

Evidence That $\gamma\delta$ versus $\alpha\beta$ T Cell Fate Determination Is Initiated Independently of T Cell Receptor Signaling

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Abstract

Two types of T cells, $\alpha\beta$ and $\gamma\delta$, develop in vertebrates. How these two T cell lineages arise from a common thymic T progenitor is poorly understood. Differentiation of $\alpha\beta$ lineage T cells requires the surrogate α chain (pT α), which associates with the T cell receptor (TCR) β chain to form the pre-TCR. $\gamma\delta$ lineage development does not appear to involve an obligatory surrogate chain, but instead requires productive rearrangement and expression of both TCR γ and δ genes. It has been proposed that the quality of signals transmitted by the pre-TCR and $\gamma\delta$ TCR are distinct and that these “instructive” signals determine the lineage fate of an uncommitted progenitor cell. Here we show that the thymic T progenitor cells (CD25⁺CD44⁺c-kit⁺CD3⁻CD4⁻CD8⁻ thymocytes, termed pro-T cells) from young adult mice that have yet to express TCRs can be subdivided based on interleukin 7 receptor (IL-7R) expression. These subsets exhibit differential potential to develop into $\gamma\delta$ versus $\alpha\beta$ lineage (CD4⁺CD8⁺ cells) in the thymus. Upon intrathymic injection, IL-7R^{neg-lo} pro-T cells generated a 13-fold higher ratio of $\alpha\beta$ lineage to $\gamma\delta$ lineage cells than did IL-7R⁺ pro-T cells. Much of this difference was due to a fivefold greater potential of IL-7R⁺ pro-T cells to develop into TCR- $\gamma\delta$ T cells. Evidence indicates that this biased developmental potential is not a result of enhanced TCR- γ gene rearrangement/expression in IL-7R⁺ pro-T cells. These results indicate that the pro-T cells are heterogeneous in developmental potential before TCR gene rearrangement and suggest that in some precursor cells the initial lineage commitment is independent of TCR-mediated signals.

Key words: T cell development • IL-7 • T precursor cells • lineage commitment • T cell receptor gene rearrangement

Introduction

The mechanism of T cell lineage commitment leading to the development of two functionally distinct T cell subsets expressing either TCR- $\alpha\beta$ or TCR- $\gamma\delta$ heterodimers is unknown. A key question has been whether and how the expressed TCR determines the lineage fate of precursor cells. During the early stages of adult T cell development, the V(D)J recombinase assembles TCR- γ , - δ , and - β genes nearly contemporaneously in the precursor cells that give rise to both $\gamma\delta$ and $\alpha\beta$ cells. Normal development of the $\alpha\beta$ lineage requires the expression of the surrogate α chain (pT α) which associates with a TCR β chain to form the pre-TCR (1). Only thymocytes that express the pre-TCR

efficiently traverse a developmental checkpoint at the CD25⁺CD44⁻ stage of thymocyte development (TCR β chain selection; reference 2). Subsequent differentiation of pre-TCR⁺ cells to immature CD4⁺CD8⁺ thymocytes involves extensive proliferation comprising 8–10 cell divisions (3). Rearrangement of TCR- α genes initiates only after the cells have successfully passed through the TCR β chain selection checkpoint. In contrast, no surrogate chains for $\gamma\delta$ lineage development have been identified, and evidence suggests that both chains are required for differentiation along this lineage. Furthermore, $\gamma\delta$ T cell development apparently involves much less proliferation than development of $\alpha\beta$ lineage cells (4).

Models of T cell lineage commitment vary in the extent to which TCR signals skew the fate decision itself. At one end of the spectrum, the instructive view of $\gamma\delta/\alpha\beta$ lineage commitment suggests that the $\gamma\delta$ TCR and pre-TCR provide distinct signals that stimulate uncommitted precursor

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cells to differentiate into $\gamma\delta$ or $\alpha\beta$ lineage cells, respectively (5, 6). At the other end of the spectrum is the view that lineage commitment is initially TCR independent, with committed cells able to subsequently rearrange all three relevant TCR genes (γ , δ , and β) (4, 6–9). In this model, TCR signals that are appropriate to the predetermined lineage ensure cell survival and/or expansion, rather than the initial fate choice. Indirect evidence argues against a strict instructive role of the TCR in lineage commitment. For example, in both normal and TCR- $\beta^{-/-}$ mice, precursor cells expressing $\gamma\delta$ TCR can differentiate into CD4⁺CD8⁺ $\alpha\beta$ lineage cells, albeit inefficiently (4, 8). Furthermore, TCR- β expression can lead to the formation of cells with the phenotype of $\gamma\delta$ cells (10–12). However, the issue remains highly controversial, especially because there has been no direct evidence that T precursor cells, at a stage before TCR gene expression, are divisible into subsets that differ in their potential to differentiate into $\gamma\delta$ versus $\alpha\beta$ lineage cells.

T cell precursors are contained within a CD4⁻CD8⁻ thymic subset that does not express the TCR-CD3 complex. This triple negative (TN)¹ population can be further subdivided into four subsets based on cell surface expression of c-kit, CD25, and CD44 molecules, with the following order of maturity: c-kit⁺CD25⁻CD44^{hi} (thymic lymphoid precursor) to c-kit⁺CD25⁺CD44^{hi} (pro-T) to c-kit^{lo}CD25⁺CD44⁻ (pre-T) to c-kit⁻CD25⁻CD44⁻ (13). TCR- γ , - δ , and - β gene rearrangements occur as cells differentiate from pro-T to pre-T cells (14–16). Previous reports (15, 16) and our unpublished data indicate that complete (VDJ or VJ) rearrangements of TCR- γ , - δ , and - β alleles are very rare in adult pro-T cells. Quantitative PCR assays demonstrate that <0.5% of pro-T cells contain VJ γ or VJ δ rearrangements, while VDJ β rearrangements could not be detected (15). In pre-T cells, 10–75% of TCR alleles are completely rearranged (VDJ or VJ) depending on the TCR locus (16). Hence, the vast majority of CD44^{hi}CD25⁺ pro-T cells are T lineage precursors lacking functional TCR gene rearrangements. In addition to having T cell developmental potential, pro-T cells can give rise to dendritic cells (DCs), whereas pre-T cells are strictly committed to the T cell lineage (17). In its simplest form, the instructive model of the $\gamma\delta/\alpha\beta$ lineage commitment predicts that all pro-T cells would be uniform in their developmental potential since they have yet to express functional TCR complexes. In contrast, a TCR-independent mode of lineage commitment can accommodate distinct $\alpha\beta$ versus $\gamma\delta$ lineage developmental potential in subsets of pro-T cells before TCR expression. As a first step in testing these predictions we show here that in normal young mice, pro-T cell subsets with distinct developmental potential can be defined based on differential expression of the IL-7R α .

¹Abbreviations used in this paper: APC, allophycocyanin; BrdU, bromodeoxyuridine; B6, C57BL/6; DC, dendritic cell; DN, double negative; FTOC, fetal thymic organ culture; RT, reverse transcription; SCF, stem cell factor; TN, triple negative.

Materials and Methods

Mice. C57BL/6 (B6), B6-Ly5.1 congenic, B6-G8TCR γ transgenic (18), and B6-IL-7R $\alpha^{-/-}$ mice (The Jackson Laboratory) were bred and maintained in specific pathogen-free facilities at the University of California at Berkeley.

Antibodies and Flow Cytometry. Anti- δ TCR (GL-3), anti- β TCR (H57), anti-CD3 ϵ (500A2), anti-IL-7R α (A7R34), anti-CD45.2 (104), and anti-CD45.1 (A20) mAbs were purified and conjugated with FITC or biotin using standard protocols. Anti-CD44-FITC, anti-CD25-PE, anti-c-kit (CD117)-allophycocyanin (APC), anti-CD8-Tricolor mAbs, and streptavidin-Tricolor were purchased from Caltag. Anti-CD44-Cy5, anti-CD11c-biotin, anti-CD25-FITC, anti-CD45.2 (Ly5.2)-FITC, anti-c-kit-FITC, anti-IL-7R α (CD127, B12-1)-biotin, anti-mouse I-A^b-PE mAbs, and streptavidin-PE were supplied by BD PharMingen. Anti-CD4-613, anti-CD8-613, anti-CD25-613, and streptavidin-613 were acquired from GIBCO BRL. Rabbit polyclonal antibody against IL-7R α (D20) was supplied by Santa Cruz Biotechnology, Inc. A7R34 rat anti-mouse IL-7R α mAb was a gift from Dr. S.-I. Nishikawa and was obtained from Dr. M. Kondo (Stanford University, Palo Alto, CA). Anti-rabbit IgG-biotin and anti-bromodeoxyuridine (BrdU)-FITC were purchased from Vector Laboratories and Becton Dickinson, respectively. Incorporation of BrdU into dividing thymocytes in vivo was carried out by injecting mice intraperitoneally twice at a 2-h interval with 0.9 mg of BrdU (3, 4). Thymocytes from injected mice were analyzed 1 h later by flow cytometry. For the analysis of IL-7R α expression on thymic precursor populations, CD4⁻CD8⁻ thymocytes were isolated by rabbit and guinea pig complement-mediated lysis of CD4⁺ and/or CD8⁺ thymocytes using anti-CD4 (RL172) and CD8 (AD4-15) ascites fluids. After one or two rounds of complement lysis >99% pure double negative (DN) cells were obtained. Cells were incubated with 2.4G2 to block Fc receptors, then stained with mAbs specific for CD25-613, CD44-Cy5, IL-7R α -biotin/streptavidin-PE, and CD3 ϵ -FITC (or c-kit-FITC), and analyzed by four-color flow cytometry on an XL-MCL flow cytometer (Beckman Coulter). Since virtually all CD25⁺ DN cells were CD3⁻, the combination of anti-CD25-FITC/CD44-Cy5/IL-7R α (B12-1)-biotin-PE and CD8-613 or c-kit-APC mAbs was used for the analysis of pro-T and pre-T subsets. IL-7R⁺ and IL-7R^{neg-lo} pro-T cells were sorted with an ELITE cell sorter (Beckman Coulter) using >99% pure DN thymocytes stained with the mAbs as above and then gating on CD44⁺CD25⁺ (3% of total DN thymocytes) population and sorting for the cells expressing high levels of IL-7R (25–30% of total pro-T cells) and non- or low expressing cells (20–25% of total pro-T cells). Of the anti-IL-7R α mAbs, B12-1 gave the best separation and was used for all sorting experiments. Cell yield for each pro-T cell subset was 3–5 \times 10⁵ cells/mouse.

Cell Culture, Fetal Thymic Organ Culture, and Intrathymic Injection. Sorted pro-T cells were cultured for 4 d at 10⁵ cells/ml in medium (RPMI 1640 supplemented with 10% FCS, 50 μ M 2-ME, 2 mM L-glutamine, 20 mM HEPES, and antibiotics) containing 10 ng/ml of rIL-7 (Genzyme) or a 1:100 dilution of cell culture supernatants from J558 plasmacytoma cells transfected either with IL-7 or stem cell factor (SCF) cDNAs (19). For fetal thymic organ culture (FTOC), 1.5 \times 10⁴ viable, sorted cells were used in a 2-d hanging drop culture to reconstitute fetal day 14 thymic lobe that had been previously depleted of resident thymocytes by treatment with 1.35 mM 2'-deoxyguanosine for 5 d. Repopulated thymi were transferred to a Transwell plate and cultured for 9–24 d. For some experiments, thymi from B6-Ly5.1 mice were used to check for host thymocyte survival in FTOC. Invariably,

recipient thymocytes were not detected during FTOC in these experiments. At the early phase of culture (days 9–11) three to five thymi were pooled to obtain sufficient cells for flow cytometric analysis using appropriate mAbs. At a later phase of culture (day 21–22) individual thymi were analyzed. For intrathymic injection, sorted cells from B6 mice were washed and resuspended in PBS/0.1% BSA and injected into thymi of 4–6-wk-old sublethally irradiated (750 rads) B6-Ly5.1 congenic mice. For pro-T cells and pro-T subsets $\sim 2 \times 10^4$ sorted viable cells were injected. For pre-T cells $\sim 2 \times 10^5$ cells were injected. Reconstituted thymi were analyzed by flow cytometry 8–11 d after injection. DCs were isolated as described (20). In brief, thymi were digested for 1 h at 37°C in serum-free medium containing 1.6 mg/ml collagenase and 0.1% DNase. DCs were incubated with biotinylated anti-CD11c mAb and subsequently with streptavidin-coupled magnetic beads. They were then positively selected by magnetic cell sorting (MACS; Miltenyi Biotec) and analyzed by flow cytometry after staining with Ly5.2 (donor)-FITC and streptavidin-Tricolor that binds to the remaining free biotin of CD11c mAb on DCs.

Reverse Transcription PCR. RNA samples were reverse transcribed using oligo dT primer and avian reverse transcriptase as described (4). PCRs of serially diluted (three- or fourfold) cDNA and genomic DNA samples were performed using published primers (4, 21) in the presence of 1.0 μ Ci of [α - 32 P]dCTP. All PCRs entailed 28 cycles except those for TCR- γ or δ gene rearrangements/transcripts, which entailed 35–38 cycles. The starting input concentration for positive control tubulin PCRs was three- or fourfold lower than the starting concentrations for the gene of interest. Quantitation was performed using a PhosphorImager (Molecular Dynamics).

Results

Nonuniform Expression of IL-7R by Pro-T Cells in Young Mice. IL-7R-mediated signals are necessary for the survival of the earliest T progenitors (22–24), and play an independent, critical role in $\gamma\delta$ cell development. In IL-7R $\alpha^{-/-}$ mice the development of $\gamma\delta$ cells is completely

abolished (25, 26), even when a Bcl2 transgene is expressed (23, 24), whereas the development of $\alpha\beta$ T cells is blocked incompletely and can be partially rescued by expression of the Bcl2 transgene. The complete block in $\gamma\delta$ cell development in IL-7R $\alpha^{-/-}$ mice was shown to result specifically from a deficiency in TCR γ chain synthesis (18), consistent with the evidence that IL-7R α signaling stimulates rearrangement (18, 27) and expression (18, 28) of TCR- γ genes. TCR- δ and TCR- β gene rearrangement and expression are largely unaffected by IL-7R α deficiency (27).

We examined the expression of IL-7R α chain on T precursor populations of 3–4-wk-old B6 mice. Similar results were obtained with three different IL-7R α -specific mAbs (B12-1, A7R34 [29], and D20 [21], Fig. 1 using B12-1, and data not shown). IL-7R α was expressed homogeneously at a relatively high level on the earliest thymic T cell progenitors (c-kit $^+$ CD25 $^-$ CD44 $^+$ TN). In contrast, pro-T cells (CD25 $^+$ CD44 $^+$ c-kit $^+$ TN) exhibited a much broader expression pattern of IL-7R α chain (Fig. 1, A and B). Using pro-T cells from IL-7R $\alpha^{-/-}$ mice (22) as a negative staining control, only $\sim 40\%$ of the pro-T cells stained above background, with 15–30% accumulating in the lowest fluorescence intensity channels (Fig. 1 A). Many of the latter cells are not visible in the figure, as they accumulated in the channel corresponding to the left axis of the histogram. At the subsequent pre-T cell stage (c-kit $^{\text{lo}}$ CD25 $^+$ CD44 $^-$ TN), IL-7R α was expressed at lower levels than the bright subset of pro-T cells (Fig. 1 A). Four-color cell sorting of TN thymocytes was employed to purify the 25% of pro-T cells expressing the highest levels of IL-7R α (IL-7R α^+ cells) and the 25% of pro-T cells expressing the lowest levels of IL-7R α (IL-7R $^{\text{neg-lo}}$ pro-T cells; Fig. 1 B). Both sorted subsets were Thy-1 $^{\text{med}}$ CD3 $^-$ CD4 $^-$ CD8 $^-$ IL-2R β^- CD25 $^+$ CD44 $^+$ c-kit $^{\text{hi}}$ (Fig. 1 C, and data not shown). The purity of sorted pro-T subsets ranged from 86 to 95% in 23 independent sorting experiments. The majority

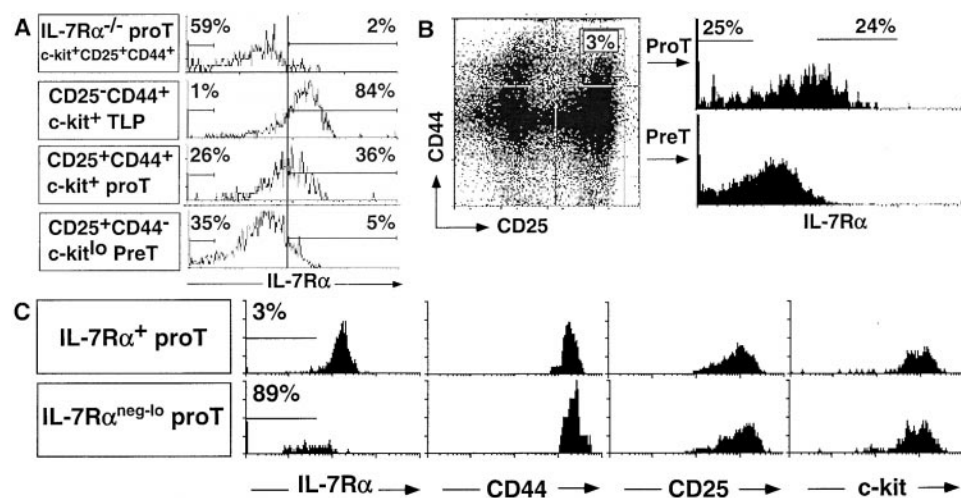


Figure 1. Subdivision of pro-T cells based on IL-7R α expression. (A) CD4 $^-$ CD8 $^-$ thymocytes from B6 and B6 IL-7R $\alpha^{-/-}$ mice were stained with mAbs for CD25, CD44, IL-7R α (B12-1), and c-kit. IL-7R α chain levels on indicated precursor subsets are shown. The level of positive staining for IL-7R α chain was determined by comparing to the fluorescence signal from IL-7R $\alpha^{-/-}$ thymocytes (top). An irrelevant, isotype-matched (to B12-1) control mAb did not stain T precursor subsets and CD25 $^+$ cells were not stained by mAb specific for CD3 ϵ chain (data not shown). (B) Sorting gates for IL-7R $^+$ and IL-7R $^{\text{neg-lo}}$ CD44 $^+$ CD25 $^+$ TN pro-T cells are shown. IL-7R α staining of CD25 $^+$ CD44 $^-$ TN pre-T cells is

presented for comparison. (C) Postsort analysis of similar numbers of purified populations with the indicated mAbs. Sorted cells were additionally stained with anti-c-kit-APC and analyzed on an ELITE flow cytometer. Many of the IL-7R $\alpha^{\text{neg-lo}}$ cells in A and C accumulate in the lowest fluorescence intensity channel, characteristic of Beckman Coulter flow cytometers, especially when using four-color compensation settings.

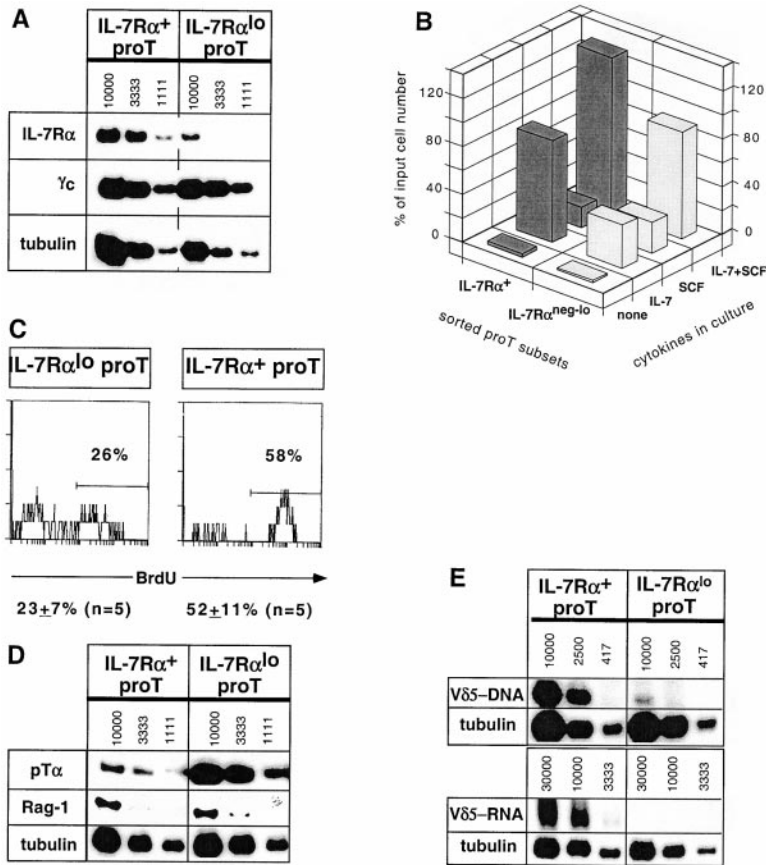


Figure 2. IL-7R α^+ and IL-7R $\alpha^{\text{neg-lo}}$ pro-T subsets exhibit distinct properties. (A) A representative radioactive RT-PCR analysis for IL-7R α , γc , and tubulin transcripts in the sorted pro-T subsets of similar purity. Numbers indicate approximate cell equivalents used for IL-7R α and γc -specific PCR. For tubulin PCR, the starting concentration was 3,333 cell equivalents, which was subject to two serial threefold dilutions. The IL-7R α and γc autoradiographs were exposed for 3 d or overnight, respectively. No PCR products were detected when the RT step was omitted. (B) Proportion of input sorted cells surviving culturing the pro-T subsets for 4 d in the presence of IL-7 and/or SCF. (C) BrdU incorporation after a 3-h pulse with BrdU in vivo. Treated DN thymocytes were stained with mAbs for CD25-613, CD44-Cy5, IL-7R α -biotin/streptavidin-PE, and BrdU-FITC. The levels of BrdU staining on gated CD25 $^+$ CD44 $^+$ IL-7R α^+ and CD25 $^+$ CD44 $^+$ IL-7R $\alpha^{\text{neg-lo}}$ TN thymocytes are presented. (D) RT-PCR analysis for pT α and Rag-1 transcripts. Results from one of four independent sorting experiments are shown for pT α expression analysis; two experiments showed a marginal difference (two- to threefold), whereas two others showed a larger (more than ninefold) difference. (E) Levels of V δ 5-J δ 1 rearrangements in genomic DNA (top) and V δ 5-J δ 1 transcripts in total RNA (bottom) from sorted pro-T cell subsets, determined by semiquantitative PCR or RT-PCR, respectively. Genomic DNA PCR was for 35 cycles; RT-PCR entailed 38 cycles for V δ 5-J δ 1 and 28 cycles for tubulin. No PCR products were detected when the RT step was omitted. The autoradiographs shown for V δ 5-J δ 1 and tubulin transcripts were exposed for 5 d and 4 h, respectively.

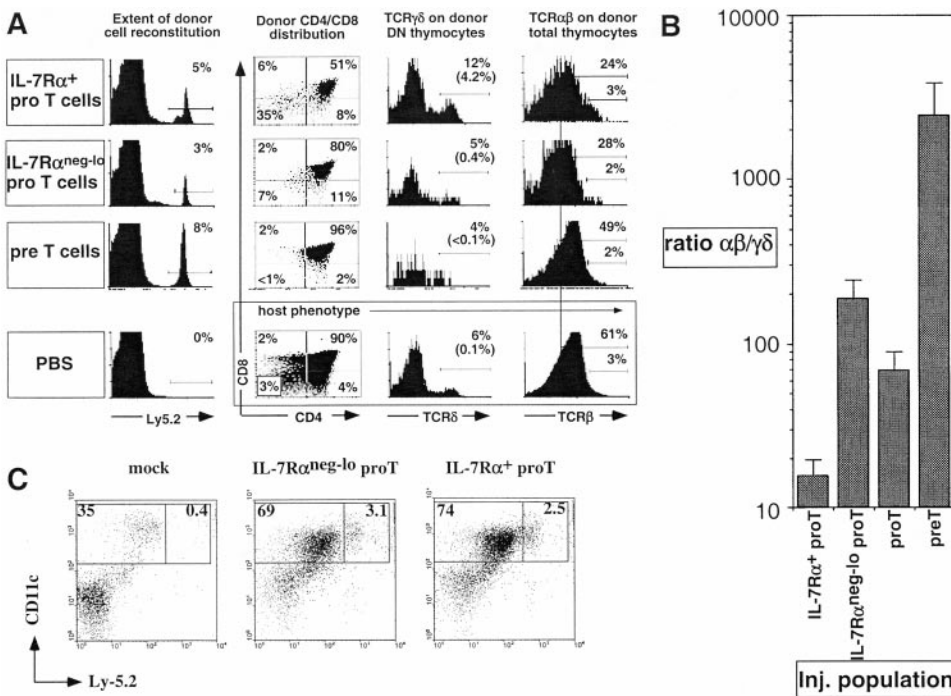


Figure 3. Intrathymically injected distinct $\gamma\delta$ versus $\alpha\beta$ lineage developmental potential. (A) Representative profiles of thymocytes generated from donor (Ly5.2 $^+$) precursor cells. CD4/CD8 profiles of donor-type thymocytes, $\gamma\delta$ TCR expression on gated donor-type CD4 $^+$ CD8 $^-$ thymocytes, and $\alpha\beta$ TCR expression on donor thymocytes are illustrated. Host thymocyte profiles are presented for a mouse injected with saline alone. Percentages in brackets represent the percentage of $\gamma\delta$ TCR $^+$ cells of all donor thymocytes. In the profiles compared, IL-7R α^+ and IL-7R $\alpha^{\text{neg-lo}}$ pro-T cells generated similar numbers of donor-derived thymocytes. (B) The ratio of $\alpha\beta$ to $\gamma\delta$ thymocytes generated from the respective sorted populations 8–11 d after injection. The bars represent averages of $\alpha\beta/\gamma\delta$ thymocyte ratio of individual mice (see Table I). Total donor thymocyte numbers were higher in the thymi injected with IL-7R $\alpha^{\text{neg-lo}}$ pro-T cells, but the broad range (0.2–10.6%) of donor cell reconstitution level in

each experimental group makes the interpretation of this difference ambiguous. (C) Representative profiles of five independent experiments show similar proportions of DCs generated from the pro-T subsets (average proportions of donor DCs in CD11c $^+$ population \pm SEM: IL-7R $\alpha^{\text{neg-lo}}$, 10.4 \pm 2.6%; IL-7R α^+ , 12.2 \pm 2.4%). Thymic DCs were purified from mice 9 d after intrathymic injection of pro-T cells or no cells (mock), as indicated. The cells were analyzed for CD11c expression, which identifies all DCs, and for Ly-5.2 expression, which distinguishes DCs of donor (Ly-5.2 $^+$) versus host (Ly-5.2 $^-$) origin. Purified DCs (all MHC class II $^+$) from mice with 4% and 1.5–1.9% donor cell reconstitution from injected IL-7R $\alpha^{\text{neg-lo}}$ and IL-7R α^+ pro-T cells, respectively, are shown. For comparison, cells from a mouse that underwent the same surgical procedure but was not injected are presented (mock).

(>80%) of sorted IL-7R^{lo} pro-T thymocytes again accumulated on the lowest fluorescent intensity channel (Fig. 1 C) as a result of the compensation setting used for four-color flow cytometric analysis.

Sorted IL-7R α^+ pro-T cells contained approximately fivefold more IL-7R α mRNA on average than IL-7R $\alpha^{\text{neg-lo}}$ pro-T cells, based on semiquantitative reverse transcription (RT)-PCR analysis with tubulin mRNA as a control (Fig. 2 A). Expression of the common γ chain (γ c) of the IL-2, -4, -7, -9, and -15R complex, on the other hand, was indistinguishable between the two subsets. When cultured for 4 d in the presence of IL-7, sorted IL-7R⁺ pro-T cells survived better than IL-7R^{neg-lo} pro-T cells (Fig. 2 B), yielding on average threefold more cells. The difference is due at least in part to a differential responsiveness to IL-7, since the survival of both populations was higher and more similar when a mixture of SCF and IL-7 was used. Analysis of BrdU incorporation during a 3-h pulse demonstrated that the IL-7R⁺ pro-T cell subset proliferates very rapidly in vivo (Fig. 2 C). More of these cells incorporated BrdU than any other thymocyte subset tested under similar conditions (4). On average, only half as many IL-7R^{neg-lo} pro-T cells incorporated BrdU in a parallel analysis (Fig. 2 C).

Semiquantitative RT-PCR analysis demonstrated that IL-7R⁺ and IL-7R^{neg-lo} pro-T cells contained similar levels of recombination activating gene (Rag)-1/2 transcripts (Fig. 2 D, and data not shown). Interestingly, expression of the pT α chain, essential for development of

most $\alpha\beta$ lineage T cells (1), was consistently elevated in IL-7R^{neg-lo} pro-T cells than in IL-7R⁺ pro-T cells by an average of fourfold (range: 2–10-fold; Fig. 2 D, and data not shown). Conversely, transcripts corresponding to completely rearranged TCR- δ genes (Fig. 2 E, similar data for V δ 4 not shown) were 6- to >10-fold more abundant in sorted IL-7R⁺ pro-T cells than in IL-7R^{neg-lo} pro-T cells. In line with the latter finding, the rare V δ 5-D δ J δ rearrangements (<0.2% of alleles [15]) were confined almost exclusively to the IL-7R⁺ pro-T cell subset (Fig. 2 E). In contrast, the rare complete V γ 2-J γ 1 rearrangements in the population were equally represented in the two subsets (data not shown). Thus, the IL-7R^{neg-lo} subset exhibited properties suggestive of $\alpha\beta$ lineage cells whereas rare cells in the IL-7R⁺ subset exhibited at least one characteristic of $\gamma\delta$ lineage cells, TCR- δ rearrangement and expression.

IL-7R⁺ and IL-7R^{neg-lo} Pro-T Cells Exhibit Differences in Developmental Potential. The developmental potential of sorted IL-7R⁺ and IL-7R^{neg-lo} pro-T cells (from B6-Ly5.2 mice) was examined after intrathymic injection into adult B6-Ly5.1 mice. 7–11 d after engraftment, the reconstituted thymi were analyzed for the content of differentiated $\gamma\delta^+$ cells and CD4⁺CD8⁺ ($\alpha\beta$ lineage) cells that had arisen from donor cells (Fig. 3 A, and Table I). Strikingly, the two pro-T cell populations differed substantially in their lineage potential. In the experiment shown in Fig. 3 A, IL-7R⁺ pro-T cells gave rise to 12-fold more $\gamma\delta$ thymocytes than did IL-7R^{neg-lo} pro-T cells; the average dif-

Table I. Summary of Intrathymic Injection of T Cell Precursor Subsets

T precursor cells	Donor cells		Host cells	
	$\gamma\delta$	$\gamma\delta$ cells	$\gamma\delta$	$\gamma\delta$ cells
	%	$\times 10^3$	%	$\times 10^3$
IL-7R α^+ pro-T	5.0 \pm 0.7 (11)	21.8 \pm 8.2 (7)	0.5 \pm 0.1 (11)	124 \pm 32 (7)
IL-7R α^{lo} pro-T	0.9 \pm 0.3 (8)*	6.3 \pm 1.9 (6)‡	0.2 \pm 0.0 (8)	53 \pm 12 (6)
Pro-T	1.5 \pm 0.4 (5)	5.0 \pm 1.5 (5)	0.2 \pm 0.1 (5)	59 \pm 6 (5)
Pre-T	0.4 \pm 0.1 (9)	2.4 \pm 1.4 (8)	0.6 \pm 0.1 (9)	123 \pm 42 (8)
PBS/none	0 (3)	0 (3)	0.4 \pm 0.0 (3)	131 \pm 22 (3)
T precursor cells	Donor cells		Host cells	
	CD4 ⁺ CD8 ⁺	CD4 ⁺ CD8 ⁺ cells	CD4 ⁺ CD8 ⁺	CD4 ⁺ CD8 ⁺ cells
	%	$\times 10^5$	%	$\times 10^5$
IL-7R α^+ pro-T	54.3 \pm 8.2 (11)	3.0 \pm 1.3 (7)	84.0 \pm 4.4 (11)	267.2 \pm 57.4 (7)
IL-7R α^{lo} pro-T	83.8 \pm 6.3 (8)§	14.6 \pm 6.7 (6)‡	90.8 \pm 2.5 (8)	242.9 \pm 41.9 (6)
Pro-T	74.3 \pm 8.3 (5)	3.6 \pm 1.0 (5)	91.0 \pm 2.2 (5)	293.2 \pm 50.9 (5)
Pre-T	97.6 \pm 0.7 (9)	12.2 \pm 6.0 (8)	78.9 \pm 5.6 (9)	143.6 \pm 26.2 (8)
PBS/none	0 (3)	0 (3)	94.7 \pm 2.6 (3)	298.2 \pm 30.5 (3)

Numbers in brackets represent the sample size; cell numbers are averages \pm SEM. Significance is versus the corresponding IL-7R⁺ pro-T injected group. Statistics are based on Student's *t* test.

**P* < 0.0005.

‡*P* < 0.05.

§*P* < 0.01.

ference was fivefold (Table I). Unseparated pro-T cells exhibited an intermediate capacity to generate $\gamma\delta$ cells (Table I). Conversely, IL-7R^{neg-lo} pro-T cells gave rise to more $\alpha\beta$ lineage cells than did IL-7R α^+ cells, by an average of more than fivefold numerically, although the difference was less dramatic when expressed as a percentage of donor-derived cells (Table I). Overall, the average ratio of the percentage of CD4⁺CD8⁺ cells and the percentage of $\gamma\delta^+$ cells in individual reconstituted mice was >13-fold lower with IL-7R α^+ pro-T cells than with IL-7R^{neg-lo} pro-T cells (Fig. 3 B). When the host cell types were analyzed in the same animals, the difference in the ratio was limited to twofold (Table I). It is important to note that the sorted donor pro-T cell subpopulations differentiated in the presence of a large excess (20–100-fold; Table I) of developing host thymocytes. Thus, thymic “niches” and other undefined factors that might influence development of the cells should be equivalently available to both populations of injected cells. Therefore, the different outcomes observed indicate that the IL-7R α^+ and IL-7R^{neg-lo} pro-T cell subpopulations differ intrinsically in their developmental potential.

Intrathymically injected pre-T cells efficiently differentiated into $\alpha\beta$ lineage cells, but yielded extremely few $\gamma\delta$ cells (Table I; Fig. 3, A and B). In some experiments pre-T cells yielded no detectable $\gamma\delta$ T cells. Hence the $\alpha\beta/\gamma\delta$ ratio for pre-T cells was 10-fold higher than that of IL-7R^{neg-lo} pro-T cells, and 100-fold higher than IL-7R α^+ pro-T cells. The results suggest that most pre-T cells are restricted to the $\alpha\beta$ lineage. It should also be noted that ~ 10 times as many pre-T cells as IL-7R^{neg-lo} pro-T cells had to be injected to generate similar numbers of $\alpha\beta$ lineage cells (Table I). The much lower cell generative capacity of pre-T cells compared with pro-T cells excludes the possibility that contaminating pre-T cells can account for the $\alpha\beta$ lineage preference of the IL-7R^{neg-lo} pro-T cell subset.

In contrast to the apparent $\gamma\delta/\alpha\beta$ lineage bias in pro-T subsets, injected IL-7R α^+ and IL-7R $\alpha^{\text{neg-lo}}$ pro-T cell subsets, like unseparated pro-T cells (17), generated similar proportions of lymphoid DCs (Ly5.2 donor type, MHC class II⁺ and CD11c⁺; Fig. 3 C). Thus, the two pro-T subsets do share some developmental properties associated with an early T progenitor population, but specifically differ in their ability to generate $\gamma\delta$ versus $\alpha\beta$ lineage cells.

The lineage bias observed in the pro-T subsets developing in the adult thymic environment *in vivo* was recapitulated in FTOC. Host thymocyte-depleted E14 thymi were repopulated with sorted pro-T cells. At a relatively early stage of the cultures (days 9–11), IL-7R α^+ pro-T cells yielded approximately three times the percentage of $\gamma\delta$ thymocytes as IL-7R $\alpha^{\text{neg-lo}}$ pro-T cells (Fig. 4 A and Table II). Conversely, IL-7R α^+ pro-T cells generated fewer $\alpha\beta$ lineage cells than IL-7R $\alpha^{\text{neg-lo}}$ cells, although the difference was not striking. The total numbers of thymocytes generated from the two pro-T subsets in FTOC were not significantly different (Table II). Thus, as was observed in the intrathymic injection studies, the average ratio of $\alpha\beta/\gamma\delta$ lineage cells generated by IL-7R $\alpha^{\text{neg-lo}}$ pro-T cells was higher than that of IL-7R α^+ pro-T cells, in this case by a

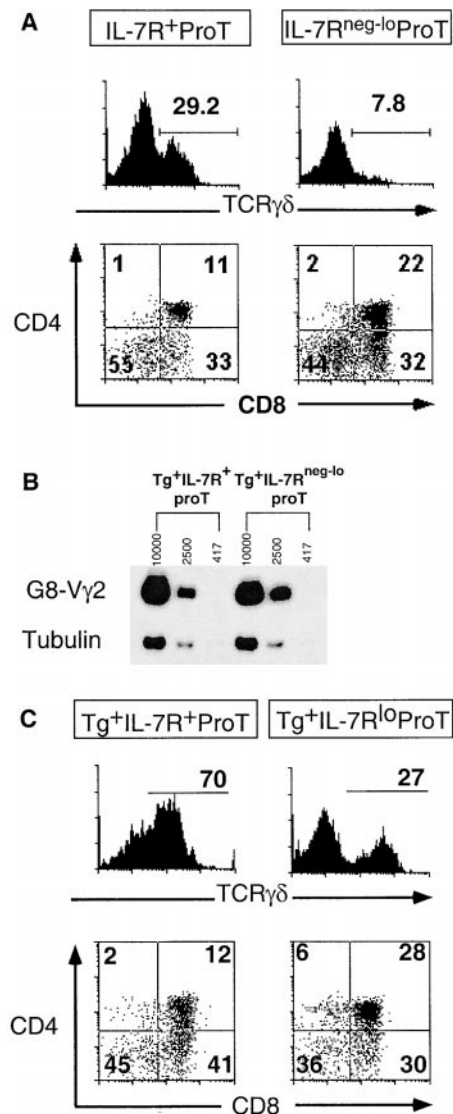


Figure 4. The sorted pro-T subsets display distinct $\gamma\delta/\alpha\beta$ lineage developmental potential in FTOC. (A) Thymocytes from FTOCs reconstituted with sorted pro-T cell subsets were analyzed for $\gamma\delta$ TCR and CD4/CD8 expression after 11 d of culture. Representative profiles are from three thymic lobes that were pooled. Many of the cells are accumulating on the axes of the profiles as a result of four-color compensation settings. (B) Similar transgene expression in sorted pro-T cell subsets from low (1–2) transgene copy line as determined by semiquantitative RT-PCR using transgene-specific primers. Numbers indicate approximate cell equivalents. Two additional experiments yielded comparable results. No PCR products were detected when the RT step was omitted. (C) Thymocytes from FTOCs reconstituted with sorted pro-T cell subsets from high copy G8 transgenic mice were analyzed for $\gamma\delta$ TCR and CD4/CD8 expression after 10 d of culture. The levels of IL-7R α expression on the pro-T progenitor populations in the transgenic mice were not notably different from those in nontransgenic littermates. Three thymic lobes of each type were pooled for analysis.

factor of five on average (Table II). At a later stage of FTOC (21–22 d) both input populations yielded somewhat lower percentages of $\gamma\delta$ cells and higher percentages of $\alpha\beta$ lineage cells, probably because of greater expansion of $\alpha\beta$ lineage cells. Nevertheless, the IL-7R $\alpha^{\text{neg-lo}}$ pro-T cells still

Table II. Summary of FTOC of T Cell Precursor Subsets

Day of culture	T precursor cells	<i>n</i>	Total cells	$\gamma\delta$	$\gamma\delta$ cells	CD4 ⁺ CD8 ⁺	CD4 ⁺ CD8 ⁺ cells
			$\times 10^3$	%	$\times 10^3$	%	$\times 10^3$
Day 9–11	IL-7R α^+ pro-T	14	26.1 \pm 3.7	21.1 \pm 3.0	5.8 \pm 1.2	26.8 \pm 7.1	6.8 \pm 1.9
	IL-7R α^{lo} pro-T	11	37.2 \pm 2.6	7.8 \pm 0.4*	2.9 \pm 0.3‡	34.1 \pm 5.1§	12.8 \pm 2.2‡
	ProT	4	13.6	12.3	1.7	16.2	2.2
	PreT	4	34.7	1.1	0.4	61.4	23.1
	TG ⁺ IL-7R α^+ pro-T	7	14.6 \pm 2.4	64.0 \pm 5.7	9.2 \pm 0.7	15.2 \pm 2.8	2.3 \pm 0.8
	TG ⁺ IL-7R α^{lo} pro-T	8	20.0 \pm 1.2	25.4 \pm 2.1*	5.1 \pm 0.1*	30.2 \pm 2.0*	6.0 \pm 0.7*
Day 21/22	IL-7R α^+ pro-T	12	40.6 \pm 3.5	11.6 \pm 0.5	4.7 \pm 0.4	44.2 \pm 3.4	18.1 \pm 2.4
	IL-7R α^{lo} pro-T	12	46.8 \pm 3.0	3.5 \pm 0.2*	1.6 \pm 0.1*	62.1 \pm 3.0‡	29.1 \pm 2.1‡

TG, G8 TCR- γ transgenic; cell numbers/lobes are averages \pm SEM; for pro-T and pre-T cultures, results represent analysis of four pooled thymic lobes. Significance is versus the corresponding IL-7R α^+ pro-T injected group. Statistics are based on the Student's *t* test.

**P* < 0.0001.

‡*P* < 0.0005.

§*P* < 0.05.

yielded fewer $\gamma\delta$ cells and more $\alpha\beta$ lineage cells than IL-7R α^+ pro-T cells, resulting in an average threefold difference in the $\alpha\beta/\gamma\delta$ ratio (Table II). Thus, the lineage bias of the two pro-T cell subsets is apparent at both early and late stages of thymic reconstitution.

The Two Subsets of Pro-T Cells Support TCR- γ Expression Equally. It was possible that the IL-7R α^+ population generates more $\gamma\delta$ cells simply because the higher IL-7R α levels enhance responsiveness of the cells to IL-7, leading to augmented rearrangement and/or transcription of TCR- γ genes. Semiquantitative PCR data indicated, however, that TCR- γ rearrangements were equally prevalent in the two pro-T cell subsets, though very rare in both cases (data not shown). Since most cells in both populations did not contain rearranged TCR- γ alleles, an alternative approach was developed to assess whether the subsets differ in the capacity to express rearranged TCR- γ genes. A previous study of mice with a prerrearranged TCR- γ transgene present at low copy number (<2) demonstrated that transgene transcription was reduced by 26-fold when the mice lacked a functional IL-7R α gene (18). These data suggest that transgene transcription is an excellent assay for IL-7R α -dependent TCR- γ transcription. Semiquantitative RT-PCR analysis revealed that the TCR- γ transgene was transcribed equally in IL-7R α^+ and IL-7R $\alpha^{\text{neg-lo}}$ pro-T cell subsets (Fig. 4 B). This result suggests either that the low IL-7R α level on IL-7R $\alpha^{\text{neg-lo}}$ pro-T cells is sufficient to stimulate TCR- γ gene rearrangement and transcription, or that IL-7R α signaling at the earlier c-kit⁺CD25⁻CD44⁺ stage activates the TCR- γ locus in a manner that is sustained in later stages. Most significantly, the data suggest that despite having different levels of IL-7R α , the two subsets do not differ in the capacity to express TCR- γ genes.

Another approach was employed to address the role of IL-7R-dependent TCR- γ rearrangement/transcription. If the distinct developmental potentials of the two pro-T cell subsets was due simply to differential expression and/or rear-

angement of TCR- γ genes, the difference should be abolished by equipping both subsets with a TCR- γ gene that is expressed adequately even in the absence of IL-7R α signaling. Previous analysis of a high copy (~33 copies) TCR- γ transgenic line crossed into an IL-7R $\alpha^{-/-}$ background showed that the transgene is expressed at a higher level than endogenous TCR- γ genes are in normal mice (18). The high number of transgene copies compensates for reduced transcription of each copy, resulting in a high aggregate transcription level. Importantly, this level of transcription was shown to be sufficient to restore development of $\gamma\delta$ T cells in IL-7R $\alpha^{-/-}$ mice. As expected from the previous results with the low copy transgene, the high copy transgene was expressed equally in sorted IL-7R α^+ and IL-7R $\alpha^{\text{neg-lo}}$ pro-T cells (data not shown). As assayed in FTOCs, both subsets yielded more $\gamma\delta$ cells than wild-type subsets, presumably because the in-frame transgene provides functional γ chains to all cells whereas productive γ rearrangements occur in only a fraction of cells in nontransgenic mice. However, the high copy transgene did not equalize the developmental potential of the pro-T cell subsets as assayed in FTOCs (Fig. 4 C, and Table II). The IL-7R α^+ pro-T cells yielded an average of 2.6 times the percentage of $\gamma\delta$ cells and half the percentage of $\alpha\beta$ lineage cells as did IL-7R $\alpha^{\text{neg-lo}}$ pro-T cells. The collective data indicate that the two subsets exhibit distinct developmental potential even when TCR- γ expression is driven in all cells at higher than normal levels, and further, that the two subsets do not differ in the extent of TCR- γ rearrangement or the capacity to support TCR- γ expression. Clearly, other differences between the subsets must account for their distinct developmental capacities.

Discussion

We present data demonstrating that pro-T cells are heterogeneous in phenotype and that the subpopulations of pro-T cells do not generate equivalent progenies. A trivial

explanation of the data would be that the sorted IL-7R $\alpha^{\text{neg-lo}}$ subset was selectively contaminated with more mature IL-7R α^{neg} pre-T cells. Our data indicate that the latter cells exhibit a strong bias to the $\alpha\beta$ lineage (Fig. 3 B). This possibility is highly unlikely, however, because pre-T cells in the intrathymic assay exhibited ~ 10 -fold less cell generative capacity compared with IL-7R $\alpha^{\text{neg-lo}}$ pro-T cells (Table I) and would therefore not be capable of generating sufficient numbers of $\alpha\beta$ lineage cells to account for the results. For example, after intrathymic injection of low numbers of pre-T cells ($\sim 10^4$), equivalent to half the number of pro-T cells that were injected, negligible progeny were detected (data not shown). We therefore consider various substantive explanations for the observed lineage bias of pro-T cell subsets.

Are the Pro-T Cell Subsets Sequentially Related Populations That Exhibit Distinct TCR Rearrangement Potential, but No Other Intrinsic Lineage Bias? One possibility is that the IL-7R α^+ pro-T cell population precedes the IL-7R $\alpha^{\text{neg-lo}}$ population in a developmental sequence. If cells at the IL-7R α^+ stage rearrange TCR- γ and - δ genes in preference to TCR- β genes, the population would exhibit a relative bias in favor of $\gamma\delta$ cell development. Failure to successfully initiate $\gamma\delta$ lineage development at this stage would result in subsequent differentiation of IL-7R $\alpha^{\text{neg-lo}}$ pro-T cells. At this stage TCR- γ , - δ , and - β genes may rearrange more or less equivalently. Equivalent rearrangement of the three genes would marginally favor $\alpha\beta$ lineage development, which requires only one in-frame rearrangement (of TCR- β) as opposed to the two in-frame rearrangements (of γ and δ) required for $\gamma\delta$ lineage development. This scheme differs substantially from models in which the two populations represent divergent paths, though it does imply different lineage potential in the two populations due to an early preference for TCR- γ and - δ rearrangements.

Several lines of evidence are inconsistent with this sequential model. First, if all developing T cells traverse the IL-7R α^+ pro-T cell stage, the latter cells should ultimately yield $\alpha\beta$ and $\gamma\delta$ lineage progeny in physiological proportions, i.e., a ratio of 100 or more, and should not exhibit a significant bias for $\gamma\delta$ lineage development. In fact, the IL-7R α^+ pro-T cell subset yielded an $\alpha\beta/\gamma\delta$ ratio of ~ 15 (Fig. 3 B). The discrepancy is unlikely to reflect differences in developmental kinetics in the populations, because the lineage bias was evident at both early and late times after initiation of FTOCs (Table II). In contrast, unseparated pro-T cells yielded $\alpha\beta$ and $\gamma\delta$ progeny in physiological proportions. A second persuasive argument against the sequential scheme arises from its prediction that many cells in the IL-7R $\alpha^{\text{neg-lo}}$ population should have rearrangements at the TCR- δ and/or - γ loci. This is because the majority (67%) of the TCR- γ and - δ rearrangements that would occur at the “early” IL-7R α^+ stage are predicted to be nonproductive. Many cells with productive rearrangements of one of the genes (γ or δ) would have nonproductive rearrangements of the other, and some cells would have only nonproductive rearrangements at both loci. All of these cells would be unable to differentiate into $\gamma\delta$ cells. If they

have no intrinsic lineage bias they should then convert to the “subsequent” IL-7R $\alpha^{\text{neg-lo}}$ population, contributing numerous chromosomes with rearranged TCR- γ and - δ alleles. In strong contrast to this expectation, the latter population is essentially devoid of cells with TCR- δ rearrangements (Fig. 2 E), and contains a very low frequency of rearranged TCR- γ alleles similar to that in the IL-7R α^+ population. Yet, $\alpha\beta$ lineage cells are known to contain high levels of TCR- γ and - δ rearrangements, most of which are nonproductive (30–32). It is likely that these rearrangements occur subsequent to the IL-7R $\alpha^{\text{neg-lo}}$ stage rather than before. These data argue against the proposal that IL-7R α^+ pro-T cells preferentially undergo TCR- γ and - δ rearrangements before differentiating into IL-7R $\alpha^{\text{neg-lo}}$ pro-T cells. Although the results do not rule out all conceivable models in which the two subsets are sequentially related, the simplest interpretation is that the two subsets represent alternative, as opposed to sequential, developmental stages.

Is Differential IL-7R Signaling in Pro-T Cells Responsible for the Observed Lineage Bias? Previous reports demonstrate that IL-7R signaling plays a unique role in $\gamma\delta$ cell development, as it promotes rearrangement and expression of TCR- γ genes but is not necessary for rearrangement or expression of the other TCR genes (18, 27, 28, 33). Thus, an obvious possibility was that the bias of the IL-7R α^+ subset for $\gamma\delta$ lineage development resulted from enhanced rearrangement and expression of TCR- γ genes in this subset. Conversely, the $\alpha\beta$ lineage bias of the IL-7R $\alpha^{\text{neg-lo}}$ pro-T cell subset might be because of impaired rearrangement and expression of TCR- γ genes. However, several lines of evidence indicate that IL-7R $\alpha^{\text{neg-lo}}$ pro-T cells and pre-T cells have the capacity to support high levels of TCR- γ gene expression, despite the low or absent levels of IL-7Rs on these cells. First, the low copy prerearranged TCR- γ transgene, whose transcription was previously shown to be IL-7R α dependent, was expressed at a similar level in the pro-T subsets (Fig. 4 B). Second, in pre-T cells, endogenous TCR- γ rearrangements are abundant, and these are expressed at a high level despite negligible IL-7R on these cells (data not shown). Third, V $\gamma 2$ -J $\gamma 1$ rearrangements, although very rare at the pro-T cell stage, are equally represented in the two pro-T cell subpopulations as determined by semiquantitative PCR (data not shown). Finally, it is notable that $\alpha\beta$ lineage cells contain very high levels of mostly nonproductive TCR- γ rearrangements (34, 35); it is therefore implausible to argue that rearrangement of TCR- γ genes is impaired in the progenitors of these cells. Collectively these data suggest either that IL-7R α signaling at an earlier stage of development allows TCR- γ gene activation at a later stage, or that low levels of IL-7R are sufficient to activate the locus at the pro- and pre-T cell stages.

Although these arguments suggest that the two pro-T cell subsets are equally capable of activating the TCR- γ locus, we sought a more direct test of whether the lineage bias was attributable to impaired TCR- γ gene expression. The results indicated that the lineage bias was still evident even in the presence of high levels of functional TCR γ

chain in both pro-T subsets, directed by a high copy transgene (Fig. 4 C). These findings represent a strong argument against the possibility that the lineage bias of the pro-T cell subsets results from differential TCR- γ gene activation as a consequence of distinct IL-7R α levels. Nevertheless, IL-7R α levels correlate with lineage-biased subsets in young adult mice. It should be noted that pro-T cells in newborn mice uniformly express high levels of IL-7R α (data not shown). Thus, the correlation between IL-7R levels on pro-T cells and lineage potential may hold true only in a homeostatic steady-state thymic environment.

Are Pro-T Cells Committed to Developing into $\gamma\delta$ or $\alpha\beta$ Cells before TCR Gene Rearrangement? The selective differences in $\alpha\beta/\gamma\delta$ composition, but not in DCs, in the progenies of the pro-T subsets appear to be most consistent with the possibility that pro-T cells already contain $\gamma\delta$ and $\alpha\beta$ lineage-biased precursor cells. This lineage bias is TCR independent since a negligible fraction of pro-T cells express functional TCR. It should be noted that even the relatively $\gamma\delta$ -biased IL-7R α^+ population generated more $\alpha\beta$ lineage cells than $\gamma\delta$ cells, probably because $\alpha\beta$ lineage cells undergo 8–10 rounds of cell division (3), whereas $\gamma\delta$ lineage cells undergo much less expansion (4). Thus, the IL-7R α^+ pro-T cell population probably contains fewer $\alpha\beta$ lineage precursors than $\gamma\delta$ lineage precursors, with subsequent expansion of the $\alpha\beta$ lineage cells eventually leading to an excess of these cells.

A caveat of our results is that the two sorted populations appear to be incompletely restricted in their lineage potential. Subsets defined by differential IL-7R expression generate cells of both $\alpha\beta$ and $\gamma\delta$ lineages, albeit at different relative ratios. The precursor cells may be only biased rather than completely restricted in their lineage potential, but other possibilities must be considered. For example, the IL-7R α marker may not precisely define restricted precursor cells, or the cell sorting may have been imperfect. It is also possible that an initial bias in lineage potential is subject to modification by signals occurring later in development, including signals through the TCR. Nevertheless, our data do argue strongly against a simple instructive model in which the lineage fate decision of T cell precursors is determined primarily by which TCR, $\gamma\delta$, or pre-TCR is expressed. Obviously, a further refinement of our understanding of T cell lineage commitment at the pro-T cell level awaits in vivo clonal analyses of developmental potential of single pro-T cells sorted from adult mice.

We propose that a thymic lymphoid progenitor generates two distinct lineage-biased populations at or before the pro-T cell stage. Most $\gamma\delta$ lineage-committed precursor cells that express a functional TCR- $\gamma\delta$ will develop into $\gamma\delta$ lineage cells whether or not they have previously or concomitantly expressed TCR β chain, consistent with published data (10, 11). Conversely, $\alpha\beta$ lineage-committed precursor cells that express the pre-TCR will proliferate and give rise to a predominant population of double positive thymocytes (3). It has been demonstrated that $\alpha\beta$ lineage-biased precursors that express functional $\gamma\delta$ TCRs instead of the pre-TCR can differentiate into double posi-

tive thymocytes (4, 8). However, TCR- γ and δ gene expression is then extinguished in these cells (4, 36, 37), presumably leading to their eventual death. Hence, although lineage commitment may be initiated well before functional TCR expression, subsequent expression of the corresponding type of TCR is normally necessary for the ultimate maturation of the cells.

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