The Majority of Postselection CD4+ Single-positive Thymocytes Requires the Thymus to Produce Long-lived, Functional T Cells

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Summary

We have previously isolated, and characterized in vitro, two subsets of $CD4^{hi}$ T cell receptor $(TCR)^{hi}$ single positive (SP) thymocytes: $CD8^-$ and $CD8^{lo}$. In this report, we have analyzed phenotypic, functional, and developmental characteristics of these "late" $CD4^{hi}$ SP thymocyte subsets. The TCR^{hi} phenotype and the elimination of T cells expressing TCR V_β segments reactive with endogenous mouse mammary tumor virus (MMTV) products suggested that both subsets had undergone positive and negative selection. $CD8^-4^{hi}$ thymocytes were functional, as judged by their ability to: (*a*) induce lethal graft versus host disease (GVHD); (*b*) survive and expand in peripheral lymphoid organs; and (*c*) proliferate, rather than undergo apoptosis, in response to in vitro TCR cross-linking. By contrast, $CD8^{lo}4^{hi}$ cells could not induce GVHD, were unable to expand (and perhaps even survive) in peripheral organs and underwent apoptosis upon TCR cross-linking. However, when reintroduced into the thymus, these cells matured into functional, long-lived $CD8^-4^{hi}$ lymphocytes. These results document an obligatory requirement for the thymic microenvironment in the final maturation of the majority of $CD4^{hi}$ SP postselection thymocytes, and demonstrate the existence of a previously unrecognized control point in T cell development.

TCR-α/β T cells develop in the thymus from immature precursors via an ordered sequence of phenotypic and functional changes (reviewed in 1–3). The majority of the early T cell precursors express neither CD8 nor CD4 coreceptor molecules and are therefore described as $CD8^-4^-$ double-negatives (DN)[1]. After successful TCR-β rearrangement (4–6), and other less well-defined events, these cells begin to express both CD4 and CD8 on the cell surface, initially at very low (7, 8) and then at high membrane density, to become $CD8^{hi}4^{hi}$ double-positive (DP) thymocytes. In the course of further maturation, positively selected DP cells downregulate one of the accessory molecules and upregulate the TCR to become TCR^{hi} cells of the $CD8^-4^+$ or $CD8^+4^-$ single-positive (SP) phenotype.

SP thymocytes have generally been regarded as functional equivalents of mature, peripheral SP T cells, despite phenotypic differences between thymic and peripheral subsets in the expression of several molecules, including CD24 (heat stable antigen [HSA], low on SP thymocytes and negative on peripheral SP, except for recent thymic emigrants), and Qa-2 (low on SP thymocytes and high on peripheral SP) (9–12). However, we have demonstrated that ~70% of the $CD4^+$ TCR^{hi} SP thymocytes express very low levels of CD8 (undetectable by flow cytometry [FCM]), intermediate levels of HSA, and are hyporesponsive to in vitro allogeneic stimulation in the absence of exogenous IL-2 (13). By contrast, the remaining 30% of $CD4^+TCR^{hi}$ SP cells do not express CD8, are HSA^{lo} and are self-sufficient in an in vitro allogeneic response. Ramsdell et al. (14) obtained similar results, using Qa-2, rather than CD8, as a criterion for subdividing the $CD4^+$ SP thymocytes. To better understand the final steps in intrathymic maturation, we further examined the in vivo and in vitro behavior of these subsets. Throughout the text, we shall use the term $CD4^{hi}$ SP for both cell subsets, since they are indeed SP by FCM; when the $CD8^{lo}$ phenotype is mentioned, it refers to levels of CD8 undetectable by FCM, and detectable by panning; see Materials and Methods, Fig. 1, and reference 13). We show that purified $CD8^{lo}4^{hi}$ thymocytes neither function nor expand in the periphery. However, these cells can develop into mature, functional $CD4^{hi}$ cells if reintroduced into the thymus. Thus, the majority of $CD4^{hi}TCR^{hi}$ SP thymocytes still depend on the thymus for terminal maturation.

[1] *Abbreviations used in this paper:* DN, double negative; DP, double positive; FCM, flow cytometry; GARIG, goat anti-rat IgG; HSA, heat stable antigen; MFI, mean fluorescence intensity; MMTV, mouse mammary tumor virus; SP, single positive.

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Materials and Methods

Mice. BALB/c, C57BL/6 (B6), $CB6F_1$, and $B6D2F_1$ female mice were purchased from the National Cancer Institute animal facility (Frederick, MD). B6.PL and BALB/c *nu/nu* breeding pairs were originally obtained from The Jackson Laboratory (Bar Harbor, ME) and were maintained at the Memorial Sloan-Kettering Cancer Center. Animals were used at 5–10 wk of age, and were age- and sex-matched for each experiment.

Reagents. mAbs against CD8 (3.168, reference 15), CD4 (RL 172.4) and CD25 (PC-61) (16), CD24 (J11d) and Thy-1.2 (J1J) (17), CD24 (M1/69, reference 18) TCR-β (H57-597, reference 19), $V_\beta 8$ (F23.1, reference 20), Thy-1.1 (19E12, reference 21), and CD44 (IM 7.8, reference 22) were produced in our laboratory as tissue culture supernatant or ascites fluid, and were used without modifications or following conjugation to FITC. Goat anti-rat IgG (GARIG; cat. no. R2129) and Con A (both from Sigma Chemical Co., St. Louis, MO), Thy-1.2 FITC and CD4-PE (Becton Dickinson & Co., Mountain View, CA), CD8-PE (Coulter Corp., Hialeah, FL) CD69-FITC, MEL-14-FITC, $V_\beta 3$-FITC, $V_\beta 6$-FITC, and $V_\beta 11$-FITC (Pharmingen, San Diego, CA), anti-Fc-FITC (Sigma Chemical Co.) anti-mouse IgG-FITC (Fisher Biotech, Malvern, PA), and the guinea pig complement (GIBCO BRL, Gaithersburg, MD) were purchased from manufacturers. Dexamethasone was obtained via the Memorial Sloan-Kettering Cancer Center pharmacy.

Isolation of Cell Subsets. Cytotoxic elimination by mAb and complement (C'), positive and negative selection of thymocytes by panning, and FCM sorting were performed as described previously (13). Thymocyte subsets were purified from αCD8 + C' treated thymocytes by panning on GARIG, and both adherent (αCD8-coated cells that survived complement treatment, since they are $CD8^{lo}$) and nonadherent ($CD8^-$) cells further selected for $CD4^{hi}$ expression by either panning or FCM sorting (97–99.5% purity, by FCM analysis). This standard procedure was varied in order to confirm the phenotype of isolated cells and exclude the masking of CD8 molecules at the surface of DP or $CD8^{int}$ cells. Thymocytes were separated into CD8-adherent and CD8-nonadherent fractions by panning, and were then sorted without cytotoxic depletion into $CD8^{lo}4^{hi}$ cells (CD4 SP gate from CD8-adherent cells) and $CD8^-4^{hi}$ cells (CD4 SP gate from CD8-nonadherent cells). This isolation procedure yielded cells with identical phenotype to $CD8^{lo}4^{hi}$ and $CD8^-4^{hi}$ cells obtained by a combination of cytotoxic depletion and panning. Splenic $CD8^-4^{hi}$ cells (>96% pure) were obtained by eliminating $CD24^+$ (B cells and macrophages) and $CD8^+$ cells by mAb + C' and subsequent positive selection of surviving cells by panning.

FCM Analysis. Staining protocols, controls and marker placement were described in (13), except that experiments were performed using a FACScan® instrument, equipped with the LYSYS II software (Becton Dickinson & Co.). 5–20 × 10^3 events were analyzed per sample. TCR V_β expression was determined by single-color staining using directly conjugated anti-V_β mAb. A lack of expression of certain V_β's owing to MMTV-mediated deletions was used as an internal negative control. Results were normalized to the expression of TCR.

For the analysis of DNA fragmentation, 10^6 cells from each population were incubated overnight in 24-well plates (Costar Corp., Cambridge, MA) in 2 ml of standardly supplemented RPMI/10% FCS. The wells were precoated with 100 μg/ml of mAb 597 or with PBS. Dexamethasone (10^{-9} M) was used as a positive control. The percentage of cells displaying subdiploid DNA content was determined by FCM, exactly as described previously (23). For the cell cycle analysis, purified thymocyte subsets were fixed overnight in 70% ethanol, washed, resuspended in PBS containing 1,000 Kunitz units RNase, and stained with propidium iodide (500 ng/ml). These samples were acquired using the doublet-discrimination module of the FACScan®, by dually gating on forward and side scatter and FL-2 area vs. width, to eliminate the inclusion of cell doublets. Cell cycle distribution was then analyzed using Multicycle software (Phoenix Flow Systems, San Diego, CA).

"Lethal" GVHD. 1 or 2 × 10^6 cells of each subset isolated from BALB/c ($H\text{-}2^d$) donors were injected into the tail vein of lethally irradiated (9.5 Gy) F1 hosts ($CB6F_1$ or $B6D2F_1$, both $H\text{-}2^{b\times d}$), reconstituted with 10^7 T cell–depleted (J1J + C') bone marrow cells of donor type. The animals were then observed daily until euthanized when it was judged necessary, or for over 100 d. Clinical signs of the disease included raised fur, weight loss, and loss of motility. Euthanasia was performed when the GVHD became so severe that the animals were no longer able to move around the cage and take food or water. This stage was defined as the endpoint, and the GVHD was deemed lethal. Uninjected, but irradiated and reconstituted animals were used as controls. These mice did not exhibit any symptoms of the GVHD.

T Cell Subset "Parking" into nu/nu Recipients. 2 × 10^6 purified thymocytes or splenocytes from BALB/c +/+ mice were injected intravenously into BALB/c *nu/nu* recipients. Recipients were prescreened against the presence of T cells in peripheral blood by FCM. Spleen and mesenteric lymph node cells of injected and control animals were analyzed for CD4 and CD8 expression at indicated times. Uninjected mice were used as controls.

Intrathymic Injection of T Cell Subsets. 1–2 × 10^6 C57BL/6 ($H\text{-}2^b$, $Thy\text{-}1.2^+$) cells of each subpopulation were injected intrathymically into lethally irradiated (9.5 Gy), syngeneic, T cell–depleted (19E12 + C') bone marrow–reconstituted B6.PL ($H\text{-}2^b$, Thy-1.1) recipients, exactly as described (24). On day 7 post injection, and every third day thereafter, the mice received an intraperitoneal injection of 19E12 ascites (100 μl/animal) to deplete host-type T cells. At indicated times, spleen and mesenteric lymph node cells were analyzed for the presence of $CD4^+$ $Thy\text{-}1.2^+$ and $CD8^+$ T $Thy\text{-}1.2^+$ expression by two-color FCM.

Proliferation Assay. Spleen cells from mice intrathymically injected with thymocyte and splenocyte subsets >36 d before were analyzed for their ability to proliferate in response to 4 μg/ml of Con A. 10^5 spleen cells were seeded in 96-well flat-bottomed plates (Falcon Labware; Becton Dickinson & Co.) in 0.2 ml of completely supplemented RPMI medium in the presence or absence of Con A. 90 h later, each well was pulsed with 1 μCi [^{3}H]TdR (New England Nuclear, Boston, MA). 6 h later, cells were harvested and the degree of incorporation of radiolabeled thymidine determined in a beta-counter (Pharmacia LKB, Piscataway, NJ). Results are represented as the mean of triplicate wells ± standard error.

Results

Expression of Developmental Markers and the TCR V_β Repertoire of $CD4^{hi}$ Thymocyte Subsets. A thymocyte subset named $CD8^{lo}4^{hi}$ was previously described by two groups (25–27). In these experiments, $CD8^{lo}4^{hi}$ cells were defined by electronic FCM gating as the cells bearing CD8 levels four to five times lower than those on typical DP thymocytes, but higher than those seen on SP cells, and were shown to bear the $TCR^{int}CD24^{hi}CD44^{lo}CD69^{hi}$ phenotype (25, 26). Furthermore, this population contained cells bearing V_β TCRs reactive with products of endogenous mouse mammary tumor viruses (MMTVs), that are eliminated from the

mature T cell pool, suggesting that negative intrathymic selection may be incomplete in this intermediate subset (25). Based on this finding, it was concluded that $CD8^{lo}4^{hi}$ cells may have undergone positive but not negative selection (25). In 1990, we described a delineation of $CD4^{hi}$ SP cells into $CD8^-$ and $CD8^{lo}$ subsets and showed that they express high levels of CD4 and TCR, similar to those found on fully mature peripheral cells (13). CD8 expression was undetectable by FCM on $CD8^{lo}$ cells, and, as discussed previously, could only be detected by panning. By phenotypic and functional criteria, both subsets looked like bona fide SP thymocytes, but differed in requirements for the in vitro proliferation (13). We therefore hypothesized that $CD8^{lo}4^{hi}$ phenotype marks a late stage in the intrathymic development, immediately before complete downregulation of CD8 and migration to the periphery. Because the $CD8^{lo}4^{hi}$ cells described by other groups bear CD8 levels clearly detectable by FCM (25–27), these cells were different from our $CD8^{lo}4^{hi}$ thymocytes, and would better be characterized as $CD8^{int}$.

It was, however, formally possible that our $CD8^{lo}4^{hi}$ cells actually were $CD8^{int}$, i.e., identical to $CD8^{lo}4^{hi}$ cells described by other groups, but that their CD8 was masked by cytotoxic treatment used to deplete $CD8^{hi}$ (DP) cells. We considered this possibility unlikely because secondary reagents (e.g., GARIG–FITC), as well as noncompeting antibodies (e.g., anti-CD8α for depletion and anti-CD8β for staining), revealed no such blocking (not shown). Furthermore, direct sorting of $CD4^{hi}$ SP cells from thymocyte suspensions that have not been subjected to cytotoxic treatment yielded cells bearing an identical phenotype to cells obtained by a combination of αCD8 + C′ and panning (not shown). However, to independently test this possibility, we performed an extended phenotypic analysis of gated $CD8^{int}4^{hi}$ cells (using the gates described by Guidos et al. [25], and Bendelac et al. [26, 27]) and $CD8^-4^{hi}$ and $CD8^{lo}4^{hi}$ thymocytes (isolated as described in Materials and Methods), using total thymocytes as the reference point. In a typical experiment using BALB/c thymocytes (Fig. 1 *A*), all populations were generated from the same starting population (total thymocytes), either by selective gating ($CD8^{int}$) or by a combination of positive and negative selection by mAb + C′ and panning ($CD8^{lo}$ and $CD8^-$ cells). The cells were triple-stained to reveal the ex-

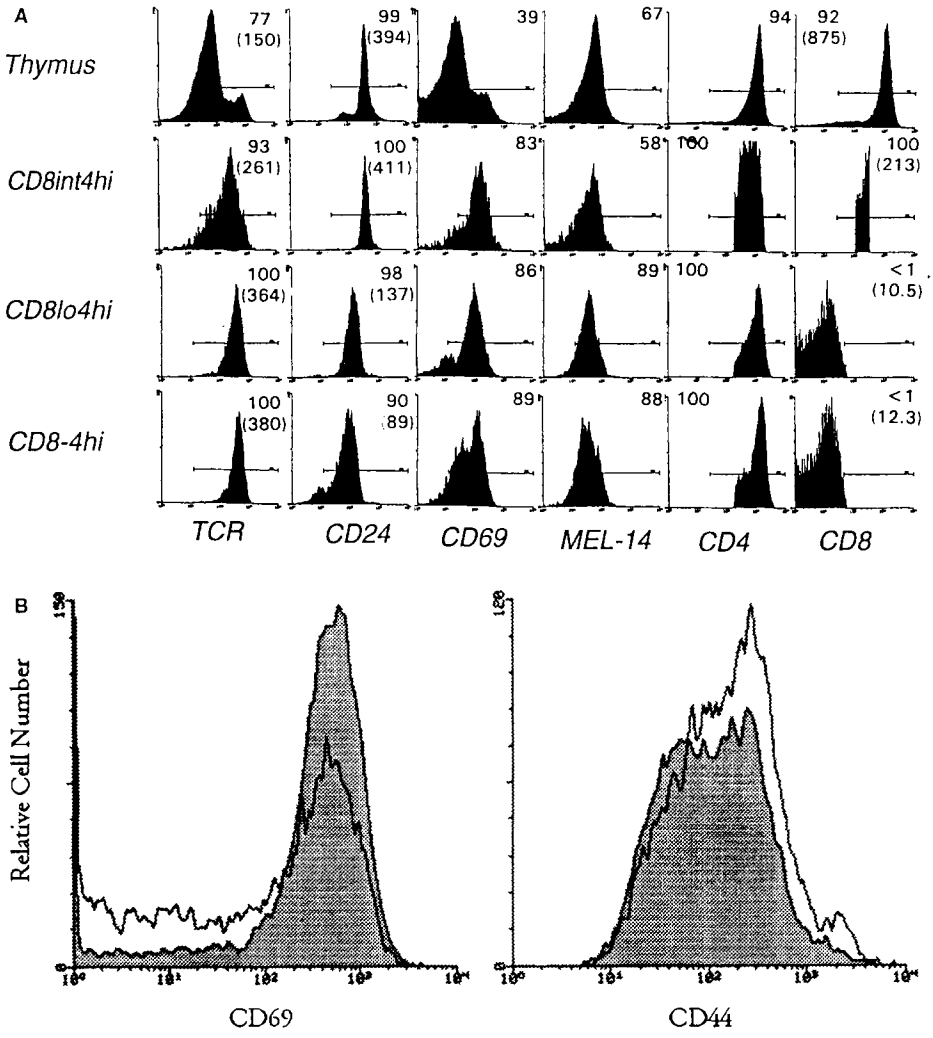

Figure 1. Expression of developmental markers on $CD4^{hi}$ SP thymocyte subsets of B6 mice. (*A*) Total BALB/c thymocytes (*Thymus*), thymocytes gated on $CD8^{int}4^{hi}$ cells ($CD8^{int}4^{hi}$, gated as in references 25 and 26) or purified $CD8^{lo}4^{hi}$ and $CD8^-4^{hi}$ thymocytes were analyzed after three-color staining for CD8, CD4, and the indicated molecule. Markers were set to denote fluorescence higher than that of the background. Numbers represent percent of cells positive for the given molecule. Numbers in brackets represent MFI, and are provided to facilitate comparisons in the intensity of expression of TCR, CD24, and CD8. (*B*) B6 $CD8^{lo}4^{hi}$ (*shaded histograms*) and $CD8^-4^{hi}$ (*open histograms*) thymocytes were purified as described in Materials and Methods, and analyzed by three-color FCM for the expression of CD4, CD8, and either CD69 (*left*) or CD44 (*right*). In this experiment, $CD8^{lo}4^{hi}$ cells were 82.3% $CD69^+$, 79.9% $CD44^+$, and $CD8^-4^{hi}$ cells were 54.7% $CD69^+$, 86.1% $CD44^+$, based on the marker placement to demarcate fluorescence higher than that of isotype-stained control. Both cell preparations were <1% $CD8^+$ and >98% $CD4^{hi}$.

pression CD4, CD8, and the third marker, as shown in Fig. 1 *A*. Total thymocytes contained the major TCR^-/TCR^{lo} population, a minor TCR^{hi} population, and a TCR^{int} population between those two. While $CD8^{int}4^{hi}$ cells were TCR^{int} (mean fluorescence intensity [MFI] = 261), both $CD8^{lo}4^{hi}$ and $CD8^-4^{hi}$ cells were TCR^{hi}, with a slight difference between the two in TCR intensity (364 vs. 380 MFI). CD24 showed the opposite trend; levels were very high and virtually identical on total thymocytes and $CD8^{int}4^{hi}$ cells, were reduced to a third on $CD8^{lo}4^{hi}$ cells, and to less than a fourth in $CD8^-4^{hi}$ cells, in accordance with previous reports (13, 14). The expression of an early "activation" marker CD69 (25–28) correlated well with the expected maturational status of thymocyte subsets; its intensity was the highest on the most immature $CD8^{int}4^{hi}$ cells, and progressively decreased in $CD8^{lo}4^{hi}$ and $CD8^-4^{hi}$ cells, to reach ~45–55% of $CD8^-4^{hi}$ cells that are intermediate to low for CD69 expression (Fig. 1, *A* and *B*), consistent with previous results (25–27). The intensity of expression of Mel-14 (L-selectin) was relatively uniform on all subsets analyzed, although $CD8^{lo}4^{hi}$ thymocytes showed the most homogenous MEL-14^+ staining pattern. CD4 expression was high in all populations, while that of CD8 was intermediate (as gated) on $CD8^{int}4^{hi}$ cells (roughly fourfold lower than that on total thymus) and negative on $CD8^{lo}4^{hi}$ and $CD8^-4^{hi}$ cells. Expression of CD44 increased with maturation, so that $CD8^{int}$ cells were largely low, while $CD8^{lo}$ and $CD8^-$ cells were almost identically high (Fig. 1 *B* and not shown). That trend was more obvious in B6 mice (Fig. 1 *B*), where the CD44 expression is generally lower, than in BALB/c (not shown). Qa-2, a late differentiation antigen, was expressed at low levels on the most mature, $CD8^-4^{hi}$ cells and was negative on other populations analyzed (not shown), in accordance with previous results of Ramsdell et al. (14).

The expression of endogenous MMTV-reactive V_β TCRs on thymocyte subsets is shown in Fig. 2. This analysis revealed that the elimination of self-reactive V_β's ($V_\beta 3$ and $V_\beta 11$ in this experiment) had occurred in both $CD8^{lo}4^{hi}$ and $CD8^-4^{hi}$ thymocytes. This feature further distinguishes our $CD8^{lo}4^{hi}$ cells from the $CD8^{lo}4^{hi}$ (more accurately $CD8^{int}4^{hi}$) cells described by Guidos et al. (25) and Bendelac et al. (26, 27), in which the elimination was incomplete (Fig. 2). Therefore, both the $CD8^{lo}4^{hi}$ and $CD8^-4^{hi}$ thymocyte subsets have the TCR phenotype of postselection thymocytes, based on the high TCR expression and the elimination of cells bearing self-reactive V_βs.

Together, these results show that $CD8^{lo}4^{hi}$ cells are a distinct thymocyte subset, phenotypically more mature than the previously identified $CD8^{int}4^{hi}$ cells, but less mature than $CD8^-4^{hi}$ thymocytes. These three stages probably represent a continuum of phenotypic changes that occur as a consequence of intrathymic (selectional) contacts at an earlier stage (perhaps DP) in development. In the remaining part of this work, we sought to determine whether additional intrathymic contacts were required for the progression through the final stages of intrathymic development.

In Vivo Responsiveness of $CD4^+$ Thymocyte Subsets. In our previous experiments, both $CD8^{lo}4^{hi}$ and $CD8^-4^{hi}$ thymocytes were shown to be restricted by MHC class II and not class I molecules. However, while $CD8^-4^{hi}$ thymocytes were fully capable of proliferating to allogeneic stimulation in vitro, $CD8^{lo}4^{hi}$ cells required exogenous IL-2 (13). To establish whether the in vitro responsiveness of $CD8^{lo}4^{hi}$ and $CD8^-4^{hi}$ thymocytes was indeed representative of their function in vivo, thymocyte subsets were injected intravenously into semiallogeneic irradiated hosts. In such a test, mature splenic $CD8^-4^{hi}$ cells (positive control) readily induced lethal GVHD (Table 1). In all experiments performed to date,

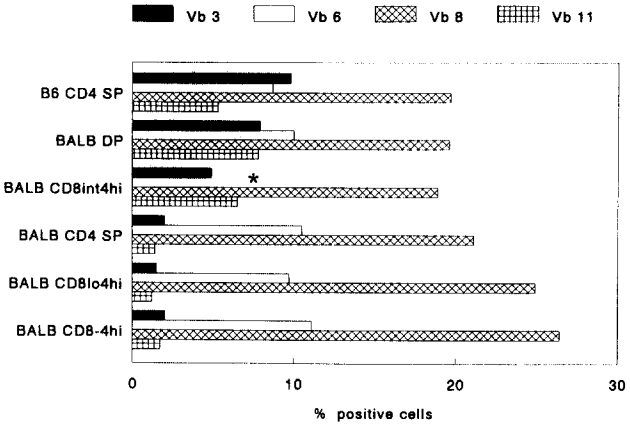

Figure 2. Expression of V_βs in thymocyte subsets. Thymocyte subpopulations were isolated/obtained as described for Fig. 1, and were stained with FITC-conjugated mAb against V_βs and PE-conjugated αCD4. All populations were selectively gated on $CD4^{hi}$ cells, and analyzed for the expression of TCR $V_\beta 3$ (*solid bars*), $V_\beta 6$ (*open bars*), $V_\beta 8$ (45°-angle *hatched bars*) and $V_\beta 11$ (90° angle-*hatched bars*). V_β expression is normalized to the expression of TCR. Similar results were obtained in two other experiments. Asterisk (*), not determined.

Table 1. *GVHD Mediated by $CD4^{hi}$ SP Thymocyte Subsets*

Subset	Recipient	Survival
Experiment 1		*d*
BALB/c $CD8^{lo}4^{hi}$ Thy	B6D2 F_1	3/3 (>100)
BALB/c $CD8^-4^{hi}$ Thy	B6D2 F_1	0/3 (5;5;6)
BALB/c $CD8^-4^{hi}$ Spl	B6D2 F_1	0/3 (7;7;9)
nil	B6D2 F_1	3/3 (>100)
Experiment 2		
BALB/c $CD8^{lo}4^{hi}$ Thy	CB6 F_1	3/3 (>100)
BALB/c $CD8^-4^{hi}$ Thy	CB6 F_1	0/3 (13;15;28)
BALB/c $CD8^-4^{hi}$ Spl	CB6 F_1	0/3 (12;15;32)
nil	CB6 F_1	3/3 (>100)

Purified cell subsets (2×10^6 cells/recipient) were injected into lethally irradiated (9.5 Gy) and reconstituted F_1 recipients as described in Materials and Methods. Animals were maintained on acidified water and observed daily for signs of GVHD. Lethal GVHD was determined as described in Materials and Methods. Results are expressed as the number of mice surviving/total number of tested mice, with length of survival shown in parentheses.

$CD8^-4^{hi}$ thymocytes were at least as efficient as their splenic counterpart in inducing acute GVHD. By contrast, the $CD8^{lo}4^{hi}$ subset failed to mount a GVHD response, and the animals that received these cells enjoyed a long-term survival equivalent to the control group (Table 1). Note that the kinetics of GVHD was more rapid when BALB/c ($H\text{-}2^d$) cells were injected into B6D2 F_1 ($H\text{-}2^{b\times d}$) mice (from which they differ at additional minor histocompatibility loci, including MMTVs), compared to truly semiallogeneic CB6 F_1 ($H\text{-}2^{b\times d}$) animals, but that $CD8^{lo}4^{hi}$ cells did not react in either case. These results indicate that $CD8^{lo}4^{hi}$ cells are not only hyporesponsive to antigenic stimulation in vitro, but also cannot respond to antigenic stimulation in vivo.

$CD8^{lo}4^{hi}$ Thymocytes Fail to Expand (and May Fail to Survive) in Peripheral Lymphoid Organs. Several hypotheses, including the functional incompetence, the inability to survive in the periphery and the inability to properly home to lymphoid organs, could explain the failure of $CD8^{lo}4^{hi}$ cells to induce GVHD. To address these possibilities, we asked whether $CD8^{lo}4^{hi}$ cells could populate the peripheral lymphoid organs of athymic syngeneic hosts after intravenous transfer. Transfer of either splenic or thymic $CD8^-4^{hi}$ cells resulted in a selective and progressive increase of $CD4^+$ cell numbers in lymphoid organs of syngeneic nude recipients (Table 2). This result indicated that donor cells not only populated host lymphoid organs, but also proliferated there. For example, 2×10^6 cells injected on day 0 yielded by day 42 an average increase in $CD4^+$ cell number by 6.3×10^6 cells (thymic $CD8^-4^{hi}$) and 5.7×10^6 cells (splenic $CD8^-4^{hi}$ cells) in donor spleens alone (experiment 2, Table 2). Assuming that the injected cells distributed equally throughout the lymphoid system, the proliferation of donor cells was substantial. By contrast, lymphoid organs of animals injected with $CD8^{lo}4^{hi}$ thymocytes failed to show any increase in $CD4^+$ cells by 3–4 wk post injection, although some increase could be detected on days 10 and 15 post transfer (Table 2 and not shown). The percentage and numbers of total $CD4^+$ cells in animals injected with $CD8^{lo}4^{hi}$ cells suggest that, before becoming

Table 2. *$CD8^{lo}4^{hi}$ Thymocytes Cannot Populate Peripheral Lymphoid Organs*

	Percent cells in lymphoid organs of *nu/nu* recipients			
	Spleen		Lymph nodes	
"Parked" subset	$CD8^+$	$CD4^+$	$CD8^+$	$CD4^+$
Experiment 1				
Day 10				
nil	0.4	2.0	ND	ND
$CD8^{lo}4^{hi}$ Thy	0.2	**4.7**	ND	ND
$CD8^-4^{hi}$ Thy	0.3	**4.3**	ND	ND
Day 15				
nil	1.0	2.0	ND	ND
$CD8^{lo}4^{hi}$ Thy	0.8	**5.7**	ND	ND
$CD8^-4^{hi}$ Thy	0.5	**10.3**	ND	ND
Day 29				
nil	0.8	1.8	ND	ND
$CD8^{lo}4^{hi}$ Thy	0.7	1.8	ND	ND
$CD8^-4^{hi}$ Thy	1.1	**11.2**	ND	ND
Experiment 2				
Day 42				
nil	0.7 (0.5)	1.4 (0.5)	0.4 (0.3)	3.6 (1.0)
$CD8^{lo}4^{hi}$ Thy	0.6 (0.5)	1.3 (0.4)	0.6 (0.5)	3.3 (2.3)
$CD8^-4^{hi}$ Thy	0.8 (0.4)	**9.7 (3.4)**	0.3 (0.2)	**14.1 (2.3)**
$CD8^-4^{hi}$ Spl	1.2 (1.0)	**8.3 (1.2)**	0.6 (0.5)	**12.9 (5.1)**

Syngeneic (BALB/c) *nu/nu* animals were injected with 2×10^6 cells of indicated phenotypes, and their peripheral lymphoid organs analyzed. For experiment 1, individual mice were analyzed at indicated times. For experiment 2, four to five animals were analyzed per group, and the results are expressed as mean percentage (SE). Normal BALB/c animals, used as controls in these experiments contained 11.1–15.4% $CD8^+$ and 30.2–41.6% $CD4^+$ T cells. Absolute cell numbers are discussed in the text. Results where $CD4^+$ cells were overrepresented by more than twofold compared with the control are boldfaced. Results are representative of four experiments. ND, not determined.

undetectable, these cells probably did not undergo significant proliferation. For example, on day 15 (experiment 1), the spleens of animals injected with $CD8^{lo}4^{hi}$ cells were estimated to contain 2.1×10^6 cells of donor origin (after correction for the background).

Since we did not have a congenic marker to follow the injected cells directly, we had to rely on numbers of CD8 and CD4 T cells as an indicator of the appearance, survival, and proliferation of T cells in recipient spleen and lymph nodes. To avoid possible misinterpretation due to leakiness of *nu/nu* mice in our colony, we selected our recipients among mice containing less than 2% of T ($CD4^+$ and $CD8^+$) cells in the peripheral blood. The results of our experiments clearly segregated into two groups. Uninjected and $CD8^{lo}4^{hi}$-injected animals had identical baseline levels of T cells 4 wk and later, after the transfer. This was in contrast to animals injected with thymic and splenic $CD8^-4^{hi}$ cells, which displayed a selective increase in $CD4^+$ and not $CD8^+$ T cells. Furthermore, a small and selective increase in $CD4^+$ cells was detected in animals receiving $CD8^{lo}4^{hi}$ cells (but not control animals) at early time points. Our assay therefore measured the proliferative ability (and, most likely, survival; see the next section) of transferred $CD4^{hi}$ subsets, rather than their putative indirect effect on host T cells. Based on these results and on the results of apoptosis experiments (see below), we favor the explanation that $CD8^{lo}4^{hi}$ cells are probably not capable of surviving in peripheral lymphoid organs. However, since our approach could not formally prove that no $CD8^{lo}4^{hi}$ cells can be detected in recipient lymphoid organs, we have to allow an alternative explanation, namely that some $CD8^{lo}4^{hi}$ cells may have survived after transfer. Nevertheless, these cells are clearly incapable of expanding (Table 2) or functioning (Table 1) in peripheral lymphoid organs. Thus, we conclude that $CD8^{lo}4^{hi}$ thymocytes were capable of homing to, but were unable to proliferate (and may have failed to survive) in peripheral lymphoid organs.

$CD8^{lo}4^{hi}$ Thymocytes Are Susceptible to TCR-induced Apoptosis Despite Their "Postselection" Phenotype. The inability of $CD8^{lo}4^{hi}$ thymocytes to expand, (and, possibly, survive) upon syngeneic adoptive intravenous transfer prompted us to examine whether these cells would undergo apoptosis after TCR stimulation. TCR-induced DNA fragmentation was used as a quantitative readout of apoptosis. (The nature of apoptosis was confirmed by DNA "ladder" analysis, but this method was not quantitative for our purposes; data not shown.) The results of this analysis (Fig. 3) show a susceptibility of $CD8^{lo}4^{hi}$ thymocytes to TCR-induced apoptosis. By contrast, both $CD8^-4^{hi}$ thymocytes and splenocytes were resistant. The apoptotic behavior of $CD8^{lo}4^{hi}$ cells was thus reminiscent of DP thymocytes in normal (29) and TCR transgenic (30, 31) mice. This was a surprising finding, given that $CD8^{lo}4^{hi}$ thymocytes have almost completely downregulated their CD8 expression (which is detectable only by panning and not by direct FCM) and that they express the V_β repertoire associated with completed negative selection (32, 33). Nevertheless, this finding may explain the failure of $CD8^{lo}4^{hi}$ cells to mediate GVHD, expand and perhaps even survive outside the thymus.

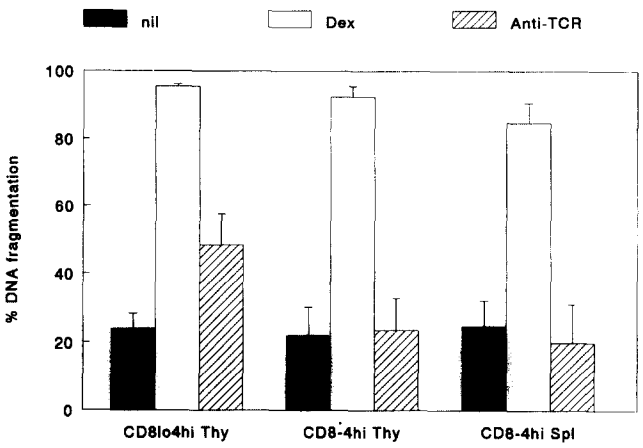

Figure 3. TCR-induced apoptosis in $CD4^+$ SP subsets. Purified $CD4^{hi}$ subsets were incubated without stimulation (*solid bars*), with dexamethasone (*open bars*) or immobilized anti-TCR-βmAb (*striped bars*) overnight. Cells were then washed, stained with propidium iodide, and analyzed for the number of cells containing subdiploid DNA content, diagnostic of apoptosis, as described in Materials and Methods. Results are represented as mean (SD) for two experiments.

Intrathymic Differentiation of $CD8^{lo}4^{hi}$ Cells. We originally hypothesized that $CD8^{lo}4^{hi}$ thymocytes are precursors of $CD8^-4^{hi}$ cells, but other explanations were also plausible. For example, high susceptibility of $CD8^{lo}4^{hi}$ thymocytes to apoptosis, and their inability to survive in peripheral lymphoid organs, suggested a possibility that $CD8^{lo}4^{hi}$ thymocytes are not precursors of thymic and peripheral $CD8^-4^{hi}$ cells, but are instead a dead-end population, marked for intrathymic death. However, this explanation is difficult to reconcile with the postselection phenotype of these cells. An alternative to this explanation, and the one in line with our initial hypothesis (13) was that $CD8^{lo}4^{hi}$ cells will become $CD8^-4^{hi}$, but can only complete their differentiation in the presence of the thymic microenvironment. We sought to distinguish between these possibilities by performing intrathymic injections of Thy-1.2^+ thymocyte and splenocyte subsets and analyzing the phenotype and function of their progeny in peripheral lymphoid organs of congenic (Thy-1.1^+) recipients. Intrathymic transfer of thymic and splenic $CD8^-4^{hi}$ cells gave rise to long-lived (>35 d) $CD8^-4^{hi}$ cells in the spleen (not shown) and mesenteric lymph nodes (Table 3), with CD4/CD8 ratios among donor-derived cells of 20–60:1. It is likely that these cells simply migrated out of the thymus, populated the periphery, and proliferated there (1.75×10^6 injected cells yielded $1.5–2 \times 10^6$ cells in mesenteric lymph nodes and $5–8 \times 10^6$ cells in the spleen after 42 d; Table 3 and not shown). Similar numbers of donor-derived cells were obtained by transferring $CD8^{lo}4^{hi}$ cells, except that we consistently noted the presence of a small number of $CD4^-8^+$ cells. In this case, CD4/CD8 ratios varied between 8:1 and 14:1 (Tables 3 and 4, and not shown). By contrast, intrathymic transfer of purified $CD4^-8^-$ precursor thymocytes yielded peripheral progeny consisting of a physiological mixture of $CD4^-8^{hi}$ and $CD8^-4^{hi}$ SP cells in a CD4/CD8 ratio of 2:1, typically found in the donor strain

Table 3. *$CD8^{lo}4^{hi}$ Thymocytes Require the Thymic Microenvironment for Final Maturation*

Cells transferred	Percent donor-derived (Thy 1.2) cells (SD) CD4	CD8	CD4/8 index
nil (B6)	[46.0	23.2	2.0]
$CD8^{lo}4^{hi}$ Thy	23.9 (5.6)	2.2 (0.7)	10.9
$CD8^{-}4^{hi}$ Thy	12.4 (5.1)	0.2 (0.1)	62.0
$CD8^{-}4^{hi}$ Spl	14.1 (1.4)	0.5 (0.1)	28.2

Intrathymic injections of purified thymocyte subsets (1.75×10^6/thymus) into Thy-1 congenic mice were performed as described in Materials and Methods. From day 7 after injection, host T cells were depleted by in vivo mAb treatment (19E12, intraperitoneally). On day 42, the presence of donor-derived (Thy-1.2^+) cells within CD4 and CD8 T cell subsets in individual mesenteric lymph nodes (results expressed as mean [SE], $n = 3$) was determined by two-color FCM. The absolute donor-derived cell numbers recovered from the nodes varied from $1.3–3.6 \times 10^6$ cells, and were comparable between different groups. These results indicated an expansion of intrathymically injected progeny. Similar results were obtained upon analysis of host splenocytes, and in three other experiments.

of mice. Thus, although some cells in our $CD8^{lo}4^{hi}$ preparation developed into peripheral $CD4^{-}8^{hi}$ cells (most likely due to DN contamination, as discussed below), most of these cells gave rise to long-lived $CD8^{-}4^{hi}$ cells that populated peripheral lymphoid organs.

A possible caveat of the above experiments lies in their long time course. This could magnify the contribution of occasional cross-contaminants to the outcome of these experiments. For example, if $CD8^{lo}4^{hi}$ cells were contaminated with $CD8^{-}4^{hi}$ cells at the moment of injection, it was possible that we actually detected the progeny of proliferating $CD8^{-}4^{hi}$ cells in the periphery. Since the results shown in Table 2 failed to show any evidence of peripheral expansion of such putative contaminants, the possibility remained that these contaminants proliferated intrathymically. Several lines of evidence speak against this possibility. (*a*) Panning, which was used to separate these two subsets, is an exquisitely sensitive and successful positive selection technique, but a relatively poor negative selection technique (7, 24, 34). Therefore, a contamination would mostly occur in the reverse direction, such that the $CD8^{-}4^{hi}$ cells might contain some $CD8^{lo}4^{hi}$ cells, and not vice versa. Indeed, our results in Tables 1 and 2 strongly support this notion, since even the long-term (over 100 d) functional assays failed to reveal contamination of $CD8^{lo}4^{hi}$ cells by $CD8^{-}4^{hi}$ cells. (*b*) A number of reports from different laboratories, using thymocyte labeling with three different markers ([^{3}H]TdR, bromodeoxyuridine (BrdU), and 7-amino-actinomycin D) had concluded that adult $CD4^+$ SP thymocytes contain a negligible population of cycling cells (35–38). It is therefore extremely unlikely that $CD4^+$ SP cells could proliferate to a significant degree intrathymically. (*c*) Our results (Dyall, R., and J. Nikolić-Zugić, unpublished results) show that CD4 SP thymocytes become undetectable in the thymus 10 d after the intrathymic injection. The long duration of our assay, which was necessary in order to ascertain that we are looking at bona fide long-lived progeny of injected $CD8^{lo}4^{hi}$ cells, and not transient emigrants that will disappear (like those in experiment 1, Table 2), was therefore probably not a factor in light of low numbers of cycling SP cells and their relatively short intrathymic life. (*d*) Finally, our interpretation could indeed be questioned if all cycling $CD4^+$ SP cells were to segregate with $CD8^{-}4^{hi}$ and none with $CD8^{lo}4^{hi}$ subset. To address this possibility, we purified thymocyte subsets and determined their cell cycle status using propidium iodide. This analysis

Table 4. *Progeny of Intrathymically Injected $CD8^{lo}4^{hi}$ Cells Is Immunocompetent*

Subset transferred	Unstimulated cells Thy-1.2^+ CD4/8 Percent	Index	Stimulation Con A response (SI)*	After stimulation Thy-1.2^+ CD4/8 Percent	Index
BALB/c control	27.8/9.3	3.0	328.2	25.1/8.3	3.0
nil (B6.PL)	NA	NA	1.2	NA	NA
DN Thy	3.0/1.6	1.9	42.3	9.4/3.8	2.4
$CD8^{lo}4^{hi}$ Thy	5.1/0.6	8.5	39.7	24.3/2.3	10.5

Intrathymic injection (10^6 cells/recipient) and antibody treatment were performed as described in Fig. 3. 5 wk later, pooled splenocytes ($n = 3$) were phenotyped for the coexpression of Thy-1.2 and either CD4 or CD8 (Unstimulated cells) and tested for their ability to proliferate in response to mitogenic stimulation (Stimulation) as described in Materials and Methods. Proliferating cells were also stained for the presence of donor-derived CD8 and CD4 cells (After culture). Results of phenotypic analysis are displayed as donor-type CD4/8 ratios and indices obtained by dividing the two. These parameters could not be determined for control B6.PL mice, (NA, not applicable) since their spleen cells underwent a pronounced depletion of T cells by the in vivo mAb treatment.

* Results of the proliferation assay are displayed as stimulation indices, where SI = mean experimental cpm/mean control cpm. Actual cpm ($\times 10^{-3}$) were: 430.0 and 1.3 for BALB/c; 31.8 and 0.7 for DN; and 14.4 and 0.3 for the $CD8^{lo}4^{hi}$ group, for experimental and control triplicates, respectively. Cultures without Con A were used as a control.

(Fig. 4) reveals that CD8$^-$4^{hi} cells contain very low numbers of cycling cells (3.6%; 2.3% when corrected for the presence of DN contaminants), even lower than CD8^{lo}4^{hi} cells (4.4%; 3.3% when corrected for the presence of DN contaminants). By contrast, total thymocytes, and, in particular, DN cells, contained much higher numbers of cycling cells (13.4 and 37.1%, respectively). (Above percentages of cycling cells may actually be an overestimation, owing to slight overestimation of the S phase by most DNA cycle programs.) It is therefore clear that rare CD8$^-$4^{hi} contaminants are not capable of significant intrathymic expansion. Furthermore, when monitored for the incorporation of BrdU into DNA over 24 h, both subsets revealed identical low labeling (3–4%), consistent with a noncycling nature of these cells. Together, these results effectively rule out the role of contamination, and strongly suggest that CD8^{lo}4^{hi} thymocytes require the thymus for further development.

Progeny of Intrathymically Injected CD8^{lo}4^{hi} Thymocytes Is Functionally Immunocompetent. Despite the phenotypic evidence that CD8^{lo}4^{hi} thymocytes can develop into long-lived (>35 d), mature peripheral CD8$^-$4^{hi} cells, it was critical to demonstrate that these cells were immunocompetent, and not paralyzed or anergized. We therefore tested the peripheral progeny of intrathymically injected CD8^{lo}4^{hi} cells for their ability to proliferate in response to mitogenic and antigenic stimulation. We stimulated total splenic lymphocytes from control and intrathymically injected animals, measured their proliferation and determined the phenotype of proliferating cells. Control BALB/c splenocytes (consisting of 37.1% T cells before stimulation, with a CD4/8 ratio of 3:1) proliferated vigorously in response to Con A, and the ratio of Thy-1.2$^+$ CD4/CD8 among proliferating cells remained 3:1 (Table 4). B6.PL animals, treated with αThy-1.1 mAb in vivo, contain <3% T (Thy-1.1$^+$) cells and, under the experimental conditions in Table 4, these cells failed to proliferate in response to Con A (very low proliferation was only noted with 5 × 10^5 spleen cells/well; stimulation index = 2.9). Even though by FCM analysis these cells survived the in vivo depletion, most of them were probably damaged or functionally altered by such a treatment. Therefore, the extent of functional in vivo depletion was nearly complete, and allowed us to measure the proliferation of donor-derived cells in other experimental groups. Indeed, B6.PL animals injected intrathymically with either B6 DN or B6 CD8^{lo}4^{hi} thymocytes contained ~5% Thy-1.2$^+$ thymocytes, and these cells vigorously responded to Con A (Table 4). When corrected for the input of T cells, the response of the progeny of DN and CD8^{lo}4^{hi} cells was comparable on a per cell basis to that of intact BALB/c T cells. The proliferating progeny of DN and CD8^{lo}4^{hi} dramatically differed in CD4/CD8 ratios. The CD4/8 ratio was 2.4:1 for the DN and 10.5:1 for the CD8^{lo}4^{hi} progeny. Of note, the representation of Thy1.2$^+$ cells within proliferating cells reflected a significant increase compared with their representation before proliferation (Table 4). (Comparable results, although with quantitatively smaller proliferation [SI = 3-5], were obtained when allogeneic splenocytes were used to stimulate the progeny of intrathymically injected thymocytes [not shown].) These results unequivocally establish that CD8^{lo}4^{hi} thymocytes develop into functional CD8$^-$4^{hi} progeny after intrathymic injection, and demonstrate an obligatory requirement for the thymic microenvironment in the development of the majority of TCR^{hi}CD4^{hi} SP thymocytes.

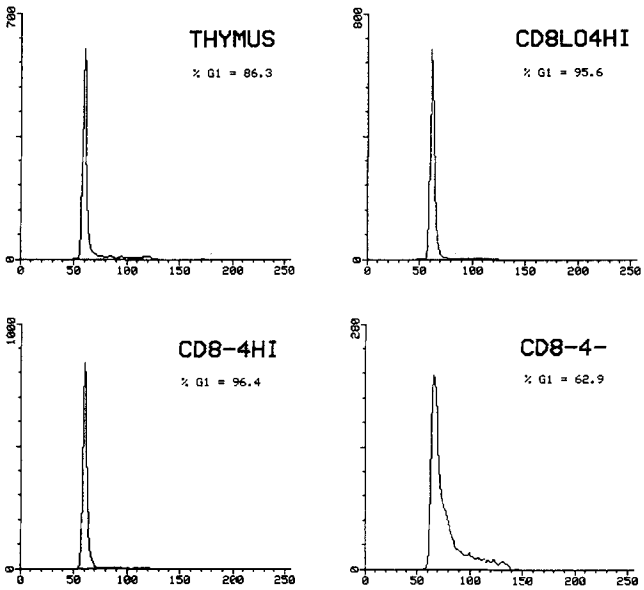

Figure 4. Both CD4^{hi} SP subsets contain few cycling cells. Purified CD4^{hi} thymocyte subsets were fixed in ethanol and DNA stained with PI. Total thymocytes and CD8$^-$4$^-$ thymocytes are shown as controls. Doublets and cell debris were gated out, and results from 5 × 10^3 cells represented as DNA content histograms. Numbers represent percent of cells in the G_1 phase, as determined by Multicycle software (Phoenix Flow Systems). Similar results were obtained in two independent experiments.

Discussion

In this study, we have further characterized two subsets of CD4^{hi}TCR^{hi} SP thymocytes. We extended their phenotypic characterization to show that our CD8^{lo}4^{hi} cells, which appear CD8$^-$ by direct FCM, clearly differ from previously described CD8^{lo}4^{hi} cells (25–27) that bear intermediate levels of CD8 (4–5× lower than those on DP thymocytes), and that we therefore call CD8^{int}. In addition to the expression of CD8, the most pronounced differences between these two subsets were observed in the expression of TCR, CD24, and CD44. CD8^{lo}4^{hi} cells were TCR^{hi}CD24^{int}CD44^{hi}, in contrast to CD8^{int}4^{hi} cells which were TCR^{int}CD24^{hi}CD44^{lo}. Furthermore, the elimination of T cells bearing self-reactive Vβs was complete in CD8^{lo}4^{hi} cells, but not in CD8^{int}4^{hi} cells. This is consistent with the idea that our CD8^{lo}4^{hi} cells are phenotypically more mature than CD8^{int}4^{hi} cells, although neither subset could produce IL-2 (13, 26) or IL-4 (Chiocchia, G. and R. Dyall, unpublished results). On the other hand, we witnessed high IL-2 and IL-4 production by CD8$^-$4^{hi} thymocytes in response to TCR ligation in vitro (Chiocchia, G., and R. Dyall, unpublished results), and, in that respect, our CD8$^-$4^{hi} cells correspond to the analogous

subset described by Bendelac et al. (26, 27) although the latter cells would also contain our $CD8^{lo}4^{hi}$ cells which are inactive in cytokine assays.

The main objective of this study was to test functional and developmental potential of $CD8^{lo}4^{hi}$ and $CD8^-4^{hi}$ cells in vivo. We documented the elimination of thymocytes bearing endogenous MMTV-reactive V_β TCRs in both subsets. This elimination is a late event in the thymocyte development (31, 32) and we can therefore conclude that, at least by phenotypic criteria, both subsets have undergone positive (TCR upregulation, reference 30) and negative selection. However, an in vivo examination of the functional behavior of these two subsets revealed profound differences. Whereas $CD8^-4^{hi}$ thymocytes and control mature peripheral $CD8^-4^{hi}$ cells induced lethal GVHD in allogeneic recipients and populated lymphoid organs of syngeneic recipients, $CD8^{lo}4^{hi}$ cells could not mount an immune response in vivo, and could not expand (and probably not survive longer than 3–4 wk) in peripheral lymphoid organs. Furthermore, $CD8^{lo}4^{hi}$ cells were sensitive to TCR-induced apoptosis. It will therefore be of interest to examine the expression of the apoptosis-protective protooncogene *bcl-2* and related genes in these subsets, since the expression of this molecule correlates very well to the stage of T cell development (39–41) and to the susceptibility of thymocyte subsets to Ca^{2+}-induced apoptosis (41).

After intrathymic injection, nonfunctional $CD8^{lo}4^{hi}$ thymocytes could mature into long-lived $CD4^{hi}$ cells that populated peripheral lymphoid organs. Low numbers of donor derived cells precluded us from distinguishing whether the peripheral progeny of $CD8^{lo}4^{hi}$ cells was $CD8^-$ or $CD8^{lo}$, but their longevity (Table 3) and immunocompetence (Table 4) suggested that these cells were $CD8^-$. In most experiments, we could also observe a small population of $CD4^-8^{hi}$ cells that developed from the donor inoculum. These cells could develop from a few DN contaminants (between 0.2 and 2%, or 0.4–4 $\times$ 10^4 cells in our experiments) or could originate from a novel $CD4^{hi}8^{-/lo}TCR^{hi}$ thymic intermediate. This latter possibility would be supported by a finding of a similar thymocyte intermediate, but of $TCR^{lo/int}$ phenotype, in TCR transgenic and MHC-deficient mice (42–46). Experiments are in progress to distinguish between these possibilities. Nevertheless, the presence of small numbers of contaminants could not influence the difference in CD4/CD8 ratios between the progeny of control DN and experimental $CD8^{lo}4^{hi}$ thymocytes. This difference was drastic (1.9 vs. 8.5), and it revealed that the latter cells were heavily biased towards becoming $CD4^{hi}$ SP.

Most importantly, we could directly demonstrate that intrathymically injected $CD8^{lo}4^{hi}$ thymocytes yield functional $CD4^{hi}$ SP progeny. Thus, our results demonstrate that most of the post-selectional $CD4^{hi}$ SP thymocytes still need intrathymic signal(s) for final maturation. A recent report (31) has indicated that TCR^{hi} DP cells from *bcl-2* transgenic and normal mice require the thymus to be processed into the $CD8^-4^{hi}$ lineage. We show here that a requirement for the thymic microenvironment exists very late in the T cell ontogeny, at the $CD8^{lo}4^{hi}$ stage, that is by a number of phenotypic and functional features very different from DP thymocytes. $CD8^{lo}4^{hi}$ thymocyte subset had undergone positive selection (as measured by the upregulation of TCR expression) on class II molecules (13, and this study); negative selection (as measured by the deletion of thymocytes bearing endogenous MMTV-reactive V_βs) and an almost complete downregulation of $CD8^+$, but still requires the presence of the thymic microenvironment for final phenotypic and functional maturation. When $CD8^{lo}4^{hi}$ cells were taken out of the thymus, and allowed to populate peripheral lymphoid organs of a syngeneic recipient, they failed to complete development and become functional $CD8^-4^{hi}$ cells. These findings suggest the existence of a novel developmental control point that controls the final maturation of thymocytes.

The nature of the late signal(s) required by $CD8^{lo}4^{hi}$ cells is not known at present. Such a signal could be transmitted by class II molecules, in order to verify the correct pairing of class II–restricted TCR and CD4 at the surface of late thymocytes. If this is true, it would prove the stochastic model of accessory molecule exclusion (reviewed in 47, 48) correct. However, class II signal is probably insufficient by itself, since class II molecules cannot induce TCR^{hi} DP thymocytes from *bcl-2* transgenic mice to mature into $CD8^-4^{hi}$ cells (31). An alternative hypothesis, and the one we favor, would be that $CD8^{lo}4^{hi}$ cells have indeed received all signals for downregulation of CD8, and that no further checks are required to test the TCR/accessory molecule matching. Instead, thymocytes at $CD8^{lo}4^{hi}$ (or $CD4^{lo}8^{hi}$) stage would require intrathymic signal(s) to become functionally competent. This signal(s) would restore the capacity of late thymocytes to secrete cytokines (3) and could be mediated by a cytokine, a membrane contact, or both. The above hypotheses are testable in experiments with normal and class II–deficient animals and experiments are underway to distinguish between them.

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