RAPID COMMUNICATION

Phenotypic Characterization of a Novel Bone Marrow-Derived Cell That Facilitates Engraftment of Allogeneic Bone Marrow Stem Cells


Bone marrow transplantation is an accepted therapy for hematologic malignancies, aplastic anemia, metabolic disorders, and solid tumors. However, graft-versus-host disease (GVHD) and failure of engraftment have limited the widespread application of this technology to nonmalignant disease states. The use of purified bone marrow stem cells has been suggested as an approach to promote engraftment yet avoid GVHD. Although bone marrow stem cells, purified by cell sorting, engraft and repopulate lethally irradiated genetically identical recipients, they do not engraft in major histocompatibility complex (MHC)-disparate allogeneic recipients. We report for the first time the characterization of a novel cell population of donor bone marrow origin, separate from the hematopoietic stem cell, that facilitates engraftment of purified allogeneic bone marrow stem cells in an MHC-specific fashion without causing GVHD. Although 1,000 purified stem cells (c-kit+/Sca-1+/ lineage-) reliably repopulate syngeneic mouse recipients, 10 times that number could not effectively remove the GVHD-producing cells without adversely affecting engraftment led to an intense interest in purifying BM stem cells. It was believed that the administration of purified stem cells might provide an approach to optimize conditions that promote engraftment yet avoid GVHD. Transplantation of a highly enriched or purified stem cell population rather than unfractionated BM might also safely extend the clinical application of BMT to full major histocompatibility complex (MHC)-mismatched situations. However, this approach was soon shown to suffer from the same limitations as TCD failure of BM engraftment. Highly purified stem cells have only been shown to reliably engraft in MHC-matched genetic combinations. However, purified stem cells have not been shown to engraft in allogeneic recipients. This failure of stem cell engraftment has been traditionally attributed to rejection by the host microenvironment. However, an alternative hypothesis to these observations is that a non-stem cell component in donor BM may be required to facilitate stem cell engraftment in allogeneic recipients.

Bone marrow transplantation (BMT) is an accepted therapy for hematologic malignancies, aplastic anemia, metabolic disorders, and solid tumors. Despite advances in the field, graft-versus-host disease (GVHD) and failure of engraftment continue to cause significant morbidity and mortality. Thirty to 70% of recipients of HLA-identical BM from living related donors develop GVHD. Among the 70% of patients who lack a suitably matched family member donor, the incidence of GVHD may approach 80%. Clinical trials in the 1970s and early 1980s that removed T cells from the donor BM inoculum prevented GVHD, but were associated with a significant increase in failure of engraftment in up to 70% of patients. These clinical data, as well as data from rodent models, suggested that T cells are required for engraftment of the BM stem cell in an allogeneic environment. Because failure of engraftment is usually lethal, many T-cell depletion (TCD) protocols were abandoned and GVHD was accepted as an unavoidable complication of allogeneic stem cell therapy. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

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the donor stem cell in an allogeneic environment. CD4+, CD8+, MAC-1+, or natural killer (NK) cells can be removed from the allogeneic component of the BM inoculum using MoAb plus complement-mediated lysis without loss of the facilitating effect. This effect is mediated by an MHC-specific donor cell fraction that lacks the potential to produce GVHD and is not caused by depletion of BM stem cells.

We have now used rare events multiparameter live sterile cell sorting to identify a novel cell population purified from BM that facilitates engraftment of purified BM stem cells in an allogeneic environment. We show for the first time that, although purified hematopoietic stem cells alone do not engraft in allogeneic recipients, the addition of a small number of phenotypically unique donor BM-derived cells, distinct from the pluripotent hematopoietic stem cell, permits reliable and reproducible engraftment of purified stem cells in an allogeneic environment without causing GVHD.

MATERIALS AND METHODS

Animals. Four- to 6-week-old male C57Bl/10SnJ (B10), BALB/c, and B10.BR SgSnJ (B10.BR) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Animals were housed in a specific pathogen-free facility at the Pittsburgh Cancer Institute (Pittsburgh, PA).

Chimera preparation. Chimeras were prepared as previously described. Briefly, 4- to 6-week-old B10 mice were lethally irradiated with a dose of 950 cGy from a Cesium source (Nordion, Ontario, Canada). Using sterile technique, BM was flushed with Media 199 (GIBCO, Grand Island, NY) containing 50 μL/mL Gentamicin (henceforth called minimal essential medium [MEM]) from the femurs and tibias of mice donors with a 22-gauge needle. The marrow was mechanically resuspended and counted. TCD of the syngeneic donor BM was performed using RAMB (ramose) serum as previously described.

In antibody plus complement depletion studies, animals received 5 × 10⁶ RAMB B10 plus 15 × 10⁶ B10.BR BM cells treated with K-AMB, anti-Thy 1.2 or other monoclonal antibodies as described. Recipient animals were reconstituted within 4 to 6 hours following irradiation via the lateral tail veins using a 27 gauge needle. For the cell sorting studies, lethally irradiated animals received a mixture of 5 × 10⁶ RAMB treated B10 (syngeneic) plus 5 × 10⁶ RAMB-treated B10.BR (allogeneic) BM cells plus the sorted fraction (see below). Controls received the same mixed inoculum of RAMB-treated BM plus an equivalent number of RAMB-treated B10.BR BM cells administered with the sorted fractions.

Characterization of chimeras by flow cytometry. Recipients were characterized for engraftment with syngeneic and/or allogeneic donor lymphoid elements using flow cytometry to determine the percentage of PBLs bearing H-2b (B10) or H-2a (B10.BR) markers. PB was collected into heparinized plastic serum vials. After thorough mixing, the suspension was layered over 3 mL of room temperature LSM (Organon Technica, Kensington, MD) and centrifuged at 23°C (30 minutes at 1,700 rpm). The lymphocyte layer was aspirated from the saline-LSM interface and washed with medium. The resulting population was stained with directly labeled antihuman CD3 (Leu-4) as a control as well as directly labeled anti-H-2b and H-2a. Arbitrary levels on log scale were selected based on the inflection point at which staining of the control negative population was minimized while retaining maximal numbers of positive cells. The percentage of PBL chimerism was normalized to 100% as previously described. This represents the mean ± standard deviation of allogeneic chimerism for all experiments performed.

Depletion of BM with antibodies and complement. Mouse BM was isolated from the long bones as previously described. After isolation, BM cells were resuspended at a concentration of between 1 × 10⁶ to 1 × 10⁷ cells/mL in Media 199 (GIBCO) containing 50 μg/mL Gentamicin plus an appropriate concentration of MoAbs. MoAbs were previously titrated to determine saturating concentrations. Cells were incubated for 45 minutes at 37°C, washed once using MEM, and centrifuged for 10 minutes at 1,000 rpm. Cells were resuspended in a 1:8 dilution of rabbit complement (prepared in our laboratory), incubated for 30 minutes at 37°C, and washed. After a second round of complement treatment, cells were washed twice in MEM, resuspended, and counted. Confirmation of depletion was performed using flow cytometric analysis (FACStar; Becton Dickinson, Mountain View, CA). BM was analyzed before and after depletion using directly labeled antibodies specific for the populations being depleted as well as antibodies specific for the isotype and species of origin for the depleting antibodies. Antibody and complement depletion generally yielded a 1 to 1.5 log reduction in populations of interest.

MoAbs. MoAbs used in cell sorting experiments were all directly labeled and consisted of the following (CD marker, clone, species of origin, isotype, source): CD3-Epsilon-FITC, 145-2C11, hamster IgG, Pharmingen (San Diego, CA); CD4-FITC, RM 4-4, rat IgG2b, Pharmingen; CD5-FITC, 57-3-7, rat IgG2a, Pharmingen; CD8α-FITC, 53-6-7, rat IgG2a, Becton Dickinson; CD8αa-PE, 53-6-7, rat IgG2a, Pharmingen; CD8β-β, 53-5-8, rat IgG1, Pharmingen; CD45-FITC, 30F11.1, rat IgG2b, Pharmingen; CD45R-FITC, 23G2, rat IgG2a, Pharmingen; B220-FITC, RA3 6B2, rat IgG2a, Pharmingen; NK1.1-PE, PK 136, mouse IgG2a, Pharmingen; Thy 1.2-PE, 30-H12, rat IgG2b, Pharmingen; β-β-TCR-FITC or biotin, H57-597, hamster IgG, Pharmingen; I-A-FITC, 11-5-2, BALB/c IgG2b, Pharmingen; H-2b-FITC, 15-5-5, C3H IgG2a, Pharmingen; H-2a-FITC, KH95, BALB/c IgG2b, Pharmingen; H-2d-FITC, 34-12-2, C3H IgG2a, Pharmingen; Gr-1, RB6-8C5, Rat IgG2b, Pharmingen; MAC-1-FITC, M170.1, rat IgG2b, Boehringer Mannheim (Indianapolis, IN); Ly6/9E-PE, D7, rat IgG2a, Pharmingen; and c-kit-biotin, 3C1, rat IgG2b, Pharmingen. In the three-color sorts, biotinylated β-TCR, γδ-TCR, or C-kit mAb was counterstained with streptavidin Cy-5 (Pharmingen).

Multiparameter live sterile cell sorting. Populations were positively selected from BM using multiparameter, live sterile cell sorting (FACStar Plus; Becton Dickinson). Briefly, BM was isolated as previously described and resuspended at a concentration of 70 to 150 × 10⁶ cells/mL in sterile cell sort media (CSM), which consisted of sterile Hanks' Balanced Salt Solution without phenol (GIBCO), 2% heat-inactivated fetal calf serum (FCS; GIBCO), 2 μL/mL HEPES buffer (GIBCO), and 150 μg/mL of Gentamicin (GIBCO). Directly labeled MoAbs were added at saturating concentrations and the cells were incubated for 45 minutes. If biotinylated antibodies were used, cells were washed once for 10 minutes at 1,000 rpm using sterile CSM and then Streptavidin Cy-chrome was added. Cells were incubated with antibody for 15 to 20 minutes, washed twice, and resuspended in CSM at 5 to 10 × 10⁶ cells/mL. BM cells were then aliquoted into 12 × 75 mm tubes (Falcon; 1 mL/tube) coated with 10% FCS to prevent cells from sticking to the sides of the tubes.

All cells, samples, and collecting tubes were maintained on ice throughout the sorting process whenever possible. Seventy percent ETOH was run through the machine to sterilize it at the start of the sort. Cells were collected into 1.5 mL of CSM. As controls, and to calibrate the flow cytometer, separate aliquots of BM were stained with directly labeled nonspecific MoAbs (anti-Leu-4; SK7; mouse IgG; Becton Dickinson) as well as antibodies directly labeled with the same fluorochrome used in the experiment. An additional aliquot of BM with no antibody added analyzed to quanititate autofluorescence. After the background controls were run, the stained sample was used for the sort.
was collected. In initial experiments, cells were collected from the regions consistent for lymphoid and myeloid regions. After determining that the populations of interest resided in the region of low to intermediate forward and side scatter (lymphoid gate), in all subsequent experiments cells were gated on forward and side scatter consistent with the conventional lymphoid gate. The populations of interest were collected with respect to FL1, FL2, and FL4 fluorescence. Fluorescein isothiocyanate conjugate (FITC) and phycoerythrin (PE) were collected on FL1 and FL2, respectively, using a 488-nm wavelength on an argon laser and Cy-chrome fluorescence was detected using a krypton laser. After the cells were sorted, each population was reanalyzed with respect to the cell surface markers used as criteria for sorting. After collection, tubes were placed on ice, pooled at the end of the day, and centrifuged at 1,000 rpm for 10 minutes. Isolated cells were resuspended and injected into lethally irradiated mice in a total volume of 1 mL MEM. Purity of the sorted populations was calculated by first analyzing the sorted populations with respect to forward and side scatter to exclude debris and then analyzing cells with respect to fluorescence. Cell populations which were less than 85% pure for the sorting criteria were not used in experiments. The average purity of cells sorted for these experiments has been 94% (range, 86% to 98%). Sorting time for the isolated cell populations varied between 8 and 12 hours. Yields for three-color experiments ranged from 7 × 10^3 to 1.2 × 10^6 cells per hour. In general, the CD8αβ-TCR+ population can be collected at an approximate rate of 1 × 10^6 cells per hour.

**Stem cell purification.** BM stem cells were purified by a modification of the approach previously described. Briefly, marrow was flushed from long bones, washed in MEM, treated with 0.1 mL ACK/40 × 10^6 cells for 15 to 20 seconds, and washed again before staining with directly conjugated MoAbs. The MoAbs chosen to isolate murine stem cells were Ly6A/E-PE, c-kit-biotin, and the following mixture of fluorescein-conjugated antilinesage (Lin) MoAbs: B220-FITC, αβ-TCR-FITC, CD8+FITC, GR-1-FITC, and MAC-1-FITC (described previously). After 45 minutes of incubation at 4°C, cells were washed, resuspended at 50 × 10^6 cells/mL, and incubated with streptavidin Cy-chrome at 4°C for an additional 20 to 30 minutes. Cells were again washed twice and then sorted by multiparameter live sterile cell sorting for Sca1+/c-kit<sup>dim</sup>-intermediate/Lin<sup>−</sup> cells within the conventional lymphoid gate. No “carrier” cells were added to support the isolated stem cells in any of these studies.

**RESULTS**

Phenotype of cellular fraction that facilitates engraftment: Antibody plus complement depletion studies. Reconstitution of lethally irradiated mice with a mixture of 5 × 10^6 TCD syngeneic plus 15 × 10^6 TCD allogeneic BM cells (A + B → A) results in stable engraftment of both syngeneic and allogeneic stem cells with production of multiple lineages. TCD of the allogeneic component of the mixed BM inoculum by complement mediated lysis using RAMB, a polyclonal sera directed against the theta (Θ) antigen expressed on brain tissue, or anti-Thy 1.2 plus complement (C) treatment results in a level of donor-derived cells averaging 50% (Table 1). When only the syngeneic component of the mixed BM inoculum is TCD and the allogeneic component administered is untreated, allogeneic engraftment is significantly enhanced, approaching 100% of peripheral blood lymphocytes in all recipients. These data therefore suggest that conventional methods for TCD may remove a significant proportion of the donor BM inoculum by antibody plus complement-mediated lysis without loss of the facilitating effect (Table 1). Adequacy of cellular depletions was confirmed using flow cytometric analysis with non-blocking as well as species and isotype-specific sandwich staining and was always below the limits of detection by flow cytometric analysis (0.5%). These data therefore, suggested that the facilitating cell population was Thy 1<sup>+</sup> and RAMB-reactive, but did not have a high density of cell surface expression for Mac-1<sup>+</sup>, B220<sup>+</sup>, CD8<sup>+</sup>, or CD4<sup>+</sup> (B220<sup>+</sup>) could be removed from the donor BM inoculum by antibody plus complement-mediated lysis without loss of the facilitating effect (Table 1). Adequacy of cellular depletions was confirmed using flow cytometric analysis with non-blocking as well as species and isotype-specific sandwich staining and was always below the limits of detection by flow cytometric analysis (0.5%). These data therefore, suggested that the facilitating cell population was Thy 1<sup>+</sup> and RAMB-reactive, but did not have a high density of cell surface expression for Mac-1<sup>+</sup>, B220<sup>+</sup>, CD8<sup>+</sup>, or CD4<sup>+</sup>.

**Purification of the facilitating cell fraction using one-color live sterile cell sorting: 5 × 10<sup>6</sup> RAMB B10 + 5 × 10<sup>6</sup> RAMB B10.BR + sorted fraction → B10.** To further characterize the facilitating cell population, live sterile rare event cell sorting was performed. A model was established in which the majority of lethally irradiated recipients repopulated with a very low level of allogeneic chimerism. When the ratio of RAMB-treated syngeneic to RAMB-treated allogeneic BM cells was 1:1 (5 × 10^6 TCD syngeneic plus 5 × 10^6 TCD allogeneic BM cells), 57% of lethally irradiated recipients populated as syngeneic and the overall mean for allogeneic chimerism was 13% (Table 2). Facilitation of allogeneic stem cell engraftment (>95% allogeneic chimerism) occurred reliably and reproducibly if class 1<sup>low</sup>/intermediate, CD45<sup>−</sup>, CD45R<sup>+</sup>, or CD8<sup>+</sup> BM cells were purified and coadministered with the established mixed BM inoculum (Table 2). The facilitating cell fraction was found to reside in the...
characteristic lymphoid gate, defined by intermediate forward scatter (size) and low side scatter (granularity) properties shown in red (Fig 1A). Cells stained for the facilitating markers (CD45R+ and class II*αβ) but sorted from the myeloid region (high forward and side scatter) did not facilitate engraftment (data not shown). The facilitating fraction must be matched to the MHC of the stem cell, because MHC-disparate BALB/c (H-2d) cells sorted for the same facilitating population did not express CD8. The use of the same MoAb for cell sorting experiments showed surprisingly negative for γδ-TCR, as well as for αβ-TCR. The expression of CD8 in the cellular fraction that was αβ-TCR+ but CD45R- depicted in blue (Fig 1B and C) was a separate population that was not quite as bright as that for the γδ-TCR+/CD8+/CD45R+ population depicted in black (Fig 1B). When sorted for γδ-TCR+/CD8- and CD45R+ (Fig 1C), this cellular fraction comprised approximately 0.4% (range, 0.04% to 0.62%) of the total BM and less than 1.6% of cells in the lymphoid gate. None of the recipients of the cellular fraction that facilitated engraftment developed external or histologic features of GVHD, whereas the majority of recipients (4/7) showed histologic changes in tongue and other external features of GVHD, along with weight loss and an unkempt appearance.

Although the facilitating effect was not removed by anti-CD8 antibody plus complement treatment of the BM, the use of the same MoAb for cell sorting experiments showed that the facilitating cell population did express CD8. The

<table>
<thead>
<tr>
<th>Phenotype of Sorted Population</th>
<th>Allogenic Donor</th>
<th>No. of Animals</th>
<th>Mean Allogeneic Engraftment (±SD)*</th>
<th>Mean Cell Dose x106 (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control groups</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No sorted fraction added</td>
<td>163</td>
<td>13 ± 17</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td>RAMB B10.BR added</td>
<td>B10.BR</td>
<td>40</td>
<td>7.5 (5-10)</td>
<td></td>
</tr>
<tr>
<td>Facilitating populations†</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Class II*αβ</td>
<td>B10.BR</td>
<td>12</td>
<td>98 ± 4</td>
<td>2.8 (1.9-3.7)</td>
</tr>
<tr>
<td>CD8+</td>
<td>B10.BR</td>
<td>6</td>
<td>99 ± 4</td>
<td>0.05 (0.01-0.09)</td>
</tr>
<tr>
<td>CD45+</td>
<td>B10.BR</td>
<td>4</td>
<td>98 ± 4</td>
<td>5.4 (4.0-8.7)</td>
</tr>
<tr>
<td>CD45R+</td>
<td>B10.BR</td>
<td>2</td>
<td>98 ± 2</td>
<td>2.6 (2.4-2.7)</td>
</tr>
<tr>
<td>Nonfacilitating populations</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Class II*αβ</td>
<td>B10.BR</td>
<td>12</td>
<td>10 ± 13</td>
<td>0.6 (0.1-1.9)</td>
</tr>
<tr>
<td>Class II-</td>
<td>B10.BR</td>
<td>12</td>
<td>7 ± 9</td>
<td>3.4 (1.2-5.7)</td>
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<tr>
<td>CD8-</td>
<td>B10.BR</td>
<td>2</td>
<td>25 ± 7</td>
<td>0.45 (0.4-0.5)</td>
</tr>
<tr>
<td>CD4-</td>
<td>B10.BR</td>
<td>2</td>
<td>5 ± 6</td>
<td>3.8 (3.7-3.9)</td>
</tr>
<tr>
<td>B220+</td>
<td>B10.BR</td>
<td>4</td>
<td>24 ± 30</td>
<td>4.1 (3-5.1)</td>
</tr>
<tr>
<td>CD41</td>
<td>B10.BR</td>
<td>6</td>
<td>33 ± 29</td>
<td>0.29 (0.20-0.36)</td>
</tr>
<tr>
<td>Mac 1</td>
<td>B10.BR</td>
<td>6</td>
<td>18 ± 21</td>
<td>3.7 (3.6-3.9)</td>
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<tr>
<td>NK 1.1</td>
<td>B10.BR</td>
<td>2</td>
<td>11 ± 1</td>
<td>0.04 (0.03-0.05)</td>
</tr>
<tr>
<td>CD8+</td>
<td>BALB/c</td>
<td>2</td>
<td>27 ± 31</td>
<td>0.09 (0.05-0.12)</td>
</tr>
<tr>
<td>CD45R+</td>
<td>BALB/c</td>
<td>2</td>
<td>24 ± 16</td>
<td>4.9 (4.7-5.1)</td>
</tr>
<tr>
<td>CD8+ /CD45R+ /γδ-</td>
<td>BALB/c</td>
<td>4</td>
<td>13 ± 1</td>
<td>0.07 (0.02-0.11)</td>
</tr>
</tbody>
</table>

All mice were conditioned with total body irradiation and received 5 x 10⁶ RAMB-treated B10 + 5 x 10⁶ RAMB-treated B10.BR bone marrow cells plus a sorted fraction from donor bone marrow. Each experiment was repeated at least twice. The purity for cell sorting ranged from 85% to 99%. All sorts were performed using a Becton Dickinson FACStar Plus with forward and side scatter profile of intermediate forward scatter and low side scatter characteristic for the lymphoid gate shown in Fig 1A.

* Percentage of PBL chimerism was normalized to 100% as previously described. This represents the mean ± SD of allogeneic chimerism for all experiments performed.

† The level of engraftment was statistically higher in the facilitating populations than the nonfacilitating populations (P = .019, using two-tailed Student's t-test).
level of CD8 expression on the facilitating cell population was slightly less than that for \( \alpha\beta\)-TCR\(^+\)/CD8\(^+\) T cells (Fig 1B and C). The disparity between the cell sorting and complement lysis experiments may be caused by an insufficient number of CD8 molecules on the cell surface to allow efficient complement lysis or by the inefficiency of complement lysis compared with cell sorting techniques. Because as few as 10,000 CD8\(^+\) cells are sufficient to facilitate engraftment, this number of cells may have remained as a residual after complement depletion.

Two distinct isoforms of the CD8 molecule have been identified: CD8 \( \alpha\alpha \) homodimers and CD8 \( \alpha\beta \) heterodimers. \(^{29}\) CD8\(\alpha\alpha\) is acquired extrathymically, whereas CD8\(\alpha\beta\) is believed to be acquired only in the thymus. Donor-
Table 3. Effect of Addition of Cellular Subsets Purified by Live Sterile Cell Sorting on Facilitation of Allogeneic Engraftment

<table>
<thead>
<tr>
<th>Phenotype of Sorted</th>
<th>Allogeneic Donor</th>
<th>No. of Animals</th>
<th>Mean Allogeneic Engraftment (±SD)</th>
<th>Mean Cell Dose x 10^9 (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Facilitating populations</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD4^+CD45R^+ B10.BR</td>
<td>2</td>
<td>97 ± 1</td>
<td>5.7 (4.6-6.8)</td>
<td></td>
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<tr>
<td>B220^-/CD45R^+ B10.BR</td>
<td>4</td>
<td>99 ± 2</td>
<td>2.4 (0.9-3.8)</td>
<td></td>
</tr>
<tr>
<td>CD8^-/CD3^- B10.BR</td>
<td>10</td>
<td>97 ± 4</td>
<td>0.8 (0.4-1.2)</td>
<td></td>
</tr>
<tr>
<td>CD8^+/CD45R^+ B10.BR</td>
<td>3</td>
<td>98 ± 9</td>
<td>0.04 (0.02-0.07)</td>
<td></td>
</tr>
<tr>
<td>CD4^-/CD8^- B10.BR</td>
<td>2</td>
<td>98 ± 1</td>
<td>0.05 (0.03-0.07)</td>
<td></td>
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<tr>
<td>CD8^-/CD45R^-/αβ-TCR^- B10.BR</td>
<td>4</td>
<td>98 ± 8</td>
<td>0.04 (0.03-0.06)</td>
<td></td>
</tr>
<tr>
<td>CD8^-/CD45R^-/γδ-TCR^- B10.BR</td>
<td>2</td>
<td>99 ± 6</td>
<td>0.05 (0.03-0.08)</td>
<td></td>
</tr>
<tr>
<td>CD8^-/CD3^-/αβ-TCR^- B10.BR</td>
<td>4</td>
<td>96 ± 2</td>
<td>0.03 (0.017-0.05)</td>
<td></td>
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<tr>
<td>Nonfacilitating populations</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD4^-/CD45R^- B10.BR</td>
<td>2</td>
<td>8 ± 7</td>
<td>0.07 (0.03-0.11)</td>
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<tr>
<td>B220^-/CD45R^- B10.BR</td>
<td>4</td>
<td>8 ± 13</td>
<td>3.1 (1.7-4.2)</td>
<td></td>
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<tr>
<td>CD8^-/CD45R^- B10.BR</td>
<td>10</td>
<td>14 ± 18</td>
<td>3.7 (2.1-4.9)</td>
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<td>CD8^-/CD3^- B10.BR</td>
<td>3</td>
<td>39 ± 21</td>
<td>0.9 (0.6-1.5)</td>
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<td>CD8^-/CD45R^-/αβ-TCR^- B10.BR</td>
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<td>6 ± 5</td>
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<tr>
<td>CD8^-/CD45R^-/γδ-TCR^- B10.BR</td>
<td>2</td>
<td>31 ± 1</td>
<td>0.2 (0.1-0.08)</td>
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<tr>
<td>CD8^-/CD3^-/αβ-TCR^- B10.BR</td>
<td>2</td>
<td>17 ± 4</td>
<td>0.02 (0.01-0.03)</td>
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<tr>
<td>CD8^-/CD8^- B10.BR</td>
<td>2</td>
<td>21 ± 1</td>
<td>0.06 (0.015-0.048)</td>
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To further characterize the cell surface phenotype of the facilitating population, two- and three-color flow cytometric sorting was performed on donor bone marrow. All mice were lethally conditioned and received a mixed bone marrow inoculum of T-cell-depleted syngeneic and allogeneic bone marrow, as well as populations sorted from allogeneic bone marrow, as described in Table 1. In these experiments, the cells were sorted by first gating on the conventional lymphoid region as shown in Figs 1A and 2A. Usually one population was excluded from the collection gate based on FL4 and the remaining cells were gated based on FL1 and FL2 fluorescence, using a Becton Dickinson FacStar Plus.

derived CD8^-+/CD8^- cellular fractions facilitated engraftment, whereas CD8^-+/CD8^- fractions did not, suggesting that cells expressing CD8^-/αβ heterodimers are responsible for facilitation of stem cell engraftment (Table 3).

The CD8^- cells that facilitated engraftment were CD3^- but negative for αβ-TCR as well as γδ-TCR. The CD8^-/CD3^-/TCR^- fraction of BM-derived cells obtained from the lymphoid gate represented in red (Fig 2A) facilitated engraftment, whereas the CD8^-/CD3^-/TCR^- fraction did not (Table 3). CD3 expression was slightly lower in the TCR^- fraction (shown in green; Fig 2C) than in the TCR^- fraction shown in black (Fig 2B). The populations of CD8^-+/αβ-TCR^- (Fig 3B) and CD3^-+/αβ-TCR^- (Fig 3C) cells in the BM are distinct and separate from their T-cell counterparts as analyzed by flow cytometry.

Purified facilitating cells are sufficient to permit engraftment of purified allogeneic stem cells. To confirm that the facilitating cell population was sufficient to permit engraftment of purified stem cells in an allogeneic recipient, purified stem cells were coadministered with purified facilitating cells (Table 4). Although 1,000 stem cells purified from B10.BR BM reliably reconstituted lethally irradiated syngeneic B10.BR recipients (group D), none of the B10 recipients of allogeneic stem cells alone, even at 10-fold higher doses, were rescued from radiation-induced aplasia (group C). In striking contrast, all recipients of 10,000 puri-
fied allogeneic stem cells plus 30,000 facilitating cells MHC-matched to the donor stem cell engrafted and repopulated as fully allogeneic (group A). Equivalent numbers of purified facilitating cells alone did not rescue syngeneic recipients from radiation-aplasia, suggesting that the facilitating cell population does not contain stem cell activity (group E). These data support the concept that a component of donor BM, distinct from the hematopoietic stem cell, is required for engraftment of the stem cell in an allogeneic environment.

Evidence for multilineage production in recipients of purified stem cells plus facilitating cells. The pluripotent stem cells produces at least 11 lineages. In the mouse, hematolymphoid progenitors survive at most 4 months.29 To examine whether engraftment of the pluripotent allogeneic stem cell and/or self-renewing progenitors had occurred, recipients of purified stem cells plus facilitating cells as well as other facilitated recipients were analyzed at 5 months after reconstitution for the presence of multilineage allogeneic chimerism in the spleen, BM, and thymus. It is not of note that, in some animals, donor chimerism was not 100% (range, 64% to 99%) and varied between lineages, especially T cells (31% to 99%). Nonetheless, in all recipients, donor-derived cells of lymphoid (T cells, B cells, and NK cells) and myeloid (monocyte and macrophage) lineages were consistently present at greater than 4 months, suggesting that the pluripotent hematopoietic stem cell had engrafted.

Morphology of facilitating population. Sorting for either αβ-TCR/CD8+/CD45R- or the class IIint/intermediate cellular population yielded a mixture of cells including a granular lymphoid-appearing cells plus a predominance of unique granule-filled cells with a characteristic morphology (Fig 4A and B). The granule-filled cells were homogeneous in morphology (8 to 10 μm in diameter) with a high concentration of centrally placed granules and a pericytoplasmic skirt relatively free of granules. The presence of large numbers of highly dense granules and the horseshoe-shaped lobulated nucleus is uncharacteristic of any T-cell or B-cell population, because lymphoid cells have a rounded nucleus with scant granular cytoplasm and a high nuclear:cytoplasmic ratio. Studies are in progress to analyze the content of the granules as well as to further purify the facilitating cell fraction phenotypically using four- and five-color cell sorting.

DISCUSSION

The field of allogeneic BMT has progressed dramatically over the past 20 years, resulting in an expansion in the scope of diseases that can be treated by this modality. BMT is now considered an accepted therapy for numerous hematologic malignancies, aplastic and lineage-deficient anemias, metabolic disorders, and solid tumors. Despite major advances in the field, two major limitations exist: GVHD and a delay in or failure of engraftment. GVHD has posed a significant and longstanding limitation to allogeneic BM transplantation and remains the primary cause of morbidity and mortality. Thirty to 50% of recipients of genotypically HLA-identical untreated (T-cell replete) BM from living related donors develop severe, and potentially life-threatening, GVHD.1-6 At least 15% of these patients die of complications related to acute or chronic GVHD.1-6 The occurrence of GVHD in recipients of HLA-mismatched BM grafts, or HLA-identical grafts from unrelated donors, is even greater, approaching 80% in some reports.1-6 Because only 20% of patients who require BMT have a suitably matched family member donor, a significant fraction of all patients who are potential candidates for BMT are at high risk for GVHD. Strategies to prevent GVHD would allow the indications for BMT to expand significantly in scope for nonmalignant diseases in which such high morbidity cannot currently be justified.

After T cells were identified as the primary effector cell in GVHD,30-34 strategies to deplete the donor BM of T cells using combinations of agents were applied in clinical protocols.6 GVHD was successfully prevented. However, this triumph was accompanied by the tragedy of a 20% to 40% incidence of failure of engraftment for HLA-identical and a 50% to 70% incidence in graft failure when one or two HLA haplotype mismatches were present.1,6 Because failure of engraftment is often a life-threatening complication, TCD protocols were abandoned and GVHD was accepted as an unavoidable complication of BMT. These clinical data, as well as data from rodent models, strongly suggested that T cells were required for engraftment of the BM stem cell in an allogeneic environment. In most of the clinical trials for TCD in which graft failure occurred, antibodies were used against at least one cell surface marker present on the facilitating cell population described in this manuscript.1,6

The role of T cells in engraftment has been studied using a number of mouse models. In experiments using mice with a hereditary stem cell deficiency has recipients (Ww'), Sharkis et al14 showed that the defect in red blood cell production could be cured with BMT from hematopoietically normal littersmates without radiation or cytoablation. Thus,

Table 4. Facilitating Cells Are Sufficient to Permit Engraftment of Purified Stem Cells in Allogeneic Recipients

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<td>30,000</td>
<td>B10.BR</td>
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<td>6</td>
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<td>D</td>
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<td>1,000</td>
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<td>8</td>
<td>100% B10.BR</td>
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Lethally irradiated B10 or B10.BR mice received purified stem cells (c-kit+/Sca-1+/lin-) or a mixture of stem cells plus facilitating cells. No additional cells were administered. Survival was observed daily. Surviving animals were typed for PBL chimerism at 1 month and then monthly thereafter.

Abbreviation: GF, failure of engraftment defined by death in the same time as radiation controls.

References
FACILITATION OF ALLO-STEM CELL ENGRAFTMENT

In another rodent model, Lapidot et al.\textsuperscript{16,17} showed that as few as 50,000 CD8\textsuperscript{+} BM cells from nude mice donors coadministered with BM stem cells permitted engraftment in normal F1 recipients. CD4\textsuperscript{+} BM cells from the same nude mice donors did not mediate this effect. In a model of BMT dependent on the presence of T cells for donor engraftment, the addition of as few as 0.5 × 10\textsuperscript{5} CD8\textsuperscript{+} cells to TCD donor BM allowed donor reconstitution.\textsuperscript{13} In these studies, panning techniques were used to isolate heterogeneous cells expressing markers of interest from donor BM. Populations of CD4 cells did not mediate that same effect, providing further evidence for a facilitating population that is CD8\textsuperscript{+} and CD4\textsuperscript{−}.\textsuperscript{35} Others showed that depletion of T cells from the allogeneic BM inoculum resulted in NK cell-mediated graft rejection of marrow in SCID mice, but this effect could be overcome by the addition of donor-specific thymocytes to the inoculum.\textsuperscript{17}

We have now applied the model for mixed chimerism to establish the cell surface phenotype of the cell(s) that facilitate engraftment of the allogeneic BM stem cell. Because it is well known that in vitro colony assays do not reflect the conditions necessary or sufficient for stem cell engraftment, we elected to use an in vivo assay for engraftment to characterize the facilitating cell population. Most in vivo models of engraftment have been limited by relying on death as an endpoint for failure of engraftment. However, in mixed reconstitution, one can examine the level of allogeneic engraftment. We report here for the first time that engraftment of BM stem cells is facilitated by a phenotypically unique cellular population in donor BM that expresses CD45\textsuperscript{+}, CD45R\textsuperscript{+}, CD8\textsuperscript{+}, CD3\textsuperscript{+}, class II\textsuperscript{dull/intermediate}, but is TCR\textsuperscript{−}. The coadministration of as few as 10,000 of these cells, MHC-matched to the stem cell donor, is sufficient to mediate stem cell engraftment in an allogeneic environment.

Using live sterile rare-event multiparameter cell sorting, we have characterized the phenotype of a unique cell population that comprises approximately 0.4% of total BM and ≤1.6% of total cells in the lymphoid gate that reliably and reproducibly facilitates engraftment of purified stem cells in allogeneic recipients. As few as 30,000 of these cells was sufficient to facilitate engraftment of highly purified stem cells (c-kit\textsuperscript{+}/Sca-1\textsuperscript{−}/Lin\textsuperscript{−}), whereas 10,000 purified stem cells alone did not engrant in genetically disparate recipients. The presence of multilineage progeny of donor origin at ≥5 months after reconstitution with purified stem cells plus facilitating cells strongly suggests that the pluripotent hematopoietic stem cell has engrafted rather than committed progenitors. Moreover, the facilitating cell fraction is not a stem cell population because syngeneic recipients of purified facilitating cells alone were not rescued from radiation-induced aplasia.

Although the facilitating effect was not removed by anti-CD8 MoAb plus complement treatment, the use of the same MoAb for cell sorting experiments showed that CD8 was a critical marker for facilitation of stem cell engraftment. One might postulate that the disparity between cell sorting and complement-mediated lysis experiments may be caused by a somewhat lower level of CD8 expression on the facilitating cell surface, resulting in an insufficient number of CD8 molecules to allow efficient complement-mediated cell lysis. This is supported by the flow cytometry profiles in Figs 2 and 3.
If very few cells are required for the facilitating effect, enough cells may have escaped complement-mediated lysis to mediate the effect yet not be detected by flow cytometric analysis.

Although the facilitating cell population is CD8+/CD3−, it is TCR−. T cells comprise a relatively small percentage of unfractionated murine BM cells. The T cells present in marrow have been attributed to contamination by PBLs. More recently, a dichotomy with regard to BM cells that express T-cell markers has been observed.30−33 Although it is thought that the TCR is required to stabilize the expression of CD3 in mature T cells, other rare cell types selected from BM have been identified as TCR− yet CD3+ and Thy 1+ in normal, as well as nude mice.30,31,35 Unlike mature T lymphocytes, cells of this phenotype exhibit MHC-unrestricted cytolytic activity and do not proliferate well in vitro. The population of TCR+/CD3+ cells present in BM appears distinct from the TCR−/CD3+ fraction that facilitates engraftment of allogeneic stem cells, and intensity of CD3 expression is not quite as bright as for the TCR− fraction. Studies are in progress to identify whether a receptor unique to the facilitating cell population serves as a surrogate molecules to stabilize CD3 expression.

One may hypothesize that a cell–cell interaction is required for stem cell engraftment to occur, because small numbers of MHC-matched cellular fractions facilitated engraftment of allogeneic stem cells, but third-party cellular fractions did not. One could hypothesize at least three mechanisms of action. The facilitating cell may provide a nurturing environment for allogeneic stem cells, but third-party cellular fractions did not. The facilitation of programmed cell death of T lymphocytes was shown to block engraftment or interfere with survival of the host stem cells. Studies have shown that the veto effect may be mediated in part by cells that express CD8.34,35 The initiation of programmed cell death of T lymphocytes was shown to be mediated by the CD8 molecule independent from the TCR using transgenic cell lines with CD8 uncoupled from TCR expression.36 Studies are currently in progress using ex vivo live cell imaging and long-term BM culture to characterize the mechanism of action of the facilitating cell population. A third mechanism of action could be a direct cytotoxic effect on host stem cells. Data using the αβ-TCR+/CD8+ fractions argues against this hypothesis, because TCR+/CD8+ are sufficient to mediate GVHD, but do not routinely facilitate allogeneic stem cell engraftment.

It is well recognized that the BM microenvironment exerts an influence on stem cell engraftment and survival.37,38 Unfractionated BM contains a considerable proportion of stromal and adherent cells. Interaction of the stem cell with these other cells may explain why successful engraftment and function of allogeneic BM occurs when less-pure stem cell fractions are administered. The marrow stroma is believed to provide an environment for stem cell “seeding” and has been shown to influence proliferation, differentiation, and long-term hematolymphopoiesis. Recent data suggest that it is the microenvironment that prevents the pluripotent stem cell from undergoing one of two undesirable fates: apoptosis or terminal differentiation. In a long-term BM culture (LTC) system, it was shown that host stroma cells could support donor-derived stem cells only if there was MHC-genotypic identity or haploidentity between the donor-recipient pair.39-41 Another more recent study suggests that stromal cell–stem cell contact was not necessary for the short-term maintenance and differentiation of CD34+/DR+ stem cells over an 8-week period in vitro.42 However, additional “local” factors produced by the stroma were necessary for the stem cell to survive and function. Studies are in progress to examine the mechanism of action of the facilitating cell on stem cell survival, propagation, and differentiation in LTC and to determine the optimum ratio of cell types as well as whether facilitating cells alone are sufficient to promote survival of purified stem cells in LTBMCS.

The coexpression of class II and CD45R on the facilitating cell suggests that these cells may represent a subset of cells of dendritic-type lineage. Dendritic cells, with their characteristic morphology of elongated interdigitating processes, were initially believed to be of stromal (non-BM derived) origin.42-48 They have since been proven to be of BM origin and are probably derived from the myeloid lineage.49 The distribution of these cells is widespread and includes all of the tissue from which cellular isolates which promote engraftment of the BM stem cells have been obtained.49 Mature dendritic cells are CD45+, CD45R+, and class II(high).49,50 Although previously believed to be CD8− and Thy 1+, recent reports identified a subset of precursor dendritic cells isolated from both thymus and spleen that were CD8−,49 The thymic, but not splenic, dendritic cells also expressed Thy 1. Precursor dendritic cells isolated from PB were shown to express CD45 and CD45R and were class II(dim/intermediate) but not class II(high), but were CD8−.49 The phenotype of the cellular population that facilitates engraftment of the allogeneic BM stem cell shares many of these markers and may, therefore, represent a subset of cells from the same lineage. However, the morphology for the facilitating cell fraction differs significantly from that for dendritic cell precursors in the BM.49 Moreover, dendritic cells propagated in vitro from BM by the method described by Inaba et al42 did not facilitate engraftment from day 1 to day 10 in culture (data not shown).

The present studies strongly suggest that the facilitating cell fraction is not a T, B, or NK cell, because removal of these cellular fractions from the BM inoculum did not remove the facilitating effect. Moreover, most T cells in the mouse do not express class II, even in the activated state. Recipients of highly purified facilitating cell fractions did not develop clinical or histologic features of GVHD, whereas most of the animals that received αβ-TCR+ fractions did. This finding is important to potential clinical application of this observation for prevention of GVHD because these are the cells that are the primary effectors of GVHD in all species examined. Therefore, it may be possible to coadminister purified MHC-specific facilitating cells plus purified stem cell fractions to promote engraftment across allogeneic and possibly even xenogeneic barriers without the morbidity and
mortality associated with GVHD. The use of a cellular component therapy similar to that for blood banking may allow for a focused approach to achieve reliable engraftment and yet overcome the limitation of GVHD currently encountered in clinical BMT. As a result, the scope of diseases that could be treated by BMT could be expanded significantly.

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Phenotypic characterization of a novel bone marrow-derived cell that facilitates engraftment of allogeneic bone marrow stem cells

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