The Final Maturation of At Least Some Single-positive CD4hi Thymocytes Does Not Require T Cell Receptor-Major Histocompatibility Complex Contact
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Summary
The majority (~70%) of postselection CD4 single-positive (SP) thymocytes are CD8loCD4hi. These cells express very low levels of CD8, undetectable by flow cytofluorimetric (FCM) analysis, but sufficiently high to allow purification by panning. Unlike the fully mature CD8−CD4hi thymocytes, which account for the remaining ~30% of the SP CD4+ thymocytes, CD8loCD4hi cells are functionally immature and short-lived unless they receive an unidentified maturation signal from the thymus. In this study, we tested the hypothesis that this signal is provided by a T cell receptor (TCR)–major histocompatibility complex (MHC) class II interaction. Using intrathymic transfer, we show that the immature CD8loCD4hi cells could complete their intrathymic maturation and populate the peripheral lymphoid organs in the absence of MHC class II (and class I) molecules. Furthermore, in mice devoid of class II (and class I) molecules, the progeny of CD8loCD4hi cells was long-lived and functionally reactive to allogeneic class II molecules, although their numbers in the spleen and the mesenteric lymph node were ~40–50% lower than those in class II+ mice 5 mo after transfer. Control experiments demonstrated that the surviving cells did not originate from the contaminating mature thymocytes. These results demonstrate that the final maturation, proliferation, and peripheral survival (up to 5 mo) of at least some postselection CD4+ SP cells do not require the TCR–MHC class II interaction. They also indicate that the TCR–MHC class II interaction(s) required for the intrathymic development of long-lived CD4+ SP cells occurs before the CD4hi SP stage of development.

Key words: CD4 thymocytes • T cell receptor • T cell development • major histocompatibility complex class II

...cell receptor (TCR)-a/b lymphocytes develop in the thymus from immature bone marrow-derived precursors, via a series of phenotypically identifiable maturational steps (1–3). After intrathymic development, successfully selected T cells are exported to the periphery where they play a central role in the immune response (4). The most mature intrathymic T cells comprise the CD4−CD8− and CD4+CD8− thymocytes, which recognize Ag presented by MHC class I and II molecules, respectively. Both cell subsets were initially believed to be fully immunocompetent cells on their way to the periphery (for a review, see reference 4). Subsequently, several findings demonstrated that such a view was oversimplified. First, many murine single-positive (SP)1 thymocytes remain in the thymus for ~13–14 d (5, 6), which accounts for about half the time they spend in this organ. Second, only ~6% of the SP thymocytes are potentially exportable at any given time, suggesting that selective requirements for export of the other 94% are yet to be satisfied (7, 8). SP thymocytes (9) were also found to proliferate in the fetal organ culture (10) and in vivo (11), suggesting that these cells may still be receiving signals from the thymus. In parallel, several studies showed that most of the CD4 SP cells in the thymus are functionally immature (12–15). Finally, it was demonstrated that in vivo the immature, CD8−CD4hi thymocytes require signals from the thymic microenvironment to become immunocompetent long-lived CD8−CD4hi cells (9).
In this study, we investigated the role of the TCR-MHC class II contact in the interaction between the incompletely mature CD8\(^+\)CD4\(^+\) thymocytes and the thymic microenvironment. The design of these experiments also led to insights into the longevity and function of the resulting CD4\(^+\) lymphocytes in a peripheral environment devoid of MHC class II or both class I and II molecules. Our results clearly demonstrate that at least some of the CD8\(^-\)CD4\(^+\) thymocytes can complete their maturation in the absence of such contacts, and that they only require intrathymic contact with class II molecules before the CD4\(^+\) SP stage to become functional CD4\(^+\) SP lymphocytes. Furthermore, these cells were able to proliferate and survive in substantial numbers in the peripheral compartment of MHC class I/II-deficient (CI/II\(^-\)) mice for at least up to 5 mo after transfer, indicating that at least some class II-restricted CD4\(^+\) T cells do not depend on MHC class II molecules for peripheral survival.

**Materials and Methods**

Mice. C57BL/6 (B6) mice and their Ly-5.2 congenic variant (B6.Ly-5.2) were purchased from the National Cancer Institute animal facility (Frederick, M.D.). Class II-deficient (CI/II\(^-\)) mice and class I/II-deficient mice on a B6 background (12th backcross, CII/II\(^-\)) were purchased from Taconic Farms. The B6 Thy-1 congenic variant strain, B6.PL-thy-1\(^+\) Cy, as well as the mice genetically deficient in the CD4 gene (18), which were used as class II\(^-\) recipients, and the natural H-2K\(^b\) and H-2L\(^a\) coisogenic variants of B6 mice, B6.C-H-2\(^m\)(bm1) and B6.C-H-2\(^m\)(bm12), respectively, which were used as stimulators in the MLR, were purchased from The Jackson Laboratory. Female mice, between 7 and 10 wk of age, were used in all experiments.

Antibodies, Reagents, Cell Separation, and Flow Cytometry. The allele-specific anti-Ly5.1 (104-2.1) and anti-Ly5.2 (A20-7.1) mAbs (19; obtained from Dr. U. Hammerling, Memorial Sloan-Kettering Cancer Center) were produced as ascitic fluid, purified, and conjugated to FITC or biotin in our laboratory. All other antibodies and reagents, including the cytotoxic elimination of CD8\(^{hi}\) and CD8\(^{lo}\) cells by mAb \(\text{Cy}^+\) and separation of the resulting population into CD8\(^{lo}\) and CD8\(^{hi}\) by panning, were described previously (9). CD4\(^{hi}\) populations from each subset were then purified to >99% purity, either by flow cytometry (FCM) using a Mo-Flo sorter (Cytomation), or by two sequential immunomagnetic bead sorting steps using the VS\(^+\) separation columns and anti-CD4-conjugated microbeads (Miltenyi Biotec), as indicated. For the cell sorting, cells were stained with mAbs against CD4, CD11c\(^+\), and MHC class II, and were sorted to obtain a CD4\(^{hi}\) class II-CD11c\(^-\) fraction. Upon reanalysis, these cells contained <0.1% cells positive for class II, CD11b, or CD11c, and were used for Exp. 2 in Tables I–III. CD8\(^-\)CD4\(^+\) cells were used in the intravenous transfer experiment to confirm the stringency of cell purification, and in the intrathymic “spiking” experiment aimed to test the role of contaminating cells; the CD8\(^-\)CD4\(^+\) cells were used for intravenous and intrathymic transfers.

Intrathymic Injection, Adult Thymectomy, and In Vivo Antibody Depletion. B6 mice were depleted of CD4 cells using two intraperitoneal injections of 100 \(\mu\)l (–500 \(\mu\)g) mAb GK1.5 in the form of ascites, 2 d apart. Antibody-injected mice were then rested for 4 wk before they entered the experiment to allow elimination of the antibody. They were subsequently used as recipients for intrathymic transfers or were also thymectomized and used as recipients for the intravenous transfer. Genetically deficient mice (CD4\(^-\), CD11c\(^-\), and CI/II\(^-\)) as used as recipients were not pretreated before intrathymic transfer. Intrathymic injections were performed as described (20, 21), with 0.85–2 \(\times\) 10\(^6\) thymocyte subsets of the B6.6Ly-5.2 and/or B6.PL origin injected into each thymic lobe. The thymectomies were performed using the same surgical approach as for the intrathymic injections, except that vacuum suction was applied to remove the organ. Killed mice were dissected to verify the removal of the thymus.

Phenotypic and Functional Analysis of the Peripheral CD4\(^+\) T Cells. 4–5 mo after intravenous or intrathymic transfer, spleens and mesenteric lymph nodes (mLN) from the recipient mice were used to analyze the phenotype and function of CD4 T cells. Phenotypic analysis was performed by FCM, using anti-CD4 and donor- and recipient-specific Ly-5 mAbs, using a FAC Scan™ instrument (Becton Dickinson) equipped with the Lyss™ software, as described previously (9).

For functional analysis, donor-derived CD4\(^+\) cells were magnetically purified from the pooled spleens of the recipients, using FITC-labeled donor allele-specific mAbs, followed by the anti-FITC–conjugated paramagnetic microbeads and the RS\(^+\) separation columns (Miltenyi Biotec). 10\(^5\) cells/well were seeded in a flat-bottomed 96-well plate, alone or with 2 \(\times\) 10\(^3\) irradiated (30 Gy) nucleated spleen cells of bm1, bm12, or B6 origin. The assays were done in quadruplicate. Culture supernatants were collected 72 h later and frozen for subsequent IL-2 quantification by using the IL-2-dependent CTLL indicator line. 5 \(\times\) 10\(^3\) CTLL cells were incubated in a 1:1 dilution of the supernatants for 18 h, the last 6 h in the presence of 1 \(\mu\)Ci of \(\text{H-TdR}\) (New England Nuclear). The cells were then harvested, and the degree of incorporation of radioactive thymidine was determined in a beta-counter (Amersham Pharmacia Biotech).

**Results**

To investigate the role of MHC class II molecules in the final maturation of incompletely mature CD8\(^-\)CD4\(^+\) thymocytes, these cells were intrathymically transferred into recipients genetically deficient in MHC class II molecules.

To that effect, CD8\(^-\)CD4\(^+\) and CD8\(^-\)CD4\(^-\) thymocytes were purified (>99%) from B6.Ly-5.2 mice, as described in Materials and Methods. The key separation step was achieved by panning, and, in our hands, this was found to be the most reliable method of separating these two functionally nonoverlapping subsets. By FCM, the phenotype of these two subsets was characteristic of that described previously (Fig. 1 of this paper, data not shown, and Figure 1 in reference 9); both subsets were CD8\(^-\)CD4\(^+\)TCR\(-\alpha\beta\) by FCM, but the CD8\(^-\)CD4\(^+\) cells contained more CD69\(^+\) cells and expressed higher levels of CD24 and somewhat lower levels of CD44 than CD8\(^-\)CD4\(^+\) thymocytes (not shown). However, as shown previously (9), most of these molecules are expressed by both subsets in at least partly overlapping fashion. We observed that the most
intrathymic and intravenous transfer in Exp. 2 shown in Tables I–III. Percentages denote the distribution of cells into the four major CD8/4-defined populations. (B) Histograms depicting expression of CD45RB on CD8loCD4hi (thick line, MFI = 151) and CD8hiCD4lo (thin line, MFI = 341) thymocytes isolated as described in Materials and Methods. Cells were stained to reveal expression of CD4, CD8, and CD45RB, and the results were obtained using a FACScan (Becton Dickinson). (A) Obtained cells were stained for the expression of CD4 and CD8, and the FCM profile is shown in the figure as the 12% probability plot using the Lysys II® software program (Becton Dickinson). These cells were then used for the consistent difference in the phenotype of these two subsets was in the level of expression of CD45RB: the levels observed on CD8loCD4hi cells were more than twice higher than those on CD8hiCD4lo thymocytes (mean fluorescence intensity [MFI] 151 and 341, respectively; Fig. 1 B), similar to the findings of Penit’s group (15). This feature correlates very well with the difference in functional responsiveness of the two subsets and the function of CD45: the former subset is functionally mature, whereas the latter cannot respond with the full spectrum of functional activities (12).

To further confirm the identity of the above purified cell populations, both subsets were transferred intravenously into thymectomized and CD4-depleted syngeneic recipients. Under such circumstances, only the CD8loCD4hi thymocytes can give long-term (>1 mo) repopulation of the peripheral lymphoid organs, whereas the CD8hiCD4lo cells disappear 3–4 wk after transfer (9). Results shown in Table I confirmed this finding, and demonstrated that the CD8loCD4hi thymocyte subset isolated in this study was phenotypically and functionally indistinguishable from the previously described cells (9, 12). Moreover, results of this experiment demonstrate that the cross-contamination and outgrowth of the contaminating cells between the two subsets isolated as described in Materials and Methods was negligible or nonexistent, as the putative progeny of the cross-contaminating CD8loCD4hi cells was not detectable 4–5 mo after injection of purified CD8loCD4hi cells.

CD8loCD4hi thymocytes from the same cellular preparation were also injected into the thymi of control class II+ CD4– recipients, which express wild-type levels of the MHC class II molecules (but lack CD4+ T cells [18], thus facilitating the detection of CD4+ progeny of the injected cells), or of the C1– recipients, which genetically lacked these molecules (and consequently had only very low CD4+ numbers, owing to a severe defect in CD4+ T cell positive selection [16]). In both types of recipient, injected immature CD8loCD4hi thymocytes yielded long-lived progeny that populated the peripheral lymphoid organs (Table II, Exp. 1). Therefore, class II molecules appeared not to be necessary either for the terminal differentiation of CD4hi thymocytes or for their survival in the periphery. Again, a caveat to this conclusion was the long time course of our experiment, which was conducive for the outgrowth of a minor population of contaminating CD8loCD4hi thymocytes. Although the results shown in Table I strongly argue against this possibility, we performed another control experiment in which we deliberately spiked the transferred CD8loCD4hi cells with Thy-1 congenic (Thy-1.1) CD8hiCD4lo thymocytes. When we added 1% CD8loCD4hi thymocytes to the sorted >99% pure CD8loCD4hi thymocytes, we could not detect the progeny of the admixed Thy-1.1 cells (Table II). The progeny of 10% contaminating cells was detectable, but these cells showed no selective proliferative advantage: their representation remained at or below the 10% level among the transferred thymocytes even after 5 mo in vivo (Table II). Therefore, the above results cannot be accounted for by the contaminating mature thymocytes. Another possibility was that we transferred a substantial number of class II+ cells with our thymocytes. However, cells transferred in Exp. 2 (Table II) were sorted to exclude class II+ and CD11c+ cells, and, after sorting, contained no cells positive for class II, for the dendritic cell (DC) marker CD11c, or the macrophage (M-φ) marker CD11b (<0.1% in Exp. 2, Table II [see legend]). Coupled with the fact that the turnover of class II+ cells (DC, B cells, and M-φ) is relatively rapid and certainly complete within 1 mo, it is extremely unlikely that those cells could have promoted the final maturation of CD4 thymocytes in the thymus and/or the periphery.
were derived from the class I injected CD4 cells themselves expressed class I, as they cally. However, one caveat of this experiment is that the that were tenfold higher than those injected intrathymi- from the spleen only we recovered amounts of CD4 cells still reliably conclude that the presence of the MHC mole-

To test whether the peripheral CD4 progeny of the CD8<sup>+</sup>CD4<sup>+</sup> thymocytes was functional in the absence of MHC molecules, we isolated ~99% pure CD4<sup>+</sup>Ly-5.2<sup>+</sup> donor-derived cells from the recipient mice, stimulated them with allogeneic cells in vitro, and measured the IL-2 release in response to such stimulation (Table III). CD4 cells of donor origin produced IL-2 at levels comparable to those produced by unmanipulated peripheral CD4 cells, es-

Table I. Functional Characterization of the CD8<sup>+</sup>CD4<sup>+</sup> and CD8<sup>-</sup>CD4<sup>+</sup> Thymocyte Subsets Isolated in This Study

| Exp. 1 Donor cells | Mouse no. | Exp. 2 Percent donor CD4<sup>+</sup> cells in mLN Spleen | CD8<sup>-</sup>CD4<sup>+</sup> | CD8<sup>-</sup><br>CD4<sup>+</sup> | CD8<sup>-</sup><br>CD4<sup>+</sup> | Mean 9.0 ± 2.0 2.5 ± 0.3 | CD8<sup>-</sup><br>CD4<sup>+</sup>
|------------------|-----------|-----------------------------------------------------|------------------|------------------|------------------|-------------------------------|------------------|
| CD8<sup>-</sup>CD4<sup>+</sup> | 1         | 7.0 2.0 | CD8<sup>-</sup><br>CD4<sup>+</sup> | 1 | 0.2 0.0 | CD8<sup>-</sup><br>CD4<sup>+</sup> | Mean 9.0 ± 2.0 2.5 ± 0.3 | CD8<sup>-</sup><br>CD4<sup>+</sup>
| 2 | 11.0 3.0 | | 2 | 0.6 0.1 | | Mean 9.0 ± 2.0 2.5 ± 0.3 | |
| Mean | 9.0 ± 2.0 2.5 ± 0.3 | | 3 | 0.8 0.0 | | Mean 9.0 ± 2.0 2.5 ± 0.3 | |
| 4 | 0.5 0.0 | | 4 | 0.5 0.0 | | Mean 9.0 ± 2.0 2.5 ± 0.3 | |
| 5 | 0.0 0.0 | | 5 | 0.0 0.0 | | Mean 9.0 ± 2.0 2.5 ± 0.3 | |
| Mean | 0.4 ± 0.4 0.0 ± 0.1 | | Mean | 0.4 ± 0.4 0.0 ± 0.1 | | Mean 0.4 ± 0.4 0.0 ± 0.1 | |

2 × 10<sup>6</sup> donor B6.Ly-5.2 cells of either subset were injected in the tail vein of B6 mice that were previously thymectomized, and depleted of CD4 cells by mAb treatment. 4 mo (Exp. 1) or 5 mo (Exp. 2) later, the recipients were analyzed by FCM, using CD4 and Ly-5 allele-specific antibodies, as described in Materials and Methods.

A possibility also existed that MHC class I molecules may provide critical signals to keep CD4<sup>+</sup> T cells alive in the peripheral organs. To test this possibility, we used CI/II<sup>-</sup> mice as recipients for thymocytes. Interestingly, upon such transfer, we recovered more CD4 T cells than when recipients only lacked class II molecules (Table II), similar to the results of a recently published study (22). It is possible that the complete lack of T cells, observed in CI/II<sup>-</sup> mice (17), may be conducive to an even more extensive expansion than the one occurring in CI/II<sup>-</sup> mice. Indeed, from the spleen only we recovered amounts of CD4 cells that were tenfold higher than those injected intrathymically. However, one caveat of this experiment is that the injected CD4 cells themselves expressed class I, as they were derived from the class I<sup>+</sup> mice (this was the only way to unambiguously ascertain the donor origin of these cells); therefore, their contact with each other could have provided the survival signal. However, at a minimum, we can still reliably conclude that the presence of the MHC mole-

cules on stromal cells or on APCs is not necessary for either the terminal CD4 thymocyte differentiation or the survival of at least some CD4 T cells in the periphery.

To test whether the peripheral CD4 progeny of the CD8<sup>-</sup>CD4<sup>+</sup> thymocytes was functional in the absence of MHC molecules, we isolated ~99% pure CD4<sup>+</sup>Ly-5.2<sup>+</sup> donor-derived cells from the recipient mice, stimulated them with allogeneic cells in vitro, and measured the IL-2 release in response to such stimulation (Table III). CD4 cells of donor origin produced IL-2 at levels comparable to those produced by unmanipulated peripheral CD4 cells, es-

Discussion

The primary objective of this study was to investigate the dependence of the final phase of intrathymic maturation on MHC class II molecules. Several reports indicated that the TCR–MHC contact must be maintained throughout several discrete stages in T cell development, but none of these has addressed the importance of this contact for terminal maturation of SP cells. As pointed out in our previous publications (9, 12), the CD8<sup>-</sup>CD4<sup>+</sup> cells in our studies differ significantly from the cells called CD8<sup>-</sup>CD4<sup>+</sup> (23–25); theirs correspond to the CD8<sup>-</sup>CD4<sup>+</sup> cells, which bear levels of CD8 lower than those on double-positive (DP) thymocytes but clearly detectable by FCM. Many of these latter cells actually belong to the CD8 lineage. The CD8<sup>-</sup>CD4<sup>+</sup> cells studied here belong to the CD4 lineage and score within the CD4 SP population by FCM; their CD4 levels are undetectable by FCM (for a full phenotypic difference between our CD8<sup>-</sup>CD4<sup>+</sup> cells and the CD8<sup>-</sup>CD4<sup>+</sup> cells of the other authors, see reference 9). Our data resolve the class II dependence of the last phase of intrathymic maturation of SP CD4 thymocytes unambiguously: no MHC class II–TCR contact is required for this late differ-

Survival and peripheral homeostasis of T cells were not the key subjects of this study. However, our results beg a discussion of these issues. Perhaps surprisingly, the perip-
Table II. CD8<sup>+</sup>CD4<sup>+</sup> T lymphocytes Do Not Require MHC Class II Molecules for Final Intrathymic Maturation and Survival in the Peripheral Lymphoid Organs

| Exp. 1 | Recipients | Mouse no. | Percent donor CD4<sup>+</sup> cells in | N. of donor CD4<sup>+</sup> cells |
|--------|-------------|-----------|-------------------------------------|----------------------------------|
|        |             |           | mLN                                | Spleen                           | (Spleen + mLN × 10<sup>6</sup>) |

| Class II<sup>+</sup> | 1 | 12.5 | 5.4 | 4.7 |
|----------------------|---|------|-----|-----|
|                      | 2 | 12.9 | 3.4 | 3.7 |
|                      | 3 | 12.0 | 4.6 | 5.1 |
| Mean                 |   | 12.5 ± 0.5 | 4.5 ± 1.1 | 4.5 ± 0.8 |

| Class II<sup>-</sup> | 1 | 6.9  | 2.3 | 2.7 |
|----------------------|---|------|-----|-----|
|                      | 2 | 5.5  | 2.3 | 3.3 |
|                      | 3 | 5.3  | 2.1 | 2.3 |
| Mean                 |   | 5.9 ± 1.0 | 2.2 ± 0.2 | 2.8 ± 0.5 |

| Exp. 2 | Intrathymic transfer | Mouse no. | CD4<sup>+</sup> B6 cells in spleen | CD4<sup>+</sup> B6.PL cells in spleen |
|--------|----------------------|-----------|----------------------------------|-------------------------------------|
|        |                      |           | Percent | N. x 10<sup>6</sup> | N. x 10<sup>6</sup> |

|                       | 100% B6 | 1 | 1.9 | 2.5 | N/A |
|-----------------------|---------|---|-----|-----|-----|
| → MHC I<sup>-</sup>II<sup>-</sup> |         | 2 | 2.3 | 2.9 | N/A |
|                       |         | 3 | 2.0 | 2.3 | N/A |
| Mean                  |         |   | 2.1 ± 0.2 | 2.6 ± 0.3 | N/A |

|                       | 99% B6<sup>ly</sup> | 1 | 1.7 | 2.5 | N/D |
|-----------------------|---------------------|---|-----|-----|-----|
| 1% B6.PL CD8<sup>+</sup> | 2 | 2.1 | 2.9 | N/D |
| → MHC I<sup>-</sup>II<sup>-</sup> | | 3 | 1.7 | 2.1 | N/D |
| Mean                  |         |   | 2.2 ± 0.5 | 2.5 ± 0.4 | N/D |

|                       | 90% B6<sup>ly</sup> | 1 | 2.1 | 1.9 | 0.07 |
|-----------------------|---------------------|---|-----|-----|-----|
| 10% B6.PL CD8<sup>-</sup> | 2 | 1.7 | 1.5 | 0.05 |
| → MHC I<sup>-</sup>II<sup>-</sup> | | 3 | 1.8 | 1.8 | 0.06 |
| Mean                  |         |   | 1.9 ± 0.3 | 1.7 ± 0.2 | 0.07 ± 0.01 |

|                       | 100% B6 | 1 | 4.2 | 9.0 | N/A |
|-----------------------|---------|---|-----|-----|-----|
| → MHC I<sup>-</sup>II<sup>-</sup> |         | 2 | 4.1 | 7.3 | N/A |
|                       |         | 3 | 3.8 | 11.2 | N/A |
|                       |         | 4 | 3.5 | 8.8  | N/A |
|                       |         | 5 | 2.9 | 6.9  | N/A |
| Mean                  |         |   | 3.7 ± 0.6 | 8.6 ± 1.7 | N/A |

In Exp. 1, 2 × 10<sup>6</sup> donor B6.Ly-5.2 CD8<sup>+</sup>CD4<sup>+</sup> cells were injected intrathymically into CD4<sup>−/−</sup> class II<sup>+</sup> (Class II<sup>+</sup>) or class II<sup>−/−</sup> (Class II<sup>−</sup>) Ly-5.1 recipients. For Exp. 2, CD8<sup>+</sup>CD4<sup>+</sup>CD11c<sup>−</sup> class II<sup>−</sup> cells were isolated from B6 mice by cell sorting at >99% purity (with class II<sup>+</sup>, CD11b<sup>+</sup>, and CD11c<sup>−</sup> cells undetectable by FCM) and were injected into MHC class II<sup>+</sup> recipients alone, or after mixing with 1 or 10% CD8<sup>−</sup>CD4<sup>−</sup> cells from B6.PL mice, to control for the level of possible contamination and outgrowth. An aliquot of CD8<sup>+</sup>CD4<sup>+</sup> cells was also injected into CI/II<sup>+</sup> recipients. Cell number was always kept constant at 8.5 × 10<sup>6</sup>. The recipients were analyzed 5 mo later, as described in Materials and Methods. Results are representative of two experiments. The percentage of CD4<sup>+</sup>Ly-5.1<sup>−</sup> (host) cells in unmanipulated control mice of the recipient type was 0.0 in the peripheral organs of CD4<sup>−/−</sup> mice, up to 1.7 ± 0.5 in the class II<sup>−</sup> mice and <0.1 in CI/II<sup>+</sup> mice. This background was subtracted from the results. N/A, not applicable; N.D, not detectable.

*The number of CD4<sup>+</sup> cells of donor origin in the spleen and mLNs was calculated from the percentages of CD4<sup>+</sup>Ly-5.2<sup>+</sup> cells obtained by FCM and the cellularity of the organs, obtained by trypan blue vital dye counting. The cellularity of the spleens was in the range of 50-85 × 10<sup>6</sup> cells/spleen, whereas that of the mLNs ranged 9-17 × 10<sup>6</sup> cells/mL. 

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| Exp. 1 | CD4 donors | Control | B6 | bm12 | bm1 |
|--------|------------|---------|----|------|----|
| B6     | 420        | 580     | 8,580 | N D |
| CD 8\(^{a}\)→class II\(^{+}\) | 340 | 870 | 8,010 | N D |
| CD 8\(^{a}\)→class II\(^{-}\) | 340 | 380 | 5,520 | N D |
| bm12   | 400        | 9,880   | 890 | N D |

| Exp. 2 |
|--------|
| B6     | 1,560     | 1,180   |
| CD 8\(^{a}\)→class II\(^{+}\) | 560 | 6,750 |
| CD 8\(^{a}\)→class II\(^{-}\) | 790 | 1,200 |
| bm12   | 1,060     | 17,660  | 570 | 870 |

Tissue culture supernatants from the above MLRs were collected after 96 (Exp. 1) or 72 h (Exp. 2), and assayed for the IL-2 content as described in Materials and Methods. Shown are the mean cpm from triplicate determinations. SD did not exceed 10% in any combination. Results are representative of three experiments. The numbers denoting significant proliferation are in bold.


everal progeny of intrathymically transferred CD 8\(^{a}\)CD 4\(^{hi}\) cells was able to survive for \(~5\) mo in the absence of MHC class II molecules (albeit the numbers were \(~50–60\)% of those in class II\(^{+}\) mice 5 mo after the transfer). This survival was not due to the outgrowth of contaminating mature CD 8\(^{a}\)CD 4\(^{hi}\) thymocytes (Table II) nor to the contaminating class II\(^{-}\) cells (which were undetectable in the inoculum). We conclude that MHC class II molecules are not necessary for the long-term survival of at least some CD 4 T cells, but are required for their optimal expansion and homeostasis, since both the percentages and the absolute numbers of CD 4\(^{+}\) T cells were about twofold higher in the presence of class II molecules. The issue of whether MHC molecules are required and necessary for the survival of peripheral T cells has recently gained much attention. Although it appears that MHC class I molecules are necessary for the survival of CD 8\(^{+}\) cells (27–30), the consensus is more tenuous in the case of CD 4\(^{hi}\) cells. Takeda et al. (31) using the transfer of fetal thymic lobes into CII\(^{-}\) mice, concluded that class II molecules are not essential for survival but affect the half-life of CD 4 cells. Rook et al. (22) reported on a model of retroviral reconstitution of class II expression in CII\(^{-}\) mice, and found that the half-life in reconstituted mice that expressed no class II was similar to that of Takeda et al. (31). These authors concluded that the lack of class II molecules curtailed (but did not abolish) the survival of CD 4\(^{+}\) cells, and showed that the lack of class I molecules did not further pronounce this curtailment. In these experiments, the uneven expression of intrathymically injected and retrovirus-driven class II molecules could have caused a situation in which class II molecules were not expressed in all the intrathymic compartments relevant to the T cell longevity. This could have resulted in a paucity of intrathymic signals that regulate the half-life of T cells, thus reducing the numbers of long-lived cells or their half-life. By contrast, the study of Brocker (32) argued for an absolutely essential role of class II on peripheral APCs in the survival of CD 4 T cells, based on the results of transplantation of the APC-depleted class II\(^{-}\) thymi into CII\(^{-}\) mice bearing no transgenes or expressing an MHC class II transgene on DCs alone. In these experiments, good repopulation and intrathymic differentiation of CD 4 T cells were observed, but no extrathymic CD 4 T cells were detected unless the DCs expressed the class II transgene. Brocker postulated that class II molecules are essential for survival of CD 4 T cells. Kirberg et al. (33) showed that CD 4 T cells bearing a TCR transgene required self class II molecules for long-term survival in the periphery. Finally, Viret et al. showed that the CD 4 T cell repertoire is incompletely maintained when a single peptide is present on the majority of MHC class II molecules (34).

Our data certainly agree with all of the above studies in that class II molecules are required for optimal CD 4\(^{+}\) expansion and the filling up of the peripheral compartment. But our results, together with those of Takeda et al. (31), also clearly show that a substantial proportion of peripheral CD 4 T cells do not require MHC class II molecules. Results of Viret et al. (34) are also compatible with this notion, since these authors saw a reduction, but not disappearance, of CD 4 T cells in mice unable to exchange the class II–associated invariant chain peptides for other peptides, owing to a lack of H-2M\(\alpha\). However, the period of observation in these experiments was rather short. In distinction to the studies of Kirberg et al. (33), we used polyclonal T cells and it is quite possible that only some cells from the original inoculum have survived, reflecting the requirement of some, but not all, cells for the particular MHC class II ligand. It is possible that in our system, we have selected for only those cells that do not require class II for peripheral survival, and we are testing this possibility at present. We are also investigating whether these cells express a diverse repertoire, as suggested by their vigorous response to alloantigens (Table III). At face value, our data appear contrary to those of Brocker (33). However, these differences can be reconciled if class II molecules expressed on hematopoietic cells rather than being essential for CD 4 T cell survival, are necessary for the export of selected CD 4 cells from the thymus. If this is the case, both studies would have to be reinterpreted slightly. In our study, then, the transferred CD 8\(^{a}\)CD 4\(^{hi}\) cells either would already have received class II–mediated signals before transfer, or would have been contaminated with sufficient numbers of class II\(^{-}\) cells to provide this signal after transfer. We believe that the latter possibility is highly unlikely, for two reasons. First, the magnetic bead separation retains most DCs and M\(\phi\) on the columns nonspecifically, and since the largest theoretical contamination of the inoc-
With CD4− cells in the range of 600–800 cells (see Fig. 1), even assuming all of these cells were MHC class II+, this number appears too small to permit substantial proliferation and export of maturing thymocytes. Second, the presence of class II+, CD11c+, or CD11b+ cells was undetectable among FCM-sorted inoculated thy-
num with CD4− cells in the range of 600–800 cells
mice grafted with class II− epithelium.
Finally, with regard to CD4 T cell homeostasis, our study demonstrated that the proliferation of the injected thymocytes can occur in the absence of class II molecules, as well as in the absence of class I+ APCs. Interestingly, this proliferation was particularly pronounced in CI/II− mice, paralleling the findings of Rooke et al. (22). At present, it is unclear whether the SP thymocytes in our study proliferated intrathymically (10, 11) or whether the proliferation occurred in the periphery, and additional studies will be needed to address this issue.

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References

1. Nikolić-Žugić, J. 1994. Intrathymic T-Cell Development. R.G. Landes Co., Austin, TX. 1–172.
2. Shortman, K., and L. Wu. 1996. Early T lymphocyte progenitors. Annu. Rev. Immunol. 14:29–47.
3. Aguila, H.L., K. Akashi, J. Domen, K.L. Gandy, E. Lagasse, R.E. Mebius, S.J. Morrison, J. Shizuru, S. Strober, N. Uchida, et al. 1997. From stem cells to lymphocytes biology and transplantation. Immunol. Rev. 157:13–40.
4. Sprent, J., and S.R. Webb. 1987. Function and specificity of T cell subsets in the mouse. Annu. Immunol. 41:39–133.
5. Scollay, R., E. Butcher, and I. Weissman. 1980. T lymphocyte migration: quantitative studies on the rate of migration of cells from the thymus to the periphery in mice. Eur. J. Immunol. 10:210–218.
6. 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. 104:81–120.
7. Gabor, M.J., D.I. Godfrey, and R. Scollay. 1997. Recent thymic emigrants are distinct from most medullary thymocytes. Eur. J. Immunol. 27:1005–1015.
8. Rocha, B., N. Dautigny, and P. Pereira. 1989. Peripheral T lymphocytes expansion potential and homeostatic regulation of pool sizes and CD4/CD8 ratios in vivo. Eur. J. Immunol. 19:905–911.
9. Dyall, R., and J. Nikolić-Žugić. 1995. T cell proliferation and export signal intrathymically (indeed, DCs are well known to reside in the thymus [2]), and would also help the CD4 expansion in the periphery. The first function would be absolutely critical, and its lack would mean that no CD4 T cells could be detected in CII− mice grafted with class II+ epithelium.

The majority of post-selection CD4+ single-positive thymocytes requires the thymus to produce long-lived, functional T cells. J. Exp. Med. 181:235–245.
decreased helper cell activity in mice lacking CD4. Nature. 353:180-184.

19. Shen, F.-W. 1981. Monoclonal antibodies to mouse lymphocyte differentiation antigens. In Monoclonal Antibodies and T Cell Hybridomas. U.H. Hammerling, editor. Elsevier Science B.V., Amsterdam. 25-31.

20. Goldschneider, I., K.I. Komschlies, and D.L. Greiner. 1986. Studies of thymocyteopoiesis in rats and mice. I. Kinetics of appearance of thymocytes using a direct intrathymic adoptive transfer assay for thymocyte precursors. J. Exp. Med. 163:1-17.

21. Nikolic-Zugic, J., and M.J. Bevan. 1988. Thymocytes expressing CD8 differentiate into CD4+ cells following intrathymic injection. Proc Natl. Acad. Sci. USA. 85:8663-8667.

22. Rooke, R., C. Waltzinger, C. Benoist, and D. Mathis. 1997. Targeted complementation of MHC class II deficiency by intrathymic delivery of recombinant adenoviruses. Immunity. 7:123-134.

23. Guidos, C.J., J.S. Danska, C.G. Fathman, and I.L. Weissman. 1990. T cell receptor-mediated negative selection of autoreactive T lymphocyte precursors occurs after commitment to the CD4 or CD8 lineages. J. Exp. Med. 172:835-845.

24. Kearse, K.P., Y. Takahama, J.A. Punt, S.O. Sharrow, and A. Singer. 1995. Early molecular events induced by T cell receptor (TCR) signaling in immature CD4+CD8+ thymocytes increased synthesis of TCR-α protein is an early response to TCR signaling that compensates for TCR-α instability, improves TCR assembly, and parallel other indicators of positive selection. J. Exp. Med. 181:193-202.

25. Lucas, B., and R.N. Germain. 1996. Unexpectedly complex regulation of CD4/CD8 coreceptor expression supports a revised model for CD4+CD8+ thymocyte differentiation. Immunity. 5:461-467.

26. Hare, K.J., E.J. Jenkinson, and G. Anderson. 1999. CD69 expression discriminates MHC-dependent and -independent stages of thymocyte positive selection. J. Immunol. 162:3978-3983.

27. Tanchot, C., F.A. Lemonnier, B. Perarnau, A.A. Freitas, and B. Rocha. 1997. Differential requirements for survival are proliferation of CD8 naive or memory T cells. Science. 276:2057-2062.

28. Nesic, D., and S. Vukmanovic. 1998. MHC class I is required for peripheral accumulation of CD8+ thymic emigrants. J. Immunol. 160:3705-3712.

29. von Boehmer, H., and K. Hafen. 1993. The life span of naive α/β T cells in secondary lymphoid organs. J. Exp. Med. 177:891-896.

30. Benoist, C., and D. Mathis. 1997. Selection for survival? Science. 276:2000-2001.

31. Takeda, S., H.-R. Rodewald, J. Arakawa, H. Bluethmann, and T. Shimizu. 1996. MHC class II molecules are not required for survival of newly generated CD4+ T cells, but affect their long-term life span. Immunity. 5:217-228.

32. Brocker, T. 1997. Survival of mature CD4 T lymphocytes is dependent on major histocompatibility complex class II-expressing dendritic cells. J. Exp. Med. 186:1223-1232.

33. Kirberg, J., A. Berns, and H. von Boehmer. 1997. Peripheral T cell survival requires continual ligation of the T cell receptor to major histocompatibility complex-encoded molecules. J. Exp. Med. 186:1269-1275.

34. Viret, C., F.S. Wong, and C.A. Janeway, Jr. 1999. Designing and maintaining the mature TCR repertoire: the continuum of self-peptide:self-MHC complex recognition. Immunity. 10:559-568.