Induction of Donor-Type Chimerism and Transplantation Tolerance Across Major Histocompatibility Barriers in Sublethally Irradiated Mice by Sca-1+Lin- Bone Marrow Progenitor Cells: Synergism With Non-Alloreactive (Host × Donor)F1 T Cells

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Transplantation tolerance by means of bone marrow (BM) transplantation could become a reality if it was possible to achieve engraftment of hematopoietic stem cells under nonlethal preparatory cytoreduction of the recipient. To that end, BM facilitating cells, veto cells, or other tolerance-inducing cells, have been extensively studied. In the present study, we show that BM cells within the Sca-1+Lin- cell fraction, previously shown to be enriched for early hematopoietic progenitors, are capable of reducing specifically antidonor CTL-p frequency in vitro and in vivo, and of inducing split chimerism in sublethally 7-Gy-irradiated recipient mice across major histocompatibility complex barriers. The immune tolerance induced by the Sca-1+Lin- cells was also associated with specific tolerance toward donor-type skin grafts. The minimal number of cells required to overcome the host immunity remaining after 7 Gy total body irradiation is very large and, therefore, it may be very difficult to harvest sufficient cells for patients. This challenge was further addressed in our study by demonstrating that nonalloreactive (host × donor)F1 T cells, previously shown to enhance T-cell-depleted BM allografts in lethally irradiated mice, synergize with Sca-1+Lin- cells in their capacity to overcome the major transplantation barrier presented by the sublethal mouse model. © 1999 by The American Society of Hematology.
TBI from a Gamma beam 150-A60 Co source (produced by the Atomic
non-alloreactive T cells, previously shown 23 to enhance engraft-
quantity in humans. Therefore, we explored other cells
that might synergize with the Sca-1"Lin- cells in overcoming
the challenge of the marked host immunity typical of the sublethal
mouse model. To that end, the potential of (host × donor)F1
non-alloreactive T cells, previously shown21 to enhance engr-
agement of T-cell–depleted BM allografts in lethally irradiated
recipients, was evaluated.

MATERIALS AND METHODS

Animals. Mice used were female 6- to 12-week-old C3H/HeJ and
4- to 6-week-old BALB/c, C57BL/6, C57BL/Beige, and (C3H/
HeJ × C57BL/6)F1, obtained from the Roscoe B. Jackson Memorial
Laboratory (Bar Harbor, ME) or the Weizmann Institute Animal
Breeding Center (Rehovot, Israel). All mice were kept in small cages (5
animals in each cage) and fed sterile food and acid water containing
ciprofloxacin (20 µg/mL).

Purification of murine stem cells by magnetic sorting. BM cells
were prepared as previously described,23 and then subjected to a stem
cell purification procedure based on their expression of stem cell
antigen-1 (Sca-1)25 and the lack of expression of cell-surface antigens
associated with differentiated hematopoietic cell lineages typical for B
cells (CD45/B220), myelomonocytic cells (CD11b/mac-1), and T cells
(CD4/Ly2, CD8/Ly-2). Sca-1–positive, lineage-negative cells (Sca-
1 "Lin") were purified from 6 to 10 × 10^9 BM cells (obtained from 100
C57BL/6 donors 5 to 9 weeks old, respectively), which were initially
enriched for monoclonal cells by separation on Ficoll-Paque Plus
(Pharmacia Biotech AB, Uppsala, Sweden). BM cells (50 × 10^9/mL) were
layered on Ficoll (50 mL) in 50-mL Falcon tubes (Becton
Dickinson, San Jose, CA). The cells were centrifuged at room tempera-
ture, at 800g for 30 minutes, and the cells in the Ficoll interface were
collected. This cell fraction was magnetically labeled with anti–Sca-1
antibodies conjugated to microbeads using a multi-parameter magnetic
cell sorting (MACS) Sca-1 Multisort Kit (Miltenyi Biotec, Bergisch
Gladbach, Germany). Sca-1–labeled cells were then purified by a
positive selection column in a magnetic field. The microbeads were then
removed from the Sca-1 cells using Multisort release reagent, to allow
subsequent magnetic labeling and separation of Sca-1 cells according
to expression of lineage markers. The depletion of Sca-1+ cells
expressing lineage markers was performed by positive selection follow-
ing labeling of Sca-1+ cells with MACS microbeads conjugated to antibodies directed against CD45/B220, CD8/Ly2, CD4/Ly3, CD11b/
mac-1, and anti-NK (DX5), when natural killer (NK) cells were
depleted. The negative and positive fractions of this separation, the
Sca-1 "Lin-" and Sca-1 "Lin+" cell fractions, were collected. In a typical experiment starting with 6 × 10^8 BM cells, cell recovery after Ficoll
fractionation and in the Sca-1-, Sca-1 "Lin-" or in the Sca-1 "Lin+" cell
fraction was 2 × 10^7, 1 × 10^7, 7.5 × 10^6, and 5 × 10^6, respectively.
Cytotoxicity analysis of the fractionated cells was performed by
double-immunofluorescent staining, using the following directly la-
beled antibodies (obtained from Pharmingen San Diego, CA): fluores-
cienc isothiocyanate (FITC)-Sca-1/Ly6A/E (clone D7), R-phycocery-
thrin (PE)-CD11b (clone M1/70), PE-CD8a/Ly-2 (clone 53-6.7),
PE-CD45/B220 (clone RA3-6B2), PE-CD4/Ly3 (clone RM-4.5), and
PE-pan-NK cells (clone DX5) for NK cell detection. Nonspecific
staining was analyzed with rat Ig isotype controls: FITC-rat IgG1,
PE-rat IgG2a, and PE-rat IgG2b.

Preparation of non-alloreactive F1 T cells. Thymocytes from
(C3H/HeJ × C57BL/6)F1 mice were separated by differential agglutina-
tion with peanut agglutinin into mature and immature fractions as
described.26

Irradiation and BM transplantation. Mice were exposed to a single
dose of 7 Gy (sublethal conditioning) or 10 Gy (lethal conditioning)
TBI from a Gamma beam 150-A60 Co source (produced by the Atomic
Energy of Canada, Kanata, Ontario, Canada) with focal skin distance of
75 cm, at a 0.65 Gy/min dose rate. The following day the mice received,
intravenously, BM subpopulations as described in Results. In the
experiments studying the facilitating effect of non-alloreactive T cells,
mice were injected with Sca-1 "Lin-" cells supplemented with F1 T cells.

Chimerism analysis. Chimerism was determined 30 days posttrans-
plant by cytofluorometry. Mice were bled from the retro-ocular vein
using heparin-coated glass capillaries. Peripheral blood cells were
fractionated on Ficoll-Paque plus, and the isolated mononuclear cells of
each mouse were triple-stained by direct immunofluorescence, with
FITC anti-H2b monoclonal antibody specific for the donor, PE anti-H2k
for host-type major histocompatibility complex (MHC) antigens, and
Cy-chrome-anti-CD3 antibody. The following antibodies were obtained
from Pharmingen (San Diego, CA): FITC-H-2b (clone 34-2-12),
PE-H-2k (clone 36-7-5), FITC-H-2k (clone AF6-88.5), and Cy-
Chrome-CD3e (clone: 145-2C11).

Skin grafting. Skin grafting was performed as previously de-
scribed,27 with one additional step. Briefly, a circular piece of skin was
removed from the recipient mouse and replaced by skin taken from the
donor’s trunk after it had been cleaned of fat and connective tissue. The
attachment of the skin graft to the recipient was achieved by spraying
several thin layers of acrylic plastic spray (Nobucourt Spray; Astra,
Sodertalje, Sweden) without any accessories. After the plastic dried (3
minutes), several layers of antibiotics (Rikospray antibiotic; Riker
Laboratories, Loughborough, UK) were sprayed on the graft, and the
mice were then transferred separately into individual small cages in
which they were kept throughout the observation period. The trans-
planted skin grafts were observed daily and the graft status was
recorded, with an acceptor score based on size, color, contraction, and
hair growth of the graft. Rejection started with the loss of hair and
culminated in necrosis of the graft epithelium that was occasionally
associated with ulcer formation. Definite rejection was taken as the
time of complete sloughing, or when a dry scab was formed.

Veto activity of Sca-1 "Lin-" cells. To determine whether mouse
Sca-1 "Lin-" cells possess veto activity, spleen cells from C3H/HeJ mice
(2 × 10^9/mL) were incubated for 5 days with irradiated (25 Gy)
allogeneic spleen cells (1 × 10^6/mL) from C57BL/6 (stem-cell matched)
or BALB/c (third party) mice. C57BL/6 Sca-1 "Lin-" or Sca-1 "Lin+" cells
were added to the primary MLC at a 0.5:1 veto:responder cell ratio.
The responder cells were then recultured under limiting dilution conditions
for 7 days. The CTL-p activity was determined by 51Cr-release assay.

Direct limiting dilution culture of CTL-p.Responder cells of the
veto activity cultures or splenocytes of C3H/HeJ chimeric mice
(irradiated by 7 Gy TBI and transplanted with 2 × 10^7 Sca-1 "Lin-" cells
from C57BL/6 donors) were fractionated on Ficoll-Paque plus and the
isolated mononuclear cells were plated in a limiting dilution culture.
Responder cells (40 to 0.16 × 10^5) were cultured for 7 to 8 days in
round-bottomed 96-well plates (Nunc, Roskilde, Denmark) in 16
replicates, in the presence of 10^5 cells/well of irradiated (20 Gy)
allogeneic (C57BL/6, BALB/c) or syngeneic (C3H/HeJ) splenocytes
and human recombinant interleukin-2 (10 U/mL; Eurocetus, Milan,
Italy) in a final volume of 0.2 mL complete tissue culture medium
(CTCM) at 37°C in a 10% CO2 incubator. CTCM is RPMI 1640
which contains 2 mM/L L-glutamic acid, 100 U/mL penicillin, 0.1 mg/mL
streptomycin, 2 mM/L HEPES, 1 mM/L sodium pyruvate, 0.1
mM/L nonessential amino acids, and 5 × 10^-5 mol/L 2-mercaptoetha-
nol, supplemented with 10% fetal calf serum (Biological Industries,
Kibbutz Bet Hekem, Israel).

Estimate of cytotoxic cell frequency. Splenocytes were harvested
from individual limiting dilution culture wells and were assayed for
cytotoxic activity by transferring a fixed volume (100 mL) to conical-
bottomed 96-well plates (Greiner, Frickenhausen, Germany) containing
5 × 10^5 51Cr-labeled Concanavalin A (Sigma, St Louis, MO) blasts of
C3H/HeJ, C57BL/6, or BALB/c, respectively, as target cells. As
described by Ryser and MacDonald,28 microwells were considered
positive for cytolytic activity when they exceeded the mean spontane-
ous release value (determined in a group of parallel wells that contained irradiated stimulating cells and CTCM, but no responding cells) by at least 3 standard deviations of the mean.

RESULTS

Induction of split chimerism by purified Sca-1<sup>+</sup>Lin<sup>-</sup> progenitor cells in sublethally irradiated allogeneic mice. To test the potential of cells within the hematopoietic progenitor cell fraction to overcome host resistance, we attempted to purify (using magnetic beads) Sca-1<sup>+</sup>Lin<sup>-</sup> BM cells from C57BL/6 donors, and we tested their capacity to induce chimerism in fully allogeneic C3H/HeJ recipients exposed to sub-lethal 7 Gy TBI. Figure 1 shows an analysis on a fluorescence-activated cell sorter (FACS) of a typical purification of Sca-1<sup>+</sup>Lin<sup>-</sup> cells afforded by the MACS double-step procedure. The average frequency of these cells in the initial BM fraction that was applied to the MACS procedure (depleted of red blood cells and neutrophils by Ficoll separation) was 2.7% ± 1.1% (range, 1.8% to 5.1%), and after the 2-step fractionation procedure it was enriched to an average of 68.3% ± 9.8% (range, 53.5% to 84.4%).

Determination of the different subpopulations within the Lin<sup>+</sup> phenotype (Table 1) showed more than 1 log depletion of CD4, CD8, CD11b, and CD45/B220 cells from the Sca-1<sup>+</sup>Lin<sup>-</sup> cell fraction compared with that found in the Sca-1<sup>+</sup> cell fraction. Determination of donor-type chimerism after infusion of different cell numbers from the Sca-1<sup>+</sup>Lin<sup>-</sup> cell fraction showed an exponential dose-response curve similar to those

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<td>&lt;0.1</td>
<td>0</td>
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<tr>
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<td>3.8</td>
<td>0.7</td>
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BM cells were subjected to a stem cell purification procedure based on their expression of stem cell antigen-1 (Sca-1) and the lack of expression of cell surface antigens associated with differentiated hematopoietic cell lineages typical for B cells (CD45/B220), myelomonocytic cells (CD11b/mac-1), and T cells (CD4/L3T4, CD8/Ly-2), using MACS Sca-1 Multisort kit (see Materials and Methods). The fractions were analyzed by flow cytometry. The results represent 2 experiments in which the purity of Sca-1<sup>+</sup>Lin<sup>-</sup> was 56.6% and 63.4%, compared with 1.7% and 1.4% in the unseparated fraction and 4.2% and 5.0% in the Sca-1<sup>+</sup> cell fraction.

Fig 1. Purification of Sca-1<sup>+</sup>Lin<sup>-</sup> BM cells. Cells in the different cell fractions obtained by MACS purification (see Materials and Methods) were analyzed by FACS for the presence of Sca-1<sup>+</sup>Lin<sup>-</sup>, Sca-1<sup>+</sup>Lin<sup>-</sup>, and Sca-1<sup>+</sup> Lin<sup>-</sup> cells. Nonspecific staining was determined by FITC- and PE-conjugated isotype control antibodies. Percentage of each subpopulation is shown in the appropriate area of the dot plot.
previously described for T-cell–depleted transplants. The pooled results of 8 different experiments illustrating the chimerism level induced in each individual mouse exposed to 7 Gy TBI clearly show that substantial chimerism (>20%) was initially noticed upon infusion of $3 \times 10^5$ Sca-1$^+$Lin$^-$/cells (9 of 20), whereas recipients of $4 \times 10^5$ to $2 \times 10^5$ Sca-1$^+$Lin$^-$ cells very rarely exhibited low levels of donor-type chimerism (2 of 48) (Fig 2). In contrast, when C3H/HeJ recipients of C57BL/6 Sca-1$^+$Lin$^-$ BM cells were exposed to 10 Gy lethal TBI, about 75-fold fewer cells ($4 \times 10^3$ Sca-1$^+$Lin$^-$) were required for the induction of over 50% donor-type chimerism in 10 of 11 recipients (Fig 2). It seems that a large proportion of the cells, required to establish chimerism in the sublethal model, are involved in overcoming the larger number of host immune cells remaining after 7 Gy TBI, compared with the miniscule numbers surviving 10 Gy TBI. Considering that, in C3H/HeJ recipients of C57BL/6 BM, resistance is largely mediated by T-cell–mediated responses while NK-cell–mediated resistance is minimal, it should be noted that spleens of C3H/HeJ mice, 1 week after exposure to 7 Gy TBI, contain about 1 log more T cells compared to mice treated with 10 Gy TBI (unpublished results, January 1999). Indeed, FACS analysis of the chimeric mice obtained after exposure to sublethal TBI showed a substantial number of host-type T cells coexisting with donor-type T cells, as well as non-T cells (Fig 3). No GVHD, as defined by measurement of body weight and the general appearance of the mice, was observed in recipients of Sca1$^+$Lin$^-$ cells, in agreement with previous studies, defining the threshold for GVHD in lethally irradiated mice by more than an order of magnitude above the numbers that might have been infused in the present study (ie, $<1 \times 10^5$ T cell/mouse). Furthermore, considering that antihost donor alloreactivity generally manifests itself by eradication of host-type blood cells, our finding of durable long-term substantial mixed chimerism strongly suggests that if there is any T-cell contamination in the infused Sca1$^+$Lin$^-$ cell fraction (ie, below detection of our FACS analysis; Table 1), it is not associated with any form of clinical or subclinical GVHD.

Limit dilution analysis of CTL-p in the spleen of chimeric mice showed no detectable CTL-p against donor type cells (C57BL/6) while the frequency of CTL-p against a third party (BALB/c) was not significantly different from that found in irradiated mice that were not transplanted with Sca-1$^+$Lin$^-$ cells (Fig 4). To test the capacity of Sca-1$^+$Lin$^-$ cells to affect postthymic T cells that survive the sublethal irradiation and that present a barrier to engraftment during the immediate period posttransplant, we chose to evaluate the level of chimerism at 30 days after transplantation. However, we also tested donor-

![Fig 2](image_url)

**Fig 2.** Induction of donor type chimerism after transplantation of C57BL/6 Sca-1$^+$Lin$^-$ cells into lethally (10 Gy TBI) or sublethally (7 Gy TBI) irradiated C3H/HeJ recipients. The number of Sca-1$^+$Lin$^-$ cells infused was calculated based on FACS analysis.

![Fig 3](image_url)

**Fig 3.** Split chimerism after transplantation of C57BL/6 Sca-1$^+$Lin$^-$ cells (H2b) into sublethally irradiated (7 Gy) C3H/HeJ recipients (H2k). Percentages of donor and host T cells (determined by gating of CD3 stained cells) and non-T cells are shown in the appropriate FACS dot plots. Peripheral blood chimerism was determined 30 days posttransplant by cytofluorimetry (see Materials and Methods).
the selected phenotype and the level of chimerism associated with this enrichment. In 2 experiments, no chimeric mice were found upon infusion of \(1 \times 10^6\) unseparated cells, while following the first step of positive selection of Sca-1\(^+\) cells, infusion of \(1 \times 10^6\) cells of the Sca-1\(^+\) fraction led to substantial donor type chimerism (\(>20\%\)) in 10 of 14 recipients (Fig 5). The second step of negative selection led to further enhancement of engraftment (9 of 9) compared with that observed in the Sca-1\(^+\) cell fraction. While the latter enhancement is not statistically significant, there is clearly no reduction of chimerism induction associated with the more than 1 log depletion of Lin\(^+\) cells (Table 1), despite the exponential nature of our chimerism induction curves (Fig 2). It seems that, regardless of other cell phenotypes that might possess tolerizing or facilitating activity, the enrichment of Sca-1\(^+\)Lin\(^-\) cells, associated with depletion of different cell subpopulations sharing the Sca-1\(^+\)Lin\(^+\) phenotype, is also associated with the capacity to achieve engraftment in the face of marked host immunity remaining after the sublethal conditioning. Because several previous studies have shown that different cell subpopulations bearing CD8 are capable of enhancing BM allografts, we examined the presence of such cells in the different cell fractions. The FACS data of a typical experiment are illustrated in Fig 6, and the level of each cell phenotype (based on the FACS analysis), found in 2 independent experiments, is shown in Table 1. The percentage of CD8\(^+\) cells in the unseparated fraction was 0.6% to 0.7%. After purification for Sca-1\(^+\) cells, the level of CD8\(^+\) cells was enriched to 2.7% and 3.4%, respectively. After depletion of Lin\(^+\) cells it was reduced to <0.1% and <0.2%, respectively, in the Sca-1\(^+\)Lin\(^-\) cell fraction. Thus, although the enhancement of donor-type chimeras in recipients of Sca-1\(^+\) cells could be mediated by either Sca-1\(^+\)CD8\(^+\) or Sca-1\(^+\)Lin\(^-\) cells, both of which are enriched in this fraction, the high level of chimerism found after the second fractionation step, which is associated with at least a 20-fold depletion of CD8\(^+\) cells along with a 12-fold enrichment of Sca-1\(^+\)Lin\(^-\) cells, strongly suggests that a CD8-negative cell in the Sca-1\(^+\)Lin\(^-\) cell fraction is responsible for the ability of

**Fig 5.** Donor type chimerism after transplantation of different BM cell fractions into allogeneic recipients exposed to sublethal (7 Gy) TBI: effect of positive selection for Sca-1\(^+\) cells and subsequent depletion of Lin\(^+\) cells. The figure shows peripheral blood chimerism at 30 days after transplantation of \(1 \times 10^6\) cells from each cell fraction.
Sca-1\(^+\)Lin\(^-\) cells to induce marked donor-type chimerism in face of the substantial host resistance remaining in sublethally irradiated recipients.

Donor NK cells with antihost alloreactivity that might contaminate the Sca-1\(^+\)Lin\(^-\) cell fraction or emerge upon engraftment and differentiation of the Sca-1\(^+\)Lin\(^-\) cells could also contribute in part to the tolerance observed, by eradicating residual host peripheral T cells. However, addition of anti-NK antibody to the Lin\(^-\) depletion step did not abrogate the capacity of Sca-1\(^+\)Lin\(^-\) cells to engraft, nor did the use of Sca-1\(^+\)Lin\(^-\) cells from NK-deficient C57BL/6-Beige donors (data not shown).

**Specific reduction of antidonor CTL-p by Sca-1\(^+\)Lin\(^-\) cells.** To evaluate whether the Sca-1\(^+\)Lin\(^-\) cell fraction is enriched for cells possessing veto activity, we analyzed different cell fractions for their capacity to specifically reduce antidonor CTL-p in comparison with anti–third-party CTL-p. Mixed lymphocyte reaction (MLR) was set up using spleen cells from C3H/HeJ mice as responder cells and spleen cells from C57BL/6 or BALB/c (third-party) mice as stimulator cells. To evaluate the veto activity of C57BL/6 Sca-1\(^+\)Lin\(^-\) cells compared with Sca-1\(^-\) cells, these cells were added to the MLR at a veto-to-responder cell ratio of 0.5:1, and the CTL-p levels were determined by limiting dilution analysis. As can be seen in Table 2, addition of Sca-1\(^+\)Lin\(^-\) cells completely abrogated CTL-p directed against their H-2 antigens (C57BL/6) while substantial cytotoxic activity could be monitored when no cells were added, or upon addition of Sca-1\(^-\) cells. In contrast, when the same Sca-1\(^+\)Lin\(^-\) cells were added to MLR against third-party (BALB/c) stimulators, no reduction of CTL activity was...
Spleen cells from C3H/HeJ mice (2 × 10⁶/mL) were incubated for 5 days with irradiated (25 Gy) allelogeneic spleen cells (1 × 10⁶/mL) from C57BL/6 (stem cell matched) or BALB/c (third-party) mice. C57BL Sca-1⁺Lin⁻ or Sca-1⁻ cells were added to the primary MLC at a 0.5:1 veto:responder cell ratio. The responder cells were then recultured under limiting dilution conditions for 7 days. Cells were then harvested from individual limiting dilution culture wells and assayed for cytotoxic activity by ⁵¹Cr-release assay as described in Materials and Methods.

Non-alloreactive (host × donor)F₁ T cells synergize with Sca-1⁺Lin⁻ BM cells of donor origin in the induction of donor-type chimerism. Although we are encouraged by the ability of Sca-1⁺Lin⁻ cells to overcome the host T cells remaining after sublethal TBI, it is clear that the number of cells required is very high and that it may be quite difficult to collect such large numbers of cells from human donors. For example, in the experiments described above, transplantation into sublethally irradiated recipients required about 75-fold more Sca-1⁺Lin⁻ cells compared with the number engrafting lethally irradiated mice. To reduce the effective number of Sca-1⁺Lin⁻ cells needed for overcoming resistance to engraftment, we were interested in studying the potential role of other facilitating or veto cells. Therefore, we attempted to test whether the addition of non-alloreactive (donor × host)F₁ T cells, previously shown to exhibit marked enhancement of engraftment of T-cell-depleted transplants in mismatched lethally irradiated recipients, could also help to reduce the minimal number of progenitor cells required for chimerism induction in the sublethal model. As can be seen in Fig 7, in 5 experiments monitoring the engraftment of 1 × 10⁶ infused F₁ T cells (H₂b⁺H₂k⁺) in sublethally irradiated C3H/HeJ (H₂b) recipients by their unique H₂b⁺H₂k⁺ phenotype, we found that only 16.6% of the mice were engrafted, suggesting that infusion of this number of F₁ T cells alone could overcome resistance only in a relatively small fraction of the recipients. However, engraftment of F₁ T cells was markedly increased to 65% by adding 2.0 × 10⁵ purified Sca-1⁺Lin⁻ cells. Likewise, engraftment of a suboptimal number of 2.0 × 10⁵ purified (Sca-1⁺Lin⁻) cells, monitored by the presence of donor-type cells (stained by anti-H₂b) and not by anti-H₂k, was enhanced, by adding 1 × 10⁷ F₁ T cells, from 12.9% of the recipients to 74.0%.

The resulting chimeric mice exhibited stable split chimerism in which significant levels of host (H₂b⁺H₂k⁻) and donor-type T cells (H₂b⁺H₂k⁺) as well as F₁ T cells (H₂b⁺H₂k⁺) coexisted with host and donor non-T cells (Fig 8).

**DISCUSSION**

Our results demonstrate that cells within the BM Sca-1⁺Lin⁻ subpopulation, previously shown to comprise the pluripotent hematopoietic stem cells as well as more differentiated progenitors, possess a marked capacity to overcome the substantial host resistance found in recipients conditioned by sublethal 7 Gy TBI. Considering that it is virtually impossible to achieve homogeneous purity by any methodology of BM cell fractionation, we resorted in our analysis to evaluation of specific activities before and after each of the purification steps. We found that the initial step of positive selection of Sca-1⁺ cells contributes significantly to the enhancement of donor-type chimerism and that over 1 log depletion of Lin⁺ cells from the Sca-1⁺ cell fraction does not retrace from the engraftment...
potency of the resulting Sca-1\(^+\)Lin\(^-\) cell fraction but, rather, further enhances donor-type chimerism.

In particular, our demonstration that the chimerism-inducing activity is enhanced upon purification of Sca-1\(^+\)Lin\(^-\) cells from the Sca-1\(^+\) cell fraction, while the frequency of CD8\(^+\) cells is reduced by about 20-fold, suggests that the ability of Sca-1\(^+\)Lin\(^-\) cells to overcome the marked host immunity is probably mediated by a non-CD8–mediated mechanism. Likewise, the use of NK-deficient “beige” donors or NK-depleted Sca-1\(^+\)Lin\(^-\) cells rule out the possibility that the latter cells are critical for the effect exerted by cells in the Sca-1\(^+\)Lin\(^-\) cell fraction.

It could be argued that chimerism induction associated with purification of Sca-1\(^+\)Lin\(^-\) cells might involve not only the capacity to overcome the host immune system, but it may also be mediated by their capacity to compete with host hematopoietic stem cells.\(^{31,32}\) This inherent duality of Sca-1\(^+\)Lin\(^-\) cells was addressed by our demonstration that Sca-1\(^+\)Lin\(^-\) cells facilitate not only their own engraftment, which could be reflecting, in part, donor-versus-host stem cell competition, but also the engraftment of (donor × host)\(F_1\) T cells, as well as acceptance of donor-type but not third-party skin. Thus, we were able to separate in vivo the potent capacity to overcome host immunity associated with the purified Sca-1\(^+\)Lin\(^-\) cells from their hematopoietic repopulating capacity, which might also contribute to donor-type chimerism induction. We have shown recently that escalation of human CD34 progenitor cell transplants enables overcoming major HLA barriers in the treatment of heavily conditioned leukemia patients.\(^{18-20}\) The capacity of the “mega dose” transplants to neutralize residual host antidonor CTL-p was attributed, in part, to the veto activity shown in the hematopoietic progenitor CD34 cell compartment.\(^{21,22}\)

Our present study, finding great similarity between the Sca-1\(^+\)Lin\(^-\) and the human CD34 cell fraction, suggests that further insight into the mechanism of tolerance induction by
hematopoietic progenitor cells might be facilitated by using different natural or genetically engineered mutant strains of mice.

Our demonstration that resistance to fully allogeneic stem cell transplants found in sublethally irradiated recipients can be overcome by escalation of cell dose is in accordance with the recent study of Uchida et al,33 which showed that lethally irradiated allogeneic recipients transplanted with highly purified \( c^-{\text{Kit}}^-{\text{Thy-1}}^-{1}^{1^-}{\text{Lin}}^-{\text{Sca-1}}^- \) stem cells require about 10-fold more cells compared with congenic recipients. However, the comparison between the allogeneic and the congenic groups in that study was made using animals pretreated by a different TBI dose. Moreover, there are no data regarding recipients treated with sublethal TBI. Thus, further studies are required to define whether the \( c^-{\text{Kit}}^-{\text{Thy-1}}^-{1}^{1^-}{\text{Lin}}^-{\text{Sca-1}}^- \) cells within the Sac-1^-Lin^- subpopulation will exhibit a similar capacity to overcome BM allograft rejection to that exhibited in the present study by the Sca-1^-Lin^- cells in the sublethal model.

The characterization of new cell phenotypes that are capable of enhancing acceptance of T–cell-depleted BM allografts is of great relevance to the continuing efforts to achieve HLA disparate hematopoietic engraftment in humans. Our present results in the mouse model, although demonstrating that major transplantation barriers can be overcome by megadoses of BM progenitors, also suggest that it might be difficult to harvest sufficient cells in humans. We found that the minimal number of Sca-1^-Lin^- cells required to achieve donor type chimerism in 7 Gy TBI treated mice was about 75-fold higher than that needed in lethally irradiated (10 Gy) TBI recipients.

One approach to overcome this quantitative problem might be afforded if it were possible to expand the veto cells within the CD34^+ progenitor cell fraction. Thus, while it is still very difficult to expand ex vivo the most primitive pluripotent hematopoietic stem cells, it may be possible to expand in vitro the CD34^+ cells possessing veto activity and use them together with a small number of pluripotent cells for transplantation. Further studies to elucidate the cell subpopulations that are most relevant for the induction of tolerance, in the human CD34 or in the mouse Sca-1^-Lin^- subpopulation, might help to find simple means to expand these valuable cells in vitro, and could help develop strategies for the elucidation of the mechanism by which their effect is mediated.

An alternative approach to achieving engraftment in the face of marked residual host immunity is based on the use of CD34 cells, together with other veto cells or facilitating cells. One example for such cells is shown in the present study by using (host × donor)F_{1} non-alloreactive T cells, previously shown to synergize with suboptimal doses of T–cell-depleted BM allografts in the lethal mouse model.30 In humans, non-alloreactive T cells can be generated by purging interleukin-2 receptor (CD25) MLR reactive T cells or by anergy induction upon incubation with CTLA-4.35,36

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Induction of Donor-Type Chimerism and Transplantation Tolerance Across Major Histocompatibility Barriers in Sublethally Irradiated Mice by Sca-1+Lin− Bone Marrow Progenitor Cells: Synergism With Non-Alloreactive (Host × Donor)F1 T Cells

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