# Small Cortical Thymocytes Are Subject to Positive Selection

## By Katarina Lundberg and Ken Shortman

From The Walter and Eliza Hall Institute of Medical Research, Melbourne, Victoria 3050, Australia

## Summary

To determine the developmental stages at which positive selection can act to produce mature T cells, $CD4^+8^+3^{lo}$ thymocytes of large dividing type and of small nondividing type were sorted and transferred into the thymus of nonirradiated Thy-1 congenic recipient mice. In contrast to earlier studies, the small as well as the large thymocytes produced mature $CD4^+8^-3^{hi}$ and $CD4^-8^+3^{hi}$ progeny, although production was less efficient from the small cells. The relative efficiency of small cells was increased and was close to that of large cells when $bcl$-$2$/anti–HY T cell receptor (TCR) $\alpha\beta$ transgenic donors were used to improve cell survival, overcome stress effects of the transfer process, and increase the frequency of selectable cells. The results from transferring small $CD4^+8^+3^{lo}$ thymocytes expressing a TCR transgene from a nonselecting to a selecting thymic MHC environment also confirmed that the small cells were capable of being selected and maturing. Thus the developmental window available for positive selection includes the small $CD4^+8^+3^{lo}$ thymocytes. The results also showed a striking difference in the kinetics of production of mature progeny from the transferred $CD4^+8^+3^{lo}$ precursors. $CD4^+8^-3^{hi}$ cells appeared several days before $CD4^-8^+3^{hi}$ cells, apparently because the $CD4^-8^+$ lineage cells spent several days in transit as $CD4^+8^+3^{hi}$ intermediates before losing CD4. Most $CD4^+8^-$ lineage cells on the other hand, either passed very rapidly through this intermediate stage, or lost CD8 before increasing the expression of CD3.

The role in T cell development of the small $CD4^+8^+3^{lo}$ cortical thymocyte, the most common cell in the thymus, has been the subject of much debate and controversy. The earliest proposed developmental model (1), and some later variants (2), logically had all cortical thymocytes differentiating directly into mature medullary thymocytes, and thence to peripheral T cells. However, kinetic and balance sheet assessments (3–6) indicated that most cortical thymocytes were unproductive, their fate being intrathymic death. It had therefore been argued (3, 7) that cortical thymocytes were end cells, production of mature T cells branching off from some earlier stage of the developmental process. This dispute seemed to be resolved when experiments with TCR–transgenic mice showed that the crucial step of positive selection for self-MHC recognition was required for the final step of maturation to a T cell (8, 9). The consensus model (10) was that, although all cortical thymocytes indeed had intrathymic death as an inbuilt default pathway, a small proportion with a TCR recognizing self-MHC molecules would be positively selected and these few cells would then develop into mature medullary thymocytes.

Subsequent studies by Guidos et al. (11) suggested that the developmental window available for positive selection is quite narrow and does not include the small, nondividing cortical thymocytes, which they argued are end cells, beyond the stage where rescue by positive selection is possible. However, this seems in conflict with the high levels of recombination activating gene products and terminal deoxynucleotide transferase in cortical thymocytes, expression only ceasing after positive selection (12–15). Such expression of the enzymatic apparatus for DNA recombination suggests that, although they are nondividing cells, the small cortical thymocytes may keep rearranging their $V_\alpha$ genes to produce a selectable TCR. There is evidence from T cell clones (16, 17) and a thymocyte cell line (18) for multiple rounds of TCR $V_\alpha$ gene rearrangements. In addition, expression of a rearranged $\alpha$ gene in TCR $\alpha\beta$ transgenic mice did not block rearrangement at the endogenous $\alpha$ locus (13, 19) and unselected small cortical thymocytes originally expressing one TCR could lose this and apparently substitute a TCR with a different $\alpha$ chain (15). Is all this a mere relic of the cells' past enzymic history, or might it give small cortical thymocytes a further chance to be positively selected?

We sought evidence of precursor activity by testing the capacity of small $CD4^+8^+3^{lo}$ thymocytes from normal and transgenic mice to produce mature progeny after intrathymic transfer. We found that highly purified populations of the small thymocytes did give rise to mature T cells. In addition

we found a marked difference in the time taken to produce $CD4^+8^-$ and $CD4^-8^+$ progeny from the $CD4^+8^+3^{lo}$ precursor cells, indicating a difference in the terminal maturation process for these two classes of T cells.

## Materials and Methods

*Mice.* Normal mice were bred under specific pathogen free $(SPF)^1$ conditions at the Walter and Eliza Hall Institute (WEHI) animal facility. The transgenic mice were bred and maintained under clean conventional conditions. The normal strains used were C57BL/6J (Thy 1.2), C57BL/Ka (Thy 1.1), and CBA/CaH WEHI (Thy 1.2). The following transgenic mice were kindly supplied by Drs. A. Harris and A. Strasser of this Institute: DBA/2 (Thy 1.2) mice transgenic for anti-HY TCR $\alpha\beta$, restricted to H-2D$^b$, were descended from breeding stock obtained from Dr. H. von Boehmer (Basel Institute for Immunology, Basel, Switzerland) (20). C57BL/6J mice hemizygous for the E$\mu$-bcl-2-36 transgene (21) were produced by two serial backcrosses with C57BL/6J from the initial bcl-2 genetic background of (C57BL/6J $\times$ SJL/J)F$_2$. DBA/2 mice expressing transgenic anti-HY TCR $\alpha\beta$ and bcl-2 were produced by serially crossing bcl-2-36 mice with DBA/2 mice for four generations, typing them as H-2D$^d$, and then crossing them with anti-HY TCR $\alpha\beta$ transgenic DBA/2 mice. Transgenic mice were used only as cell donors, whereas C57BL/6J and C57BL/Ka were used interchangeably as donors and recipients. Mice of both sexes were used at 6-8 wk of age.

*Cell Preparation.* Mice were killed with $CO_2$ and the thymuses were removed 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 washed once through FCS.

*Monoclonal Antibodies and Fluorescent Reagents.* The antibodies used for cytotoxic depletion were derived from the following hybridoma clones: anti-CD3, 17A2 (22); anti-CD4, 172.4 (23); anti-IL-2R$\alpha$, 7D4 (24); and anti-CD44, IM7.81 (25). The antibodies used for magnetic bead depletion were: anti-CD3, KT3-1.1 (26); anti-CD4, GK1.5 (27); anti-IL-2R$\alpha$, PC/61 (23); anti-CD44, IM7.81; anti-Mac-1, M1/70.15 (28); anti-GR-1, RB6-8C5 (29); anti-B220, RA3-6B2 (30); anti-MHC class II, M5/114 (31); anti-erythrocyte antigen, TER-119 (provided by Dr. T. Kina, Chest Disease Research Institute, Kyoto University, Kyoto, Japan); anti-Thy-1.1, 19F12 (32); and anti-Thy-1.2, 30H12 (33). All the above antibodies were used as either culture supernatants or ascites fluid. The purified antibodies used for immunofluorescent staining were: PE-conjugated anti-CD4, GK1.5 (Becton Dickinson and Co., San Jose, CA); anti-CD8, 53-6.7 (33) conjugated with either FITC (Becton Dickinson and Co.) or with allophycocyanin (APC); biotinylated anti-CD3, KT3-1.1; biotinylated anti-heat-stable antigen (HSA), M1/69 (28); FITC-conjugated anti-Thy-1.1, 19F12; FITC-conjugated anti-Thy-1.2, 30H12; and PE-conjugated goat anti-rat IgG (Caltag Lab., San Francisco, CA). As a second stage fluorescent reagent, Texas red–streptavidin (Tr-Av) (Amersham Int., Buckinghamshire, England) was used.

*Purificiation of Thymocyte Subsets.* For purification of $CD4^+8^+3^{lo}$ small and large cells, thymocytes were stained with anti-CD4, anti-CD8, and anti-CD3 using procedures previously described (34). The cells were then sorted in a modified FACS® II instru-

ment (Becton Dickinson & Co.). Dead cells were gated out on the basis of forward light scatter and propidium iodide staining. For purification of late $CD4^-8^-$ double negative cells ($CD3^-4^-8^-IL$-$2R^-CD44^-$) and immature $CD4^-8^+$ cells ($CD3^-4^-8^+IL$-$2R^-CD44^-$) thymocytes were first depleted of $CD4^+8^+$ cortical thymocytes, $CD3^+$ mature medullary thymocytes, early $CD4^-8^-$ cells, and non-T lineage cells, by using a combination of complement-mediated cytotoxicity and immunomagnetic bead depletion, for which the procedures are described in detail elsewhere (34). The remaining population was stained with FITC-conjugated anti-CD8 and PE-conjugated goat anti-rat IgG. The immature blasts of $CD4^-8^-$ and $CD4^-8^+$ type were then sorted as described above. By setting fluorescence intensity gates for PE negative cells, the small percentage of cells that escaped depletion were excluded from the sort. Reanalysis showed that the purity of the sorted $CD4^-8^-$ and $CD4^-8^+$ immature populations was >98%.

*Intrathymic Transfer.* 6-8-wk-old unirradiated recipient mice were anesthetized and intrathymically injected as described previously (35). Briefly, various numbers of purified thymocytes, 1.8-3.0 $\times$ $10^6$ small $CD4^+8^+3^{lo}$ cells, 0.2-0.5 $\times$ $10^6$ large $CD4^+8^+3^{lo}$ cells, 0.2 $\times$ $10^6$ $CD4^-8^-$ cells, or 0.4 $\times$ $10^6$ immature $CD4^-8^+3^{lo}$ cells, in 10 $\mu$l of BSS, were injected into a single lobe of the recipient thymus.

*Analysis of Recipient Thymuses for Donor-derived Thymocytes.* At 2, 3, or 4 d after transfer, cell suspensions were prepared from the injected thymus lobes and most of the host thymocytes and non-T lineage cells were removed before analysis for donor-derived cells. Depletion was done by coating the cells with antibodies against host thymocytes (anti-host Thy-1), erythrocytes (TER-119), granulocytes (anti-Gr-1), macrophages (anti-Mac-1), B cells (anti-B220), and dendritic cells (anti-MHC class II), 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 Ig- and anti-mouse Ig-coupled Dynabeads (Dynal Inc., Oslo, Norway). The depleted suspensions were stained in four fluorescent colors with FITC-anti-Thy-1, PE-anti-CD4, APC-anti-CD8, and biotin-anti-CD3, or biotin-anti-HSA, followed by Tr-Av. The stained cells were then analyzed by flow cytometry, gating for Thy-1$^+$ donor-derived cells.

## Results

*Purification of Small and Large $CD4^+8^+3^{lo}$ Thymocytes.* The phenotype of unsorted thymocytes from normal C57BL/6J mice, stained with antibodies against CD4, CD8, and CD3 is shown in Fig. 1. The box and the markers indicate the sorting gates for small and large cells. Similar staining intensity profiles were observed in all donor mice used. Sorting gates similar to that shown in Fig. 1 were used throughout this study.

Reanalysis of the sorted populations after a single sort showed that small $CD4^+8^+3^{lo}$ thymocyte preparations (Fig. 1) were always more than 99% pure by all four parameters. The same purity, regarding the $CD4^+8^+3^{lo}$ phenotype, was obtained with large thymocytes, but by cell size the purity of this population ranged from 65 to 90%. Since the blasts in this study mainly served as positive controls, and the contaminants were entirely small $CD4^+8^+3^{lo}$ thymocytes, a higher purity with regard to cell size was not required.

Unsorted thymocytes    Small CD4⁺8⁺3ˡᵒ thymocytes
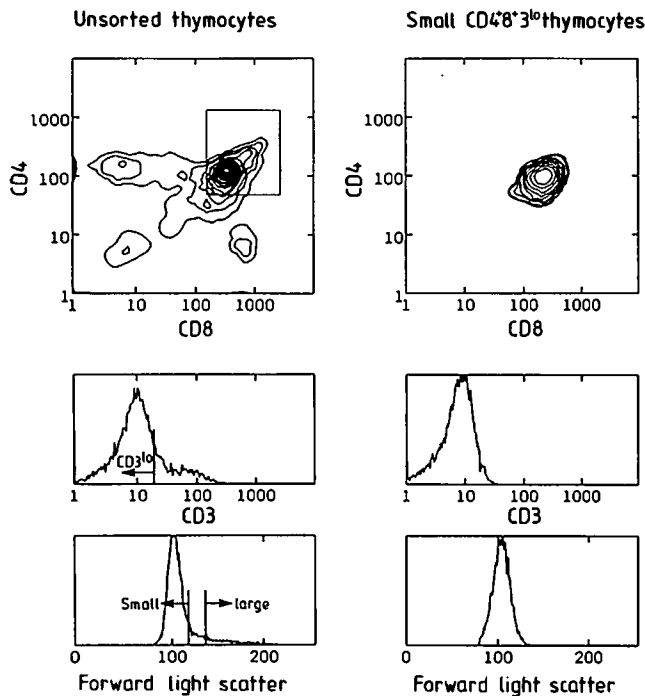
**Figure 1.** CD3, CD4, and CD8 expression on unsorted and sorted thymocytes from normal C57BL/6J mice. Thymocytes were stained and sorted as described in Materials and Methods. Staining intensity profiles and sorting gates for unseparated thymocytes and sorted CD4⁺8⁺3ˡᵒ small thymocytes are shown.

*Intrathymic Development of Normal CD4⁺8⁺3ˡᵒ Small and Large Thymocytes Transferred to Congenic Recipient Mice.* To determine the potential of small CD4⁺8⁺3ˡᵒ thymocytes to be positively selected and to produce mature T cells, they were purified, in parallel with large CD4⁺8⁺3ˡᵒ cells as a positive control, and injected intrathymically into nonirradiated Thy-1 congenic recipient mice. After a maturation period of 2–4 d, suspensions of the recipient thymus lobes were depleted of host cells and the donor-derived cells were analyzed.

As with previous experiments of this type (11, 36), there was an extensive loss of donor cells within the first 2 d after transfer (Table 1). Part of the loss was probably due to the normal high rate of cell death within the CD4⁺8⁺ population (37). There was substantial additional cell death due to the stress of the intrathymic injection procedure, since the number of host cells in the injected mice dropped from 200 ± 50 × 10⁶ cells/lobe initially to 120 ± 20 × 10⁶ cells/lobe at day 2, 90 ± 40 × 10⁶ cells/lobe at day 3, and 110 ± 30 × 10⁶ cells/lobe at day 4. The loss of donor cells could not be attributed to engagement of the cell surface CD3 with antibody, since the same low recovery was obtained using sorted large and small CD4⁺8⁺ thymocytes with or without labeling with anti–CD3 antibody (data not shown). Despite the extensive and variable loss of donor cells, sufficient cells were recovered for analysis after depletion of recipient cells (Fig. 2, Table 1).
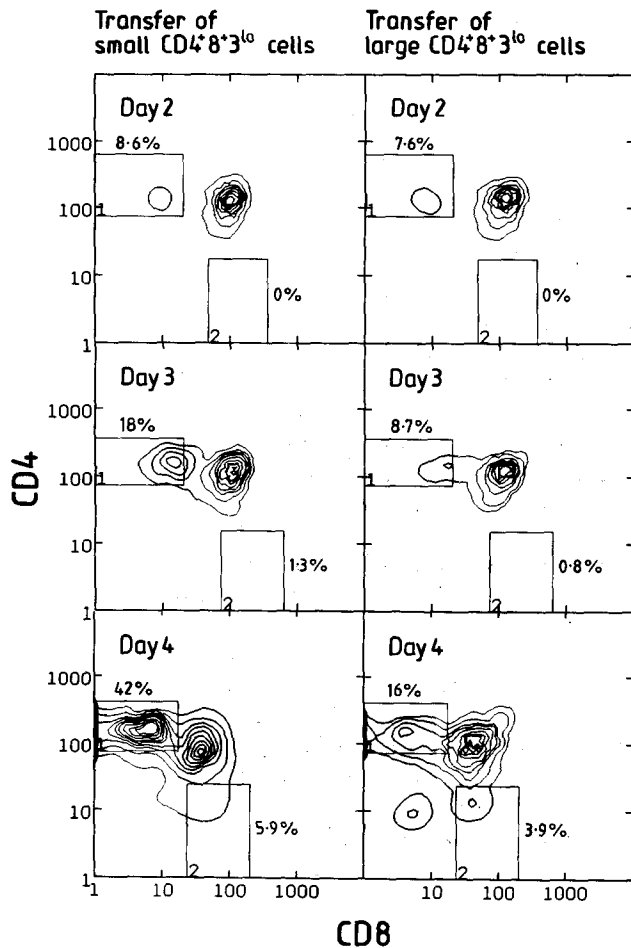
Both small and large CD4⁺8⁺3ˡᵒ thymocytes produced single positive CD4⁺8⁻ and CD4⁻8⁺ progeny. In addition a proportion of CD4⁺8⁺ cells persisted (Fig. 2, Table 1). From both groups a relatively high proportion of CD4⁺8⁻ progeny (6–8%) was already seen at day 2, the earliest sampling time. In absolute terms the number of CD4⁺8⁻ progeny did not increase after this time, although the proportion increased due to the loss of CD4⁺8⁺ cells. In contrast, a significant proportion of CD4⁻8⁺ progeny (6–7%) was not apparent until day 4 after transfer, and the absolute numbers of CD4⁻8⁺ progeny increased continually over the 2–4-d period. A normal thymic CD4⁺8⁻:CD4⁻8⁺ ratio was not reached within the 4-d period studied.

Although the proportion of mature progeny and the rela-

**Table 1.** *Mature Progeny Formation by Normal Small and Large CD4⁺8⁺3ˡᵒ Thymocytes after Intrathymic Transfer*

| Days after transfer | Population transferred | Total recovery | CD4⁺8⁻ progeny | | CD4⁻8⁺ progeny | |
|---|---|---|---|---|---|---|
| | | Percent of cells transferred | Percent of recovered cells | Progeny per 10⁵ cells transferred | Percent of recovered cells | Progeny per 10⁵ cells transferred |
| 2 | Small | 0.8 ± 0.2 | 8.1 ± 1.1 | 75 ± 30 | 0.5 ± 0.4 | 2 ± 1 |
| | Large | 5.9 ± 2.4 | 6.2 ± 2.2 | 300 ± 170 | 0.4 ± 0.2 | 7 ± 4 |
| 3 | Small | 0.4 ± 0.3 | 14 ± 3 | 53 ± 22 | 0.9 ± 0.2 | 3 ± 1 |
| | Large | 2.0 ± 0.8 | 9.1 ± 1.1 | 160 ± 60 | 1.0 ± 0.6 | 15 ± 7 |
| 4 | Small | 0.1 ± 0 | 46 ± 2 | 33 ± 9 | 6.6 ± 1.2 | 5 ± 1 |
| | Large | 1.7 ± 0.5 | 16 ± 2 | 280 ± 80 | 5.7 ± 0.8 | 80 ± 19 |

Small (1.8–3.0 × 10⁶) and large (0.2–0.5 × 10⁶) CD4⁺8⁺3ˡᵒ thymocytes from normal mice were injected intrathymically into Thy-1 congenic recipient mice. After depletion of most host-thymocytes, donor cells were stained with anti–donor Thy-1, anti–CD4, anti–CD8, and anti–CD3 and analyzed. Results are the mean ± SEM of at least three experiments, each with three mice per group.

Figure 2. Development of normal small and large CD4⁺8⁺3ˡᵒ thymocytes. Small and large CD4⁺8⁺3ˡᵒ thymocytes from normal mice were injected into the thymus of Thy-1 congenic recipients. Representative profiles of CD4 and CD8 expression on donor-derived cells 2, 3, and 4 d after transfer are shown. The boxes define mature CD4⁺8⁻ and CD4⁻8⁺ thymocytes and were based on the analysis of normal thymus controls. When analyzed, donor-derived cells within the gates were of mature phenotypes by other criteria, including size, Thy-1, and CD3 expression.

tive rate of production of CD4⁺8⁻ compared to CD4⁻8⁺ cells was roughly similar for the two groups, the proportion of the total recovery from the small cells was 5–20-fold lower than from the large cells. On an absolute recovery per injected cell basis, the large cells produced 3 to 10 times as many mature progeny as did the small cells (Table 1).

The CD4⁺8⁻ and CD4⁻8⁺ progeny were mature cells by other criteria (data not shown). Compared to the CD4⁺8⁺ cells, they expressed high levels of CD3, lower levels of Thy-1, and were larger based on forward light scatter measurements, all features of mature cells. In addition, CD4⁻8⁺ progeny expressed low levels of HSA, whereas most CD4⁺8⁻ progeny still expressed high levels of HSA; this is also the case in the normal CD57BL/6 thymus.

Although the purity of the sorted cells was high (>99%), there was a small contamination of mature CD4⁺8⁻ and CD4⁻8⁺ cells, always <0.2%. To test the possibility that
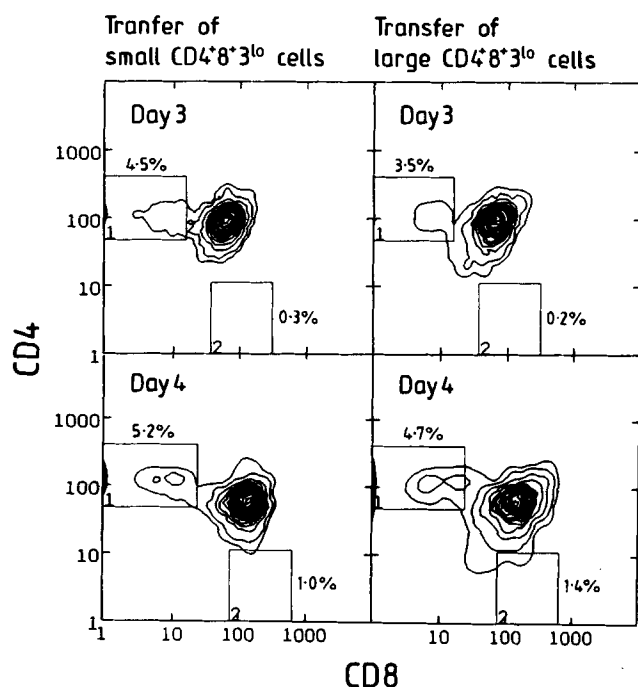
the mature cells found after transfer were simply these contaminants, we sorted CD4⁺8⁻3ʰⁱ and CD4⁻8⁺3ʰⁱ mature cells and injected them at a level 10 times higher than that found in a sorted population of small cells. The recovery of mature cells ranged from 1.5–6.4% at day 3 and 0.8–1.2% at day 4 after injection. Based on these recoveries, we calculated that the mean number of cells in the mature single positive populations derived from injections of small CD4⁺8⁺3ˡᵒ cells was 10 times higher than would have been expected from contaminating cells at day 3, and 40 times higher at day 4. In the mature single positive populations derived from large CD4⁺8⁺3ˡᵒ cells, these ratios were even higher. Thus, we conclude that contaminating cells did not significantly contribute to the recoveries of mature thymocytes.

*Intrathymic Transfer of Small and Large CD4⁺8⁺3ˡᵒ Thymocytes from bcl-2 Transgenic Mice.* Intrathymic transfer of thymocytes from normal mice resulted in a very low recovery of cells, <1% from small cells and 1–6% from the large (Table 1). The use of bcl-2 transgenic mice has previously been valuable in studies of CD4⁺8⁺ thymocytes (21). In such mice T cell development, function, number, and ratios are essentially normal, but their CD4⁺8⁺ cells have a longer lifespan and are less sensitive to stress than the cells from normal mice. To increase the recovery of donor cells and to see if longer survival of small postmitotic cells would affect the number of mature progeny produced, we transferred bcl-2 transgenic small and large CD4⁺8⁺3ˡᵒ thymocytes into normal mice.

The use of bcl-2 transgenic CD4⁺8⁺3ˡᵒ thymocytes indeed increased the recovery from both small cells (5–10-fold) and large cells (two- to fivefold). However, the recovery was still low (Fig. 3, Table 2). Despite a higher recovery, the absolute number of mature CD4⁺8⁻ and CD4⁻8⁺ cells produced by the two populations was not significantly different from that obtained from normal thymocytes, and because of better CD4⁺8⁺ cell survival, the proportion of recovered mature cells appeared lower. This was probably also the reason why it was difficult to resolve clear populations of CD4⁻8⁺ cells in Fig. 3, although there were clearly cells within the gates corresponding to cells with mature phenotype. Again, blast cells were at least three to four times more efficient than small cells at producing mature progeny. As for normal cells, the number of CD4⁺8⁻ cells was largely the same on days 3 and 4 after transfer, whereas an increase over time was observed for the CD4⁻8⁺ population.

*Allogeneic Intrathymic Transfer of Small and Large CD4⁺8⁺3ˡᵒ Thymocytes.* If the selecting system was very efficient, one could argue that the reason for the small cells producing mature progeny at a lower frequency than large cells was that the population of small cells had already been pruned of selectable cells, because of the longer exposure to the selecting system. To test this possibility we transferred thymocytes from either normal CBA or bcl-2 transgenic DBA/2 mice into normal C57BL/Ka mice, arguing that a different selecting MHC haplotype might allow a new spectrum of TCR specificities to be used.

Allogeneic transfer resulted in a slightly lower recovery of cells than that obtained with congenic transfers. There

**Transfer of small CD4⁺8⁺3ˡᵒ cells** / **Transfer of large CD4⁺8⁺3ˡᵒ cells**

(Contour plots: CD4 vs CD8)
Day 3 — 4·5%, 0·3% (small); Day 3 — 3·5%, 0·2% (large)
Day 4 — 5·2%, 1·0% (small); Day 4 — 4·7%, 1·4% (large)

**Figure 3.** Development of small and large CD4⁺8⁺3ˡᵒ thymocytes from bcl-2 transgenic mice. Small and large CD4⁺8⁺3ˡᵒ thymocytes from bcl-2 transgenic mice were injected intrathymically into Thy-1.1, MHC compatible recipient mice. Representative profiles of CD4 and CD8 expression on donor cells 3 and 4 d after transfer are shown. The boxes define mature CD4⁺8⁻ and CD4⁻8⁺ thymocytes and were based on the analysis of normal thymic controls. The proportion of CD4⁻8⁺ cells was too small to appear as a population in the probability plot, but there were clearly cells within the gates that had a mature phenotype, regarding size, Thy-1 and CD3 expression.

was, however, little change in the relative production of CD4⁺8⁻ and CD4⁻8⁺ mature progeny, and the large cells remained more efficient than small cells on a per cell basis, as in Tables 1 and 2 (data not shown).

*Intrathymic Transfer of Small and Large CD4⁺8⁺3ˡᵒ Thymocytes from bcl-2/Anti-HY TCR αβ Transgenic DBA/2 Mice.* In the previous experiments we carefully eliminated CD4⁺8⁺3ʰⁱ cells from the sorted populations to remove any CD4⁺8⁺ cells which, according to some models, might have already undergone positive selection before transfer (6, 38). However, it was possible that some of the CD4⁺8⁺3ˡᵒ cells transferred had already been selected but had not yet upregulated CD3. Accordingly, we transferred CD4⁺8⁺3ˡᵒ cells from female DBA/2 mice transgenic for both bcl-2 and a TCR specific for the male (HY) antigen in the context of H-2Dᵇ. No positive selection of anti-HY TCR αβ transgenic thymocytes can occur in such mice because of the absence of the H-2Dᵇ MHC, so all the CD4⁺8⁺ anti-HY TCR αβ transgenic thymocytes are in a pre-selection state (38). In addition, most of the cells are destined to become CD4⁻8⁺ in a selecting environment, with only a small proportion becoming CD4⁺8⁻ due to endogenous rearrangements (13). Thus, the sensitivity of detection of CD4⁻8⁺ cells after transfer was greatly increased, allowing a critical test of the observation that CD4⁺8⁻ progeny appear ahead of CD4⁻8⁺ progeny.

The transfer of these nonselected CD4⁺8⁺3ˡᵒ TCR-transgenic cells into an H-2Dᵇ selecting environment resulted in the production of both CD4⁺8⁻ and CD4⁻8⁺ mature progeny, with a similar yield from small and large cells (Fig. 4, Table 3). Initially, CD4⁺8⁻ progeny cells were as frequent as CD4⁻8⁺ progeny, but the proportion of CD4⁻8⁺ cells was much higher by day 4. In contrast to the number of CD4⁺8⁻ progeny, which remained constant over days 3 to 4, there was a 10-fold increase in the number of CD4⁻8⁺ progeny over the same period.

The majority of the donor cells recovered at day 3 expressed high levels of CD3, characteristic of mature T cells, although most were CD4⁺8⁺ (Fig. 4). This suggests that they were already positively selected, but only a few had yet downregulated CD4. Even by day 4 a large proportion remained CD4⁺8⁺3ʰⁱ.

**Table 2.** *Mature Progeny Formation by bcl-2 Transgenic Small and Large CD4⁺8⁺3ˡᵒ Thymocytes*

| Days after transfer | Population transferred | Total recovery | CD4⁺8⁻ progeny | | CD4⁻8⁺ progeny | |
| --- | --- | --- | --- | --- | --- | --- |
| | | Percent of cells transferred | Percent of recovered cells | Progeny per 10⁵ cells transferred | Percent of recovered cells | Progeny per 10⁵ cells transferred |
| 3 | Small | 2.1 ± 0.7 | 5.4 ± 1.2 | 100 ± 30 | 0.5 ± 0.1 | 7 ± 2 |
| | Large | 12 ± 6 | 4.9 ± 0.9 | 400 ± 180 | 0.5 ± 0.2 | 29 ± 11 |
| 4 | Small | 1.2 ± 0.2 | 7.8 ± 1.7 | 86 ± 23 | 1.2 ± 0.1 | 13 ± 2 |
| | Large | 4.6 ± 0.7 | 5.1 ± 0.3 | 220 ± 30 | 1.5 ± 0.3 | 67 ± 17 |

Small (2.6 or 3.0 × 10⁶) and large (0.3 × 10⁶) CD4⁺8⁺3ˡᵒ thymocytes from bcl-2 transgenic mice were injected intrathymically into Thy-1.1, MHC compatible recipient mice. After depletion of most host-thymocytes, donor cells were stained with anti–Thy-1.2, anti–CD4, anti–CD8, and anti–CD3 and analyzed. Results are the mean ± SEM of two experiments, each with three mice per group.

Transfer of
small CD4⁺8⁺3ˡᵒ cells

Transfer of
large CD4⁺8⁺3ˡᵒ cells
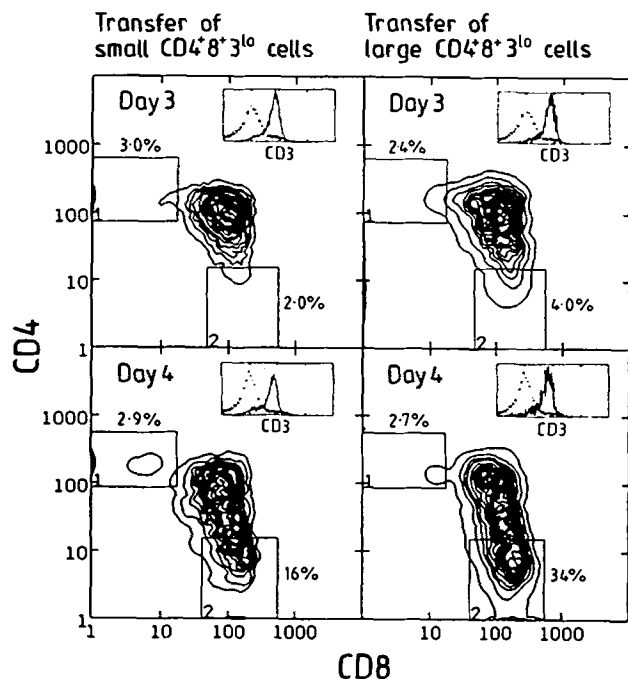
**Figure 4.** Development of small and large CD4⁺8⁺3ˡᵒ thymocytes from *bcl-2*/anti-HY TCR $\alpha\beta$ transgenic mice. Sorted small and large cells were injected into the thymus of Thy-1.1, H-2Dᵇ recipients. Representative profiles of CD4 and CD8 expression on donor-derived cells 3 and 4 d after transfer are shown. Boxes define mature CD4⁺8⁻ and CD4⁻8⁺ thymocytes and were based on the analysis of normal thymus controls. CD3 expression on total donor cells recovered is shown in the inset histograms, the dotted line shows CD3 expression on whole control thymocytes, for comparison.

*Production of Mature Thymocytes after Intrathymic Transfer of Late CD4⁻8⁻3⁻ or CD4⁻8⁺3⁻ Precursors.* In view of the relatively delayed appearance of mature CD4⁻8⁺ progeny from CD4⁺8⁺ precursors, we asked if the same delay would be obtained with earlier precursors, especially the "immature single positives" that had expressed CD8 before CD4

(39). Accordingly, "late double negatives" (CD4⁻8⁻3⁻IL-2Rα⁻CD44⁻) and "CD8 immature single positives" (CD4⁻8⁺3⁻), were isolated from normal mice and transferred. However, the results from both groups were similar and were very similar to those obtained with CD4⁺8⁺ cells, with CD4⁺8⁻3ʰⁱ progeny appearing several days ahead of CD4⁻8⁺3ʰⁱ progeny (data not shown). This confirmed earlier studies (40, 41) and indicated that early pre-selection expression of CD8 during development does not speed up the process of postselection development of CD4⁻8⁺ cells.

## Discussion

The factors known to determine if a developing thymocyte can be positively selected for further maturation are the expression and the specificity of the TCR, together with the coexpression of the appropriate CD4 or CD8 accessory molecules. A minor population of CD4⁺8⁺ dividing cortical blasts are the first cells clearly to exhibit these properties, followed by their progeny, the major thymic population of small CD4⁺8⁺ cortical thymocytes. Guidos et al. (11) could detect mature progeny after transfer of the CD4⁺8⁺3ˡᵒ blasts, but not after transfer of CD4⁺8⁺3ˡᵒ small thymocytes, implying that some additional factors limited the selection process to the narrow window of CD4⁺8⁺ blasts. They therefore proposed that the small cortical thymocytes had no other developmental fate than cell death.

We now show that normal small CD4⁺8⁺3ˡᵒ cortical thymocytes, as well as CD4⁺8⁺3ˡᵒ blasts, can give rise to mature progeny and are thus subject to positive selection. The production of mature cells by small CD4⁺8⁺3ˡᵒ *bcl-2*/anti-HY TCR $\alpha\beta$ transgenic thymocytes, when transferred from a nonselecting to a selecting environment, indicates that the formation of mature cells involved the full process of positive selection, not just terminal maturation of cells that had already received a selection signal. In addition to the down-regulation of either CD4 or CD8, the progeny cells had other features characteristic of mature thymocytes, indicating that the cells had indeed undergone the full differentiation pro-

**Table 3.** *Mature Progeny Formation by bcl-2/Anti-HY TCR $\alpha\beta$ Transgenic Small and Large CD4⁺8⁺3ˡᵒ Thymocytes*

| Days after transfer | Population transferred | Total recovery | CD4⁺8⁻ progeny | | CD4⁻8⁺ progeny | |
|---|---|---|---|---|---|---|
| | | Percent of cells transferred | Percent of recovered cells | Progeny per $10^5$ cells transferred | Percent of recovered cells | Progeny per $10^5$ cells transferred |
| 3 | Small | 2.4 ± 0.6 | 3.5 ± 0.6 | 83 ± 20 | 3.2 ± 1.3 | 64 ± 24 |
| | Large | 3.1 ± 1.3 | 3.1 ± 0.5 | 79 ± 28 | 5.2 ± 0.8 | 140 ± 50 |
| 4 | Small | 3.7 ± 1.1 | 3.4 ± 0.2 | 120 ± 40 | 21 ± 4 | 740 ± 240 |
| | Large | 2.8 ± 0.7 | 2.8 ± 0.1 | 77 ± 17 | 44 ± 8 | 1,200 ± 400 |

Small (2.7 × 10⁶) and large (0.2 or 0.4 × 10⁶) CD4⁺8⁺3ˡᵒ thymocytes from *bcl-2*/anti-HY TCR $\alpha\beta$ transgenic mice were injected intrathymically into Thy-1.1, H-2Dᵇ recipient mice. After depletion of most host-thymocytes, donor cells were stained with anti–Thy-1.2, anti–CD4, anti–CD8, and anti–CD3 and analyzed. Results are the mean ± SEM of two experiments, each with three mice per group.

cess. This result indicates that, although many cells may be already selected at the CD4$^+$8$^+$ blast stage, the window for positive selection is much wider and the entire 4-d lifespan of small cortical thymocytes may be available. This may give cells with an inappropriate receptor sufficient time for further V$_\alpha$ gene rearrangements and expression of a new, possibly selectable, receptor (13, 15–18), or give a cell with an already appropriate TCR ample time to access a selecting site. Another possibility is that the window for positive selection is limited to small thymocytes, since cortical blasts would give rise to small thymocyte progeny after transfer.

A striking feature of our results is that the peak of CD4$^+$8$^-$ mature cells appeared several days before the peak of CD4$^-$8$^+$ mature cells, regardless of whether small CD4$^+$8$^+$ thymocytes, CD4$^+$8$^+$ blasts, or earlier stages were transferred. Yet, because of the rapid and extensive loss of cells after transfer and the normal low frequency of selectable cells, the mature cells we observe must have resulted from a selection event occurring soon after cell transfer. It therefore seems that, although both mature thymocyte populations have a common immediate precursor, the time needed for terminal maturation after selection is different. The results from our anti–HY TCR $\alpha\beta$ transgenic cell transfer experiments indicate that the lag in CD4$^-$8$^+$3$^{hi}$ cell production is due to a hold-up of cells at the CD4$^+$8$^+$3$^{hi}$ intermediate stage. These results agree with earlier precursor cell transfer studies in this laboratory (40, 41), showing the appearance of CD4$^+$8$^-$ cells before CD4$^-$8$^+$ cells. It also agrees with the thymidine uptake kinetic studies of Huesmann et al. (42), and with recent bromodeoxyuridine pulse experiments of Lucas et al. (43), all of which suggest a lag in CD4$^-$8$^+$ mature cell production, compared to mature CD4$^+$8$^-$ cells. However, the present results with CD4$^+$8$^-$ cells do not accord with our own continuous thymidine uptake studies which suggest a roughly equal lag between the last cell division and the appearance of CD4$^+$8$^-$3$^{hi}$ and CD4$^-$8$^+$3$^{hi}$ progeny (5); we are investigating the basis of the discrepancy. We conclude that either CD4$^+$8$^-$3$^{hi}$ thymocytes are not formed via a CD4$^+$8$^+$3$^{hi}$ intermediate, or their transit through this intermediate stage is very rapid. This conclusion fits with other recent studies from our laboratory (36) showing that isolated CD4$^+$8$^+$3$^{hi}$ cells give rise to many more CD4$^-$8$^+$ progeny than CD4$^+$8$^-$ progeny, a reversal of the normal mature T cell ratio. This asymmetry in the terminal maturation pathway of the two types of T cells may point to basic differences in the mechanism of selection of class I MHC and class II MHC restricted T cells. A rapid transit through the CD4$^+$8$^+$3$^{hi}$ intermediate stage for cells committed to the CD4$^+$8$^-$ cell lineage may also be the reason why many of the CD4$^+$8$^-$3$^{hi}$ cells in the thymus still express high levels of HSA as opposed to CD4$^-$8$^+$3$^{hi}$ cells. Having rapidly upregulated CD3 and downregulated CD8, CD4$^+$8$^-$3$^{hi}$ thy-

mocytes may need additional time to become fully mature by other criteria, such as HSA expression.

In our experiments with normal and bcl-2 transgenic mice, the large CD4$^+$8$^+$3$^{lo}$ thymocytes were 3- to 10-fold more efficient than small thymocytes at forming mature progeny. Part of the reason for the greater efficiency of the large cells may be their potential capacity for a few rounds of division before selection, thus amplifying their capacity to produce mature cells. However, a large part of the difference may also be their potentially longer lifespan after transfer and their higher resistance to the substantial stress induced by the injection procedure. This, and the difficulty of analyzing the few progeny in an immense excess of recipient cells, may explain why Guidos et al. (11), although using a similar transfer system, could not detect any mature progeny after transfer of small cortical cells. A small drop in the efficiency of their transfer and analysis system might have dropped the level of small thymocyte mature progeny below the detection level. It was notable in the present study that when cells from bcl-2/anti–HY TCR $\alpha\beta$ transgenic mice were used, with their prolonged lifespan, resistance to steroid-induced cell death, and high frequency of selectable cells, the small CD4$^+$8$^+$3$^{lo}$ thymocytes were almost as efficient as the large CD4$^+$8$^+$3$^{lo}$ thymocytes at producing mature progeny. This may indicate that under normal conditions small cortical thymocytes with appropriate TCRs have the same chance as large cells to be selected.

While this work was in progress, Kisielow and co-workers (44, 45) reported in reviews their findings that small CD4$^+$8$^+$3$^{lo}$ thymocytes from HY-specific TCR $\alpha\beta$ transgenic mice are able to produce mature CD4$^-$8$^+$ progeny, in agreement with our bcl-2/TCR transgenic results. We have also obtained such results with "normal" TCR transgenic mice (data not shown). One possible way of reconciling the Guidos et al. (11) results with the TCR transgenic data would have been to assume that in normal mice the developing thymocytes are efficiently depleted of all selectable cells at the early CD4$^+$8$^+$ blast stage, whereas in TCR transgenic mice, where the selection process is known to be inefficient (42, 46), cells with a selectable TCR proceed into the small CD4$^+$8$^+$ stage. However, we found that normal mice do have selectable CD4$^+$8$^+$3$^{lo}$ small cells. The results using allogeneic transfer also argue against this explanation, since there was no detectable increase in the efficiency of mature cell formation by small CD4$^+$8$^+$3$^{lo}$ thymocytes, despite their exposure to a fresh selecting environment. Our results with normal CD4$^+$8$^+$3$^{lo}$ thymocytes also emphasize that both CD4$^+$8$^-$ and CD4$^-$8$^+$ progeny can be obtained from small CD4$^+$8$^+$3$^{lo}$ thymocytes. Thus, several different selection systems, in two separate laboratories, support the view that small cortical thymocytes are able to be positively selected for maturation to T cells.

## References

1. Raff, M.C. 1971. Evidence for a subpopulation of mature lymphocytes within the mouse thymus. *New Biol.* 229:182.
2. Reinherz, E.L., P.C. Jung, G. Goldstein, R.H. Levey, and S.F. Schlossman. 1980. Discrete stages of human intrathymic differentiation: analysis of normal thymocytes and leukemic lymphoblasts of T-cell lineage. *Proc. Natl. Acad. Sci. USA.* 77:1588.
3. Shortman, K., and H. Jackson. 1974. The differentiation of T-lymphocytes. I. Proliferation kinetics and interrelationships of subpopulations of mouse thymus cells. *Cell. Immunol.* 12:230.
4. Scollay, R., E. Butcher, and I. Weissman. 1980. Thymus migration: quantitative studies on the rate of migration of cells from the thymus to the periphery in mice. *Eur. J. Immunol.* 10:210.
5. Egerton, M., R. Scollay, and K. Shortman. 1990. The kinetics of mature T-cell development in the thymus. *Proc. Natl. Acad. Sci. USA.* 87:2579.
6. Shortman, K., D. Vremec, and M. Egerton. 1991. The kinetics of T cell antigen receptor expression by subgroups of CD4$^+$8$^+$ thymocytes: delineation of CD4$^+$8$^+$3$^{++}$ thymocytes as post-selection intermediates leading to mature T cells. *J. Exp. Med.* 173:323.
7. Shortman, K., R. Scollay, A. Wilson, W.-F. Chen, and T. Ewing. 1986. Mature and immature thymocytes. *In* Progress in Immunology VI. B. Cinader and R.G. Miller, editors. Academic Press, Inc., FL. 95 pp.
8. Kisielow, P., H.S. Teh, H. Blüthmann, and H. von Boehmer. 1988. Positive selection of antigen-specific T cells in thymus by restricting MHC molecules. *Nature (Lond.).* 335:730.
9. Scott, B., H. Blüthmann, H.S. Teh, and H. von Boehmer. 1989. The generation of mature T cells requires interaction of the $\alpha\beta$ T-cell receptor with major histocompatibility antigens. *Nature (Lond).* 338:591.
10. von Boehmer H. 1986. The selection of the alpha, beta heterodimeric T-cell receptor for antigen. *Immunol. Today.* 7:333.
11. Guidos, G.J., I.L. Weissman, and B. Adkins. 1989. Intrathymic maturation of murine T lymphocytes from CD8$^+$ precursors. *Proc. Natl. Acad. Sci. USA.* 86:7542.
12. Brandle, D., C. Müller, T. Rülicke, H. Hengartner, and H. Pircher. 1992. Engagement of the T-cell receptor during positive selection in the thymus down-regulates RAG-1 expression. *Proc. Natl. Acad. Sci. USA.* 89:9529.
13. Borgulya, P., H. Kishi, Y. Uematsu, and H. von Boehmer. 1992. Exclusion and inclusion of $\alpha$ and $\beta$ T cell receptor alleles. *Cell.* 69:529.
14. Turka, L.A., D.G. Schatz, M.A. Oettinger, J.J.M. Chun, C. Gorka, K. Lee, W.T. McCormack, and C.B. Thompson. 1991. Thymocyte expression of RAG-1 and RAG-2: termination by T cell receptor cross linking. *Science (Wash. DC).* 253:778.
15. Petrie, H.T., F. Livak, D.G. Schatz, A. Strasser, I.N. Crispe,

and K. Shortman. 1993. Multiple rearrangements in TCR $\alpha$ chain genes maximize the production of useful thymocytes. *J. Exp. Med.* 178:615.
16. Hue, I., J. Trucy, C. McCoy, D. Couez, B. Malissen, and M. Malissen. 1990. A novel type of aberrant T-cell receptor $\alpha$-chain gene rearrangement. *J. Immunol.* 144:4410.
17. Kumar, V., J.L. Urban, and L. Hood. 1989. In individual T cells one productive $\alpha$ rearrangement does not appear to block rearrangement at the second allele. *J. Exp. Med.* 170:2183.
18. Marollau, J.-P., J.D. Fondell, M. Malissen, J. Trucy, E. Barbier, K.B. Marcu, P.A. Cazenave, and D. Primi. 1988. The joining of germ-line V$\alpha$ to J$\alpha$ genes replaces the preexisting V$\alpha$-J$\alpha$ complexes in a T cell receptor $\alpha\beta$ positive T cell line. *Cell.* 55:291.
19. Blüthmann, H., P. Kisielow, Y. Uematsu, M. Malissen, P. Krimpenfort, A. Berns, H. von Boehmer, and M. Steinmetz. 1988. T cell-specific deletion of T-cell receptor transgenes allows functional rearrangement of endogenous $\alpha$- and $\beta$-genes. *Nature (Lond.).* 334:156.
20. Kisielow, P., H. Blüthmann, U.D. Staerz, M. Steinmetz, and H. von Boehmer. 1988. Tolerance in T-cell-receptor transgenic mice involves deletion of nonmature CD4$^+$CD8$^+$ thymocytes. *Nature (Lond.).* 333:742.
21. Strasser, A., A.W. Harris, and S. Cory. 1991. Bcl-2 transgene inhibits T cell death and perturbs thymic self-censorship. *Cell.* 67:889.
22. Miescher, G.C., M. Schreyer, and H.R. MacDonald. 1989. Production and characterization of a rat monoclonal antibody the murine CD3 molecular complex. *Immunol. Lett.* 23:113.
23. Ceredig, R., J. Lowenthal, M. Nabholz, and H.R. MacDonald. 1985. Expression of interleukin-2 receptors as a differentiation marker on intrathymic stem cells. *Nature (Lond.).* 314:98.
24. Malek, T., R. Robb, and E. Shevach. 1983. Identification and initial characterization of a rat monoclonal antibody reactive with the murine interleukin 2 receptor-ligand complex *Proc. Natl. Acad. Sci. USA.* 80:5694.
25. Trowbridge, I., J. Lesley, R. Schulte, R. Hyman, and J. Trotter. 1982. Biochemical characterization and cellular distribution of a polymeric, murine cell surface glycoprotein expressed on lymphoid tissues. *Immunogenetics.* 15:299.
26. Tomonari, K. 1988. A rat antibody against a structure functionally related to the mouse T-cell receptor/T3 complex. *Immunogenetics.* 28:455.
27. Dialynas, D., Z. Quan, K. Wall, A. Pierres, J. Quintas, M. Loken, M. Pierres, and F. Fitch. 1983. Characterization of the murine T cell surface molecule, designated L3T4, identified by monoclonal antibody GK1.5: similarity of L3T4 to the human Leu-3/T4 molecule. *J. Immunol.* 131:2445.

28. Springer, T., G. Galfre, D.S. Secher, and C. Milstein. 1978. Monoclonal xenogeneic antibodies to murine cell surface antigens: identification of novel leukocyte differentiation antigens. *Eur. J. Immunol.* 8:539.

29. Holmes, K.L., W.Y. Langdon, T.N. Fredrickson, R.L. Coffman, P.M. Hoffman, J.W. Hartley, and H.C. Morse. 1986. Analysis of neoplasms induced by CAS-BR-M MULV tumour extracts. *J. Immunol.* 137:679.

30. Coffman, R.L. 1982. Surface antigen expression and immunoglobulin gene rearrangement during mouse pre-B cell development. *Immunol. Rev.* 69:5.

31. Bhattacharya, A., M.E. Dorf, and T.A. Springer. 1981. A shared alloantigenic determinant on Ia antigens encoded by the IA and IE subregions: evidence of I region gene duplication. *J. Immunol.* 127:2488.

32. Houston, L.L., R. Nowinski, and I. Bernstein. 1980. Specific in vivo localization on monoclonal antibodies directed against the Thy 1.1 antigen. *J. Immunol.* 125:837.

33. Ledbetter, J.A., and L.A. Herzenberg. 1979. Xenogeneic monoclonal antibodies to mouse lymphoid differentiation antigens. *Immunol. Rev.* 47:63.

34. Wilson, A., A. D'Amico, T. Ewing, R. Scollay, and K. Shortman. 1988. Subpopulations of early thymocytes: a cross-correlation flow-cytometric analysis of adult mouse Ly 2⁻ L3T4⁻ (CD8⁻ CD4⁻) thymocytes using eight different surface markers. *J. Immunol.* 140:1461.

35. Scollay, R., J. Smith, and V. Stauffer. 1986. Dynamics of early T cells: prothymocyte migration and proliferation in the adult mouse thymus. *Immunol. Rev.* 91:129.

36. Petrie, H.T., A. Strasser, A.W. Harris, P. Hugo, and K. Shortman. 1993. CD4⁺8⁻ and CD4⁻8⁺ mature thymocytes require different post-selection processing for final development. *J. Immunol.* 151:1273.

37. Shortman, K., M. Egerton, G.J. Spangrude, and R. Scollay. 1990. The generation and fate of thymocytes. *In* Seminars in Immunology. J. Sprent, editor. W.B. Saunders, London. 3–12.

38. Borgulya, P., H. Kishi, U. Muller, J. Kirberg, and H. von Boehmer. 1991. Development of the CD4 and CD8 lineage of T cells: instruction versus selection. *EMBO (Eur. Mol. Biol. Organ.) J.* 10:913.

39. Hugo, P., and H.T. Petrie. 1992. Multiple routes for late intrathymic precursors to generate CD4⁺CD8⁺ thymocytes. *In* Advances in Molecular & Cell Biology. J.F.A.P. Miller, editor. JAI Press, Inc., Greenwich, CT. 37–53.

40. Scollay, R., A. Wilson, A. D'Amico, K. Kelly, M. Egerton, M. Pearse, L. Wu, and K. Shortman. 1988. Developmental status and reconstitution potential of subpopulations of murine thymocytes. *Immunol. Rev.* 104:81.

41. Petrie, H.T., P. Hugo, R. Scollay, and K. Shortman. 1990. Lineage relationships and developmental kinetics of immature thymocytes: CD3, CD4 and CD8 acquisition in vivo and in vitro. *J. Exp. Med.* 172:1583.

42. Huesmann, M., B. Scott, P. Kisielow, and H. von Boehmer. 1991. Kinetics and efficiency of positive selection in the thymus of normal and T cell receptor transgenic mice. *Cell.* 66:533.

43. Lucas, B., F. Vasseur, and C. Penit. 1993. The normal sequence of phenotypic transitions in one cohort of BrdUrd-pulse labeled thymocytes. Correlation with T cell receptor expression. *J. Immunol.* 151:4574.

44. Kisielow, P., W. Swat, and H. von Boehmer. 1993. Intrathymic selection and maturation of αβ T cells. *In* Progress in Immunology. J. Gergely, M. Benczur, A. Erdei, A. Falus, G. Füst, G. Medgyesi, G. Petrányi, and E. Rajnavölgyi, editors. Springer-Verlag, Budapest. 81 pp.

45. von Boehmer, H., W. Swat, and P. Kisielow. 1993. Positive selection of immature αβ T cells. *Immunol. Rev.* 135:67.

46. Kelly, K.A., H. Pircher, H. von Boehmer, M.M. Davis, and R. Scollay. 1993. Regulation of T-cell production in T-cell receptor transgenic mice. *Eur. J. Immunol.* 23:1922.