T-Cell Lineage Commitment and Cytokine Responses of Thymic Progenitors

By Thomas A. Moore and Albert Zlotnik

The earliest steps of intrathymic differentiation recently have been elucidated. It has been reported that both CD4^+ (CD4^+ CD25^- c-kit^- CD3^- CD44^- CD8^-) and pro-T cells (CD4^+ CD25^- c-kit^- CD3^- CD4^- CD8^+) exhibit germline T-cell receptor $\beta$ and $\gamma$ loci, suggesting that neither population is exclusively committed to the T-cell lineage. Several groups have shown that CD4^+ cells retain the capacity to generate multiple lymphoid lineages in vivo; however, the lineage commitment status of pro-T cells is unknown. To determine when T-cell lineage commitment occurs, we examined the ability of sorted CD4^+ and pro-T cells to generate lymphoid lineage cells in vivo or in fetal thymic organ cultures (FTOCs). When intravenously injected into scid mice, CD4^+ cells generated both T and B cells, whereas the progeny of pro-T cells contained T cells exclusively. Fetal thymic organ cultures repopulated with CD4^+ cells contained both T and natural killer (NK) cells, whereas cultures repopulated with pro-T cells contained T cells almost exclusively. These observations strongly suggest that T-cell lineage commitment occurs during the transition of CD4^+ to pro-T cells. Because it is likely that the thymic microenvironment plays a critical role in T-cell commitment, we compared the responses of CD4^+ and pro-T cells to various cytokine combinations in vitro, as well as the ability of the cultured cells to repopulate organ cultures. Cytokine combinations that maintained T-cell repopulation potential for both CD4^+ and pro-T cells were found. CD4^+ cells proliferated best in response to the combination containing interleukin-1 (IL-1), IL-3, IL-6, IL-7, and stem cell factor (SCF). Unlike CD4^+ cells, pro-T cells were much more dependent upon IL-7 for proliferation and FTOC repopulation. However, combinations of cytokines lacking IL-7 were found that maintained the T-cell repopulating potential of pro-T cells, suggesting that, whereas this cytokine is clearly very important for normal pro-T cell function, it is not an absolute necessity during early T-cell expansion and differentiation. © 1995 by The American Society of Hematology.

EVENTS DRIVING EXPANSION and differentiation of early intrathymic progenitor cells are poorly understood. Intrathymic precursors were initially described as being CD3^- CD4^- CD8^- (triple negative [TN]), however, subsequent studies have shown that the earliest intrathymic progenitor cells express low levels of CD4. These early progenitors (which exhibit a CD4^+ CD3^- CD44^- phenotype and are termed CD4^+) have been shown to contain T, B, natural killer (NK), and dendritic cell precursors, although lacking the ability to generate myeloid lineage cells. The observations indicate that CD4^+ cells, currently considered to be the first cells to seed the thymus from the bone marrow (BM), are not yet committed to the T-cell lineage. Therefore, this suggests that thymic microenvironmental influences play a role in T-lineage commitment. CD4^+ cells then enter the TN stage, where they undergo extensive proliferation and differentiation. TN cells can be further subdivided into various phenotypically distinct stages of differentiation based on the expression of CD25, CD44, and c-kit. The CD4^+ cells lose expression of CD4 and acquire CD25 to become CD4^+ CD25^- c-kit^- TN pro-T cells. Both CD4^+ and pro-T cells exhibit germline configuration at their $\beta$ and $\gamma$ T-cell receptor (TCR) loci. The latter observation suggests that pro-T cells may not yet be committed to the T-cell lineage either. As pro-T cells downregulate CD44 and c-kit expression to become pro-T cells (CD4^- CD25^- c-kit^- TN), they undergo rearrangements at their TCR $\beta$ and $\gamma$ TCR loci, implying that pro-T cells are committed to the T-cell lineage.

Because these early pre-CD4^- CD8^- (double-negative [DN]) thymocyte subsets have only recently been defined, very little is known about their physiology. The effects of thymically derived cytokines on these immature subsets have only begun to be examined. The thymic microenvironment is capable of producing a variety of cytokines, including interleukin-1$\alpha$ (IL-1$\alpha$), IL-1$\beta$, IL-3, IL-6, IL-7, IL-12, granulocyte-macrophage colony-stimulating factor, macrophage colony-stimulating factor, granulocyte colony-stimulating factor, and stem cell factor (SCF). Of these cytokines, the role of IL-7 in T-cell development has been the best defined. Although generation of mature T cells still occurred, in vivo injection of antibodies directed against either IL-7 or IL-7R$\alpha$ resulted in a marked decrease in thymic cellularity. These studies suggested an important role for IL-7 in early T-cell proliferation and expansion, but not in differentiation. The stages of T-cell differentiation at which IL-7 exerts its influence are poorly understood. IL-7 has been shown to act as a maintenance factor for immature (CD25^-) T thymocytes. Godfrey et al went on to show that pro-T cells respond to IL-7, whereas later stages of differentiation failed to respond. IL-1 has also been shown to be involved in T-cell differentiation; addition of anti-IL-1 antibodies to fetal thymic organ cultures (FTOCs) resulted in inhibition of T-cell maturation. Likewise, IL-6 has been reported to accelerate the differentiation of Thy-1^- CD25^- TN thymocytes.

We report on the lymphoid lineage commitment status of CD4^+ to pro-T cells. CD4^+ cells, when intravenously transferred into scid mice recipients, generate both T and B cells. However, pro-T cells only generated T cells when transferred into scid mice. Additionally, FTOCs repopulated with CD4^+ cells contain both T and NK cells, whereas repopulation of cultures by pro-T cells results in the generation of T cells almost exclusively. Taken together, these data strongly suggest that commitment to the T-cell lineage oc-
curs during the transition to or at the pro-T-cell stage of thymic differentiation. The cytokine responses of CD4<sup>hi</sup> and pro-T cells also differed significantly. CD4<sup>hi</sup> cells required combinations of three or more cytokines to proliferate while retaining their ability to repopulate FTOCs. Several cytokine combinations lacking IL-7 (eg, IL-3 plus IL-6 plus SCF and IL-1 plus IL-3 plus IL-6 plus SCF) induced CD4<sup>hi</sup> proliferation while retaining FTOC repopulation potential. Addition of IL-7 to these combinations resulted in increased cell expansion in vitro and in FTOCs. On the other hand, IL-7 alone was sufficient to maintain FTOC repopulating ability of pro-T cells. Cytokine combinations were found that induced proliferation of CD4<sup>hi</sup> cells while having little effect on pro-T cells, indicating differential cytokine responsiveness of these cell populations. These data indicate that CD4<sup>hi</sup> and pro-T cells differ in their ability to generate lymphoid lineage cells and in their response to hematopoietic cytokines, suggesting a role for thymically derived molecules in T-lineage commitment.

**MATERIALS AND METHODS**

*Mice.* Four- to five-week-old BALB/c mice (Simonsen Laboratories, Gilroy, CA) were used throughout these experiments unless otherwise noted. For lymphoid repopulation of C.B-17 scid/scid mice (Simonsen) and NK cell repopulation studies in FTOCs, CD4<sup>hi</sup> and pro-T cells were sorted from thymi of C57BL/6 x DBA/2)F1 (B6D2F1) mice (Simonsen or Jackson Laboratories, Bar Harbor, MA).

*Antibodies.* All antibodies were purchased from PharMingen (San Diego, CA) unless otherwise specified. For isolation of CD4<sup>hi</sup> and pro-T cell populations, the following antibodies were used: anti-CD3-biotin (clone 144-2C11), anti-CD8-biotin (clone 53-6.7), anti-B220-biotin (clone RA3-6B2), anti-Mac-1-biotin (clone M1/70), anti-Gr-1-biotin (clone RB6-8C5), anti-NK1.1-biotin (clone PK136), anti-CD25-fluorescein isothiocyanate (anti-CD25-FITC) (clone 7D4), anti-CD44-phycocerythrin (anti-CD44-PE) (clone IM7), anti-c-kit-allophycocyanin (clone 2B8), and UltraAvidin-Texas Red (Leinco Technologies, Ballwin, MO). For analyses of in vitro repopulation of fetal lobes and in vivo repopulation of scid recipient mice, the following antibodies were used: anti-CD4-biotin (clone RM4-5), anti-CD8-biotin, anti-NK1.1-biotin, anti-CD4-TRIColor (clone YTS 191.1, Caltag Laboratories, South San Francisco, CA), anti-CD8-PE (clone 53-6.7), anti-TCR<alpha>beta>-FITC (clone H57-597), anti-TCR<gamma>delta>-FITC (clone GL3), anti-H-2K<sup>d</sup>-PE (clone AF6-88.5), rat-anti-mouse IgM-FITC (clone R6-60.2), anti-B220-biotin, and streptavidin-TriColor (Caltag).

*Sorting and multiparameter analysis.* The identification and isolation of CD4<sup>hi</sup> (CD4<sup>+</sup> CD25<sup>-</sup> c-kit<sup>-</sup> Lin<sup>-</sup>) and pro-T (CD4<sup>+</sup> CD25<sup>-</sup> c-kit<sup+</sup> Lin<sup+</sup>) cells has been previously described. Briefly, thymocytes were depleted of CD8<sup+</sup> cell by incubation with anti-CD8 (clone AD4; Cedarlane Laboratories, Hornby, Ontario) followed by treatment with low-tox M rabbit complement (Cedarlane) and 20 mg/mL Dnase 1 (Sigma Chemical Co, St Louis, MO). Viable cells were isolated with Histopaque 1078 (Sigma), then stained as follows: panel of lineage antibodies directed against CD3, CD8, B220, Mac-1, Gr-1(-all biotinylated), anti-CD25-FITC, anti-CD4-PE, and anti-c-kit-APC. In experiments designed to analyze NK cell regeneration in FTOCs, anti-NK1.1-biotin was added to the lineage cocktail to exclude the possibility of transferring small numbers of contaminating mature NK cells contained within the sorted populations. After washing, cells were incubated with UltraAvidin-Texas Red, CD4<sup>hi</sup> cells were sorted through a combination Lin<sup>-</sup> CD25<sup>-</sup> and CD4<sup>+</sup> c-kit<sup+</sup> gate while pro-T cells were sorted through a Lin<sup+</sup> CD25<sup+</sup> and CD4<sup>+</sup> c-kit<sup+</sup> combination gate (Fig 1). Cells were sorted using a FACSStar Plus or FACS Vantage flow cytometer (Becton Dickinson, San Jose, CA). Sort purities for CD4<sup>+</sup> and pro-T cells were routinely greater than 97%.

For analysis of lymphoid repopulation in FTOCs, single cell suspensions were prepared for staining. To reduce nonspecific staining, cells were incubated with anti-FcyRII/III (clone 2.4G2) before staining for specific antigen expression. FTOCs were stained with anti-TCR<beta>-FITC or anti-TCR<gamma>-FITC, anti-CD8-PE, and anti-CD4-TRIColor, all in one step. To determine NK cell generation, FTOC cells were stained with both anti-CD4-biotin and anti-CD8-biotin, followed by anti-TCR<alpha>beta>-FITC plus anti-TCR<gamma>delta>-FITC, anti-NK1.1-PE, and streptavidin-TRIColor. Cells were gated on the CD4<sup>+</sup> population, then analyzed for TCR and NK1.1 expression. It is important to note that the host fetal lobes are from BALB/c mice that are negative for the NK1.1 marker. Therefore, all NK1.1 staining must be derived from donor B6D2F1 sorted CD4<sup>+</sup> or pro-T cells.

Lymphoid repopulation in scid recipients was examined as follows: Donor cells were detected by staining with an anti-H-2K<sup>d</sup> antibody previously titrated such that all control H-2K<sup>d</sup>-positive thymocytes were stained. Donor thymic repopulation was determined by staining cells with anti-H-2K<sup>d</sup>-PE, anti-CD8-FITC, and anti-CD4-TRIColor. B-cell repopulation in spleen and BM was determined by staining with anti-H-2K<sup>d</sup>-PE, rat-antimouse IgM-FITC, and anti-B220-biotin followed by streptavidin-TRIColor. Donor (H-2K<sup>d</sup>-positive) cells were gated and then analyzed for expression of B220 and IgM. Stained cells were collected using a FACScan cytometer (Becton Dickinson) using Lysys II software, whereas data analysis was performed using CellQuest software (Becton Dickinson).

*In vivo repopulation of C.B-17 scid mice.* C.B-17 scid/scid (H-
bicarbonate, penicillin, streptomycin, and gentamycin. To this composite group.

CD4<sup>+</sup> and pro-T-cell populations were sorted from B6DF2F1 (H-2<sup>k</sup>) donors as described in Materials and Methods and Fig 1. Cells were intravenously injected into 300R irradiated C.B-17 scid (H-2<sup>b</sup>) recipients. As a positive control for T-cell repopulation, one group of mice was injected with BM cells. On day 26, mice were killed, thymi were excised, and cellularity was determined. Cells were then phenotyped for donor repopulation (H-2<sup>k</sup>-positive cells) as well as for the expression of CD4 and CD8. Data presented as the mean (SEM) of three to four independent experiments, each consisting of two to four mice per experimental group.

2<sup>o</sup> mice were exposed to 300 cGy 4 to 5 hours before cell transfer to facilitate lymphocyte engraftment. CD4<sup>+</sup> and pro-T thymocytes were sorted from B6DF2F1 (H-2<sup>k</sup>) mice as described, then injected (3.1 to 4.8 × 10<sup>5</sup> CD4<sup>+</sup> cells/mouse, 4.7 to 9.2 × 10<sup>5</sup> pro-T cells/mouse) into the lateral tail vein of recipients. As a positive control for T- and B-cell repopulation, one group of mice received 1 × 10<sup>6</sup> whole BM cells from B6DF2F1 mice. Mice were killed on day 26 post-cell transfer, and then single cell suspensions of thymus, spleen, and BM cells were examined for repopulation as described above. Donor-derived cells were determined by staining for expression of H-2<sup>k</sup>. Data presented in Tables 1 and 2 are from three to four independent experiments, each consisting of two to four mice per experimental group.

In vitro culture. Sorted thymic populations were cultured in medium consisting of RPMI 1640 containing 10% fetal calf serum (FCS), 200 mM/L L-glutamine, 5 × 10<sup>-5</sup> mol/L 2-mercaptoethanol, minimal essential medium amino acids and vitamins, sodium bicarbonate, penicillin, streptomycin, and gentamycin. To this complete medium, the following cytokines were added in various combinations:

- IL-2 (0.2 mg/mL; R&D Systems, Minneapolis, MN), IL-7 (0.5 mg/mL; PeproTech Inc. Rocky Hill, NJ), IL-12 (1.000 U/mL; M. Gately, Hoffman-LaRoche, Nutley, NJ), and SCF (50 U/mL, DNAx). Cytokine concentrations were determined before usage. For FTOCs, complete medium containing 2% FCS was used. Both CD4<sup>+</sup> and pro-T cells were absolutely dependent upon the addition of exogenous cytokines, the culture of either cell population in complete medium alone for 3 days did not yield any viable cells (data not shown).

Sorted CD4<sup>+</sup> and pro-T cells were incubated in round-bottom 96-well plates (Corning, Corning, NY) in 200 μL complete medium containing various cytokine combinations. Cells were cultured at a concentration of 2 to 4 × 10<sup>5</sup> cells/well. After 3 days, cells were procured and viable cell counts determined by trypan blue exclusion. Cells were then used for FTOC repopulation studies.

FTOC. Fetal thymic lobes were removed at day 15 gestation and depleted of endogenous T-cell progenitors by culturing in FTOC medium containing 1.35 mM/L deoxyguanosine for 5 days as described. Depleted lobes were then individually plated with 1 × 10<sup>7</sup> cells in 30 μL volume in Terasaki plates (Nunc, Kamstrup, Denmark). Plates were then inverted to allow lobes and cells to combine at the bottom of a hanging drop. After 24 to 48 hours, recolonized lobes were transferred back into FTOC for 21 to 24 days, being refed with FTOC medium every 6 days. At the indicated time points, lobes were gently pressed under a glass coverslip in 100 μL phosphate-buffered saline containing 2% FCS to release thymocytes. Thymocytes were then phenotyped as described above.

RESULTS

Lineage potential of CD4<sup>+</sup> and pro-T cells. The CD4<sup>+</sup> thymocytes are the earliest intrathymic progenitor population so far identified. These cells are not exclusively committed to the generation of T cells. When transferred intravenously into recipient mice, CD4<sup>+</sup> cells repopulate lymphoid organs with T, B, dendritic, and NK cells, but not myeloid lineage cells. Therefore, these cells most likely represent lymphoid committed progenitors. Both CD4<sup>+</sup> (CD4<sup>+</sup> CD25<sup>-</sup> c-kit<sup>+</sup>) and CD25<sup>-</sup> c-kit<sup>+</sup> cells were phenotyped as described above.

Table 1. SCID Thymic Repopulation by CD4<sup>+</sup> and Pro-T Cells

<table>
<thead>
<tr>
<th>Cells Injected</th>
<th>Cellularity (&lt;10&lt;sup&gt;6&lt;/sup&gt;)</th>
<th>CD4&lt;sup&gt;+&lt;/sup&gt; CD8&lt;sup&gt;-&lt;/sup&gt;</th>
<th>CD4&lt;sup&gt;+&lt;/sup&gt; CD8&lt;sup&gt;-&lt;/sup&gt;</th>
<th>CD4&lt;sup&gt;+&lt;/sup&gt; CD8&lt;sup&gt;-&lt;/sup&gt;</th>
<th>CD4&lt;sup&gt;+&lt;/sup&gt; CD8&lt;sup&gt;-&lt;/sup&gt;</th>
<th>CD4&lt;sup&gt;+&lt;/sup&gt; CD8&lt;sup&gt;-&lt;/sup&gt;</th>
<th>CD4&lt;sup&gt;+&lt;/sup&gt; CD8&lt;sup&gt;-&lt;/sup&gt;</th>
<th>CD4&lt;sup&gt;+&lt;/sup&gt; CD8&lt;sup&gt;-&lt;/sup&gt;</th>
<th>CD4&lt;sup&gt;+&lt;/sup&gt; CD8&lt;sup&gt;-&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4&lt;sup&gt;+&lt;/sup&gt;</td>
<td>11.3 (1.4)</td>
<td>6.8 (2.2)</td>
<td>15.8 (1.6)</td>
<td>24.7 (2.0)</td>
<td>2.7 (0.8)</td>
<td>6.3 (2.1)</td>
<td>1.4 (0.7)</td>
<td>69.9 (8.8)</td>
<td>22.3 (8.6)</td>
</tr>
<tr>
<td>Pro-T</td>
<td>1.6 (0.3)</td>
<td>62.2 (6.2)</td>
<td>20.9 (2.6)</td>
<td>11.9 (2.8)</td>
<td>5.1 (1.7)</td>
<td>10.4 (3.0)</td>
<td>5.0 (3.2)</td>
<td>23.2 (9.1)</td>
<td>61.4 (12.2)</td>
</tr>
<tr>
<td>BM cells</td>
<td>180.0 (32.6)</td>
<td>42.9 (9.2)</td>
<td>10.3 (4.6)</td>
<td>86.9 (7.7)</td>
<td>8.1 (3.9)</td>
<td>10.0 (5.4)</td>
<td>0.7 (0.5)</td>
<td>88.3 (5.5)</td>
<td>1.1 (0.4)</td>
</tr>
<tr>
<td>Irradiated acd</td>
<td>2.1 (0.4)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>14.6 (3.7)</td>
<td>1.1 (0.5)</td>
<td>62.2 (8.6)</td>
<td>22.2 (5.4)</td>
</tr>
</tbody>
</table>

Table 2. BM and Spleen B-Cell Repopulation by CD4<sup>+</sup> and Pro-T Cells

<table>
<thead>
<tr>
<th>Cells Injected</th>
<th>% Donor</th>
<th>B220&lt;sup&gt;-&lt;/sup&gt; slgM&lt;sup&gt;+&lt;/sup&gt;</th>
<th>B220&lt;sup&gt;-&lt;/sup&gt; slgM&lt;sup&gt;-&lt;/sup&gt;</th>
<th>% Donor</th>
<th>B220&lt;sup&gt;-&lt;/sup&gt; slgM&lt;sup&gt;+&lt;/sup&gt;</th>
<th>B220&lt;sup&gt;-&lt;/sup&gt; slgM&lt;sup&gt;-&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4&lt;sup&gt;+&lt;/sup&gt;</td>
<td>5.9 (2.4)</td>
<td>3.8 (1.3)</td>
<td>6.7 (1.9)</td>
<td>16.5 (5.5)</td>
<td>6.1 (1.9)</td>
<td>3.4 (0.8)</td>
</tr>
<tr>
<td>Pro-T</td>
<td>3.4 (0.6)</td>
<td>0.3 (0.1)</td>
<td>1.1 (0.3)</td>
<td>12.1 (3.9)</td>
<td>0.5 (0.1)</td>
<td>1.4 (0.5)</td>
</tr>
<tr>
<td>BM cells</td>
<td>88.4 (6.9)</td>
<td>24.1 (1.0)</td>
<td>40.6 (0.0)</td>
<td>79.3 (4.0)</td>
<td>56.0 (6.2)</td>
<td>9.4 (1.9)</td>
</tr>
<tr>
<td>Irradiated acd</td>
<td>0</td>
<td>0.4 (0.3)</td>
<td>3.5 (2.7)</td>
<td>0</td>
<td>0.4 (0.2)</td>
<td>4.4 (3.4)</td>
</tr>
</tbody>
</table>

Sorted CD4<sup>+</sup> and pro-T cells from B6DF2F1 (H-2<sup>k</sup>) donors were intravenously injected into 300R irradiated C.B-17 scid (H-2<sup>b</sup>) recipients. As a positive control for B-cell repopulation, one group of mice was injected with BM cells. On day 26, mice were killed and lymphoid repopulation was examined. Thymic repopulation was detailed in Table 1. Single cell suspensions of BM and spleen cells were prepared and stained for expression of H-2<sup>k</sup>, B220, and slgM. The percent donor cell repopulation was determined by gating on all nucleated cells. To determine donor-derived B220<sup>-</sup> slgM<sup>+</sup> and B220<sup>-</sup> slgM<sup>-</sup> cells, BM and spleen cells were gated on lymphocyte-sized cells expressing H-2<sup>k</sup> and then analyzed for B220 and slgM expression. Data presented as the mean (SEM) of three to four independent experiments, each consisting of two to four mice per experimental group.

* Expression of B220 and slgM on irradiated scid BM cells and spleen cells is based on endogenous host scid cell expression.
and pro-T (CD44^CD25^ c-kit^) cells contain germline β and γ TCR loci,^3,4^ whereas pre-T (CD44^-CD25^- c-kit^) cells have already undergone TCR β and γ gene rearrangement. These data suggest that commitment to the T-cell lineage occurs before TCR rearrangements in pre-T cells. If this is the case, two possibilities exist: commitment occurs as CD44^-cells differentiate into pro-T cells, or commitment occurs as pro-T cells differentiate into pre-T cells, but before TCR rearrangement. To examine these possibilities, we sought to determine whether both CD44^- and pro-T cells have the capacity to generate lymphoid lineage cells.

CD44^- and pro-T cells were sorted from B6D2F1 mice and intravenously injected into lightly irradiated C.B-17 scid recipients. Twenty-six days posttransfer, mice were killed and the T- and B-cell repopulation of thymus, spleen, and BM was examined. Both CD44^- and pro-T cells repopulated the thymus of scid recipients with donor-derived T cells (Table 1). We observed a virtual absence of immature CD4^-CD8^- (DN) donor cells, a consequence of a single wave of repopulation caused by the lack of self-renewal capacity of the donor cells. CD4^- recipients had a higher percentage of immature DP cells as compared with recipients of pro-T cells. This is predictable because pro-T cells are further along the T-cell differentiation pathway and, therefore, would be expected to display more rapid repopulation kinetics than CD4^- cells. The majority of donor origin cells within the thymus of pro-T and CD4^- repopulated mice were mature (TCR^+) single-positive CD4+ or CD8+ cells (data not shown). Further evidence that pro-T cells are more differentiated was seen in the thymic cellularity of repopulated scid mice. Scid recipients of CD4^- cells contained many more thymus cells than did recipients of pro-T cells (≈11 × 10^6^ cells vs. ≈2 × 10^6^; Table 1), indicating an increased proliferative potential for CD4^- cells as compared to pro-T cells. Although we should point out that these cells never express TCR, there were variable numbers of CD4+ and/or CD8+ expressing host cells, in agreement with earlier reports that have shown that irradiation of scid mice induces expression of CD4 and CD8.^^20,21^'

Both CD4^- and pro-T cells displayed poor BM repopulating ability, with only approximately 5% of total BM cells being of donor origin (Table 2). This was expected because both cell populations were devoid of pluripotent stem cells that exhibit high BM repopulating ability.^^22,23^ To determine B-cell repopulation, we examined the expression of B220 and IgM on donor-derived cells. As shown in Fig 2, recipients of CD4^- thymocytes had donor B220^- IgM^- and B220^- IgM^- cells in their BM and spleen. However, the majority of donor cells lacked B220 and were Thy-1.2 expressing T cells (data not shown). Interestingly, when pro-T-cell recipients were examined for B-cell repopulation in BM and spleen, no B220^- donor cells were observed (Fig 2). All donor cells found in the spleen and BM of pro-T-cell recipients were T cells (data not shown). We were unable to consistently detect any donor-derived NK cells in any of the scid recipients, regardless of the cell population transferred. This was most likely caused by the normally low frequency with which NK cells are found in peripheral organs.

Because it has been reported that CD4^- cells retain the capacity to generate NK cells as well as B cells and we were unable to detect any NK cell repopulation using our scid-adoptive transfer protocol, we chose to examine NK cell repopulation potential using FTOCs. We repopulated BALB/c fetal lobes with CD4^- or pro-T cells sorted from B6D2F1 donor mice and examined T and NK cell repopulation 21 days later. Because BALB/c mice lack expression of NK1.1, any cells expressing this marker are, by default, of donor origin. As can be seen in Fig 3, NK cells are present at very low numbers within an adult thymus (≈1% of DN cells, which themselves comprise ≈2% of total thymocytes). FTOCs repopulated with CD4^- cells clearly contain a population of DN NK1.1^- TCR^- cells (Fig 3). NK cells can be detected as early as day 14 after FTOC repopulation, and the number of NK cells increases gradually for up to 35 days. (data not shown). In contrast, FTOCs repopulated with pro-T cells contain T cells almost exclusively. However, a small yet consistent number of cells with the phenotype of NK cells can be detected. In terms of absolute cell numbers, CD4^- cells generate 5 to 10 times as many NK cells as do pro-T cells.

Taken together, these data strongly suggest that commitment to the T-cell lineage occurs either during the transition from CD4^- to pro-T cells or very rapidly after arrival of cells into the pro-T cell pool. The ability to generate NK cells appears to be lost after the ability to generate B cells, suggesting a sequential loss of lymphoid lineage potential during thymocyte differentiation.

**Cytokine responses of CD4^- and pro-T cells.** Data presented thus far indicates that T-cell lineage commitment of early thymic progenitors occurs during the transition from CD4^- to pro-T cells. The most likely explanation for this commitment is the induction of multipotent CD4^- cells toward the T-cell lineage by the thymic microenvironment. Consequently, the cytokine responsiveness of multipotent CD4^- cells may vary from that of pro-T cells.

Previously, we have shown that IL-7 served as a maintenance factor for CD25^- TN thymocytes.^^14^ IL-7 did not induce proliferation of these cells, but rather maintained their viability in culture for up to 7 days. Moreover, these IL-7^-cultured TN cells maintained their T progenitor activity as measured by their ability to repopulate FTOCs. Further examination indicated that this effect of IL-7 was restricted to CD44^-CD25^- pro-T cells.^^2^ As was seen with CD25^- TN cells, IL-7 cultured pro-T cells retained their FTOC repopulating ability. Additionally, culture of pro-T cells in IL-7 plus SCF resulted in cell expansion and retention of T progenitor activity.^^3^ Taken together, these results strongly suggest a dependence of IL-7 by pro-T cells and that IL-7 alone is sufficient for the maintenance of T-cell progenitor potential.

Because it is likely that cytokine interactions play a crucial role in the commitment to the T-cell lineage, we investigated the cytokine responsiveness of CD4^- and pro-T cells. First, we examined the response of CD4^- cells to IL-7 with or without SCF. Sorted CD4^- and pro-T cells were cultured in IL-7 or IL-7 plus SCF for 3 days. As previously reported, IL-7 with or without SCF maintained the viability and FTOC repopulating potential of pro-T cells. In sharp contrast, neither IL-7 nor IL-7 plus SCF...
Fig 2. Representative phenotypic analysis of B-cell repopulation in scid recipients of CD4⁺ or pro-T cells. BM and spleen B-cell repopulation was analyzed 26 days after injection with sorted cells. To determine donor-derived B220⁺ slgM⁺ and B220⁺ slgM⁻ cells, BM and spleen cells were gated on lymphocyte-sized cells expressing H-2Kb, then analyzed for B220 and slgM expression. Mice injected with CD4⁺ cells clearly contain donor-derived B220⁺ slgM⁺ and B220⁺ slgM⁻ cells (first column). In contrast, pro-T-cell repopulated scid mice are devoid of any donor B cells, displaying identical staining patterns as irradiated scid mice negative controls (second and third columns). Mice injected with BM cells were used as a positive control for B-cell repopulation (see Table 2 for summary of B-cell repopulation).

significantly supported viability of CD4⁺ cells (Fig 4). We then asked whether the cells surviving in vitro culture retained their ability to generate mature T cells. To address this, we used FTOC to assess the T-cell regeneration potential of these cells. Interestingly, the surviving CD4⁺ cells were unable to repopulate FTOC. These data suggest that either IL-7 is not required for CD4⁺ maintenance, or that CD4⁺ cells require a broader spectrum of cytokines in addition to IL-7 and SCF.

Because it has been well documented that multipotent hematopoietic progenitor and stem cells proliferate in response to a combination of several cytokines, whereas lin-

Fig 3. NK cell generation by CD4⁺ and pro-T cells. To determine the potential to generate NK cells, deoxyguanosine-depleted BALB/c fetal lobes were repopulated with freshly sorted CD4⁺ or pro-T cells from B6D2F1 donors, FTOCs were then procured 21 days later (see Figs 5 and 6 for T-cell repopulation analysis). For analyses, FTOC cells were stained with both anti-CD4-biotin and anti-CD8-biotin, followed by anti-TCRαβ-FITC and anti-TCRγδ-FITC, anti-NK1.1-PE, and streptavidin-TriColor. Stained cells were gated on the CD4⁺ CD8⁻ population, then analyzed for TCR and NK1.1 expression. As a staining control, adult B6D2F1 thymus cells were stained for the presence of NK cells (CD4⁻ CD8⁻ TCRβγ⁺ NK1.1⁻). CD4⁺ cells clearly retain the capacity to generate NK cells in FTOCs. FTOCs repopulated with pro-T cells appear to contain a very small number of NK cells. However, in absolute numbers, CD4⁺ cells generate 5 to 10 times more NK cells than do pro-T cells. Data shown are representative of three independent experiments.
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T-cell lineage commitment appears to decrease the required number of cytokines needed to induce proliferation,\textsuperscript{3} we expanded the search for cytokine combinations capable of maintenance/expansion of multipotent CD4\textsuperscript{lo} cells while still retaining their ability to generate T cells in FTOCs. Working on the assumption that CD4\textsuperscript{lo} cells are more "stem cell like" than committed pro-T cells, we examined the effect of several cytokines that have been shown to have an effect on BM hematopoietic stem and progenitor cells (summarized in Fig 4). IL-3 plus SCF maintained the viability of CD4\textsuperscript{lo} cells; however, these cells failed to repopulate FTOCs. The addition of IL-7 to IL-3 plus SCF resulted in the ability of these CD4\textsuperscript{lo} cells to repopulate FTOC. This would suggest a requirement for IL-7 in the maintenance of T progenitor potential along with the necessity of other cytokines to induce survival and/or proliferation. Culture of CD4\textsuperscript{lo} cells in IL-3 plus IL-6 plus SCF resulted in a slight expansion in cell numbers and addition of IL-7 to this combination resulted in slightly increased cell recovery. Interestingly, CD4\textsuperscript{lo} cells cultured in IL-3 plus IL-6 plus SCF or IL-3 plus IL-6 plus IL-7 plus SCF repopulated FTOC (Figs 4 and 5). However, addition of IL-7 resulted in an approximately fourfold greater cell expansion in FTOC. This effect of IL-7 on cell expansion could be further seen when CD4\textsuperscript{lo} cells were cultured in cytokine combinations containing IL-1a. Addition of IL-7 to IL-1 plus IL-3 plus IL-6 plus SCF produced both an increase in in vitro proliferation and a twofold increase in cell expansion in FTOCs. Surprisingly, the cells recovered from lobes repopulated with IL-1 plus IL-3 plus IL-6 plus SCF-cultured CD4\textsuperscript{lo} cells were largely CD4\textsuperscript{lo} CD8\textsuperscript{-}, whereas addition of IL-7 appeared to promote differentiation (Fig 5). Similarly, addition of IL-7 to IL-1 plus IL-3 plus SCF resulted in enhanced differentiation. It is also interesting to note that the addition of IL-7 to various cytokine combinations during the initial 3 days of culture enhances the ability of the cultured CD4\textsuperscript{lo} cells to generate DN TCR\gamma\delta T cells (Fig 5). These findings are in agreement with others indicating a role for IL-7 in \gamma\delta T-cell differentiation and expansion.\textsuperscript{5,6} Thus, CD4\textsuperscript{lo} thymocytes resemble BM multilineage progenitor and stem cells in their requirement for multiple cytokines to induce proliferation. We have identified cytokine combinations lacking IL-7 that resulted in both in vitro proliferation and retention of T-repopulating ability of CD4\textsuperscript{lo} cells (Figs 4, 5). The addition of IL-7 to these cultures resulted in increased proliferation and T-cell expansion in FTOCs, indicating an important but not absolute role for IL-7 in the expansion/differentiation of the most immature thymic cell population.

Having shown in detail the responsiveness of CD4\textsuperscript{lo} cells to multiple cytokines, we wished to examine the cytokine response profile of pro-T cells. The addition of IL-3, IL-6, or IL-12 to pro-T cells cultured in IL-7 plus SCF showed no significant additional effect on cell recovery after 3 days in culture (Fig 4). These culture conditions also retained FTOC repopulation potential (Fig 4, data not shown). The addition of four or more cytokines (IL-3 plus IL-7 plus IL-12 plus SCF and IL-1 plus IL-3 plus IL-6 plus IL-7 plus SCF) induced significant cell expansion while maintaining the ability to repopulate FTOCs. In sharp contrast to CD4\textsuperscript{lo} cells, pro-T cells failed to respond significantly to any cytokine combination lacking IL-7, indicating clear differences in cytokine responsiveness between these two cell popula-

![Graph](https://www.bloodjournal.org/bloodjournal/1855.png)

**Fig 4.** Cytokine response profile and FTOC repopulation capability of CD4\textsuperscript{lo} and pro-T cells. Cells were sorted as described, then cultured with the indicated cytokine combinations for 3 days. Cells were then procured and cell recoveries determined by trypan blue exclusion. Cells recovered after culture were then tested for their T-cell progenitor activity by measuring their ability to repopulate FTOCs. FTOCs repopulated with cultured CD4\textsuperscript{lo} cells were analyzed after 24 days of culture, whereas those repopulated with cultured pro-T cells were examined 21 days later. Fetal lobes were stained for CD4, CD8, TCR\alpha\beta, and TCR\gamma\delta expression to determine repopulation potential of the in vitro cultured cells (see Figs 5 and 6 for representative phenotypic profiles). The percentage of cell recovery data is presented as the mean (SEM) of two to five independent experiments. (I.), Failure of cultured cells to repopulate FTOCs with mature T cells; (II.), FTOC repopulation by the indicated cultured cell populations; (III), FTOC repopulation by CD4\textsuperscript{lo} or pro-T cells cultured in cytokine combinations lacking IL-7; ND, not determined.
DISCUSSION

Commitment of the earliest thymic progenitors to the T-cell lineage is a poorly understood process. Although it has been shown that CD4<sup>+</sup> cells found in the thymus have the capacity to generate T, B, NK, and dendritic cells when transferred into recipient mice, it is not known if subsequent stages of thymic differentiation retain this property. Nor is it known which cytokines may be involved in the expansion of these early thymic progenitor populations. Until recently, studies addressing these issues have proven difficult to perform because of the complexity of intrathymic differentiation. The precise phenotypic delineation of the earliest steps of intrathymic maturation occurring before
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TCR β rearrangement and expression of CD4 and CD8 has allowed us to address the issues of T-lineage commitment and cytokine responses of early thymic progenitors.

Our results from intravenous injection of sorted CD4β and pro-T cells strongly suggest that, unlike CD4β cells, pro-T cells have lost the ability to generate B cells in recipient mice. To our knowledge, this is the first analysis of the repopulation potential of pro-T cells in vivo. This result was not entirely unexpected. We have previously reported that although pro-T cells have their TCR loci in germline configuration, they transcribe two TCR-β chain germline mRNAs of 1.0 and 1.6 kb. The presence of these transcripts indicates that the TCR-β chain gene locus chromatin has already opened in pro-T cells, as would be expected of cells committed to differentiation along the T-cell pathway. As pro-T cells downregulate c-kit and CD44, they undergo TCR-β and γ chain loci rearrangement to become pre-T cells (Fig 7). These characteristics strongly suggest that pro-T cells are the targets for the signals that induce TCR gene rearrangement. Furthermore, pro-T cells are capable of significant cytokine production (including IL-2 and interferon-γ) when activated with calcium ionophore, phorbol ester and IL-1. In fact, we recently cloned a new cytokine (designated lymphotactin) from an activated pro-T-cell cDNA library. Lymphotactin is an abundant T-cell-specific cytokine produced by various subsets of mature T cells. Taken together, these observations indicate that pro-T cells already exhibit a number of T-cell-like characteristics.

Several observations can be made from our data detailing the repopulation of scid recipients with CD4β or pro-T cells. It has been shown that CD4β thymocytes do not generate B cells when intrathymically transferred into recipient mice. B-cell generation was observed only when the cells were injected intravenously. After this protocol, we confirmed the ability of CD4β cells to generate B cells. We also report for the first time that pro-T cells have lost the ability to differentiate into B cells upon intravenous transfer into scid mice. However, the possibility exists that upon intravenous transfer, pro-T cells specifically home to the thymus, having lost the ability to home back to the BM. It is possible that this would extend to shared microenvironmental signals during development of both cell lineages. Alternatively, there may be separate phenotypically indistinguishable subpopulations within pro-T cells capable of differentiating into T cells and NK cells. Further studies are needed to address these possibilities.

These observations also imply that the signals which induce CD4β precursors to become T-cell lineage committed pro-T cells must exist in the thymic microenvironment. Cytokines are likely candidates to be involved in this process. Accordingly, we compared the responses to cytokines of CD4β and pro-T cells. The results of these experiments provide further evidence of the stem cell-like nature of CD4β thymic precursors. CD4β cells proliferated best in multiple cytokine combinations containing IL-1, IL-3, IL-6, IL-7, and SCF. This is reminiscent of responses of cells with similar phenotype and stem cell potential found in the BM. A cytokine of particular importance in this analysis is IL-7, because it is a likely candidate for being a lymphoid-promoting growth and differentiation factor. Overall, IL-7 was not
necessary for significant proliferation of CD4\textsuperscript{hi} progenitors in vitro or for retention of FTOC repopulation capacity; however, the cell yields both in vitro and in vivo (FTOC) were higher when IL-7 was included. In contrast with CD4\textsuperscript{hi} cells, pro-T cells were markedly more dependent upon the presence of IL-7. IL-7 alone or with SCF maintained the ability of pro-T cells to repopulate FTOCs. Furthermore, the addition of IL-7 to other cytokine combinations always improved the cell yields both in vivo and in vitro. Overall, the presence of IL-7 during culture of pro-T cells in vitro promoted the maintenance of their T-cell repertoire potential in FTOC (eg, pro-T cells cultured in IL-12 plus SCF or IL-3 plus SCF with or without IL-7; Fig 4). These observations confirm and extend our previous conclusions\textsuperscript{10,26} indicating that pro-T cells are likely a key target for IL-7 during early T-cell development. This model would be in agreement with a putative role for IL-7 in the maintenance of lymphoid-committed progenitors. Importantly, we also identified at least one cytokine combination that did not involve IL-7 (IL-1 plus IL-3 plus IL-6 plus SCF) that allowed pro-T cells to retain their FTOC repopulating ability. The latter result suggests that, whereas IL-7 may greatly augment the proliferative capacity of pro-T cells, it is not an absolute requirement during early T-cell development.

These data are consistent with earlier in vivo studies focusing on the potential role of IL-7 in T-cell development. Mice injected with anti-IL-7 antibody have greatly decreased thymic cellularities, but they still have relatively normal CD4/CD8 ratios and functional peripheral T cells.\textsuperscript{12} Likewise, injection of anti-IL-7R antibody capable of blocking the binding of IL-7 to its receptor resulted in decreased thymic cellularity, but not a complete blockade of T-cell development.\textsuperscript{13} These studies suggested an important but not absolute requirement for IL-7 in proliferation and expansion of early thymic populations. It is possible that cytokines other than IL-7 could drive early thymic progenitors to expand and differentiate at a low level. Our in vitro culture data supports this conclusion. Further support for this concept comes from the recent generation of several cytokine or cytokine receptor knockout mouse models by homologous recombination. An IL-7-deficient mouse has been generated; these mice, although having drastically decreased thymic cellularities, contain both normal CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells and mature CD4\textsuperscript{+} or CD8\textsuperscript{+} cells in the thymus and periphery.\textsuperscript{31}

An essentially identical phenotype was observed when the IL-2R\gamma chain was deleted.\textsuperscript{11} Although the IL-2R\gamma chain is used to form the IL-7R complex,\textsuperscript{23,25} it is also used to form other cytokine receptors,\textsuperscript{34-36} making it difficult to analyze the actual contribution of IL-7 to the phenotype of these mice. Likewise, an IL-7R\alpha chain knockout mouse has been recently described.\textsuperscript{37} These mice display variable effects on the actual contribution of IL-7 to the phenotype of these mice. Likewise, an IL-7R\alpha chain knockout mouse has been recently described.\textsuperscript{37} These mice display variable effects on thymocyte development, ranging from that seen in IL-7 and IL-2R\gamma knockout mice to a complete block at the CD4\textsuperscript{hi} stage. The recent discovery of a new IL-7-like cytokine termed "thymic stromal-derived lymphopoietin," whose receptor uses the IL-7R\alpha chain, is the most likely factor involved in the apparent differences between the IL-7 and IL-7Ra knockout mice.\textsuperscript{37,38}

Muegge et al\textsuperscript{39} observed that fetal thymocytes cultured with IL-7 in vitro rearrange their TCR-\beta chain locus, and suggested a role for IL-7 in the induction of TCR rearrangement. However, our data are more consistent with a role for IL-7 as a physiologic survival factor for these cells, which may have already received the signals to rearrange TCR-\beta chain genes in vivo. This is a much more likely explanation because these investigators used unseparated day 14 fetal thymocytes, some of which have probably already begun D-J rearrangements of the TCR-\beta chain locus, unlike purified pro-T cells. In fact, we have observed that pro-T cells cultured with IL-7 ± SCF do not rearrange their TCR-\beta chain locus (J. Kennedy and A. Zlotnik, unpublished observation, September 1993).

It is also worth noting that both CD4\textsuperscript{hi} and pro-T cells, when cultured in cytokine combinations differing only by the presence of IL-7, display increased TCR-\gamma T-cell repopulation of FTOCs when cultured with IL-7 before FTOC seeding (Figs 5 and 6). These data are in agreement with previous reports indicating that IL-7 appeared to promote the expansion of \gamma T cells.\textsuperscript{25,26}

Although these studies are the first to rigorously examine the cytokine responsiveness of intrathymic progenitors, others have examined the response of BM-derived thymic progenitors. Chervenak et al have reported the expansion of pre-T-cell activity when CD3\textsuperscript{–} Thy-1.2\textsuperscript{+} BM cells were cultured in IL-3 plus SCF.\textsuperscript{40} This pre-T activity was measured by thymic repopulation after intrathymic injection. However, expansion of other lineage progenitor cells was not examined; it is likely that IL-3 plus SCF maintained multilineage stem and/or progenitor cells that have been shown to be able to repopulate thymic lobes when intrathymically transferred.\textsuperscript{41,42} More recently, another study tried to compare BM and thymic CD4\textsuperscript{hi} progenitors.\textsuperscript{43} Unfortunately, the latter study is flawed because of the procedure used to purify CD4\textsuperscript{hi} thymic progenitors. Their protocol would result in major contamination with CD4\textsuperscript{hi} CD3\textsuperscript{−} CD8\textsuperscript{−} cell population, which represent a much more mature stage of direct precursors of DP thymocytes.\textsuperscript{44} This suspicion was confirmed by the authors themselves when they reported that only \approx 1% of their CD8\textsuperscript{hi} progenitors expressed c-kit, unlike CD4\textsuperscript{hi} progenitors, which are all c-kit\textsuperscript{+}.\textsuperscript{45} Thymic stem cells from fetal liver have been recently examined for their cytokine responsiveness. McKenna et al\textsuperscript{46} report that fetal liver cells cultured in IL-1beta, IL-3, IL-6, or leukemia inhibitory factor with or without SCF for up to 7 days retained their ability to repopulate thymic lobes upon intrathymic injection. Interestingly, culture in IL-7 plus SCF failed to maintain thymic repopulating ability. This is in agreement with our studies indicating that the T-cell repopulating potential of the earliest thymic CD4\textsuperscript{hi} progenitors is not maintained by IL-7 plus SCF. Most likely, progenitor cells from either BM or fetal liver, which seed the thymus, have a cytokine response pattern similar to that of CD4\textsuperscript{hi} thymic progenitors described here. A BM-derived cell population likely to seed the thymus has been recently described.\textsuperscript{46} It would be of interest to compare the cytokine responses of these BM-derived cells to thymic CD4\textsuperscript{hi} cells.

Although we have described various culture conditions capable of supporting survival and growth of CD4\textsuperscript{hi} cells,
we have not yet examined the effects of these conditions on lineage commitment. We have defined cytokine combinations with or without IL-7 that maintain T-cell progenitor activity as determined by FTOC repopulation. We are currently determining whether CD4^+ cells cultured under these conditions (eg, IL-3 plus IL-6 plus SCF and IL-1 plus IL-3 plus IL-6 plus SCF with or without IL-7) are still able to generate B cells in vivo or in vitro. One possibility is that exposure to IL-7 may induce commitment to the T-cell lineage. Preliminary studies have shown that CD4^+ cells cultured under these conditions for 6 to 8 days maintain their phenotype (CD44^+ CD25^- c-kit^-), suggesting that CD4^+ cells have not been induced to differentiate into pro-T cells (data not shown). We are also attempting to determine the clonal response of CD4^+ cells to these various culture conditions in an attempt to determine the potential heterogeneity of this population. Future experiments will aim to clarify these issues.

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T-cell lineage commitment and cytokine responses of thymic progenitors

TA Moore and A Zlotnik