Delineation of T-Progenitor Cell Activity Within the CD34+ Compartment of Adult Bone Marrow

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T-cell production is largely dependent on the presence of a thymus gland where CD34+ precursors mature into T lymphocytes. Prethymic stages of T-cell development are less defined. Therefore, this study aims to delineate T-progenitor cell potential within the CD34+ lineage (Lin-) compartment of adult bone marrow (ABM). Fractionation of CD34+ Lin- ABM cells with CD45RA, Thy-1, CD38, and HLA-DR failed to absolutely segregate T-cell reconstituting ability, indicating broad distribution of T-progenitor cell potential. Titration experiments showed that low numbers of CD34+ Lin- CD45RA+ (RA+) cells had greater thymus repopulating ability than CD34+ Lin- CD45RA- cells (RA-). The great majority (>95%) of RA+ cells expressed CD38, HLA-DR and 70% to 90% of RA+ cells lacked Thy-1 surface expression. RA+ cells contained colony-forming unit granulocyte-macrophage (CFU-GM) progenitor cells but were depleted of erythroid potential, did not provide hematopoietic reconstitution of human bone fragments implanted into SCID mice, and did not efficiently maintain CD34+ cells with secondary clonalogenic potential in bone marrow cultures. Thus, RA+ cells are oligopotent (nonprimitive) CD34+ progenitors with T-cell reconstituting ability. In contrast, these same assays indicated that CD34+ Lin- CD45RA- cells (RA-) comprised hematopoietic stem cells (HSC) with primitive multilineage (T, B, myeloid, and erythroid) hematopoietic potential. It was confirmed that HSC-containing populations, such as CD34+ Lin- CD45RA+ Thy-1+ cells had thymus repopulating ability. Culture of RA- cells on murine bone marrow stromal cells in the presence of interleukin (IL)-3, IL-6, and leukemia inhibitory factor (LIF) generated CD34+ CD45RA+ progeny engrafting in a secondary severe combined immunodeficiency (SCID)-hu thymus assay. Altogether, our results underscore the fact that T-cell reconstituting potential can be dissociated from HSC activity. Furthermore, we speculate that HSC might develop into the T lineage indirectly, via differentiation into an intermediate oligopotent CD34+ CD45RA+ stage. Finally, T-progenitor cells can be cultured in vitro.

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expression of CD45RA, Thy-1, CD38, and HLA-DR was used to fractionate CD34+ cells. Our results show that T-lymphoid progenitor cell activity is present in functionally distinct subpopulations of ABM and support the concept that the T-cell reconstituting ability of HSC is indirect.

**MATERIALS AND METHODS**

Sample processing and staining for flow cytometry sorting. ABM aspirates were obtained with informed consent from healthy adult volunteers. Low density (<1.077 g/mL) mononuclear cells (MNC) obtained by gradient centrifugation (Lymphoprep, Nycomed Pharma, Oslo, Norway) were washed twice in staining buffer (SB) consisting of phosphate buffered saline and 0.2% bovine serum albumin (Sigma, St Louis, MO) and incubated with 1 mg/mL heat-inactivated human gamma-globulin (Gamimune; Miles Inc, Elkhart, IN) to block nonspecific Fc receptor binding of mouse antibodies. Granulocytes were removed either by incubation with magnetic beads (Dynal M450, Oslo, Norway) coated with anti-CD15 mononal antibodies (MoAbs; Medarex, West Lebanon, NH) or by one freeze/thaw cycle in the presence of 10% dimethyl sulfoxide (DMSO; Sigma) and 10% fetal calf serum (FCS; Hyclone, Logan UT). Cells were incubated with Lin-specific phycoerythrin (PE)-conjugated MoAbs. The Lin panel contained anti-CD2, -CD4, -CD8, -CD56, -CD16, -CD20, -CD19, -CD14, (Becton Dickinson, Mountain View, CA) and antilycophorin A (GAMy3) antibodies (Southern Biotechnologies Associates, Birmingham, AL) and FITC-labeled anti-CD45RA MoAbs (Becton Dickinson) followed by two washes in SB. Cells were sorted on a FACStar Plus cell sorter (Becton Dickinson, San Jose, CA) equipped with dual argon laser ion lasers emitting at 488 nm and 600 nm. All cells expressing PE-Lin levels above the level of the irrelevant Ig negative control were excluded by electronic gating and the remaining Lin- cells were sorted on the basis of expression of CD34 and CD45RA. For cell sorts using CD38 MoAbs, cells were stained with anti-CD34-TR-GAMy3, FITC-CD15, and PE-anti-CD34 MoAbs (Becton Dickinson). For the study of CD34+ cells expressing HLA-DR, cells were stained with anti-CD34-TR-GAMy3, FITC-CD15, and PE-anti-CD34 MoAbs (Becton Dickinson) as reported earlier.17 For Thy-1 sorts, cells were labeled with anti-CD34 and anti-Thy-1 MoAbs (GM201, provided by Dr W. Rettig, Ludwig Cancer Research Institute, New York, NY) recognized respectively by isotype specific TR-GAMy3 and PE-antimouse IgG1 antibodies (Caltag, South San Francisco, CA); after extensive blocking with mouse IgG1 (Sigma), FITC-Lin (CD2, CD14, CD15, CD16, and GPA) MoAbs were added. The purity of all cell populations obtained by sorting was determined by reanalysis and overall found to be greater than 90%. For the CD34 CD45RA/Thy-1 isolations, two consecutive sorts were performed. First, cells were stained with anti-CD34, anti-Thy-1, and FITC-Lin MoAbs as described above. Sorted CD34+ Lin- cells were stained again with FITC-CD45RA MoAbs and a second sort was performed on the basis of CD45RA and Thy-1 expression.

Clonal assay of hematopoietic progenitor cells. Cells were plated at a concentration of 500 to 1,000 CD34+ cells per dish (35 X 10 mm; Nunc, Naperville, IL) in Iscove’s methylcellulose (Terry Fox Laboratory, Vancouver, British Columbia, Canada) supplemented with purified recombinant human cytokines including c-kit ligand (KL; 10 ng/mL), erythropoietin (1.2 U/mL; R & D Systems, Minneapolis, MN), granulocyte-macrophage colony-stimulating factor (GM-CSF) and G-CSF (each at 25 ng/mL; Amgen, Thousand Oaks, CA), and IL-3 (10 ng/mL; SANDOZ Pharma, Basel, Switzerland). For each cell type tested, duplicate or more often quadruplicate plates were incubated at 37°C, 5% CO2 in a humidified atmosphere for 2 weeks. Burst-forming unit-erythroid (BFU-E), colony-forming unit cells comprising all classes of granulocytic and monocytic progenitors (CFU-GM), and granulocytes, monocytes, and erythroid cells (CFU-mix) were scored using an inverted microscope (Nikon, Tokyo, Japan). Results are expressed as the mean number of colonies per 105 total plated cells.

Bone marrow cultures. Sys-1 murine bone marrow stromal cells support the long-term maintenance of human progenitors.12,13 Sys-1 monolayers were pre-established by plating 1 X 105 Sys-1 cells per well of a 96-well flat bottom plate in 100 μL of medium consisting of 50% Iscove’s modified Dulbecco’s medium (IMDM; JRH Biosciences, Lenexa, KS), 50% RPMI with 10% FCS (Hyclone), 4 X 10-9 rorol/L 2-mercaptoethanol. 10 mmol/L HEPES, penicillin (100 U/mL), streptomycin (100 μg/mL) (PS), and 4 mmol/L glutamine (JRH Biosciences). One week later, sorted hematopoietic cells were distributed at 100 cells per well on the preestablished Sys-1 cell monolayer in medium containing IL-3 (10 ng/mL), IL-6 (10 ng/mL), and leukemia inhibitory factor (LIF) (50 ng/mL) (Sandoz Pharma). Half of the cytokine-containing medium was replaced weekly. After 3 weeks, cells were harvested by pipetting, counted, and transferred to subsequent assays. Cells present in the 3-week old Sys-1 cultures initiated with RA+ cells were stained with PE-anti CD34 (HPCA-2) and FITC-anti-CD45RA, and sorted into CD34+ CD45RA- and CD34+ CD45RA+ populations.

SCID-hu bone assay. CB-17 scid/scid (SCID) mice were bred in our facilities and the construction of SCID-hu bone mice was performed as previously described.26 Briefly, split fetal human long bones obtained from elective abortion in compliance with state and federal regulations were implanted subcutaneously into the mammary fat pads of SCID mice under anesthesia. HLA immunophenotyping of the recipient fetal bone and of donor ABM cells was performed with fluorescein-conjugated MA2.1, BB7.2, GAP-A3, and W6/32 MoAbs derived from hybridomas obtained from ATCC (Rockville, MD). SCID-hu bone mice were used 8 weeks postimplantation as recipients for HLA-mismatched sorted cell populations following a single whole body irradiation dose (350 cGy from a 17Co source, Gamma Cell 40, L.J. Shephard & Associates, San Fernando, CA). Sorted cells (2 to 3 X 106 in 10 μL) were injected directly into the transplanted bone using a Hamilton syringe. After an additional 8 weeks, mice were killed, human bones were removed and cells mechanically released into SB. Collected cells were washed twice in SB, and counted before being stained for two-color immunofluorescence with fluoresceinated MoAbs against the specific donor HLA allotype in combination with PE anti-CD19, -CD33, and -CD34. FITC and PE-conjugated irrelevant mouse immunoglobulins were used as negative controls. Cells were analyzed on a FACScan fluorescence cell analyzer (Becton Dickinson).

SCID-hu thymus assay. HLA immunophenotyping of the recipient fetal thymus and of donor ABM cells was performed as described above. Fragments of fetal thymus were placed on nitrocellulose filters (0.8 μm; Costar Corp, Cambridge, MA) on top of gelatin rafts (Gel-foam; Upjohn, Kalazann, MI) as described.26 After 7 to 13 days of incubation at 25°C and 5% CO2, thymus fragments were irradiated with 250 Gy, washed, and immediately microinjectted with the HLA-mismatched sorted cells in a 1 μL volume using an oil-filled microinjector (Narishige, Japan) and 1 mm diameter glass micropipets (World Precision Instruments, Sarasota, FL). Fragments were

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placed back on the filters and incubated at 37°C, 5% CO₂ overnight and then inserted under the kidney capsule of anesthetized 6 to 8 week-old SCID mice bred in our facilities. Mice were killed 6 to 7 weeks after transplantation, and the thymus grafts were recovered, reduced to a single cell suspension, and subjected to three-color immunofluorescence analysis on the FACSscan with fluoresceinated anti-HLA antibodies, or mouse IgGl irrelevant control, PE anti-CD4 or mouse IgGl control (Becton Dickinson) and Tricolor-conjugated anti-CD8, -CD3, or mouse IgGl irrelevant control (Caltag). Results are expressed as the fraction of injected thymus grafts containing greater than 1% donor-derived T cells within the human (CD45⁺ W6-32⁻) population.

RESULTS

Fractionation of CD34⁺ Lin⁻ ABM with CD45RA, Thy-1, HLA-DR, CD38 fails to absolutely segregate T-progenitor cell activity. Cell surface markers including CD45RA, Thy-1, HLA-DR, and CD38 have been used with CD34 to isolate primitive hematopoietic progenitor cells. The SCID-hu thymus assay tested the ability of CD45RA⁺, Thy-1, HLA-DR, or CD38 subsets of CD34⁺ ABM to generate T cells. T-progenitor cell potential was found in CD45RA⁺ and CD45RA⁻, Thy-1⁺ and Thy-1⁻, HLA-DR⁺, and HLA-DR⁻ or CD38⁺ and CD38⁻ subsets, indicating that thymus repopulating ability was broadly distributed within the CD34⁺ Lin⁻ compartment of ABM.

T-progenitor cell potential is greater in the CD45RA⁺ cell subset than in the CD45RA⁻ cell subset. Both CD34⁺ Lin⁻ CD45RA⁺ (RA⁺) and CD34⁺ Lin⁻ CD45RA⁻ (RA⁻) cells derived T cells in the SCID-hu thymus assay, and the composition of reconstituted thymic grafts (6 weeks post-transplantation) was similar whether injected with RA⁺ or RA⁻ cells. The percentage (± standard deviation) of donor-derived T cells in thymic grafts injected with 1 × 10⁴ cells was, respectively, 70 ± 46 and 51 ± 38 for RA⁺ and RA⁻ subsets and the difference is not statistically significant (P = .12) with the nonparametric Kolmogorov-Smirnov test. Titration of injected cell numbers indicated that RA⁺ cells engrafted better than RA⁻ cells at low cell numbers (respectively, 14 of 21 v 2 of 18 grafts positive for donor cells after injection of 2,000 to 3,000 cells per graft; Fig 1A). The percentage (± standard deviation) of donor-derived T cells in thymic grafts injected with 2,000 to 3,000 cells was, respectively, 53 ± 49 and 7 ± 23 for RA⁺ and RA⁻ subsets (Fig 1B) and the difference is significant (P = .007) with Kolmogorov-Smirnov analysis. However, in all cases, the qualitative composition of the donor-derived population in grafts reconstituted with either RA⁺ or RA⁻ subsets was comparable with greater than 80% donor-derived thymocytes expressing high levels of CD1a, coexpressing CD4 and CD8 and displaying graded levels of CD3 indicating ongoing thymopoiesis. Mature T cells bearing the αβ or γδ TCR could be expanded from reconstituted thymic grafts (data not shown). Long-term T-cell reconstitution examined at 11 and 12 weeks post cell injection showed the comparable presence of donor-derived thymocytes in both RA⁺ and RA⁻ groups (data not shown).

CD45RA fractionates CD34⁺ Lin⁻ ABM cells into primitive and nonprimitive progenitor cell populations. The CD45RA cell subsets of CD34⁺ Lin⁻ ABM were isolated

| Table 1. Expression of Thy-1, CD38, HLA-DR, and T-Progenitor Cell Activity in the SCID-hu Thymus Assay |
|---------------------------------|-----------|---------------------|---------------------|
| ABM Cell Subsets               | No. of Experiments | Cell No. Tested | Engraftment Success |
| CD34⁺Lin⁻ CD45RA⁺             | 6           | 10,000             | 11/14               |
| CD34⁺Lin⁻ CD45RA⁻             | 6           | 10,000             | 9/12                |
| CD34⁺Lin⁻ Thy-1⁺              | 3           | 10,000             | 16/20               |
| CD34⁺Lin⁻ Thy-1⁻              | 3           | 10,000             | 10/20               |
| CD34⁺CD15⁻ HLA-DR⁺            | 1           | 10,000             | 6/6                 |
| CD34⁺CD15⁻ HLA-DR⁻            | 1           | 10,000             | 3/6                 |
| CD34⁺CD45RA⁺ CD38⁺            | 1           | 5,000-10,000       | 6/6                 |
| CD34⁺CD45RA⁺/CD38⁻            | 1           | 5,000-10,000       | 5/6                 |
| CD34⁺Lin⁻ CD45RA⁺ Thy-1⁺      | 2           | 2,000              | 7/8                 |
| CD34⁺Lin⁻ CD45RA⁺ Thy-1⁻      | 2           | 2,000              | 7/8                 |
MAPPING T-PROGENITOR ACTIVITY IN CD34' ABM

which varied with different fluorochromes used.

by flow cytometry sorting with high degrees of purity (93% to 98% pure on reanalysis) and with minimal overlap between the two subsets (0% to 0.5% RA' cells cross-contaminated sorted RA' cells and 0% to 3% RA' cells cross-contaminated RA' sorted cells). Approximately 20% to 40% of the CD34' Lin- cells are RA' (Fig 2). RA' cells did not generally exhibit phenotypic characteristics known to be associated with HSC. A variable proportion (70% to 90%) of RA' cells had undetectable Thy-1 expression (Fig 2B) and the great majority (>95%) of RA' cells displayed high levels of CD38 and of HLA-DR (Fig 2C and D). It was ascertained that the T-cell reconstituting activity of RA' cells was not due to the presence of minor CD38' cells or Thy-1' cells because T cells were generated from sorted RA' CD38' cells or sorted RA' Thy-1' cells (Table 1).

In methylcellulose cultures, RA- cells generated greater numbers of CFU-mix and of BFU-E than RA' cells (respectively, 3 to 7 vs 0.5 CFU-mix per 1,000 cells and 58 to 82 vs 0 to 2 BFU-E per 1,000 cells), whereas CFU-GM were derived from both RA' and RA' cells, as anticipated from earlier reports (Table 2). Upon cocultivation on murine bone marrow stromal cells (Sy5-1) for 3 weeks in the presence of IL-3, IL-6, and LIF, both the RA' and RA' cell populations proliferated with respective total cell expansion of 100 to 500 and 70 to 200 fold observed in three experiments. Phenotypic analysis of these bone marrow cocultures showed that those initiated with RA' cells consistently contained higher percentages of CD34' Lin- cells than cultures initiated with RA' cells (respectively, 1.7% to 13.6% vs 0% to 3.5% in five experiments) (Table 3) and generated higher numbers of secondary hematopoietic colonies (12 to 17 per 1,000 cells vs 2 to 3 per 1,000 cells in two experiments). The hematopoietic reconstituting potential of RA' and RA' cells was tested in the in vivo SCID-hu bone assay previously shown to support the long-term and multilineage engraftment of allelogeneic human HSC. Human bone grafts, which were injected with HLA-mismatched RA' cells, were engrafted with donor-derived cells in 5 of 7 bone grafts, 8 weeks post cell injection, while no progeny was recovered in bone grafts (0 of 6) injected with equivalent numbers of RA' cells. Representative grafts in Fig 3 show the absence of detectable donor-derived cells after injection of RA' cells, whereas donor-derived CD19' B cells, CD33' myeloid cells, and CD34' progenitor cells were found in grafts injected with RA' cells. Altogether, the data show that the RA' subset comprises primitive hematopoietic progenitor cells, whereas the RA' subset is devoid of primitive activity.

T-cell reconstituting ability of HSC-containing cell populations. Total RA' cells engrafted poorly in the SCID-hu thymus assay when low (2,000 to 3,000) cell numbers were

| Table 3. Phenotypic Analysis of Bone Marrow Cultures Initiated With CD45RA Cell Subsets |
|----------------------------------------|---------------|---------------|---------------|
| Population Initiating the Culture     | RA'           | RA'           |
|------------------------------------------------------------------------------------------------------------------------------------------|
| Experiment no. 1: CD45RA'              | 0.6           | 2             |
| CD45RA'                                | 3             | 12.5          |
| Experiments 2: CD45RA'                 | 0             | 1.7           |
| CD45RA                                | 0.5           | 4             |
| CD45RA'                                | 3.5           | 13.6          |

* Phenotypic analysis was performed by FACS analysis. Percentages were calculated by subtracting the background staining with an irrelevant control antibody.

![Fig 2. Phenotypic analysis of CD34' ABM cells. ABM cells were analyzed for their expression of CD45RA and CD34 (A), Thy-1 (B), CD38 (C), and HLA-DR (D). ABM cells are Lin- (A) and CD34' Lin- (B through D). Quadrants indicate the limits of the irrelevant Ig negative controls, which varied with different fluorochromes used.](image-url)
injected (Fig 1). However, we obtained T-cell engraftment with 2,000 RA⁻ Thy-1⁺ cells (7 of 8 positive grafts in 2 experiments) (Table 1) suggesting that the RA⁻ cell subset might be heterogeneous in its capacity to derive T cells. Cell titrations showed higher T-progenitor cell activity in the RA⁻ Thy-1⁺ subset compared to the RA⁻ Thy-1⁻ subset (Table 4). The differences in engraftment success of RA⁻ Thy-1⁺ cells presented in Table 1 (7 of 8 [87.5%] positive grafts after injection of 2,000 cells) and Table 4 (50% to 75% positive grafts after injection of 3,000 cells) are probably due to variabilities in the quality of human samples used in these different sets of experiments. As BFU-E seem enriched in RA⁻ Thy-1⁻ cells compared with RA⁻ Thy-1⁺ cells (Table 4), T-progenitor cell activity of the total RA⁻ cell subset might be diluted by the presence of progenitors of BFU-E and of other cell subsets committed to non-T lineage.

Differentiation of HSC into T-progenitor cells. We sought to describe more precisely the relationship between RA⁻ and RA⁺ cells as they represent two functionally and phenotypically distinct cell types sharing common T-progenitor cell activity. Bone marrow cultures initiated with RA⁺ cells contained CD34⁺ cells, which all expressed CD45RA (data not shown). In contrast, bone marrow cultures initiated with RA⁻ cells consistently maintained a proportion of RA⁻

**Table 4. Myeloid, Erythroid, and T-Progenitor Cell Potential of Thy-1 Subsets of CD34⁺Lin⁻ CD45RA⁻ Cells**

<table>
<thead>
<tr>
<th>Subjects of CD34⁺Lin⁻ CD45RA⁻ Cells</th>
<th>Methylcellulose Assay (CFU/1,000 cells)</th>
<th>SCID-Hu Thymus Assay (engraftment success*, %)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CFU-GM</td>
<td>BFU-E</td>
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<tr>
<td>Experiment no. 1</td>
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<td></td>
</tr>
<tr>
<td>Thy-1⁺</td>
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<tr>
<td>Experiment no. 2</td>
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</tr>
<tr>
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<td>53</td>
</tr>
<tr>
<td>Thy-1⁻</td>
<td>73</td>
<td>129</td>
</tr>
</tbody>
</table>

* The engraftment success is expressed as the fraction of injected grafts containing donor-derived T cells. The same data are expressed as a percentage in parenthesis.
cells while RA– progeny appeared (Fig 4). Although only CD34 and CD45RA markers were used to stain cells in this experiment, we verified in other cultures that RA– progenies were indeed Lin–, and therefore have a phenotype similar to the freshly isolated populations. Thus, RA+ cells derived from RA– cells further substantiates the differences in primitiveness of these two cell subsets.

CD34+ cells with T-cell reconstituting ability could be isolated by flow cytometry sorting from a bone marrow culture initiated with RA– cells 3 weeks earlier. In a subsequent experiment, we sorted the CD34+ CD45RA– and CD34+ CD45RA+ progeny, which were generated by culturing RA– cells on bone marrow stroma for 3 weeks. The sorted CD34+ CD45RA+ cells generated T cells (Fig 4). Injection of 4,000 sorted CD34+ CD45RA+ cells reconstituted 2 of 2 grafts that contained 1% and 39% donor-derived T cells. Injection of 2,000 sorted CD34+ CD45RA+ cells reconstituted 1 of 4 grafts with 35% donor-derived T cells, and this thymic graft is shown in Fig 5. Sorted CD34+ CD45RA+ cells contained CFU-GM, but no BFU-E or CFU-mix progenitors (Fig 4). The sorted CD34+ CD45RA+ cells contained CFU-GM, BFU-E, and CFU-mix progenitors, but at the cell concentration tested (2,000 per graft) failed to provide T-cell reconstitution. This finding was not unexpected because injection of equivalent numbers of freshly isolated RA– cells would have provided poor engraftment, as shown by a titration performed with the starting RA– population into three different thymus recipients (Fig 4). It was calculated that 4,000 RA+ progeny cells originated from the initial input of approximately 500 RA– cells (calculated with the proportion of RA+ cells produced [4%] and the total cellular expansion of the culture [210-fold]). Based on the titration, this number of freshly isolated RA– cells would have engrafted very poorly or not at all. Thus, a suboptimally engrafting dose of primitive RA– cells can be converted by in vitro differentiation into a T-cell reconstituting population. Altogether, the
data indicate that primitive HSC may generate T cells indirectly via differentiation into an intermediate more committed progenitor expressing CD45RA and that T-progenitor cells can be maintained in vitro. The data support the concept that the T-cell reconstituting ability of HSC is indirect.

DISCUSSION

In the present study, a combined functional analysis of T-lymphoid and myelo-erythroid progenitor potential was undertaken to delineate the prethymic T-progenitor cell activity present in the CD34+ compartment of ABM. Fractionation of CD34+ Lin- ABM cells with CD45RA, Thy-1, CD38, and HLA-DR failed to absolutely segregate T-cell reconstituting ability, indicating that our assay detects a broad distribution of T-progenitor cell potential within the CD34+ ABM compartment. This is in agreement with previous results showing the presence of T-progenitor cells in both Thy-1+ and Thy-1- or CD7+ and CD7- subsets of FBM and FL CD34+ cells. Subfractionation with CD45RA isolated two discrete and functionally distinct populations with quantitatively different T-cell reconstitution abilities. CD34+ Lin- CD45RA+ cells (RA+ population) represent nonprimitive oligopotent hematopoietic progenitors as determined by their lack of erythroid potential, their inability to provide multilineage hematopoietic reconstitution in SCID-hu bone mice, and to maintain a high proportion of CD34+ cells with secondary clonogenic activity in vitro. However, nonprimitive RA+ cells more efficiently generated T cells than RA- cells. These results underscore the fact that T-lymphoid progenitor activity could be dissociated from long-term hematopoietic reconstituting potential. Because RA+ cells are part of Thy-1-, CD38-, and HLA-DR- subsets of CD34+ cells, T-cell reconstituting ability is also found in these progenitor cell subsets. Discrepancies between T-cell reconstituting potential and primitive hematopoietic activity have already been noted in other studies. Human FBM CD34+ Thy-1+ cells or FL CD7dull cells are relatively depleted of LTC-IC or high proliferative potential colony-forming cell activity, yet have T-cell reconstituting potential fairly similar to their respective Thy-1+ and CD7+ counterparts. More recently, a subset of mouse bone marrow cells with a phenotype and activity distinct from that of HSC was identified. These mouse Sca2+ bone marrow progenitors have a thymus repopulating ability, which is intermediate between that of HSC and that of the earliest intrathymic precursor. Thus, in mouse and in man, intrathymic T-cell reconstitution can be achieved by at least three cell types with distinct levels of hematopoietic commitment: first, pluripotent HSC-containing populations such as found in the RA- compartment and which express Thy-1; second, oligopotent prethymic
bone marrow progenitors such as those expressing CD45RA; and third, intrathymic precursors expressing CD34. In our study, the T-cell reconstituting potential of primitive and nonprimitive populations, based on phenotypic analysis of donor-derived thymocytes and long-term (12 weeks) T-cell reconstitution seems qualitatively undistinguishable.

Primitive HSC-containing CD34+ populations lacking CD45RA or CD38 or HLA-DR or expressing Thy-1 had T-lymphoid differentiation capability. The T-progenitor cell content of RA- cells was lower than that of RA+ cells. Subfractionation of the RA- cell compartment suggests that progenitors committed to non-T lineage in the Thy-1 fraction might dilute the overall T-progenitor cell content of RA- cells. It is also possible that RA+ cells might represent a population more readily committed to the T lineage than RA-. Nevertheless, the T-cell reconstituting potential of HSC has already been acknowledged in studies with mice.

Direct intrathymic injection of 5 Thy-1lo, Sca+ cells, a population highly enriched for mouse HSC with pluripotent B-lymphoid, erythroid, and myeloid potential, produces T-cell reconstitution. The question arises as to whether HSC directly seed the thymus under physiologic conditions. Such a possibility is supported by the identification of a very rare cell population with primitive HSC phenotype (CD34+ Lin- CD38low) within the triple negative (CD3-, CD4-, CD8-) fetal thymocyte population. The presumed advantage for direct seeding of the thymus by HSC would be to derive multiple lineages intrathymically, which is possible because the thymus has the capacity to support the differentiation of non-T cells. However, studies in animals and with SCID-hu mice have shown that maintenance of thymopoiesis generally requires the continuous input of hematopoietic cells into the thymus gland. This would seem to be an underutilization of the extensive proliferative potential of multipotent HSC.

The physiologic relevance of stem cells seeding the thymus is also challenged by the fact that fetal intrathymic HSC are extremely rare. Therefore, their presence might result from the continuous input of circulating HSC. Indeed, it has been shown that LTC-IC can be readily retrieved (albeit, at very low frequency) from the blood of normal individuals. Also, the paradoxical existence of nonprimitive progenitors with T-cell reconstituting ability further challenges the concept that only primitive HSC could seed the thymus. Further experiments are needed to specifically determine the thymus homing properties of various populations with T-cell differentiation potential. There are at least two cellular mechanisms for the differentiation of HSC into T cells. One involves a direct commitment of HSC into the T lineage, another involves the differentiation of HSC into progenitors increasingly restricted in their lineage potential. In an in vitro culture system, we found that small numbers of primitive RA- cells, which are inefficient at providing T-cell reconstitution, can differentiate into enough RA+ cells to provide T-cell reconstitution. Practically, it shows that CD34 and CD45RA expression define a phenotype that remains faithful to the functional activity after in vitro culture. This may not be the case with all markers useful for isolating HSC. It was recently found that in contrast to freshly isolated populations, LTC-IC are enriched in cultured CD34+ HLA-DR+ cells. Importantly, we also show that ex vivo expansion of HSC into T-lymphoid progenitors is feasible. Our data support the concept that HSC indirectly differentiate into T cells via a downstream CD34+ CD45RA+ oligopotent (nonprimitive) stage, before further committing to the T-cell lineage. Further work is in progress to identify additional steps along the pathway of T-lineage development.

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