T Lymphocyte Differentiation In Vitro from Adult Human Prethymic CD34⁺ Bone Marrow Cells

By Geir Erland Tjønnfjord, Ole Petter Veiby, Rita Steen, and Torstein Egeland

From the *Institute of Transplantation Immunology and Medical Department A, The National Hospital N-0027 Oslo; and SNycomed Bioreg N-0371 Oslo, Norway

Summary

Pluripotent lymphohematopoietic stem cells are probably confined to bone marrow cells expressing CD34 surface molecules. To investigate the capacity of adult human CD34⁺ bone marrow cells to differentiate along the T lymphoid lineage, we plated purified CD34⁺ cells from healthy adults in liquid culture on adherent thymic stromal cells prepared from HLA- or blood group-mismatched postnatal thymic tissue. We show that purified CD34⁺CD3⁻CD4⁻CD8⁻ bone marrow cells contained progenitors with the ability to differentiate into CD4⁺ and CD8⁺ T lymphocytes expressing surface (s)CD3 and T cell receptor α/β in vitro. These progenitors were found in the CD34⁺CD2⁺sCD3⁻CD4⁻CD8⁻, CD34⁺CD7⁺sCD3⁻CD4⁻CD8⁻, and CD34⁺CD2⁺CD7⁺sCD3⁻CD4⁻CD8⁻, as well as in the CD34⁺CD2⁺sCD3⁻CD4⁻CD8⁻, CD34⁺CD7⁺sCD3⁻CD4⁻CD8⁻, and CD34⁺CD2⁺CD7⁺sCD3⁻CD4⁻CD8⁻ subsets, indicating that T lymphocyte progenitors sensitive to signals mediated by thymic stroma in vitro are not restricted to CD34⁺ cells already coexpressing early T lymphocyte-associated markers. Finally, we show that T lymphopoiesis was enhanced by c-kit ligand.

Materials and Methods

Cell Donors and Cell Preparation. Bone marrow samples were obtained by aspiration from consenting healthy adults. Mononuclear cells were prepared by density gradient centrifugation (1.077 g/ml, Lymphoprep; Nycomed Pharma, Oslo, Norway), washed twice, and resuspended in IMDM (Flow Laboratories, Irvine, Scotland) with 10% (vol/vol) FCS (Gibco, Paisley, Scotland), glutamine (2 mmol/liter; Gibco), penicillin (60 μg/ml), and streptomycin (100 μg/ml), referred to as complete medium (CM). CD34⁺ cells were isolated with Dynabeads M-450 coupled with a mAb against CD34 (BI-3CS) (Dynal, Oslo, Norway) as previously described (18, 19). Contaminating mature B lymphocytes (CD34⁻ CD19⁺ CD20⁺ but not mature T lymphocytes (CD34⁻ sCD3⁺CD4⁻ or CD34⁻ sCD3⁺CD8⁻) can be demonstrated among isolated CD34⁺ bone marrow cells (19). To increase the purity of the isolated CD34⁺ cells to >95% to minimize the possibility of outgrowth of contaminating T lymphocytes to account for the appearance of CD4⁺ and CD8⁺ cells after culture, immunomagnetic depletion with Dynabeads M-450 coupled with mAbs against CD4, CD8, and CD19 (Dynal) was performed (20).

Abbreviations used in this paper: CM, complete medium; hu, human; rm, recombinant murine; s, surface.
Thymic tissue specimens were obtained from two children <6 mo of age undergoing median sternotomy and corrective cardiovascular surgery (kindly provided by H. Lindberg, Surgical Department A, The National Hospital, Oslo, Norway). One donor was HLA-A2*, ARh*, and the other was HLA-A2*, ORh*. The thymic tissue was transported to the laboratory in HBSS (Gibco) with penicillin and streptomycin. The thymic tissue was minced into pieces, and single cells were eluted in HBSS with collagenase (0.5 mg/ml) (Sigma Chemical Co., St. Louis, MO) and DNase I (1 μg/ml) (Sigma Chemical Co.) at 37°C under continuous shaking for 1.5 h. After six to eight repeated cycles of elution the thymic tissue was totally disintegrated. Single cell suspensions from the last three to four eluates were washed twice and resuspended in RPMI 1640 (Gibco) with penicillin and streptomycin before cryopreservation.

**Antibodies.** The following mAbs were used for flow cytometry: FITC-labeled anti-Leu-5b (CD2), anti-Leu-4 (CD3), anti-Leu-9 (CD7), and anti-HPCA-2 (8G12, CD34), and PE-labeled anti-HPCA-2, anti-Leu-M7 (CD13), Simultest CD4/CD8 (anti-Leu-3a FITC + anti-Leu-2a PE), Simultest LeukoGATE (anti-HL-A FITC + anti-Leu-M3 PE), and Simultest control (IgG1 FITC + IgG2a PE) (Becton Dickinson Immunocytometry Systems [BDIS], Mountain View, CA). PE-labeled anti-CD2, and anti-CD34 (IC3H) were purchased from Caltag Laboratories, Inc. (South San Francisco, CA), anti-TCR α/β (BMA031) from Behringwerke AG (Marburg, Germany), and anti-CD3 (TOL1) from Novo Research Institute (Copenhagen, Denmark). Anti-HLA-A2 (ITI-8G12) was developed in our own laboratory by G. Gaudernack. Anti-A (blood group A) was purchased from Lorne Laboratories Ltd. (England). FITC-labeled anti-Leu-5b and anti-Leu-9 and PE-labeled anti-HPCA-2 from BDIS were used for cell sorting by flow cytometry. The following mAbs were used for immunofluorescence: TE-7, reactive with non-keratinized, mesodermal-derived thymic fibrous stroma, and TE-15, reactive with Hassell's bodies (a kind gift from K. H. Singer, Duke University School of Medicine, Durham, NC) (21). Fluoresceinated goat anti-mouse isotype- and subclass-specific polyclonal antibodies were purchased from Southern Biotechnology Associates, Inc. (Birmingham, AL).

**Culture Assays.** After thawing, eluted thymic cells were washed twice, resuspended in CM, and plated, with 2 × 10⁴ cells/well in 24-well tissue culture plates (Costar, Cambridge, MA). The cells were cultured in a humidified atmosphere of 5% CO₂ in air at 37°C for 6–8 d to establish a confluent layer of adherent cells. Supernatants were collected, filtered, and frozen for later use. Nonadherent cells were removed from the wells by repeated washing.

CD³⁴⁺ cells were plated on the adherent thymic stromal cells, with 10⁴ cells/well in 1 ml CM with 10% (vol/vol) thymic stromal cell supernatant, and with four to five wells per series. The cells were cultured in the absence or presence of recombinant murine c-kit ligand (2 U/ml) (kindly provided by S. C. Clark, Genetics Institutes, Cambridge, MA). Media were half-changed with fresh supernatant media twice weekly. c-kit ligand was added according to protocol. Nonadherent cells were harvested after 14 and 28 d, enumerated in a Bürker chamber, and analyzed by flow cytometry.

To more closely identify prethymic T lymphocyte progenitors, flow cytometry—sorted CD³⁴⁺CD²⁻, CD³⁴⁺CD⁷⁻, CD³⁴⁺CD²⁺CD⁷⁻, CD³⁴⁺CD²⁻, and CD³⁴⁺CD²⁻CD⁷⁻ cells (2,500–5,000 cells/well) were cultured on thymic stromal cells in the presence of c-kit ligand, with two to three wells per series. Nonadherent cells were harvested, enumerated, and phenotyped by flow cytometry after 21 d.

Bone marrow cells from the nonCD³⁴⁺ fraction (i.e., bone marrow cells not rosetting with BI-3C5-coated Dynabeads), CD³⁴⁺ T lymphocytes, or CD³⁴⁺ T lymphocytes (10⁵ cells/well) were also cultured under the same conditions. To study the necessity of thymic stroma to support T lymphopoiesis, adherent cells prepared from MRC-5 (a fibroblast cell line), Hep-2 (a hepatoma cell line), and allogeneic peripheral blood mononuclear, and nonadherent allogeneic Epstein-Barr blasts were substituted for thymic stromal cells. Furthermore, CD³⁴⁺ cells were cultured in 96-well U-bottomed tissue culture plates (Costar), with 1,000 cells/well in 200 μl CM, in the presence of irradiated allogeneic PBMC, PHA (1 μg/ml) (Welcome, Dartford, England), and rIL-2 (10 U/ml) (Genzyme, Cambridge, MA). The cultures were restimulated with irradiated allogeneic PBMC, PHA, and rIL-2 after 7 d and cultured for an additional 8 d. The cells were cultured for the last 18 h in the presence of 10 μM ³H-thymidine (2 Ci/mmol), harvested on filter mats, and counted using a scintillation counter (LKU Instruments, Turku, Finland). Non-CD³⁴⁺ bone marrow cells and PBMC served as positive controls, and irradiated PBMC served as negative control.

**Flow Cytometry and Cell Sorting.** Cell staining was performed according to the manufacturer's instructions and the cells were resuspended in HBSS with 1% paraformaldehyde. Flow cytometry analyses were performed on a FACScan® with the FACScan® Research Software (BDIS). 50,000 events were acquired and stored in list mode files.

Cell sorting was performed on an Epics Elite (Coulter Electronics, Hialeah, FL). Cells were sorted at a rate of 800–1,000 cells/s. A sort gate within a dual parameter cytogram of forward light scatter against 90° side scatter was drawn. A second amorphous gate was drawn on the two-color cytograms shown (Fig. 6). Sort equations were set to positively sort cells satisfying both gates.

**Immunofluorescence Microscopy.** To investigate the identity of the adherent cell layer, the adherent cells were fixed in ethanol, incubated with the mAbs for 30 min at room temperature, washed twice, and incubated with a goat anti-mouse fluoresceinated antibody.

**Limiting Dilution Assays.** To examine the T lymphocyte precursor frequency of CD³⁴⁺ cells expressing the CD2 and CD7 markers and CD³⁴⁺ cells devoid of these markers, we performed experiments in limiting dilution assays. Allogeneic adherent thymic stromal cells were established in 96-well flat-bottomed tissue culture plates (Costar) as described above. CD³⁴⁺CD²⁺, CD³⁴⁺CD⁷⁺, CD³⁴⁺CD²⁻, and CD³⁴⁺CD⁷⁻ cells were plated with CM supplemented with c-kit ligand (2 U/ml) and 10% (vol/vol) thymic stromal cell supernatant at 15–240 cells/well with 20 replicates per concentration. Media were doubled at day 5 and then half changed with fresh c-kit ligand and supernatant media twice weekly until week 3. Wells were harvested and the cells phenotyped by an immunomagnetic method (22). Dynabeads M-450 (Dynal) coupled with a mAb against CD3 (38,1; kindly provided by J. A. Hansen, Fred Hutchinson's Cancer Research Center, Seattle, WA) were selected for detection of T lymphocytes in the cultures. Wells were determined positive for T lymphocytes when >5% of the cells made rosettes with more than five beads and the rosetting cells were small lymphoid cells. Based on Tawell's equation, the frequency of T lymphocyte progenitors was calculated (23).

**Statistics.** The growth abilities of CD³⁴⁺ cells in the absence and presence of c-kit ligand were compared using the Student's t test for paired observations.

**Results**

**Characterization of Isolated CD³⁴⁺ Cells.** The immunomagnetically isolated cells contained >90% CD³⁴⁺ cells in
all experiments and >95% in most experiments. The flow cytometry-sorted cells contained >98% CD34+ cells in all experiments. Cells expressing CD3 surface molecules could not be detected by flow cytometry (Fig. 1), and neither could cells expressing CD4 or CD8 molecules (not shown).

Preparation of an Adherent Layer of Thymic Stromal Cells. The eluted cells were enriched for thymic stromal cells as judged by the presence of thymus rosette–forming cells. During culture of the eluted cells for 6–8 d, they developed a confluent, adherent cell layer. The adherent cell layer contained ~20% cells of epitheloid morphology reactive with the TE-15 mAb and ~80% cells of fibroblastoid morphology reactive with the TE-7 mAb.

Growth Ability. The growth ability of CD34+ cells on adherent thymic stromal cells in the absence and presence of rmc-kit ligand are shown in Fig. 2. Cell multiplication was significantly enhanced by rmc-kit ligand: from an 8- to 42-fold increase in cell number (p <0.03) after 28 d in culture. The interexperimental variations were large. A decrease in number of nonadherent cells was observed when non-CD34+ bone marrow cells, CD4+ T lymphocytes, or CD8+ T lymphocytes were cultured for 28 d on thymic stromal cells. This was also the case when CD34+ cells were cultured on adherent cells prepared from the MRC-5 cell line, the Hep-2 cell line, or allogeneic peripheral blood monocytes, or together with allogeneic EB blasts. Opposed to non-CD34+ bone marrow cells and PBMC, no proliferative response was demonstrated when CD34+ cells were cultured in the presence of PHA, rIL-2, and irradiated allogeneic PBMC (data not shown).

Characterization of Cultured Cells. The progeny of the purified CD34+ sCD3 - CD4- CD8 - cells were analyzed in flow cytometry for cell size, granularity, and expression of
CD34- and T lymphocyte-associated surface molecules after 14 and 28 d in culture. Fig. 3 shows the results of a representative analysis of size and granularity of the cells. A population of cells (R1) is discernible with relatively low light scatter signals characteristic of agranular lymphoid cells, and two larger populations are discernible (R2 and R3) with intermediate and high light scatter signals compatible with cells of intermediate size with no or a few cytoplasmic granules and large cells heavily granulated, respectively.

Table 1 shows the relative number of cells expressing CD34- and T lymphocyte-associated surface molecules of the progeny of the CD34+sCD3-CD4-CD8- cells after 28 d in culture. Apart from a slightly relative number of CD34+ cells and CD4+CD8+ cells after 14 d than after 28 d, there were no differences between the relative numbers of cells expressing the different surface molecules analyzed after 14 and 28 d. CD34 expression was mainly confined to cells in the R2 fraction (Fig. 3 and Table 1). On the other hand, cells expressing the CD2, CD7, CD3, CD4, and CD8 molecules were mainly confined to the R1 fraction (Fig. 4 and Table 1). The majority of sCD3+ cells in R1 fraction coexpressed TCR α/β (Fig. 4). The low numbers of CD4+CD8+ cells cannot account for the high numbers of sCD3+ TCR α/β+ cells demonstrated after culture (Fig. 5, B, C, and E). Although not demonstrated in a triple-color cytoagram, most if not all CD4+ and CD8+ cells had to be

### Table 1. Relative Number of Cells Expressing Different Cell Surface Markers after 28 d in Culture

| Exp. | Marker | CD34-pos. cells | CD34+ cells depleted for CD4,8,19 | c-kit ligand added to CD34+ cells |
|------|--------|-----------------|-----------------------------------|---------------------------------|
|      |        | R1* | R2* | R1 | R2 | R1 | R2 |
| 1    | CD34   | 1   | 3   | 1  | 4  | 1  | 4  |
| 2    | CD34   | 1   | 3   | 1  | 2  | 1  | 2  |
| 3    | CD34   | 1   | 7   | nt | nt | 1  | 9  |
| 4    | CD34   | nt  | nt  | 2  | 5  | nt | nt |
| 1    | CD2    | 21  | 2   | 12 | 1  | 6  | 1  |
| 2    | CD2    | 56  | 1   | 54 | 1  | 48 | 4  |
| 3    | CD2    | 22  | 4   | nt | nt | 18 | 4  |
| 4    | CD2    | nt  | nt  | 38 | 3  | nt | nt |
| 1    | CD7    | 15  | 2   | 8  | 2  | 6  | 1  |
| 2    | CD7    | 61  | 2   | 40 | 1  | 62 | 4  |
| 3    | CD7    | 20  | 5   | nt | nt | 17 | 5  |
| 4    | CD7    | nt  | nt  | 37 | 3  | nt | nt |
| 1    | CD3    | 20  | 3   | 12 | 2  | 10 | 2  |
| 2    | CD3    | 61  | 1   | 52 | 3  | 56 | 4  |
| 3    | CD3    | 29  | 5   | nt | nt | 26 | 6  |
| 1    | CD4    | 9   | 1   | 9  | 1  | 9  | <1 |
| 2    | CD4    | 16  | 1   | 13 | 1  | 20 | 4  |
| 3    | CD4    | 4   | 1   | nt | nt | 10 | 1  |
| 4    | CD4    | nt  | nt  | 7  | <1 | nt | nt |
| 1    | CD8    | 13  | 2   | 9  | 1  | 2  | <1 |
| 2    | CD8    | 22  | 1   | 24 | 1  | 20 | 1  |
| 3    | CD8    | 15  | 2   | nt | nt | 10 | 2  |
| 4    | CD8    | nt  | nt  | 33 | 3  | nt | nt |

* R1 and R2 refer to regions defined in Fig. 3.
† nt, not tested.

1534 T Lymphocyte Differentiation from CD34+ Bone Marrow Cells
Figure 4. Phenotypic characterization of cultured cells. Representative two-color cyto-
grams with a negative control (I). Cells expressing the T lymphocyte-associated CD7, CD2, and CD3 surfaces molecules were mainly located in the lymphocyte gate, R1, whereas cells expressing CD34 surface molecules were confined to R2 (A-F). A major part of the CD3+ cells expressed TCK α/β (G). All cells in the lymphocyte gate expressed HLA-A2+, i.e., they are of bone marrow donor HLA type (bone marrow donor HLA-A2+ and thymus donor HLA-A2-) (H).

sCD3+TCR α/β+. A certain number of cells coexpressed the CD4 and CD8 molecules. Data illustrated in Fig. 5 suggest that the relative number of CD4+CD8+ cells decreased from days 14 to 28. To exclude that the few CD4+CD8+ cells observed were actually doublets of single-positive cells, light scatter properties (i.e., size) of the double-positive cells were compared with single-positive cells. CD4+CD8+ cells were exclusively confined to R1, and no differences in size between single- and double-positive cells were demonstrated, excluding the CD4+CD8+ cells to be doublets. Furthermore, dots compatible with CD4+CD4+ or CD8+CD8+ doublets could not be demonstrated. A major part of the cells in R2 and R3 fractions could be shown to belong to the myeloid lineage by detection of cells expressing CD13 and CD14 molecules (data not shown).

There were no differences in distribution of T lymphocyte-associated markers whether the progeny were derived from CD34+ cells, CD34+ cells after CD19, CD4, and CD8 depletion, or CD34+ cells cultured in the absence or presence of rmc-kit ligand.

The T lymphocytes emerging after culture could be demonstrated to be of bone marrow donor origin by coexpression of bone marrow donor-specific HLA or blood group molecules and sCD3 (Fig. 4).

T Lymphocyte Progenitors. A minor proportion of CD34+sCD3-CD4-CD8- cells was found to coexpress the CD2 and/or CD7 molecule (1-2%) (Fig. 6). When cultured in bulk, these subsets gave rise to lymphoid cells of T lymphocyte lineage just like unfractionated CD34+ cells (Figs. 4 and 5). To examine whether T lymphocyte precursors were exclusively confined to the CD34+CD2+, CD34+CD7+, and CD34+CD2+CD7+ subsets, CD34+ CD2-, CD34+CD7+, and CD34+CD2-CD7- cells were cultured under the same conditions. The results of these experiments clearly demonstrate that even CD34+ cells devoid of the T lymphoid markers CD2 and/or CD7 contained T lymphocyte progenitors with the ability to proliferate and differentiate into T lymphocytes when cultured on thymic stroma in vitro. However, apart from giving rise to T lymphocyte differentiation, both CD34+CD2+ and CD34+ CD7+ cells (and the other subsets studied) also contained progenitors able to differentiate into the myeloid lineage, as ascertained by detection of cells expressing CD13 and CD14 after culture (data not shown).

To examine whether any of the subsets of CD34+ cells studied in bulk cultures were enriched for T lymphocyte precursors, limiting dilution analyses were performed. The precursor frequency of CD34+CD2+ cells was found to be
Figure 5. Phenotypic characterization of cultured CD34+ cells and subsets of CD34+ cells. (B and C) Representative two-color cyto-
grams of cultured CD34+ cells. At day 14 (B) the presence of CD4+CD8+ cells are clearly detectable, whereas at day 28 (C) double-
positive cells are hardly detectable. (D–F) Representative two-color cytograms of cultured CD34+CD7+ cells. Similar cytograms were ob-
tained from cultured CD34+CD2+, CD34+CD2+CD7+, CD34+CD7+, CD34+CD2+, and CD34+CD2−CD7− cells (not shown). (A) Negative control.

Figure 6. Characteristics of CD34+CD2+ and CD34+CD7+ cells. Representative two-
color cytograms illustrating the presence of CD34+ cells coex-
pressing CD2 or CD7 (1–2%). The sort gates are also indicated.

Discussion

T lymphopoiesis from human fetal hematopoietic stem cells has been demonstrated in vivo in the Thy SCID-hu mouse model (15–17). Furthermore, T lymphopoiesis from fetal human CD10+ bone marrow cells and from mouse hematopoietic stem cells has been achieved in vitro (24, 25). T lymphopoiesis from adult human prethymic CD34+ bone marrow cells has been demonstrated neither in vivo nor in vitro. The present study demonstrates that T lymphopoiesis from adult human CD34+ bone marrow cells can be accomplished in vitro. After culture of CD34+sCD3−CD4−CD8− cells for 14 and 28 d on allogeneic thymic stromal cells, cells of bone marrow donor origin with characteristics of lymphoid cells expressing the phenotype of mature T lymphocytes were demonstrated.

It was necessary to exclude the possibility that the purified CD34+ cells contained mature T lymphocytes, the expansion of which could account for the T lymphocytes demonstrated. We are confident that this was not the case. First, no mature T lymphocytes (sCD3+ cells) were detected by flow cytometric analyses of purified CD34+ cells. Contaminating cells were almost exclusively small agranular cells with the phenotype of mature B lymphocytes (CD34−CD19+CD37+) (19, 26). Second, culture of non-CD34+
bone marrow cells, which contained large numbers of mature T lymphocytes, did not result in any cell multiplication, nor did cultures of CD4+ nor CD8+ T lymphocytes. Third, CD34+ cells did not proliferate after stimulation with irradiated allogeneic PBMC, PHA, and rIL-2. In addition, we have previously demonstrated lack of generation of cells expressing T lymphocyte-restricted markers after stimulation of purified CD34+ cells with PHA and rIL-2 (27). This is in accordance with the finding that CD7+CD2-CD3- human thymic and bone marrow cells do not respond to PHA and/or rIL-2 (28, 29). Fourth, after depletion with mAbs against CD4CD8 and CD19CD4+ cells still gave rise to T lymphocytes. Fifth, the finding of CD4+CD8+ cells cannot be explained by contamination of mature T lymphocytes among the purified CD34+ cells. Last, limiting dilution analyses demonstrated T lymphocyte precursor frequency of 1:8–18 in sorted CD34+ cells. Thus, outgrowth of rare contaminating mature T lymphocytes, <1% according to flow cytometry, cannot account for the appearance of CD4+ and CD8+ cells after culture.

We have presented evidence that when cultured on thymic stroma, CD34+ human bone marrow cells proliferate and differentiate along the T lymphoid lineage. It might be argued that the single-positive CD4+ and CD8+ cells observed after culture could correspond to immature single-positive (i.e., preCD4+CD8+) cells and not mature CD4+ and CD8+ cells. In the presence of IL-7, highly purified human triple-negative thymocytes can proliferate and differentiate to express CD4 and/or CD8(α+/β-). These cells, which remain CD3-, are thought to precede the common cortical double-positive thymocytes that are CD3lowCD4+CD8α+/β-. In our study, the demonstration of CD3lowTCRα/β+ cells cannot be accounted for by the low number of CD4+CD8+ cells detected. Accordingly, most, if not all, CD4+ and CD8+ cells have to be CD3+ TCR α/β+. Based on this, we are entitled to state that CD34+ adult human bone marrow cells when cultured on thymic stroma possess the ability to proliferate and differentiate into mature CD4+CD3+ TCR α/β+ and CD8+CD3+ TCR α/β+ T lymphocytes. The functional ability of these CD4+ and CD8+ T lymphocytes will be addressed in ongoing studies.

It is generally accepted that development of T lymphocytes from early thymocytes (sCD3-CD4-CD8-) involves a stage of CD4+CD8- cells preceding the appearance of sCD3-CD4- and sCD3-CD8- T lymphocytes. CD4+CD8- cells were present at day 14, but they were hardly detectable at day 28. This suggests that maturation to sCD3+CD4+ and sCD3+CD8+ lymphocytes took place through an intermediate stage of CD4+CD8- cells in this in vitro assay as well.

We found that a large majority of T lymphocytes developing when adult human CD34+ bone marrow cells were cultured on thymic stroma expressed TCR α/β. This is in contrast to previous reports that postnatal sCD3-CD4-CD8- thymic T lymphocyte precursors develop nearly exclusively into TCR γ/δ+ cells in cultures without feeder cells (30). However, fetal sCD3-CD4-CD8- thymic T lymphocyte precursors develop into TCR α/β+ cells (31, 32). Human T lymphocytes found in the peripheral blood of SCID-hu (fetal thymus and liver) mice express TCR α/β (33). Finally, hematopoietic stem cells in the G0 phase from adult mice differentiate into T lymphocytes expressing TCR β chains in an in vitro organ culture assay with fetal thymic tissue (25). Taken together with the present results, this suggests that thymic stroma is necessary for the development of T lymphocytes expressing TCR α/β from postnatal progenitors. This notion is further supported by another report showing that thymic stromal cells are required for the development of both α/β and γ/δ T lymphocytes from postnatal CD7+CD45+CD1-CD2-CD3-CD4-CD8- early thymocytes (34). In previous studies where T lymphopoiesis from hematopoietic stem cells was achieved, the three-dimensional structure of the thymic microenvironment was maintained (16, 17, 25). Our studies confirm the necessity of thymic stroma, but the maintenance of the three-dimensional structure is not always necessary. In the absence of thymic stroma we were not able to obtain T lymphocyte differentiation when CD34+ cells were cultured in the presence of several combinations of growth factors (rIL-1, rIL-2, rIL-3, rIL-7, GM-CSF, and rmc-kit ligand) or supernatant from thymic stromal cells (27, Steen, R., L. Mørkrid, G. E. Tjønnfjord, and T. Egeland, manuscript submitted for publication, and unpublished observations).

Human CD34+ cells are phenotypically heterogeneous with subsets coexpressing lineage-specific markers. CD7 and CD2 are believed to be expressed by prethymic pre-T cells (7, 11, 12, 31, 35, 36). Therefore, the ability of CD34+CD2+, CD34+CD7+, CD34+CD2+CD7+, CD34+CD2-, CD34+CD7-, and CD34+CD2-CD7+ bone marrow cells to differentiate into T lymphocytes was studied in the same assay. Progenitors able to support T lymphopoiesis in vitro were found in all of these subsets. Furthermore, limiting dilution analyses did not demonstrate enrichment of T lymphocyte precursors in any of these subsets. The inability to demonstrate an enrichment for T lymphocyte precursors among different subsets of CD34+ cells may reflect thymus-dependent negative selection and apoptosis. This may be the rate-limiting step even in this in vitro assay and may preclude the finding of a possible difference in T lymphocyte precursor frequency of the subsets studied in vivo. Surface molecules like leukocyte endothelial cell adhesion molecule 1 (LECAM-1) known to be expressed by CD34+CD2+CD7+ bone marrow cells, may be involved in homing of these cells to thymus (12). The concept of homing is bypassed in this in vitro assay, and this as well as other culture-associated phenomena may account for our inability to demonstrate a difference in precursor frequency between different subsets of CD34+ cells. CD34+CD7+sCD3-CD4-CD8- and CD34+CD2+sCD3-CD4-CD8- cells as well as the other subsets studied were multipotent since they were capable of giving rise to myeloid progeny. This corresponds with the demonstration of differentiation into nonlymphoid lineages of human thymic
CD7+ sCD3+CD4+CD8− T lymphocyte precursors by IL-3, Epo, G-CSF, M-CSF, GM-CSF, and factors produced by thymic epithelial (TE) cells (37).

Both purified native and r-rmc-kit ligand show synergistic actions with other factors on whole bone marrow and isolated CD34+ human cells such as IL-3, IL-1, IL-6, G-CSF, GM-CSF, and Epo in various assays (38-40, and Steen, R., L. Mørkrid, G. E. Tjønnfjord, and T. Egeland, manuscript submitted for publication). The results presented here show that rrmc-kit ligand strongly potentiated the cell multiplication in this assay without changing the phenotypic profile of the progeny. This supports the view that the synergistic effect of rrmc-kit ligand on growth factors also includes growth factors necessary for T lymphopoiesis.

In the present study we show that purified CD34+ sCD3+CD4+CD8− bone marrow cells from healthy adults contained progenitors with the ability to proliferate and differentiate into CD3+CD4+ or CD3+CD8+ T lymphocytes expressing TCR α/β in vitro. The presence of thymic stromal cells seemed to be a prerequisite for T lymphopoiesis to be accomplished from bone marrow progenitors. T lymphocyte progenitors were found both among CD34+ cells expressing CD2 or CD7 surface markers and CD34+ cells devoid of these markers. Furthermore, c-kit ligand enhanced T lymphopoiesis.

References
1. Civin, C.I., M.L. Banquerigo, L.C. Strauss, and M.R. Loken. 1987. Antigenic analysis of hematopoiesis. VI. Flow cytometric characterization of My-10-positive progenitor cells in normal human bone marrow. Exp. Hematol. 15:10.
2. Civin, C.I., L.C. Strauss, C. Browall, M.J. Fackler, J.F. Schwartz, and J.H. Shaper. 1984. Antigenic analysis of hematopoiesis. III. A hematopoietic progenitor cell surface antigen defined by a monoclonal antibody raised against KG-1a cells. J. Immunol. 133:157.
3. Andrews, R.G., J.W. Singer, and I.D. Bernstein. 1986. Monoclonal antibody 12-8 recognizes a 115-kd molecule present on both unipotent and multipotent colony-forming cells and their precursors. Blood. 67:842.
4. Ryan, D., S. Kossover, S. Mitchell, C. Frantz, L. Hennessy, and H. Cohen. 1986. Subpopulations of common acute lymphoblastic leukemia antigen-positive lymphoid cells in normal bone marrow identified by hematopoietic differentiation antigens. Blood. 68:417.
5. Loken, M.R., V.O. Shah, K.L. Dattilio, and C.I. Civin. 1987. Flow cytometric analysis of human bone marrow. II. Normal B-lymphocyte development. Blood. 70:1316.
6. Lu, L., D. Walker, H.E. Brockmeyer, R. Hoffman, W. Hu, and E. Walker. 1987. Characterization of adult human marrow hematopoietic progenitors highly enriched by two-color cell sorting with My-10 and major histocompatibility class II monoclonal antibodies. J. Immunol. 139:1823.
7. Watt, S.M., K. Karhi, K. Gatter, A.J.W. Furley, F.E. Katz, L.E. Healy, L.J. Altass, N.J. Bradley, D.R. Sutherland, R. Levinsky, and M.F. Greaves. 1987. Distribution and epitope analysis of the cell membrane glycoprotein (HPCA-1) associated with human hemopoietic progenitor cells. Leukemia (Baltimore). 1:417.
8. Berenson, R.J., R.G. Andrews, W.I. Bensinger, D. Kalamasz, G. Knitter, C.D. Buckner, and I.D. Bernstein. 1988. Antigen CD34+ marrow cells engraft lethally irradiated baboons. J. Clin. Invest. 81:951.
9. Wagemaker, G., F.C.J.M. van Giles, J.A.K. Bart-Baumeister, J.J. Wielenga, and R.J. Levinsky. 1990. Sustained engraftment of allogeneic CD34 positive hemopoietic stem cells in rhesus monkeys. Exp. Hematol. 18:704. (Abstr.)
10. Berenson, R.J., W.I. Bensinger, R.S. Hill, R.G. Andrews, J. Garcia-Lopez, D.F. Kalamasz, B.J. Still, G. Spitzer, C.D. Buckner, I.D. Bernstein, and E.D. Thomas. 1991. Engraftment after infusion of CD34+ marrow cells in patients with breast cancer or neuroblastoma. Blood. 77:1717.
11. Gore, S.D., M.B. Kastan, and C.I. Civin. 1991. Normal human bone marrow precursors that express terminal deoxynucleotidyl transferase include T-cell precursors and possible lymphoid stem cells. Blood. 77:1316.
cells. Blood. 77:1681.
12. Terstappen, L.W.M.M., S. Huang, and L.J. Picker. 1992. Flow cytometric assessment of human T-cell differentiation in thymus and bone marrow. Blood. 79:666.
13. Ezine, S., I.L. Weissman, and R.V. Rouse. 1984. Bone marrow cells give rise to distinct clones within the thymus. Nature (Lond.). 309:629.
14. Goldschneider, I., K.L. Komschlies, and D.L. Greiner. 1986. Studies of thymocytes in rats and mice. I. Kinetics of appearance of thymocytes using a direct intrathymic adoptive transfer assay for thymocyte precursors. J. Exp. Med. 163:1.
15. McCune, J.M., R. Namikawa, H. Kaneshima, L.D. Scultz, M. Lieberman, and I.L. Weissman. 1988. The SCID-hu mouse: murine model for the analysis of human hematolymphoid differentiation and function. Science (Wash. DC). 241:1632.
16. Namikawa, R., K.N. Weilbaecher, H. Kaneshima, E.J. Yee, and J.M. McCune. 1990. Long-term human hematopoiesis in the SCID-hu mouse. J. Exp. Med. 172:1055.
17. Pélault, B., I.L. Weissman, C. Baum, J.M. McCune, and A. Tsukamato. 1991. Lymphoid reconstitution of the human fetal thymus in SCID-hu mice with CD34+ precursor cells. J. Exp. Med. 174:1283.
18. Egeland, T. 1991. Stem cell isolation and purification. In Magnetic Separation Techniques Applied to Cellular and Molecular Biology. J.T. Kemshead, editor. Wordsmiths' Conference Publications, Sommerset, UK. 135–142.
19. Smeland, E.B., S. Funderud, G. Kvalheim, G. Gaudernack, A.-M. Rasmussen, L. Rusten, M.Y. Wang, R.W. Tindle, H.K. Blomhoff, and T. Egeland. 1992. Isolation and characterization of human hematopoietic progenitor cells; an effective method for positive selection of CD34+ cells. Leukemia (Baltimore). 6:845.
20. Egeland, T., D. Albrechtsen, P.J. Martin, and J.A. Hansen. 1990. Immunomagnetic depletion of CD6+ cells from bone marrow and peripheral blood. Bone Marrow Transplant. 5:193.
21. Singer, K.H., E.A. Harden, A.L. Robertson, D.F. Lobach, and B.R. Haynes. 1985. In vitro growth and phenotypic characterization of mesodermal-derived and epithelial components of normal and abnormal human thymus. Hum. Immunol. 13:161.
22. Gaudernack, G., and K.E.A. Lundin. 1989. Rapid immunomagnetic phenotyping of cells. J. Immunogenet. (Oxford). 16:169.
23. Tarwell, C. 1981. Limiting dilution assays for the determination of immunocompetent cell frequencies. I. Data analysis. J. Immunol. 126:1614.
24. Hokland, P., M. Hokland, J. Daley, and J. Ritz. 1987. Identification and cloning of a prethymic precursor T lymphocyte from a population of common acute lymphoblastic leukemia antigen (CALLA)-positive fetal bone marrow cells. J. Exp. Med. 165:1749.
25. Toki, J., T. Kumamoto, H. Ogata, M. Kawamura, M. Fukamoto, Cherry, Y. Yamamoto, S. Than, S. Inaba, Y. Himeno, H. Imura, R.A. Good, and S. Ikehara. 1991. Analyses of T-cell differentiation from hemopoietic stem cells in the G0 phase by an in vitro method. Proc. Natl. Acad. Sci. USA. 88:7548.
26. Egeland, T., G. Tjønnfjord, R. Steen, G. Gaudernack, and E. Thorsby. 1993. Positive selection of bone marrow-derived CD34-positive cells for possible stem cell transplantation. Transplant. Proc. 25:1261.
27. Egeland, T., R. Steen, H. Quarsten, G. Gaudernack, Y.-C. Yang, and E. Thorsby. 1991. Myeloid differentiation of purified CD34+ cells after stimulation with recombinant human granulocyte-monocyte colony-stimulating factor (CSF), granulocyte-CSF, monocyte-CSF and interleukin-3. Blood. 78:3192.
28. Mossalyi, M.D., A.H. Dalloul, J.M. Bertho, J.C. Lecon, P. Goube de Laforest, and P. Debré. 1990. In vitro differentiation of purified human thymic and bone marrow CD7+CD2− T-cell precursors. Exp. Hematol. 18:326.
29. Hori, T., J. Cupp, N. Wrighton, F. Lee, and H. Spits. 1991. Identification of a novel human thymocyte subset with a phenotype of CD3+CD4+CD8α+β−1. Possible progeny of the CD3+CD4+CD8+ subset. J. Immunol. 146:4078.
30. Denning, S.M., J. Kurzberg, D.S. Leslie, and B.F. Haynes. 1989. Human postnatal CD4+CD8− thymic T cell precursors differentiate in vitro into T cell receptor β-bearing cells. J. Immunol. 142:2988.
31. Haynes, B.F., M.E. Martin, H.H. Kay, and J. Kurtzberg. 1988. Early events in human T cell ontogeny. Phenotypic characterization and immunohistologic localization of T cell precursors in early human fetal tissues. J. Exp. Med. 168:1061.
32. Haynes, B.F., S.M. Denning, K.H. Singer, and J. Kurtzberg. 1989. Ontogeny of T-cell precursors: a model for the initial stage of human T-cell development. Immunol. Today. 10:87.
33. Krowka, J.F., S. Sarin, R. Namikawa, J.M. McCune, and H. Kaneshima. 1991. Human T cells in the SCID-hu mouse are phenotypically normal and functionally competent. J. Immunol. 146:3751.
34. De la Hera, A., W. Marston, C. Aranda, M.-L. Toribio, and C. Martinez-A. 1989. Thymic stroma is required for the development of human T cell lineages in vitro. Int. Immunol. 1:471.
35. Mossalyi, M.D., J.C. Lecon, P. Goube de Laforest, G. Janossy, P. Debré, and J. Tanzer. 1988. Characterization of prothymocytes with cloning capacity in human bone marrow. Blood. 71:1281.
36. Bertho, J.M., M.D. Mossalyi, A.H. Dalloul, G. Moutet, and P. Debré. 1990. Isolation of an early T-cell precursor (CFU-T) from human bone marrow. Blood. 75:1064.
37. Kurtzberg, J., S.M. Denning, L.M. Nycom, K.H. Singer, and B.F. Haynes. 1989. Immature human thymocytes can be driven to differentiate into nonlymphoid lineages by cytokines from thymic epithelial cells. Proc. Natl. Acad. Sci. USA. 86:7575.
38. Metcalfe, D., and N.A. Nicola. 1991. Direct proliferative actions of stem cell factor on murine bone marrow cells in vitro: effects of combination with colony-stimulating factors. Proc. Natl. Acad. Sci. USA. 88:6239.
39. Bernstein, I.D., R.G. Andrews, and K.M. Zsebo. 1991. Recombinant human stem cell factor enhances the formation of colonies by CD34+ and CD34+lin− cells, and the generation of colony-forming cell progeny from CD34+lin− cells cultured with interleukin-3, granulocyte colony-stimulating factor, or granulocyte-macrophage colony-stimulating factor. Blood. 77:2316.
40. Haylock, D.N., L.B. To, T.L. Dowse, C.A. Juttner, and PJ. Simmons. 1992. Ex vivo expansion and maturation of peripheral blood CD34+ cells into the myeloid lineage. Blood. 80:1405.