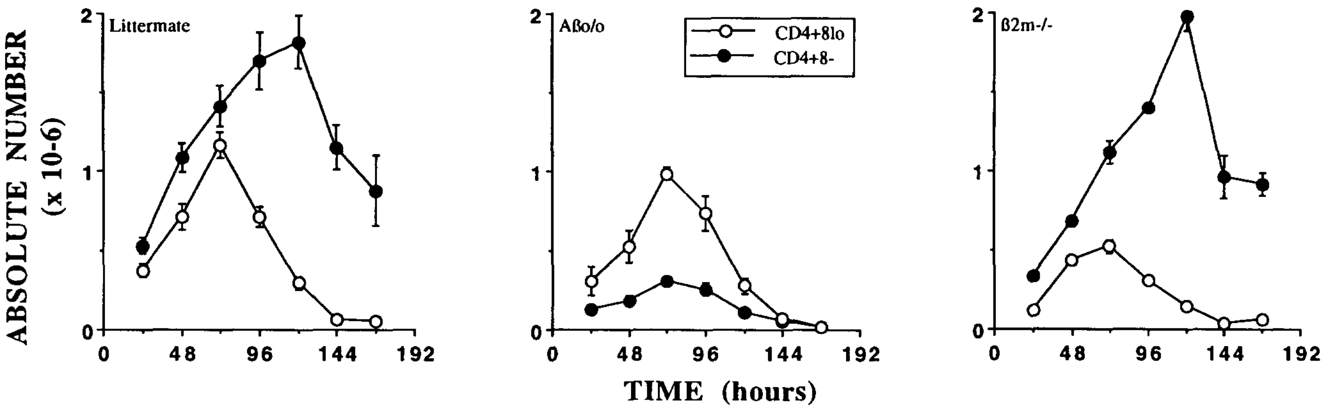

Figure 2. Generation of $CD4^+8^-$ and $CD4^+8^{lo}$ thymocytes in the progeny of cycling cells in normal and MHC-deficient mice. Absolute numbers of $BrdUrd^+$ $CD4^+8^-$ or $CD4^+8^{lo}$ were calculated each day. Calculations were done by multiplying the total number of each subpopulation by the percentage of labeled cells in each subset. These numbers are means of three to five independent determinations.

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 $CD4^+8^{lo}$ thymocytes in littermates. 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 $CD4^+8^{lo}$ 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 $CD4^+8^{lo}$ thymocytes do not give rise to $CD4^+8^-$ cells. It is also noteworthy that $CD4^+8^{lo}$ and $CD4^+8^-$ cell production were in fact simultaneous, without any real lag (Fig. 2). This observation suggests that $CD4^+8^{lo}$ 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 II dependent.

CD8 Lineage Kinetics. The differentiation of thymocyte precursors ($CD4^-8^-$ $CD3^-$) into $CD4^+8^+$ $CD3^{lo}$ immature cells is achieved through sequential acquisition of CD8 and then CD4 (18). $CD4^-8^+$ and $CD4^{lo}8^+$ 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 substraction considering that $CD4^{lo}$ $CD3^{int/hi}$ corresponded to the intermediate subset between $CD4^+8^+$ and mature $CD4^-8^+$. 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 littermates, $CD4^{lo}8^+$ $CD3^{int/hi}$ 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*). $CD4^-8^+$ $CD3^{int/hi}$ 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 $CD4^{lo}8^+$ 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, $CD4^{lo}8^+$ and $CD4^-8^+$ cell production was increased compared to littermates. 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 littermates that were again greater than those obtained in class I–deficient mice. Furthermore, in class I–deficient mice, 5.8×10^5 $BrdUrd^+$ $CD4^+8^+$ $CD3^{int/hi}$ thymocytes were found on day 3 whereas $CD4^+8^{lo}$ cell generation seemed to be finished (Fig. 2) and in the absence of $CD4^-/^{lo}8^+$ 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 $CD4^+8^{lo}$ 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

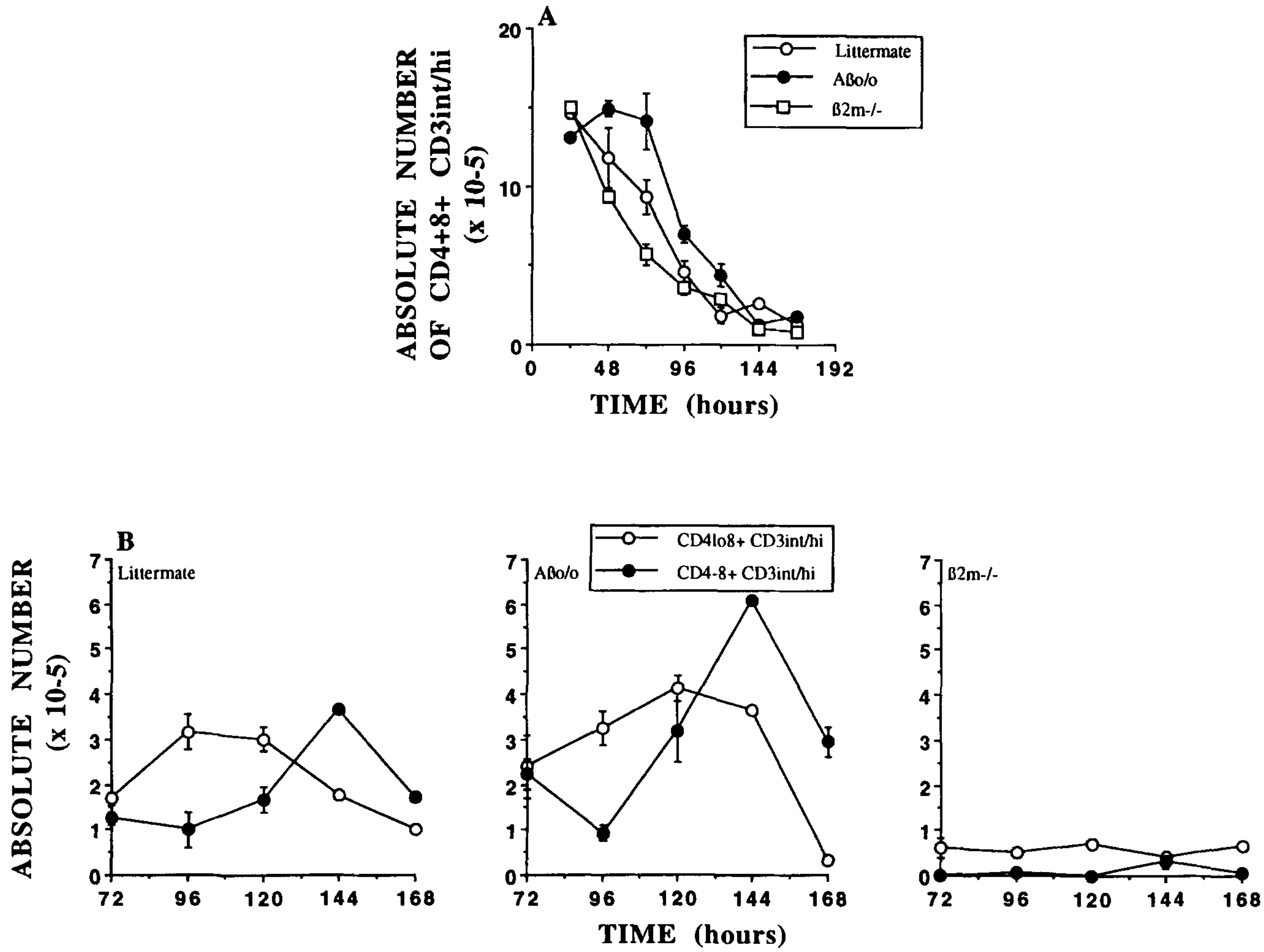

Figure 3. Absolute numbers of CD4+8+, CD4^{lo}8+, and CD4−8+ CD3$^{int/hi}$ thymocytes in the progeny of cycling cells. Thymocytes were harvested on days 1–7 after BrdUrd injections and stained with PE–anti-CD3 and with biotinylated anti-CD4 or anti-CD8 or anti-CD4+CD8. Biotinylated Abs were revelated with TC-streptavidin. Analysis was restricted to BrdUrd+ CD3$^{int/hi}$ cells and percentages of the different CD8+ subsets determined by subtraction. Absolute numbers were then calculated by multiplying the percentage of each subset among CD3$^{int/hi}$ cells by the percentage of BrdUrd+ cells in CD3$^{int/hi}$ thymocytes and by the number of total CD3$^{int/hi}$ cells per thymus.

of newly produced TCR$^{int/hi}$ 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 CD3$^{int/hi}$ 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 CD3$^{int/hi}$ BrdUrd$^+$ thymocytes by setting the gate for CD3$^{int/hi}$ cells at the inflection point of the CD3 fluorescence intensity histogram (19).

Blast-like cells were found in HSA^{hi} 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 CD3$^{int/hi}$ 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 V_β 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-V_β14. V_β6 and V_β8.2

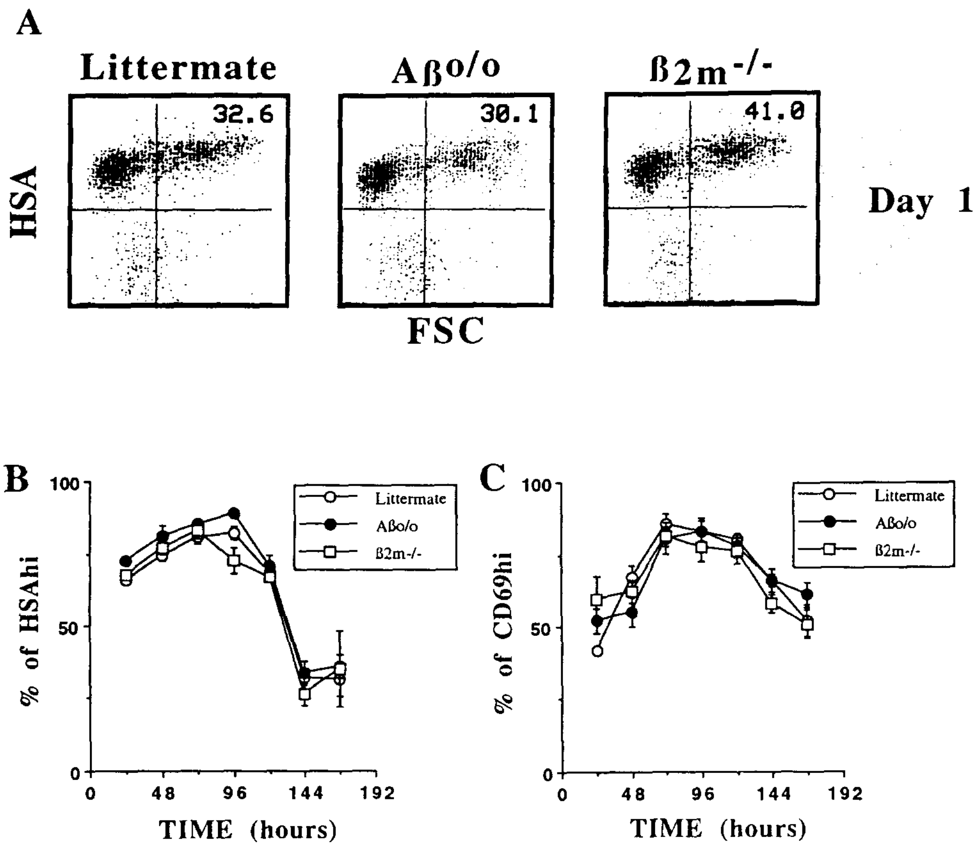

Figure 4. Cell size and phenotypic evolution of recent CD3$^{int/hi}$ thymocytes. Thymocytes were harvested on days 1–7 after BrdUrd injections and stained with biotinylated anti-HSA (*A* or *C*), or anti-CD69 (*B*). Biotinylated Abs were revealed with TC-streptavidin. Surface-stained cells were then submitted to BrdUrd detection. Analysis of the markers listed above was restricted to BrdUrd$^+$ CD3$^{int/hi}$ cells. (*A*) FSC/HSA fluorescence dot plots of day 1 BrdUrd$^+$ CD3$^{int/hi}$. (*B*) Evolution of CD69 (*B*) and HSA (*C*) expression by BrdUrd$^+$ CD3$^{int/hi}$ thymocytes in normal and MHC-deficient mice.

frequencies were also estimated. The CD4/8 subsets were defined as shown in Fig. 1. In each subpopulation, the representation of each V$_\beta$ was estimated by dividing the percentage of specific V$_\beta$-expressing cells by the percentage of TCR^{hi} thymocytes.

As shown in Table 2, the V$_\beta$6 frequency was the same in all four CD4/8 analyzed subsets. The most interesting results were obtained for V$_\beta$14 frequencies. In normal mice we noted a strong difference between CD4$^+$8$^-$ vs. CD4$^-$8$^+$ thymocytes (8.4 ± 0.3 vs. 2.3 ± 0.1) and in CD4$^+$8^{lo} cells, V$_\beta$14$^+$ 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, V$_\beta$14 frequencies in CD4$^+$8^{lo} and CD4$^+$8$^-$ 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*

	CD4$^+$8$^-$	CD4$^+$8^{lo}	CD4^{lo}8$^+$	CD4$^-$8$^+$
Vβ6				
Littermate	11.0 ± 0.5	12.8 ± 0.8	12.8 ± 0.4	12.8 ± 0.4
$A_\beta^{o/o}$	–	13.2 ± 0.4	15.6 ± 0.3	13.4 ± 0.5
$\beta_{2m}^{-/-}$	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
$A_\beta^{o/o}$	–	8.7 ± 0.2	9.3 ± 0.2	8.6 ± 0.2
$\beta_{2m}^{-/-}$	12.6	13.3	10.2	–
Vβ14				
Littermate	8.4 ± 0.3	4.9 ± 0.4	2.5 ± 0.3	2.3 ± 0.1
$A_\beta^{o/o}$	–	3.3 ± 0.2	3.3 ± 0.3	3.4 ± 0.1
$\beta_{2m}^{-/-}$	8.7 ± 0.4	8.4 ± 1.2	9.1 ± 0.2	–

the low $V_\beta14$ percentage in $CD4^+8^{lo}$ subset was due to the presence of class I–restricted thymocytes. Indeed, in class II–deficient mice, $CD4^+8^{lo}$ class I–restricted cells had the same low $V_\beta14$ representation as $CD4^-8^+$ cells (3.3 ± 0.3). The results obtained with $V_\beta8.2$ 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 $CD4^{lo}8^+$ subpopulations presented identical $V_\beta14$ 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 $CD4^{lo}8^+$ 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 $V_\beta14$ frequency was similar to that found in $CD4^+8^-$ cells (9.1 ± 0.2 vs. 8.7 ± 0.4). In fact, class II–restricted $CD4^{lo}8^+$ thymocytes were too few to be detected in class I–deficient mice and did not alter the V_β 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 $CD4^+8^{lo}$ cells in normal and class II–deficient mice.

Analysis of thymocyte V_β repertoire as a function of CD4/8 expression confirms results obtained with the BrdUrd kinetic technique: in normal mice, a large proportion of $CD4^+8^{lo}$ cells were class I restricted whereas a nonsignificant percentage of $CD4^{lo}8^+$ 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 cells and mature single-positive thymocytes. We found that $CD4^+8^{lo}$ 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 undoubtedly derived. A part of $CD4^+8^{lo}$ 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_β repertoire confirmed these results: in the absence of MHC class I proteins, $V_\beta14$ frequency was identical in $CD4^+8^-$ and $CD4^+8^{lo}$ subsets (~8.5%) whereas in MHC class II–deficient mice, we found the same percentage of $V_\beta14$ among $CD4^+8^{lo}$ and $CD4^-8^+$ subpopulations (~3%). In normal mice $V_\beta14$ frequency in $CD4^+8^{lo}$ was intermediate (4.9% ± 0.4). These results suggest that in normal mice the $CD4^+8^{lo}$ 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, $V_\beta14$ 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 $CD4^+8^{lo}$ 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 $CD4^+8^{lo}$ cell generation started simultaneously on day 2, suggesting that class II–restricted $CD4^+8^{lo}$ 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 $CD4^+8^{lo}$ subset. From $V_\beta14$ frequencies (4.9 in $CD4^+8^{lo}$ 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 $CD4^+8^{lo}$ 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 $CD4^+8^{lo}$ stage as opposed to class I–restricted ones, giving an overrepresentation of the class I–restricted $CD4^+8^{lo}$ 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 $CD4^+8^{lo}$ thymocytes (4, 21).

What is the fate of the class I–restricted $CD4^+8^{lo}$ 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 $CD4^+8^{lo}$ stage. Nevertheless, the possibility that a small number of the class I–restricted $CD4^+8^{lo}$ 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 $CD4^{lo}8^+$ and $CD4^-8^+$ cell generation was observed. Class II–restricted $CD4^{lo}8^+$ TCR^{hi} 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 $V_\beta14$ frequency among $CD4^{lo}8^+$ cells in normal versus class II–deficient mice. Van Meerwijk et al. (6) as well

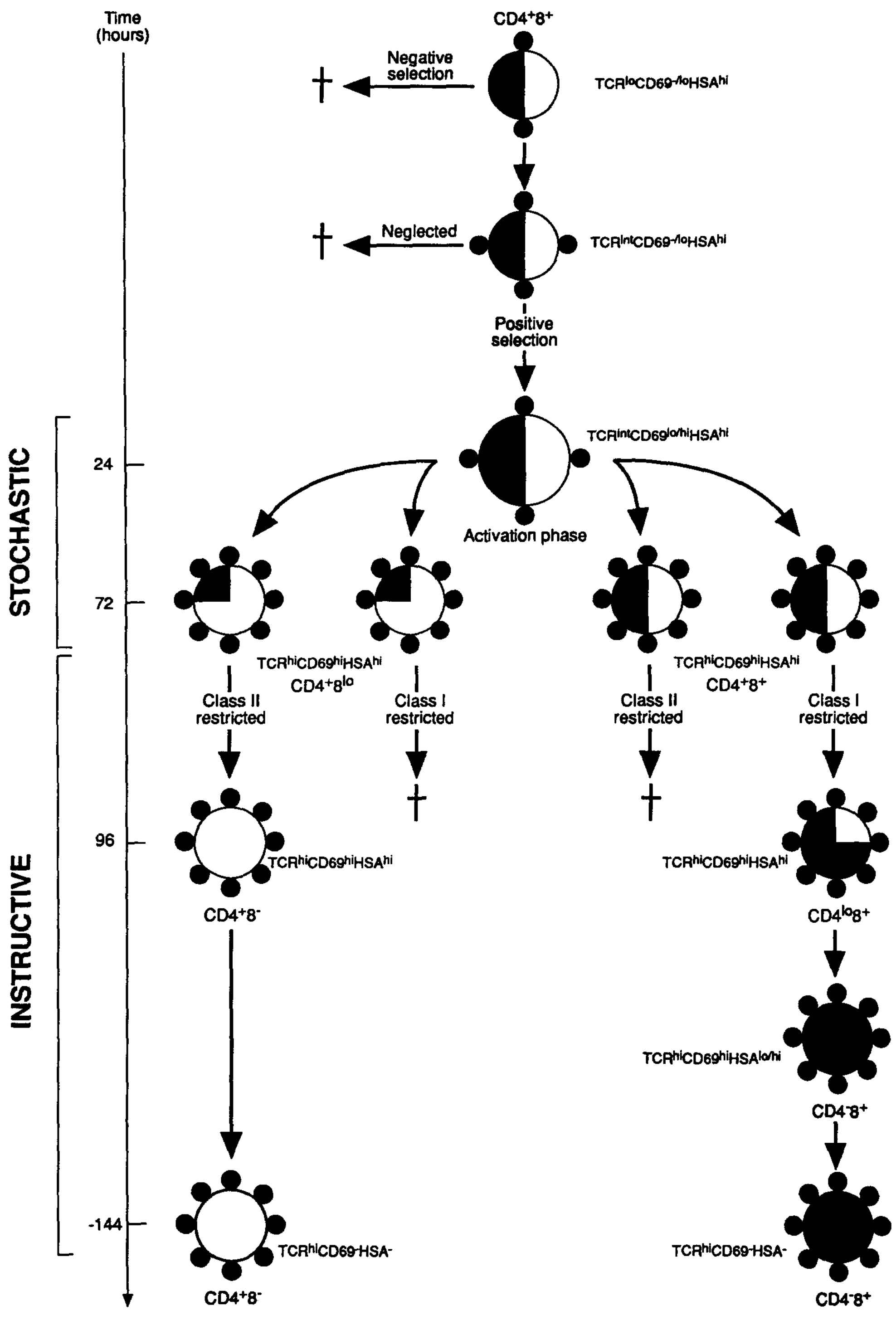

Figure 5. Kinetics of T cell commitment. Schematic representation of sequential events in CD4/8 cell commitment. Three ordered phases could be defined. Phase I corresponded to stochastic $CD4^+8^+$ TCR^{int} cell differentiation towards $CD4^+8^{lo}$ and $CD4^+8^+$ TCR^{hi} thymocytes; phase II to instructive $CD4^+8^{lo}/CD4^+8^-$ or $CD4^+8^+/CD4^+8^-$ transitions; and phase III to final maturation defined by HSA down-regulation.

as Crump et al. (7) described a $CD4^{lo}8^+$ 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 $CD4^+8^+3^{hi}$ intermediates before losing CD4. All these results suggest that only $CD4^{lo}8^+$ TCR^{hi} cells, progeny of CD4$^+$8$^+$ TCR^{hi} thymocytes are the precursors of CD4$^-$8$^+$ TCR^{hi} mature cells. Experiments are currently underway in our laboratory to characterize the CD4$^+$8^{lo} TCR^{int} thymocytes with more details (Lucas, B., G. Marodon, and C. Penit, manuscript in preparation). Nevertheless, the CD8-committed, class II–restricted cells equivalent to the class I–restricted CD4$^+$8^{lo} subset necessarily exist because they were rescued by introduction of a CD4 transgene in class I–deficient mice as well as in mice bearing a MHC class II–restricted TCR (10, 11). In class I–deficient mice, BrdUrd$^+$ CD4$^+$8$^+$ cells with high surface density of TCR were still present in significant numbers while CD4$^+$8^{lo} cell generation was terminated. These double-positive thymocytes seemed to be arrested and to disappear at this stage. Furthermore, we and others (18–20) have previously shown a 2-d lag between CD4$^+$8$^-$ and CD4$^-$8$^+$ cell generation. CD4$^+$8^{lo} cell production also preceded CD4^{lo}8$^+$ cell generation by 2 d. Then, kinetically, CD4$^+$8$^+$ TCR^{hi} rather than CD4^{lo}8$^+$ cells were the developmental equivalent of CD4$^+$8^{lo} thymocytes. Furthermore, this conclusion is also supported by the recent report of Linette et al. (39) who showed that Bcl-2 overexpression at the CD4$^+$8$^+$ stage allowed the rescue of CD4$^-$8$^+$ thymocytes in class I–deficient mice. Therefore, it was logical to postulate that in class I–deficient mice CD4$^+$8$^+$ TCR^{hi} 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 class II–restricted CD4$^+$8$^+$ TCR^{hi} 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$^-$8$^+$ cells rescued in class I–deficient mice had a cytolytic activity. More recently, Corbella et al. (17) demonstrated that class I–restricted CD4^{lo}8$^+$ intermediates were functionally committed. All these results suggest that T cell lineage commitment was functional as well as phenotypic. Perhaps, after functional commitment, a signal via the CD8 coreceptor was necessary to allow the continuation of thymic maturation. Recent papers have shown the importance of the β chain of CD8 (40, 41) and more precisely of its cytoplasmic tail in the CD4$^-$8$^+$-positive selection process (42). The α chain of CD8 was also necessary for CD4$^-$8$^+$ cell production (43, 44). Furthermore, in humans lacking Zap-70 kinase, Arpaia et al. (45) have shown the absence of CD4$^-$8$^+$ thymic selection whereas CD4$^+$8$^-$ cell generation was normal. It is possible that after stochastic functional commitment, a new signal involving Zap-70 and perhaps CD8 was necessary to allow CD4$^+$8$^+$ TCR^{hi} cells to shut-off CD4. Only class I–restricted CD4$^+$8$^+$ TCR^{hi} thymocytes would be able to receive this signal and therefore to mature whereas class II–restricted CD4$^+$8$^+$ TCR^{hi} cells that had been functionally committed could not return toward the CD4 lineage and could not mature to CD4$^-$8$^+$ cells and therefore died at this stage.

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$^+$8$^+$ TCR^{int} cell differentiation towards CD4$^+$8^{lo} or CD4$^+$8$^+$ TCR^{hi} thymocytes (this step is accompanied by CD69 overexpression and final TCR upregulation); phase II: instructive CD4$^+$8^{lo}/CD4$^+$8$^-$ or CD4$^+$8$^+$/CD4$^-$8$^+$ transitions, giving rise to single-positive cells, still in an immature state (HSA^{hi}); 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$^+$8^{lo} cells or class II–restricted TCR^{hi} CD4$^+$8$^+$ thymocytes could mature to CD4$^-$8$^+$ or CD4$^+$8$^-$ single-positive cells, respectively.

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