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Intrathymic maturation of murine T lymphocytes from $CD8^+$ precursors

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ABSTRACT **The $CD4^-8^-$ thymocyte subset contains immature precursors for phenotypically and functionally mature $CD4^+8^-$ and $CD4^-8^+$ thymocytes and peripheral T cells, as well as nonmature $CD4^+8^+$ thymocytes, most of which die *in situ*. The intrathymic death of most thymocytes is probably related to selective influences that ensure that only those precursors bearing self-major histocompatibility complex (MHC)-restricted and self-tolerant T-cell antigen receptors (TCR) survive to complete the maturation process. Interactions between surface molecules on thymocytes (TCR, CD4, and CD8) and thymic stromal cells (MHC proteins) are critical to repertoire selection. To understand this process, the lineage relationships among immature, nonmature, and mature thymocytes must be defined. We have examined directly the precursor–progeny relationships among $CD4^+8^-$, $CD4^-8^+$, and $CD4^+8^+$ murine thymocyte subsets by assessing their short-term (<5 days) developmental potentials following intrathymic injection into *Thy-1* congenic, unirradiated host mice. Our results identify $TCR^{-/lo}$ $CD4^-8^+$ and TCR^{lo} $CD4^+8^+$ blast cells as sequential intermediates in the development of mature TCR^{hi} $CD4^+8^-$ and TCR^{hi} $CD4^-8^+$ thymocytes from $CD4^-8^-$ precursors, thus defining at least one intrathymic maturation pathway for T lymphocytes.**

T lymphocytes mature intrathymically from migratory bone marrow or fetal liver precursors (1–3). The thymic microenvironment induces these precursor cells to proliferate and to express on the cell surface the recognition components (clonotypic $\gamma\delta$ or $\alpha\beta$ heterodimers) and signal-transducing components (invariant CD3 proteins) of the T-cell antigen receptor (TCR) complex, as well as the CD4 and CD8 accessory molecules (4, 5). Most mature T cells are unreactive (tolerant) to self antigens and preferentially respond to foreign antigenic peptides bound to self major histocompatibility complex (MHC) molecules (6), apparently due to negative and positive selective events during intrathymic maturation (7–11). Recent evidence suggests that these selective processes involve interactions among the TCR (12), class I and class II MHC proteins on thymic stromal cells (13, 14), and CD4 and CD8 (15–17). However, the maturational stage(s) during which selection takes place is unknown.

$CD4^+8^+$ cortical thymocytes are an early maturational stage potentially subject to selective processes, since some express low levels of surface TCR (18–20). These cells develop intrathymically from $CD4^-8^-$ precursors before mature $CD4^+8^-$ and $CD4^-8^+$ thymocytes during fetal life (2, 3) and following intrathymic injection into the adult thymus (21, 22). However, most $CD4^+8^+$ thymocytes are nonfunctional cells that die *in situ* (23, 24). Moreover, the inability of several groups to directly demonstrate a precursor function for $CD4^+8^+$ thymocytes (25, 26) conflicts with recent studies in $\alpha\beta$ TCR transgenic mice (27, 28) and lethally irradiated bone marrow chimeras (15–17) that have provided indirect evidence of $CD4^+8^+$ precursor function.

To resolve these issues, we investigated thymocyte lineage relationships directly by assessing the developmental potential of individual subsets after intrathymic injection into host mice. The standard protocol (21, 22) was modified as follows. $CD4^+8^-$, $CD4^-8^+$, and $CD4^+8^+$ donor thymocytes were obtained using a fluorescence-activated cell sorter (FACS) for the final selection step. Other methods (panning, antibody-mediated complement-dependent cytotoxicity) usually result in significant (>2%) contamination with undesired cell types. Nonetheless, even FACS-purified subsets are seldom 100% pure, and the most common contaminants ($CD4^-8^-$precursor thymocytes) expand 50- to 100-fold by 1 week after intrathymic injection (B.A. and I.L.W., unpublished data). Hence, only short-term (<5 days) developmental potential was examined. Finally, unirradiated host mice were used, since the radiation-damaged host thymus supports maturation at a significantly slower rate (unpublished data), and asynchronous maturation of different subsets has been reported (29). Our results provide direct evidence that $TCR^{-/lo}$ $CD4^-8^+$ and TCR^{lo} $CD4^+8^+$ blast cells are sequential intermediate stages in the development of both mature T-cell subsets. We speculate that repertoire selection may occur during one or both of these intermediate stages.

MATERIALS AND METHODS

Mice. Three- to six-week-old C57BL/Ka (*Thy-1.2 Ly-5.1*), C57BL/Ka BA (*Thy-1.1 Ly-5.1*), and C57BL6/J-Ly5.2 (*Thy-1.2 Ly-5.2*) mice were bred and maintained in our facility in the Department of Pathology.

Purification of Thymocyte Subsets. Sources, specificities, and fluorochrome modifications of the monoclonal antibodies used in this study are described in refs. 3 and 22. Prior to cell sorting, thymocyte suspensions were enriched for $CD4^-8^+$ or $CD4^+8^-$ cells by cytotoxic elimination of $CD4^+$ or $CD8^+$ cells (22). Fluorescein isothiocyanate (FITC)-conjugated 145-2C11 (anti-CD3 ε polypeptide) was used for separation of $CD4^-8^+$ thymocytes into $CD3^-$ and $CD3^+$ subsets. To isolate the subset consisting of large $CD4^+8^+$ cells, thymocytes were first enriched for large cells by unit-gravity sedimentation over a discontinuous calf serum gradient (40%, 30%, and 20% vol/vol). Fractions containing 30–60% large cells were then stained with allophycocyanin-GK1.5, biotin-53-6.7/avidin-Texas Red (TR), and FITC-145-2C11, and large $CD3^{-/lo}$ $CD4^+8^+$ cells were isolated by cell sorting. Small aliquots of sorted cells were reanalyzed and were always ≥98% pure. Three- to six-week-old unirradiated mice were anesthetized and intrathymic injections were performed (1–5 × 10^5 cells per lobe) as described (22).

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Abbreviations: FACS, fluorescence-activated cell sorter; FITC, fluorescein isothiocyanate; MHC, major histocompatibility complex; TCR, T-cell antigen receptor(s); TR, Texas Red.

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Detection of Donor-Derived Thymocytes. After staining with FITC- or biotin-conjugated 19XE5 (or 53-2.1), cell suspensions from individual thymic lobes were resuspended in Hanks' balanced salts solution (containing 10 mM Hepes, 2% calf serum, and 4 mM NaN_3) plus 0.05–0.3 ml of paramagnetic beads (optimal volume was determined empirically for each antibody) coupled to sheep anti-FITC antibodies or avidin (Advanced Magnetics, Inc., Cambridge, MA). After magnetic separation (22, 30), thymocytes were stained (3) with biotin- or FITC-conjugated 53-6.7 and allophycocyanin-GK1.5, followed by avidin-TR. Artificial mixtures containing 0.05% Thy-1.1 thymocytes were routinely enriched 100- to 500-fold by this procedure. Cell suspensions from intrathymically injected mice contained <0.1% donor-derived cells initially and 5–25% after immunomagnetic selection. Background staining of bead-enriched control thymocyte suspensions from saline-injected mice was always <0.5%. For each thymic lobe, 500–5000 Thy-1.1$^+$ (FITC$^+$ or TR$^+$) viable cells were analyzed for their CD8 (TR or FITC) and CD4 (allophycocyanin) phenotypes.

RESULTS

Developmental Potential of CD4$^+$8$^-$ and CD4$^-$8$^+$ Thymocytes. Although CD4$^+$8$^-$ and CD4$^-$8$^+$ thymocytes phenotypically resemble mature peripheral helper and cytotoxic T cells, CD4/8 phenotype alone is not a reliable indicator of functional maturity. Some adult CD4$^-$8$^+$ thymocytes lack alloantigen-induced cytotoxicity and, unlike peripheral CD4$^-$8$^+$ T cells, express high levels of the J11d heat-stable antigen (31). We therefore examined the "mature" thymocyte subsets for precursor activity by using a short-term intrathymic transfer system. Sorted Thy-1.1$^+$ CD4$^+$8$^-$ or CD4$^-$8$^+$ thymocytes (>98% pure, Fig. 1 *a* and *b*) were injected intrathymically into unirradiated young adult *Thy-1.2* host mice. Detection of extremely small (0.01–0.1%) donor populations was facilitated by enriching for donor-derived cells with antibody- or avidin-coated paramagnetic beads (30) prior to staining with antibodies for CD4 and CD8 and three-color FACS analysis. Using this technique, we found that a substantial proportion of CD4$^-$8$^+$ thymocytes acquired CD4 antigen by 16 hr after injection (Fig. 1*c*), whereas no detectable CD4$^+$8$^+$ cells arose from CD4$^+$8$^-$ donors (Fig. 1*d*). When the phenotype of donor-derived thymocytes was determined 38 hr after transfer, the proportion of CD4$^+$8$^+$ progeny was only slightly higher than at 16 hr (34–43% vs. 21–36%, data not shown), suggesting that the differentiation was essentially completed by 16 hr. The CD4$^+$8$^+$ progeny were probably not derived from CD4$^-$8$^-$ contaminants, since the CD4$^+$8$^-$ population had similar numbers of CD4$^-$8$^-$ cells but generated very few CD4$^+$8$^+$ thymocytes at either time point (Fig. 1 *b* and *d*; data not shown). Thus, CD4$^+$8$^-$ cells appear to be stable in the maintenance of their phenotype, whereas CD4$^-$8$^+$ cells include precursors for CD4$^+$8$^+$ cells.

The observation that some, but not all, CD4$^-$8$^+$ thymocytes converted to CD4$^+$8$^+$ cells suggested that a subset of the CD4$^-$8$^+$ population might contain most of this precursor activity. One candidate is the J11d^{hi} subset, since these cells are CD3$^-$ or CD3^{lo} blast cells (refs. 31–35; data not shown). Moreover, CD3$^-$ (and J11d^{hi}) CD4$^-$8$^+$ blasts first arise at day 15 of gestation, prior to the appearance of any CD4-expressing cells (data not shown), and are concentrated in the outer proliferative zone of the cortex in the adult thymus (32, 33). When adult CD4$^-$8$^+$ thymocytes were separated into CD3^{hi} and CD3$^-$ fractions before intrathymic injection, only the CD3$^-$ subset generated substantial numbers of CD4$^+$8$^+$ progeny (Fig. 2*a Lower left*). In two separate experiments, 76–84% of the CD3$^-$4$^-$8$^+$ progeny were CD4$^+$8$^+$ at 16 hr postinjection. CD3^{hi}4$^-$8$^+$ donor cells produced very small numbers of CD4$^+$8$^+$ cells (Fig. 2*a Lower right*), but these

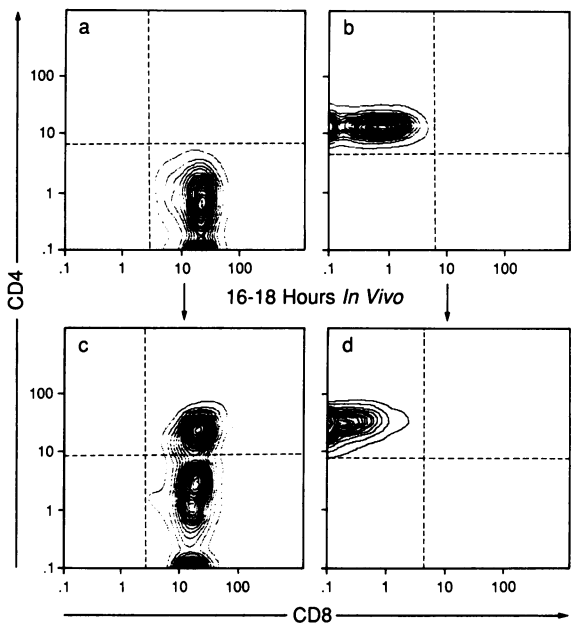

FIG. 1. Short-term developmental fate of CD4$^+$8$^-$ and CD4$^-$8$^+$ thymocytes after intrathymic injection. (*Upper*) CD4 and CD8 expression on sorted CD4$^-$8$^+$ (*a*) and CD4$^+$8$^-$ (*b*) populations prior to intrathymic injection. In both cases, contaminants (CD4$^-$8$^-$) were ≤1%. (*Lower*) Representative profiles of CD4 and CD8 expression on C57BL/Ka BA (*Thy-1.1*) CD4$^-$8$^+$ (*c*) and CD4$^+$8$^-$ (*d*) donor cells 16 hr after intrathymic injection into unirradiated C57BL/Ka (*Thy-1.2*) mice. Dotted lines show the quadrants used to define the CD4$^-$8$^-$ (lower left), CD4$^-$8$^+$ (lower right), CD4$^+$8$^+$ (upper right), and CD4$^+$8$^-$ (upper left) subsets: (*c*) CD4$^+$8$^+$, 21–36%; CD4$^-$8$^+$, 63–79%; others, <1%; (*d*) CD4$^+$8$^+$, 2–3%; CD4$^+$8$^-$, 97–98%; others, <1%.

may have been derived from the 2–3% contaminating CD3$^-$4$^-$8$^+$ cells. To determine whether the CD3$^-$4$^-$8$^+$ subset also contained precursors for CD4$^+$8$^-$ thymocytes, we examined their developmental fate at a later time point. Interestingly, by 3.5 days postinjection, CD4$^+$8$^-$ (14–20%) progeny appeared (Fig. 2*b Lower*) from CD3$^-$4$^-$8$^+$ precursors. The CD4$^-$8$^-$ progeny (5–10%) may have represented expansion of CD4$^-$8$^-$ contaminants and were not seen in later experiments. However, it is unlikely that the CD4$^+$8$^-$ progeny were derived from contaminating CD4$^-$8$^-$ thymocytes, since at this time point, very few CD4$^+$8$^-$ progeny were detectable from CD4$^-$8$^-$ donor cells (Fig. 2*b Upper*).

Phenotypic examination of the donor-derived cells 16 hr after injection revealed that the CD3^{hi}4$^-$8$^+$ cells retained high levels of CD3 (Table 1). In contrast, CD3$^-$4$^-$8$^+$ thymocytes gave rise to a small proportion of CD3^{hi} progeny at 16 hr, and a higher proportion by 88 hr. Of the CD3$^-$4$^-$8$^+$ progeny that were CD4$^-$8$^+$ at 16 hr, 8–12% became CD3^{hi} (Table 1). Similar to CD4$^+$8$^+$ cells in the unmanipulated thymus (19, 20), CD3$^-$4$^-$8$^+$-derived CD4$^+$8$^+$ progeny included CD3$^-$, CD3^{lo}, and CD3^{hi} subsets (Table 1). At 88 hr postinjection, none of the CD3$^-$4$^-$8$^+$-derived CD4$^-$8$^-$ cells were CD3^{hi}, but 40–50% of the CD4$^+$8$^-$ progeny expressed high levels of the CD3–TCR complex, and the remainder were CD3^{lo} (Table 1). High-level expression of CD3–TCR by some CD4$^+$8$^-$ and CD4$^-$8$^+$ progeny of CD3$^-$4$^-$8$^+$ cells suggests that they may be functionally mature.

Developmental Potential of CD4$^+$8$^+$ Thymocyte Subsets. The sequential appearance of CD4$^+$8$^+$ and mature CD4$^+$8$^-$ and CD4$^-$8$^+$ thymocytes during ontogeny and after intrathymic injection of adult CD3$^-$4$^-$8$^+$ (Fig. 2) or CD4$^-$8$^-$ (21, 22) cells is consistent with a precursor function for CD4$^+$8$^+$ thymocytes. Although several recent studies have been interpreted to support this idea (15–17, 27, 28), direct attempts to demonstrate such a precursor–progeny relationship have been

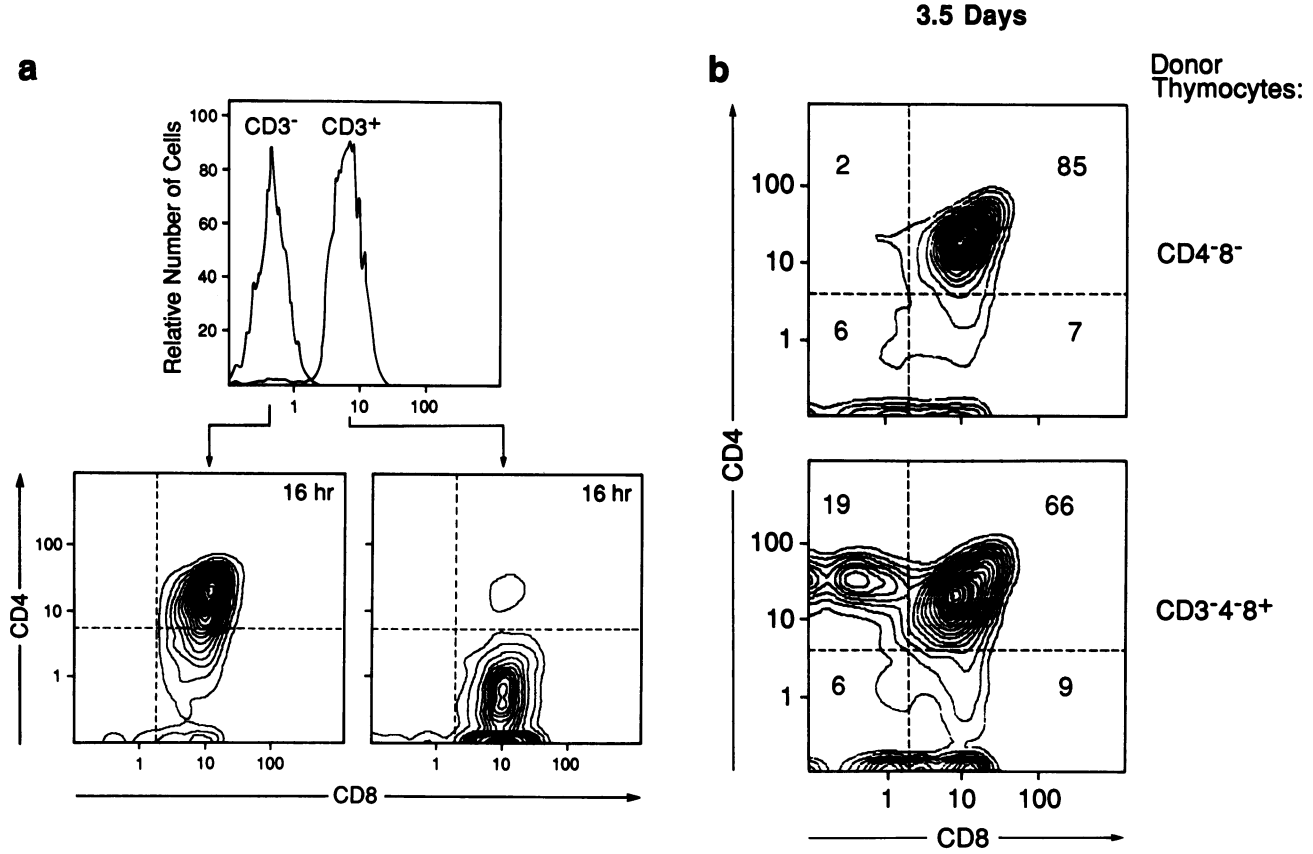

FIG. 2. Developmental potentials of $CD4^-8^+$ thymocyte subsets. (*a*) CD3 profiles of sorted $CD3^{hi}$ ($CD3^+$) and $CD3^-$ $CD4^-8^+$ Thy-1.1^+ thymocytes (*Upper*) and representative CD4 vs. CD8 profiles on donor-derived (Thy-1.1^+) cells 16 hr after intrathymic injection (*Lower*). The proportion of cells in each quadrant is shown in Table 1. (*b*) Representative CD4 vs. CD8 profiles of donor-derived thymocytes 3.5 days after intrathymic injection of $CD4^-8^-$ (*Upper*) or $CD3^-4^-8^+$ (*Lower*) thymocytes; numbers in each quadrant indicate percentages.

unsuccessful. This apparent discrepancy can be explained if only a small subset of $CD4^+8^+$ thymocytes contains precursors for mature T cells. The majority of $CD4^+8^+$ thymocytes are small, postmitotic cells with a short intrathymic life-span (3–5 days) (23, 24). It is likely that these are derived from the outer cortical $CD4^+8^+$ blast population (10–15% of total $CD4^+8^+$ thymocytes) (36, 37). To test the developmental potential of the $CD4^+8^+$ blast subset, large $CD3^{-/lo}4^+8^+$ thymocytes (99% pure) were isolated by cell sorting and injected intrathymically into unirradiated, *Thy-1* congenic host mice. By 3.5 days after injection, donor-derived $CD4^+8^-$ and "transitional" $CD4^+8^{lo}$ cells were evident (Fig. 3 *Middle*) and at higher frequencies than the $CD4^+8^-$ progeny of immature $CD4^-8^+$ thymocytes (Fig. 2*b*). At this time, $CD4^-8^+$ progeny were also detected, but most of these were $CD4^{lo}$ rather than $CD4^-$. By 4.5 days, the proportion of bona fide $CD4^-8^+$ cells had increased. The precursor activity of $CD4^+8^+$ thymocytes is confined to the blast subset; no progeny were detectable from FACS-purified small $CD4^+8^+$ thymocytes 4.5 days after intrathymic injection, even when 3-fold more cells were injected per lobe (Table 2). To serve as an internal control for injection efficiency, $CD4^-8^-$ cells (*Thy-1.2 Ly-5.2*) were coinjected with small $CD4^+8^+$ thymocytes (*Thy-1.2 Ly-5.1*) into *Thy-1.1 Ly-5.1* hosts. In four out of four lobes, only Ly-5.2^+ Thy-1.2^+ progeny were detectable, confirming the absence of precursor activity in small $CD4^+8^+$ thymocytes.

Table 1. Progenitor activity of $CD3^{hi}$ and $CD3^-$ $CD4^-8^+$ adult thymocytes

Time after injection, hr	$CD4^-8^+$ donor population	Phenotype of donor-derived thymocytes, % (% $CD3^{hi}$)			
		$CD4^-8^-$	$CD4^+8^-$	$CD4^-8^+$	$CD4^+8^+$
16	$CD3^-$	2–3	0	14–20	76–83
		(0)		(8–12)	(2–5)
16	$CD3^{hi}$	2–3	0	92–94	3–5
		(0)		(97–98)	(<1)
88	$CD3^-$	5–10	14–20	7–12	59–72
		(0)	(40–50)	(35–50)	(3–5)

Percentages shown are the ranges observed for each group. Donor cells were analyzed for CD4 vs. CD3, CD8 vs. CD3, and CD4 plus CD8 vs. CD3 expression to allow calculation of the percentage of $CD3^{hi}$ and $CD3^{lo}$ cells within each CD4/8 subset (% $CD3^{hi}$ is given in parentheses). $CD3^-4^-8^+$-derived $CD4^+8^+$ cells included 18–23% and 30–40% $CD3^{lo}$ cells at 16 and 88 hr postinjection, respectively. Fifty to 60% of $CD3^-4^-8^+$-derived $CD4^+8^-$ thymocytes were $CD3^{lo}$ 88 hr after injection.

To assess whether the $CD4^+8^-$ progeny detected 3–5 days after intrathymic injection might represent selective outgrowth of small numbers of $CD4^+8^-$ contaminants, FACS-purified large $CD4^+8^+$ cells (*Thy-1.2 Ly-5.1*) were mixed with FACS-purified $CD4^+8^-$ thymocytes (*Thy-1.2 Ly-5.2*) at a ratio of 10:1 before intrathymic injection into *Thy-1.1 Ly-5.1* hosts. Lobes injected with the mixture of Ly-5.1^+ $CD4^+8^+$ blasts and Ly-5.2^+ $CD4^+8^-$ thymocytes or with Ly-5.1^+ $CD4^+8^+$ blasts alone contained 16–23% Thy-1.2^+ $CD4^+8^-$ progeny after 4.5 days. In both cases, 92 ± 6% were Ly-5.1^+. Thus, even when the level of $CD4^+8^-$ contaminants was 5- to 10-fold higher than the maximum (2%) in our purified $CD4^+8^+$ blast populations, neither they nor their progeny were detectable.

DISCUSSION

Interactions between MHC proteins on thymic stromal cells and the CD4, CD8, and $\alpha\beta$ TCR molecules on T-cell precur-

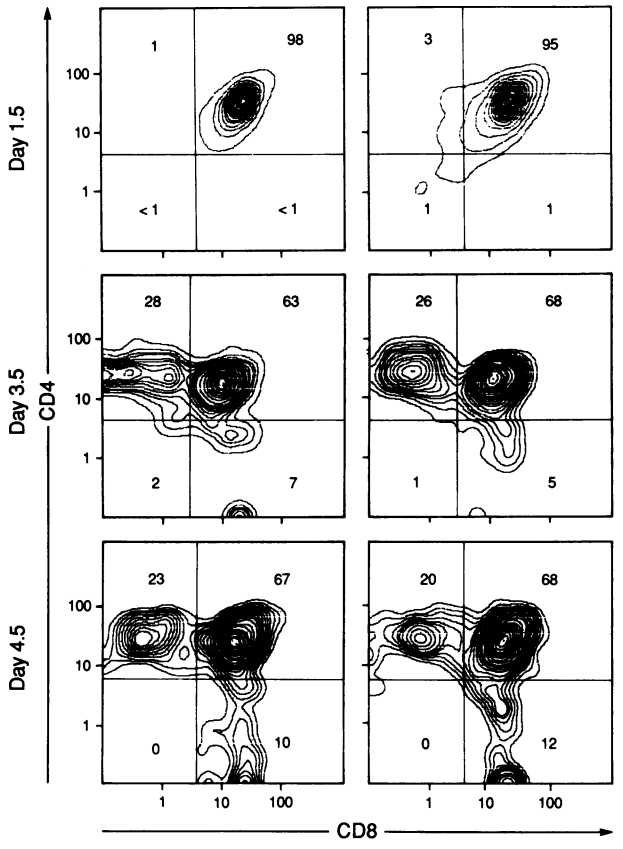

FIG. 3. Developmental potential of large $CD4^+8^+$ thymocytes. Representative CD4 vs. CD8 profiles of donor-derived thymocytes in two individual lobes analyzed 1.5, 3.5, and 4.5 days after intrathymic injection into unirradiated hosts. Numbers in each quadrant indicate percentages. Cells injected were 99% $CD4^+8^+$ and 80% large. Similar results were obtained in three different experiments.

sors are critical aspects of the intrathymic selection events that appear to shape the mature T-cell recognition repertoire (9–17). Delineation of the precursor–progeny relationships among thymocyte populations expressing different subsets of these molecules is required in order to define maturational stages during which repertoire selection might operate. We have attempted to resolve thymocyte lineage relationships by examining the short-term developmental potentials of FACS-purified normal thymocyte subsets in the unirradiated thymic microenvironment. Our results identify subsets of $CD4^-8^+$ and $CD4^+8^+$ thymocytes as sequential intermediate stages during intrathymic development of T lymphocytes from $CD4^-8^-$ precursors and provide direct evidence in favor of the maturation pathway outlined in Fig. 4. Though our data

Table 2. Precursor activity in $CD4^+8^+$ subsets

Donor cells (*Thy-1.2*)		Thy-1.2$^+$, %
Ly-5.1	*Ly-5.2*	(Ly-5.2/Thy-1.2)
0	0	<0.5
3×10^5 large $CD4^+8^+$	0	5 ± 2*
3×10^5 small $CD4^+8^+$	0	<0.5
1×10^6 small $CD4^+8^+$	0	<0.5
3×10^5 small $CD4^+8^+$	3×10^5 $CD4^-8^-$	53 ± 18* (0.98)

Four and one-half days after intrathymic injection of unirradiated host mice (*Thy-1.1 Ly-5.1*) with the indicated subset(s), cell suspensions from individual thymic lobes were immunomagnetically enriched for Thy-1.2$^+$ cells and analyzed for their Ly-5 (FITC) phenotype by two-color flow cytometry. (Ly-5.2/Thy-1.2 ratio is shown in parentheses.)
*Mean ± SD.

pertain primarily to T-cell production in the adult thymus, the results are consistent with the appearance of these subsets during fetal life (refs. 4 and 5 and data not shown).

The earliest progeny of $CD4^-8^-$ progenitors in the adult and fetal thymus are $CD4^-8^+$ cells (unpublished observations). Consistent with the immature cell surface phenotype, cell cycle status, and intrathymic location (31–35) of $TCR^{-/lo}$ ($J11d^{hi}$) $CD4^-8^+$ thymocytes, our data identify these cells as intrathymic progenitors (Fig. 2). This is a transient developmental stage; within 16 hr, the majority of these $CD4^-8^+$ blasts acquire CD4 (Fig. 2*a*). $TCR^{-/lo}$ $CD4^-8^+$ blasts may have already received signals from the thymic microenvironment necessary for transcriptional activation of the CD4 gene, since some rat and murine $CD4^-8^+$ thymocytes express surface CD4 after overnight culture in the absence of the thymic microenvironment (32, 34).

$CD4^+8^+$ blasts represent a second intermediate stage during intrathymic T-cell maturation (Fig. 3). Our data suggest that progeny of these cells have at least three possible developmental fates (Fig. 4). The majority become small $CD4^+8^+$ thymocytes that are destined for intrathymic death (Table 2). Alternatively, $CD4^+8^+$ blasts can develop into $CD4^+8^-$ or $CD4^-8^+$ thymocytes (Fig. 3) that express mature levels of TCR (data not shown). Based on recent experiments showing that most $CD4^+8^+$ blasts express very low levels of surface TCR (C.J.G., J. S. Danska, C. G. Fathman, and I.L.W., unpublished data), we propose that the specificity of the TCR expressed at this early maturational stage determines the cells' subsequent developmental fate due to TCR-mediated positive and/or negative selective events. The frequency with which $CD4^+8^+$ blast cells give rise to mature thymocytes appears to be low, since no more than 30–35% of donor-derived cells were phenotypically mature 3.5 or 4.5 days after injection. Given that small $CD4^+8^+$ progeny have short life-spans, and the probability that mature progeny soon emigrate to peripheral lymphoid tissues, it is difficult to determine the true relative frequencies of the three types of progeny. The absolute number of mature T cells that develop from a given number of $CD4^+8^+$ blasts could not be determined, since no internal control for injection efficiency was included.

Our data do not prove that all mature T cells pass through a $CD4^+8^+$ intermediate stage or that all $CD4^+8^+$ blasts arise from $CD4^-8^+$ precursors. The appearance of small numbers of phenotypically mature $CD3^{hi}4^-8^+$ progeny from intrathymically injected $CD3^-4^-8^+$ blasts within 16 hr (Table 1) suggests that cells at this maturational stage might have two possible developmental fates (Fig. 4). In both the rat and the mouse, some $CD4^-8^+$ precursors express low levels of $\alpha\beta$ TCR (ref. 35 and data not shown); therefore they could be subject to selective processes that induce those with appropriate TCR to become $CD3^{hi}$ while remaining $CD4^-$. Indeed, TCR crosslinking prevents rat $CD4^-8^+$ thymocytes from differentiating into $CD4^+8^+$ cells *in vitro* (35). Alternatively, the $CD3^{hi}4^-8^+$ progeny present at 16 hr may have already passed through a $CD4^+8^+$ intermediate stage. At present, we cannot distinguish between these two possibilities.

The existence of $CD8^+$ intrathymic precursors for mature T cells has been suggested by several recent studies, but the $CD8^+$ subset ($CD3^-4^-8^-$, $CD3^+4^-8^+$, or $CD4^+8^+$) was not identified. Nikolic-Zugic and Bevan (39) reported that $CD4^-8^+$ thymocytes obtained by panning (88–90% pure) gave rise to $CD4^+8^-$ thymocytes 1–2 weeks after intrathymic injection into lethally irradiated hosts. Smith (15) showed that chronic administration of anti-CD8 monoclonal antibody to mice following lethal irradiation and bone marrow reconstitution significantly inhibited the development of splenic $CD4^+8^-$ T cells. Similar experimental systems have been used to determine the effect of anti-CD4 antibody on the intrathymic maturation of $CD4^-8^+$ T cells bearing $V_{\beta 6}{}^+$ or

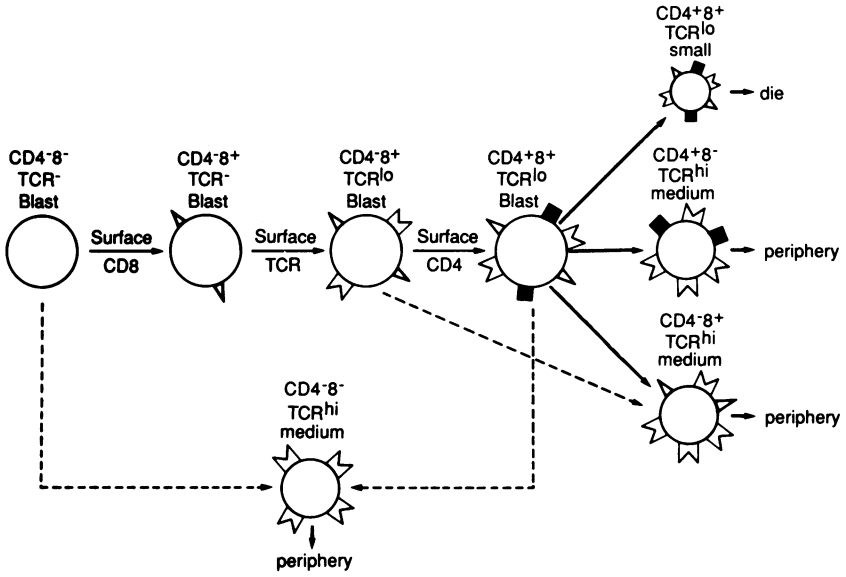

FIG. 4. Lineage relationships among thymocyte subsets. Data presented in Figs. 1–3 suggest this maturation scheme for $\alpha\beta$ TCR-bearing $CD4^+8^-$ and $CD4^-8^+$ T lymphocytes. Solid lines indicate demonstrated precursor–progeny relationships, and dotted lines indicate additional possibilities for which there is no direct evidence as yet. The TCR^{hi} $CD4^-8^-$ subset includes cells bearing $\gamma\delta$ and $\alpha\beta$ receptors. TCR^{hi} $CD4^-8^-$ ($\alpha\beta$) cells appear late in fetal life and are derived from TCR^- $CD4^-8^-$ progenitors (22, 38), but their maturation sequence has not been defined.

$V_{\beta 17a}^+$ TCR (16, 17). This approach is indirect in that the putative precursor cells cannot be isolated or identified, and it is possible that $CD4^-8^+$ cells were artificially created from TCR^{hi} $CD4^+8^+$ thymocytes (20) by antibody-induced modulation of CD4 or some other indirect mechanism. Further, this experimentally induced transition does not appear to reflect normal development, since the majority of $CD4^+8^+$ thymocytes, even those normally destined for intrathymic death, decrease CD4 and increase CD3 in response to anti-CD4 (40). Studies of thymic maturation in mice made transgenic for a self-reactive $\alpha\beta$ TCR have also provided evidence that mature T cells develop from $CD4^+8^+$ precursors (27, 28). However, circumvention of early developmental events by the use of rearranged α and β transgenes could cause maturational aberrations due to developmentally deregulated TCR expression. For example, if the immature $CD4^-8^-$ and $CD4^-8^+$ progenitors in the transgenic animals express higher than normal levels of TCR, they could be subjected prematurely to signals for selection, differentiation, and emigration. Thus, development of the downstream $CD4^+8^+$ and $CD4^+8^-$ cells would be prevented, potentially explaining the greatly increased frequencies of $\alpha\beta$-TCR^{hi} $CD4^-8^-$ and $CD4^-8^+$ peripheral T cells in these transgenic mice. The identification and purification of bipotential $CD4^+8^+$ precursors for mature $CD4^+8^-$ and $CD4^-8^+$ T cells from normal animals should enable direct studies of the repertoire selection process *in vivo*, using the intrathymic transfer technique, and *in vitro*, using cloned thymic stromal cells.

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