

# Purified hematopoietic stem cell engraftment of rare niches corrects severe lymphoid deficiencies without host conditioning

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In the absence of irradiation or other cytoreductive conditioning, endogenous hematopoietic stem cells (HSCs) are thought to fill the unique niches within the bone marrow that allow maintenance of full hematopoietic potential and thus prevent productive engraftment of transplanted donor HSCs. By transplantation of purified exogenous HSCs into unconditioned congenic histocompatible strains of mice, we show that  $\sim 0.1$ – $1.0\%$  of these HSC niches are available for engraftment at any given point and find no evidence that endogenous HSCs can be displaced from the niches they occupy. We demonstrate that productive engraftment of HSCs within these empty niches is inhibited by host  $CD4^+$  T cells that recognize very subtle minor histocompatibility differences. Strikingly, transplantation of purified HSCs into a panel of severe combined immunodeficient (SCID) mice leads to a rapid and complete rescue of lymphoid deficiencies through engraftment of these very rare niches and expansion of donor lymphoid progenitors. We further demonstrate that transient antibody-mediated depletion of  $CD4^+$  T cells allows short-term HSC engraftment and regeneration of B cells in a mouse model of B(–) non-SCID. These experiments provide a general mechanism by which transplanted HSCs can correct hematopoietic deficiencies without any host conditioning or with only highly specific and transient lymphoablation.

## CORRESPONDENCE

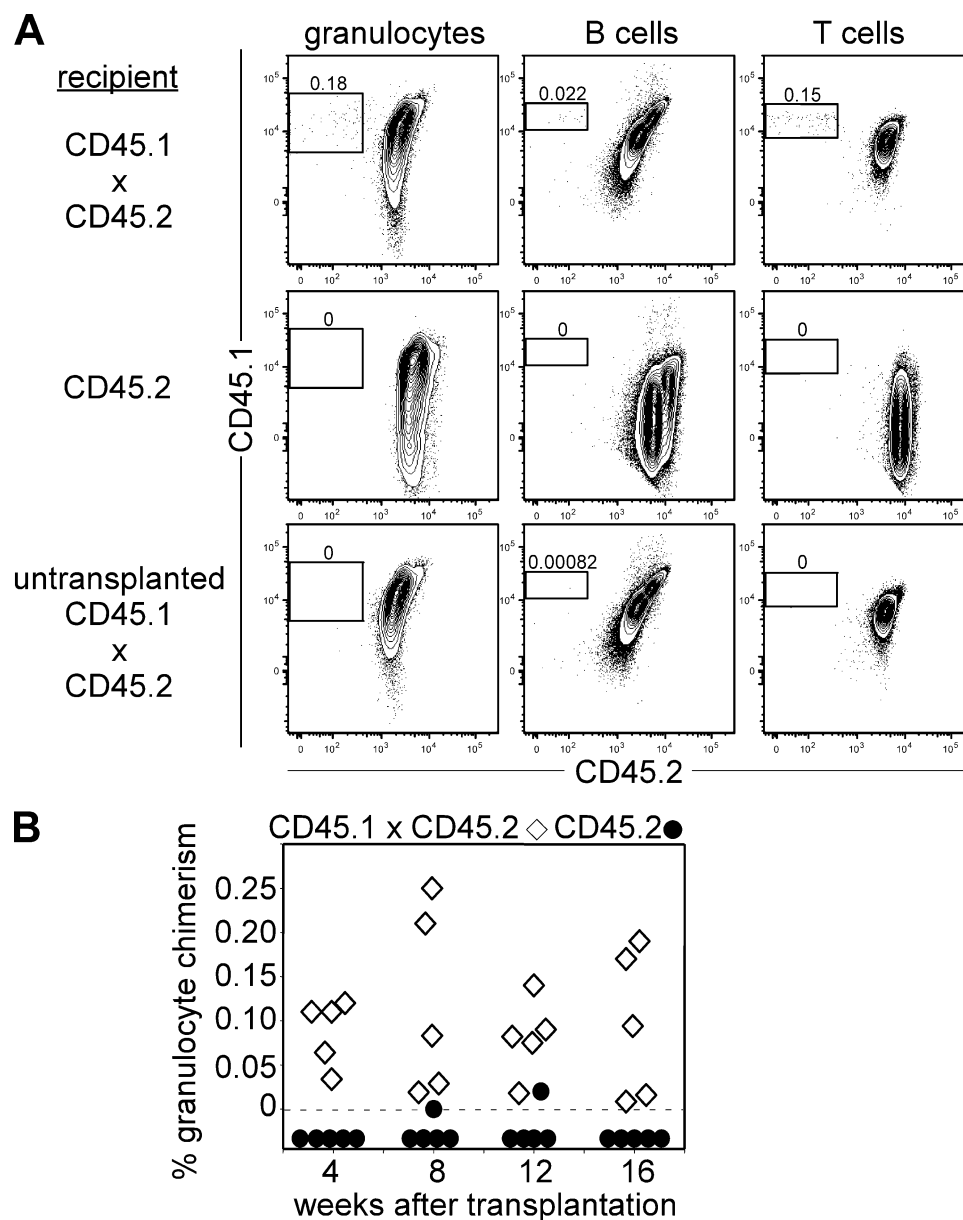
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Abbreviations used: APC, allophycocyanin; CLP, common lymphoid progenitor; CMP, common myeloid progenitor; GMP, granulocyte macrophage progenitor; GVHD, graft-versus-host disease; HSC, hematopoietic stem cell; LT, long-term; MEP, megakaryocyte erythrocyte progenitor; NP, 4-hydroxy-3-nitrophenylacetyl.

As a population, hematopoietic stem cells (HSCs) have the remarkable ability to drive hematopoiesis for the lifetime of the organism while maintaining and even expanding their numbers with age (1). The capacity of stem cells to both self-renew and yet maintain multilineage potential appears to be dependent on the localization of HSCs within specific microenvironments within the bone marrow because other organs associated with extramedullary hematopoiesis, such as the spleen, cannot maintain the self-renewal potential of HSCs (2). Although the molecular and cellular details of these HSC niches are only just beginning to be revealed (3–6), in normal settings it is likely that these specialized microenvironments are as rare as the HSCs they support. Another hallmark property of HSCs is their intrinsic ability to home to these very specific niches within the bone marrow after intravenous transplantation. Upon adoptive transfer into myeloablated recipients, single HSCs can give rise to all of the blood lineages for the

lifetime of the recipient (7, 8). Transplantation into unconditioned recipients, however, has led to mixed results. Transplantation of extraordinarily high doses of unfractionated congenic bone marrow or populations enriched for HSCs has led to hematopoietic chimerism (9, 10), although it is unclear whether this donor contribution is indicative of HSC engraftment and multilineage reconstitution, or solely the survival of lymphoid cells, which can persist for long periods of time in the absence of further input from the bone marrow (11–13). In contrast, conventional intravenous transplantation of more moderate amounts of congenically marked syngeneic or allogeneic bone marrow or purified HSCs rarely, if ever, leads to sustained multilineage engraftment without irradiation of the recipient (14, 15). Thus, the bulk of evidence has suggested that HSC niches are filled under normal conditions.

Recent evidence, however, has suggested that a small fraction of HSC niches may be available in normal animals. First,  $\sim 100$  HSCs



**Figure 1. Donor HSCs can engraft unconditioned tolerant wild-type hosts.** (A) Persistent multilineage contribution occurs only in tolerant recipients. 4,000 ckit<sup>+</sup> Thy1.1<sup>low</sup> lineage<sup>-</sup> Sca-1<sup>+</sup> Flk2<sup>-</sup> CD34<sup>-</sup> HSCs from male CD45.1 mice were transplanted into unirradiated male CD45.1 × CD45.2 (F1), CD45.2, or irradiated male CD45.1 × CD45.2 recipients. Granulocytes were pre-gated as side scatter<sup>high</sup> B220<sup>-</sup> TCRβ<sup>-</sup> cells, B cells were pre-gated as Mac-1<sup>-</sup> TCRβ<sup>-</sup> cells, and T cells

were pre-gated as B220<sup>-</sup> Mac-1<sup>-</sup> cells. Representative plots of peripheral blood at 16 wk after transplantation are presented. (B) Consistent granulocyte contribution in tolerant hosts. Granulocyte chimerism is shown at 4-wk intervals after transplantation into CD45.1 × CD45.2 and CD45.2 hosts. ◇, F1 hosts; ●, CD45.2 recipients. Data points below the dotted line represent animals with no detectable chimerism.

can be found at any given time in the peripheral blood of a normal mouse, and these cells are capable of reconstituting irradiated mice and functionally engraft unirradiated partners in a parabiotic model (16). Blood HSCs have a residence time in blood of ~1 min, and so to maintain a steady-state of 100 HSCs, upwards of 30,000 HSCs per day flux through the blood of a mouse (16). Although the etiological reasons are unclear, the presence of these cells in the

blood suggests that HSCs may exist in a dynamic equilibrium with their environment and that a certain number of bone marrow niches may be available for engraftment at any given point. Second, several recent studies using unfractionated bone marrow transplants have shown that the small congenic differences used to distinguish donor-derived hematopoietic cells from the recipient's endogenous cells constitute a significant immunologic barrier (17, 18).

These studies suggest that the absence of donor-derived cells in unconditioned hosts receiving congenic hematopoietic transplants may be a reflection of immunologic rejection rather than a lack of open HSC niches.

To assess the role of immunosurveillance and to quantify the number of available HSC niches in normal and immunodeficient animals, we performed a series of HSC transplants into histocompatible hosts and recipients with a minor histocompatibility mismatch at a single locus. We show that transplanted HSCs can productively engraft  $\sim 0.1$ – $1.0\%$  of HSC niches in H2-matched, minor histocompatibility-mismatched unconditioned hosts, but only if host  $CD4^+$  T cells are absent or genetically unreactive to the graft. Large excesses of transplanted HSCs do not significantly increase this engraftment, suggesting that endogenous HSCs residing in appropriate niches cannot be easily displaced. Despite the small numbers of available HSC niches, we show that transplantation of purified HSCs is sufficient to permanently rescue animals with severe immunodeficiencies by regenerating donor-derived lymphocytes to normal levels through expansion of progenitor cells within the unfilled lymphoid compartments. The mechanism by which purified HSC transplantation corrects lymphoid disorders is likely to be broadly applicable to the treatment of hematopoietic deficiencies.

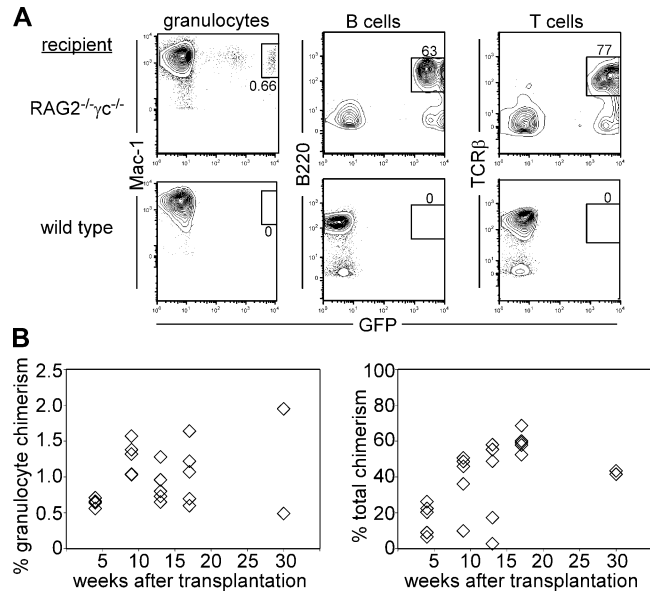
## RESULTS

### Immune surveillance prevents engraftment of subtly mismatched HSCs

HSCs in 4,000  $ckit^+$   $Thy1.1^{low}$   $lineage^-$   $Sca-1^+$   $Flk2^-$   $CD34^-$  cells from  $CD45.1$  mice were transplanted intravenously into five unirradiated  $CD45.1 \times CD45.2$  (F1) and five unirradiated congenic  $CD45.2$  mice. Every 4 wk after transplantation, peripheral blood was analyzed for granulocyte, B cell, and T cell chimerism. Donor granulocyte chimerism, which accurately reflects HSC chimerism (16), was observed only in the genetically unreactive F1 recipients at all time points analyzed with a median chimerism at 16 wk of  $\sim 0.1\%$  (Fig. 1). These data demonstrate that the subtle antigenic differences that exist between these  $CD45$  congenic strains of mice are sufficient to prevent productive HSC cross-engraftment.

### Rapid HSC-mediated correction of lymphoid deficiencies in SCID mice

To gauge the potential clinical importance of these rare available niches, we repetitively transplanted HSCs from GFP-transgenic mice into  $RAG2^-/\gamma c^-/\gamma$  mice, which lack B, T, and NK cells (19). Enormous numbers of donor-derived B and T cells were found in the blood at all time points, leading to an overall donor chimerism of  $\sim 50\%$  until at least 30 wk after the final transplantation (Fig. 2, A and B). Donor NK cells were also detected in all transplanted animals (not depicted). Moreover, all  $RAG2^-/\gamma c^-/\gamma$  mice displayed persistent donor-derived myeloid chimerism (ranging from 0.5 to 2.0% donor-derived granulocytes).

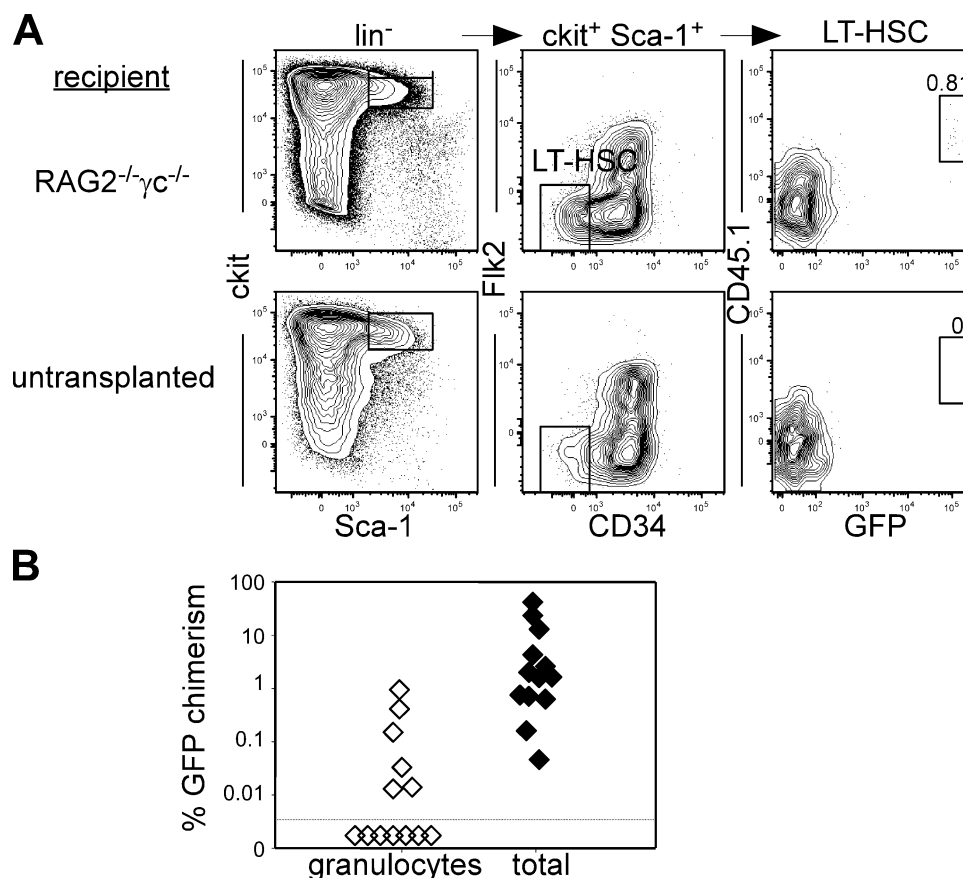


**Figure 2. Small numbers of engrafted wild-type HSCs can rescue lymphocyte deficiencies in unirradiated  $RAG2^-/\gamma c^-/\gamma$  mice.**

(A) Robust donor chimerism in unconditioned  $RAG2^-/\gamma c^-/\gamma$  recipients. Unirradiated  $RAG2^-/\gamma c^-/\gamma$  mice were repetitively transplanted six times with 1,750–4,000 HSCs from eGFP-transgenic mice, and peripheral blood was analyzed for donor myeloid and lymphoid contribution at 4 wk after the final transplant. Granulocytes were pregated as side scatter<sup>high</sup>  $B220^-$   $TCR\beta^-$  cells, B cells were pregated as  $Mac-1^-$   $TCR\beta^-$  cells, and T cells were pregated as  $B220^-$   $Mac-1^-$  cells. (B) Persistent multilineage contribution in unirradiated  $RAG2^-/\gamma c^-/\gamma$  recipients. Peripheral blood was analyzed for donor contribution periodically until 30 wk after final transplantation.

To confirm that the donor granulocyte frequencies accurately reflected bone marrow HSC chimerism, we killed animals at 30 wk after transplant and analyzed bone marrow. Donor cells comprised  $\sim 0.8\%$  of the total long-term (LT)-HSC pool (Fig. 3 A). This chimerism was essentially the same in the short-term reconstituting stem cells ( $ckit^+$   $lineage^-$   $Sca-1^+$   $CD34^+$   $Flk2^-$ ) and the multipotent progenitors ( $ckit^+$   $lineage^-$   $Sca-1^+$   $CD34^+$   $Flk2^+$ ; not depicted). We were unable to detect any cells with these surface phenotypes in the spleen, a major organ associated with extramedullary hematopoiesis (not depicted). These data confirmed that in these animals, peripheral donor granulocyte frequencies much more accurately reflect HSC chimerism than the overall donor contribution in the blood. The data also demonstrate that small numbers of bone marrow-engrafted HSCs can correct severe lymphoid deficiencies without prior cytoreductive conditioning. The HSC chimerism in the  $RAG2^-/\gamma c^-/\gamma$  mice was comparable to the chimerism seen in the genetically unreactive F1 wild-type mice (Fig. 1), demonstrating that there are not obviously greater numbers of available HSC niches in these animals.

To confirm that functional HSCs had engrafted, we isolated bone marrow at 31 wk after transplant from unirradiated  $RAG2^-/\gamma c^-/\gamma$  mice that had received GFP<sup>+</sup> HSC transplants and performed secondary transplants using either



**Figure 3. Low-level HSC engraftment in primary unconditioned  $RAG2^{-/-}\gamma c^{-/-}$  recipients.** (A) Donor HSCs can be detected in primary unirradiated  $RAG2^{-/-}\gamma c^{-/-}$  recipients. At 31 wk after the final transplantation, donor HSC contribution in the bone marrow in unconditioned primary  $RAG2^{-/-}\gamma c^{-/-}$  recipients was quantified using the markers shown. (B) Engrafted HSCs in the bone marrow of unconditioned primary  $RAG2^{-/-}\gamma c^{-/-}$  recipients can be serially transplanted.

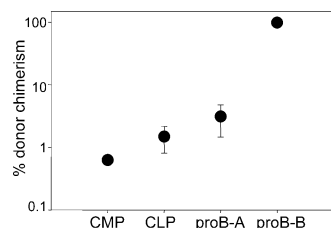
Unfractionated ( $5 \times 10^7$ ) or c-kit-enriched ( $10^6$ ) bone marrow cells from primary  $RAG2^{-/-}\gamma c^{-/-}$  recipients were secondarily transplanted into lethally irradiated (950 cGy) wild-type mice. GFP<sup>+</sup> chimerism was assessed at 25 wk after secondary transplantation. Each secondary recipient received a transplant from a distinct nonredundant primary donor. Data points below the dotted line represent undetectable chimerism.

unfractionated or c-kit-enriched marrow, which increases the frequency of HSCs by  $\sim 10$ -fold, into lethally irradiated wild-type mice, such that  $\sim 6$ – $20$  GFP<sup>+</sup> HSCs along with 1,000  $RAG2^{-/-}\gamma c^{-/-}$  HSCs were transferred into each secondary recipient. Donor-derived GFP<sup>+</sup> cells were observed in all secondary recipients until at least 25 wk after transplantation, and 6 out of 13 secondary recipients maintained detectable levels of granulocyte chimerism (Fig. 3 B). These data confirmed that rare GFP<sup>+</sup> HSCs within the bone marrow of the primary  $RAG2^{-/-}\gamma c^{-/-}$  recipients had productively engrafted. In contrast, transplantation of large numbers of splenocytes into secondary recipients did not lead to sustained multilineage stem cell reconstitution (not depicted).

#### Opportunistic expansion of donor lymphoid progenitors

To determine the developmental stage at which donor B cells overtake host B cells in  $RAG2^{-/-}\gamma c^{-/-}$  recipients, we analyzed donor frequencies in myeloid and lymphoid progenitor

cells in the bone marrow. Common myeloid progenitors (CMPs; reference 20) and common lymphoid progenitors (CLPs; references 21–23) showed donor chimerism that was comparable to HSC chimerism (Fig. 4), indicating that donor-derived cells do not have a competitive proliferative advantage at these early developmental steps. Donor chimerism at the granulocyte macrophage progenitor (GMP) and megakaryocyte erythrocyte progenitor (MEP) developmental steps were also similar to HSC chimerism (not depicted). Consistent with these results, the frequencies of endogenous HSCs, CLPs, CMPs, GMPs, and MEPs within the bone marrow are similar between untransplanted wild-type and  $RAG2^{-/-}\gamma c^{-/-}$  mice (Fig. 5). Analysis of the pro-B-A and pro-B-B cell fractions (24, 25), however, showed donor chimerism that was dramatically higher than the preceding CLP (Fig. 4). At the pro-B-B cell stage and all subsequent B cell stages, cells were exclusively donor-derived. Although IL-7 receptor, which uses  $\gamma c$  for proper signaling, is expressed at the CLP stage, these results suggest that IL-7 signaling is not a



**Figure 4. Significant donor cell expansion is not observed until the pro-B cell stage.** At 31 wk after the final transplantation, donor contribution to myeloid and lymphoid progenitor cells was analyzed. CMPs were defined as  $\text{lin}^- \text{ckit}^+ \text{Sca-1}^- \text{CD16/32}^{\text{low}} \text{CD34}^{\text{low}}$  cells. CLPs were gated as  $\text{lin}^- \text{Thy1.1}^{\text{low}} \text{IL7}\alpha^+ \text{Flk2}^+ \text{ckit}^{\text{low}} \text{Sca1}^{\text{low}}$  cells. Pro-B-A cells were defined as  $\text{B220}^+ \text{CD43}^+ \text{IgM}^- \text{CD19}^- \text{NK1.1}^-$ , and pro-B-B cells were gated as  $\text{B220}^+ \text{CD43}^+ \text{IgM}^- \text{CD19}^+ \text{Ly51}^- \text{NK1.1}^-$  cells. Mean values  $\pm$  SEM are shown from the analysis of three mice for each subset.

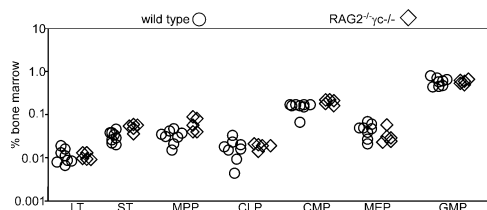
requisite pathway for CLP development or expansion, consistent with previous observations (26).

#### Normal immune responses in HSC-reconstituted SCID mice

To verify that the immune system of the HSC-reconstituted  $\text{RAG2}^{-/-} \gamma\text{c}^{-/-}$  mice had been restored and was capable of mounting appropriate immune responses, we immunized reconstituted recipients with alum-precipitated 4-hydroxy-3-nitrophenylacetyl (NP) conjugated to chicken  $\gamma$  globulin, which elicits a Th2-dependent humoral response (27, 28). Serum levels of NP-specific antibody were similar at 1 wk after immunization between HSC-transplanted  $\text{RAG2}^{-/-} \gamma\text{c}^{-/-}$  and wild-type mice, demonstrating the immunocompetence of the transplanted  $\text{RAG2}^{-/-} \gamma\text{c}^{-/-}$  recipients (Fig. 6).

#### Host $\text{CD4}^+$ T cells are essential for rejection of subtly mismatched HSCs

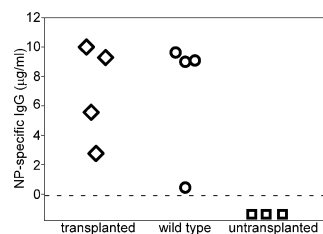
To investigate whether the absence of  $\gamma\text{c}$  was critical for HSC engraftment in unconditioned hosts, perhaps by imparting a competitive disadvantage on HSCs in  $\text{RAG2}^{-/-} \gamma\text{c}^{-/-}$  animals, we transplanted GFP $^+$  HSCs into  $\text{RAG2}^{-/-}$  mice, which have normal  $\gamma\text{c}$  expression, as well as into  $\text{RAG2}^{-/-} \gamma\text{c}^{-/-}$  mice. As shown in Fig. 7, similar levels of



**Figure 5. Stem and progenitor cell frequencies are normal in  $\text{RAG2}^{-/-} \gamma\text{c}^{-/-}$  mice.** LT-HSCs, ST-HSCs, and multipotent progenitors were stained from untransplanted mice as in Fig. 2. CLPs and CMPs were stained as in Fig. 4. MEPs and GMPs were stained according to established protocols (reference 20). Each endogenous population is presented as a percentage of total bone marrow.  $\circ$ , wild-type animals;  $\diamond$ ,  $\text{RAG2}^{-/-} \gamma\text{c}^{-/-}$  mice.

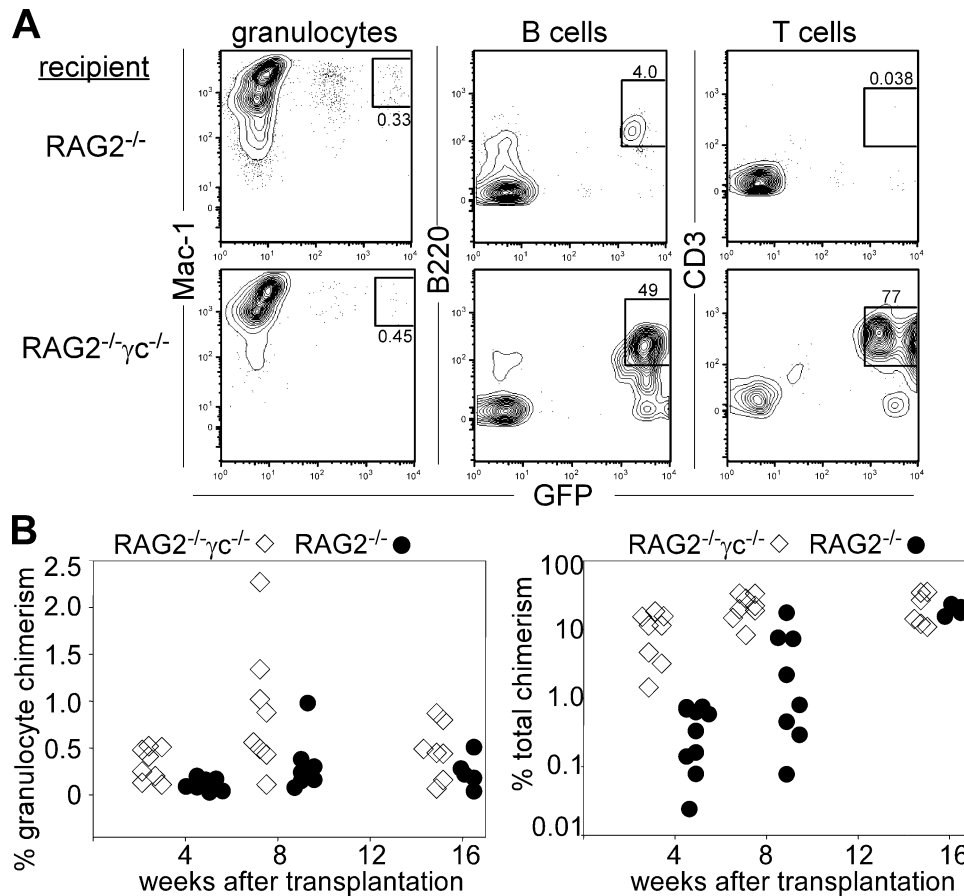
donor granulocyte contribution were seen in  $\text{RAG2}^{-/-}$  and  $\text{RAG2}^{-/-} \gamma\text{c}^{-/-}$  mice, likely excluding a direct role for  $\gamma\text{c}$  in maintaining host HSCs within their niches. However,  $\gamma\text{c}$  expression has been observed in normal HSCs (29), suggesting that there may be a slight competitive advantage for HSCs with proper  $\gamma\text{c}$  expression. The data also suggest that NK cells, which are present in normal numbers in  $\text{RAG2}^{-/-}$  mice but absent in  $\text{RAG2}^{-/-} \gamma\text{c}^{-/-}$  mice, are not mediating HSC rejection in this H2-identical system. Elimination of host NK cells is required for engraftment of HSCs that carry one or more unshared H2 haplotype (30). Interestingly, donor-derived lymphocyte frequencies were significantly reduced in  $\text{RAG2}^{-/-}$  recipients at early time points (Fig. 7 B), perhaps as a result of the occupation of the lymphoid stage-specific stromal environments by  $\text{RAG2}^{-/-}$  lymphoid progenitors (31). The number of donor-derived B cells in  $\text{RAG2}^{-/-}$  recipients was reduced more than 10-fold relative to  $\text{RAG2}^{-/-} \gamma\text{c}^{-/-}$  recipients at 4 wk after transplant, and peripheral T cells were not seen at all until 8 wk after transplant (not depicted). However, by 16 wk after transplantation, B and T cell numbers in  $\text{RAG2}^{-/-}$  recipients reached the levels seen in their  $\text{RAG2}^{-/-} \gamma\text{c}^{-/-}$  counterparts (Figs. 7 B and 8 A).

Because  $\text{RAG2}^{-/-}$  mice lack both mature B and T cells, we sought to determine which of these cell types was primarily responsible for mediating the rejection of transplanted donor HSC grafts. Therefore, we transplanted purified HSCs from GFP donor mice into unirradiated  $\text{TCR}\alpha^{-/-} \beta^{-/-}$  and  $\text{C}\mu^{-/-}$  mice. In these experiments, known antigenic differences between donor HSCs and recipient mice exist at the GFP, CD45, and Thy1 loci. Multilineage engraftment was observed in T cell-deficient mice, but not in B cell-deficient mice (Fig. 8 A). These experiments show that host  $\alpha\beta$  T cells are required for the rejection of HSC grafts with these minor histocompatibility mismatches. To determine which class of T cells is essential for this immunosurveillance, we transplanted HSCs into unconditioned  $\text{I-A}^{-/-}$  mice, which lack MHC II-restricted  $\text{CD4}^+$  T cells (32), and  $\beta_2\text{-microglobulin}^{-/-}$  ( $\beta_2\text{m}^{-/-}$ ) mice, which are deficient in MHC I-restricted  $\text{CD8}^+$  T cells (33). The HSC-transplanted



**Figure 6. Reconstituted  $\text{RAG2}^{-/-} \gamma\text{c}^{-/-}$  mice can respond normally to antigenic challenge.**  $\text{RAG2}^{-/-} \gamma\text{c}^{-/-}$  mice were immunized with 100  $\mu\text{g}$  alum-precipitated NP-chicken  $\gamma$  globulin 16 wk after HSC transplant. Serum was obtained 1 wk after immunization and tested for NP-specific antibodies. Mice with undetectable serum levels of NP-specific IgG are shown below the dotted line.





**Figure 7.**  $\gamma_c$  expression is not directly important for HSC maintenance. (A) Purified wild-type HSCs can engraft unirradiated RAG2<sup>-/-</sup> mice. Unconditioned RAG2<sup>-/-</sup> and RAG2<sup>-/-</sup> $\gamma_c$ <sup>-/-</sup> mice were transplanted with 3,000 HSCs from GFP-transgenic mice. Representative plots from peripheral blood obtained 4 wk after transplantation are shown. Granulo-

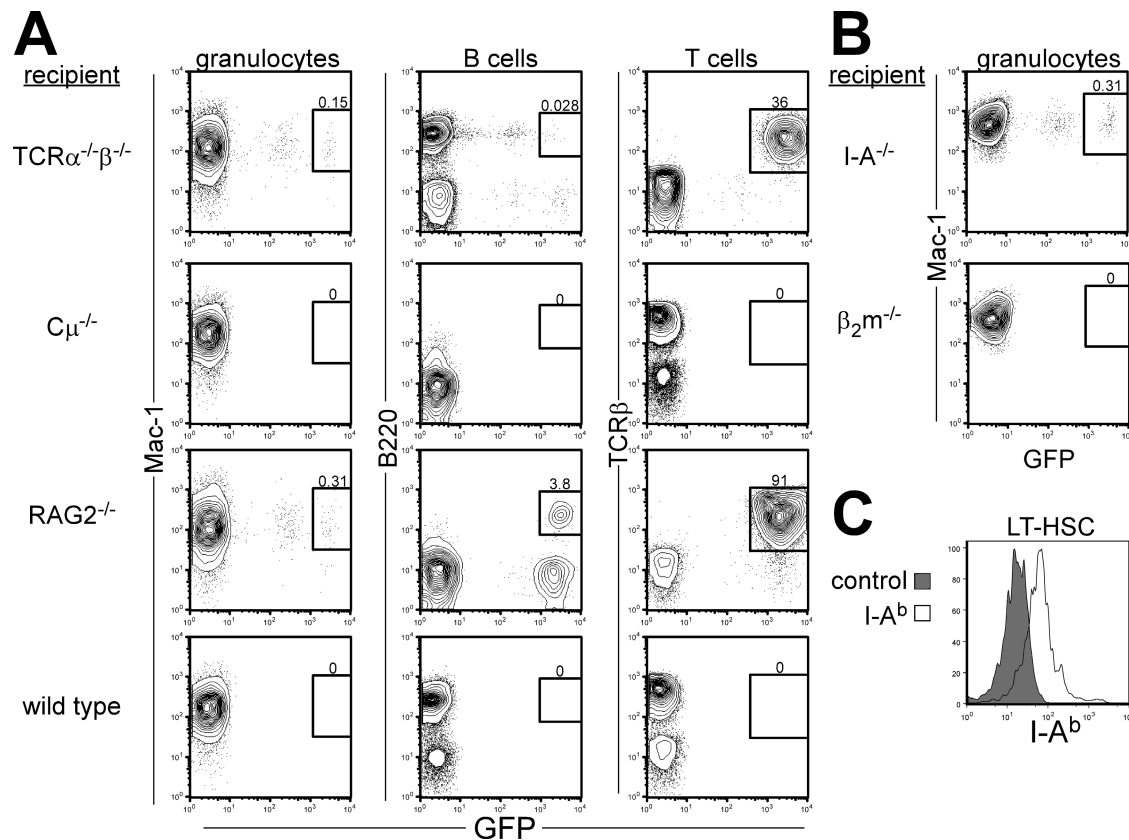
cytes were pregated as side scatter<sup>high</sup> B220<sup>-</sup> CD3<sup>-</sup> cells, B cells were pregated as B220<sup>-</sup> Mac-1<sup>-</sup> cells. (B) Persistent granulocyte chimerism but delayed lymphoid reconstitution in RAG2<sup>-/-</sup> mice. ◇, RAG2<sup>-/-</sup> $\gamma_c$ <sup>-/-</sup> animals; ●, RAG2<sup>-/-</sup> animals.

I-A<sup>-/-</sup> mice showed sustained chimerism until at least 16 wk, whereas the  $\beta_2m$ <sup>-/-</sup> recipient mice did not show chimerism at any time point (Fig. 8 B), thereby demonstrating that CD4<sup>+</sup> T cells are essential for the rejection of minor histocompatibility-mismatched HSC grafts in our system. LT-HSCs express MHC II (Fig. 8 C) and the costimulatory molecule CD86 (29), suggesting that host CD4<sup>+</sup> T cells may directly recognize HSCs with slight antigenic mismatches. Although it appears that CD8<sup>+</sup> T cells are not required for this rejection, we cannot exclude the possibility that residual hyperreactive MHC I-restricted T cells might also contribute to HSC graft rejection in the  $\beta_2m$ <sup>-/-</sup> mice (34–36). Consistent with the role for CD8 T cells in mediating bone marrow graft rejection, Xu et al. (17) have shown that host CD8 deficiency enhances engraftment.

Interestingly, the granulocyte chimerism in the RAG2<sup>-/-</sup> recipients was indistinguishable from that seen in previous experiments in which 3,000 HSCs rather than 1,000 were transplanted (Fig. 7). In contrast, although transplantation of 20 HSCs led to detectable B and T cell production in these

immunodeficient mice, granulocyte chimerism was barely detectable (not depicted). Thus, our experiments suggest that HSC engraftment and chimerism asymptotically approaches a maximum of  $\sim 0.5\%$  in a cell dose-dependent manner. The data show that in contrast to previous speculations, endogenous HSCs cannot be displaced from the niches they occupy by increasing transplanted HSC numbers above a threshold level. In repetitively transplanted mice, however, we have observed small increases in granulocyte chimerism relative to mice that were HSC-transplanted only once with doses above this threshold (Fig. 2 B vs. Fig. 7 B). This provides evidence that transplantation of an excess of HSCs does not preclude additional niches from being vacated in the future.

To determine if transient CD4<sup>+</sup> T cell removal would allow access of transplanted HSCs to appropriate niches, we treated C $\mu$ <sup>-/-</sup> mice with a depleting CD4 antibody that led to  $\sim 95\%$  depletion of peripheral blood CD4<sup>+</sup> T cells (Fig. 9 A). CD4-depleted mice were then transplanted with 800 HSCs and analyzed at various time points for donor chimerism. All mice that received anti-CD4 treatment showed

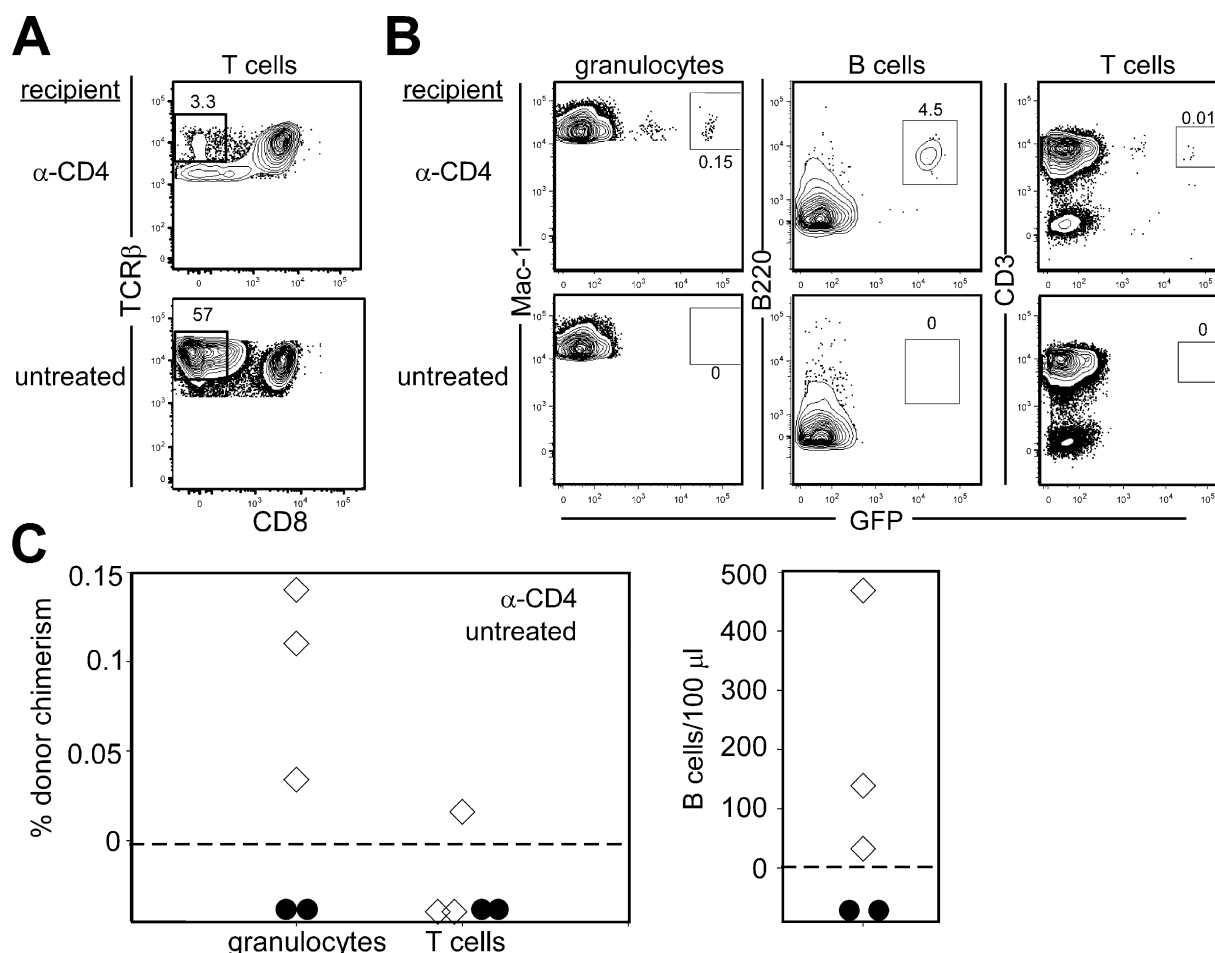


**Figure 8. Host T cells act as barriers to productive HSC engraftment.** (A)  $\alpha/\beta$  T cells are required for HSC graft rejection. 1,000 purified HSCs from eGFP-transgenic mice were transplanted into TCR $\alpha^{-/-}\beta^{-/-}$ , C $\mu^{-/-}$ , RAG2 $^{-/-}$ , and wild-type animals. Peripheral blood was analyzed for donor cell contribution at 16 wk after transplantation. Granulocytes were pregated as side scatter<sup>high</sup> B220 $^{-}$  TCR $\beta^{-}$  cells, B cells were pregated as Mac-1 $^{-}$  TCR $\beta^{-}$  cells, and T cells

were pregated as B220 $^{-}$  Mac-1 $^{-}$  cells. A representative plot for each group is shown. (B) Host CD4 $^{+}$  T cells are essential for HSC graft rejection. 800 purified HSCs from GFP-transgenic mice were transplanted into 2 I-A $^{-/-}$  and 3  $\beta_2m^{-/-}$  mice. Peripheral blood was analyzed 16 wk after transplantation for donor chimerism. Representative plots are shown. (C) LT-HSCs express MHC II. MHC II expression was analyzed on LT-HSCs from wild-type animals.

donor granulocyte chimerism, whereas none of the untreated mice displayed any detectable donor cells at 8 wk after transplantation (Fig. 9 B). Significant numbers of donor B cells were observed in the treated mice at 6–8 wk after transplantation, again demonstrating the ability of small numbers of productively engrafted HSCs to restore lymphocyte numbers in immunodeficient mice. Because host CD4 $^{+}$  T cells levels were noted to recover subsequent to immunodepletion (not depicted), these experiments demonstrate that transient depletion of these cells before transplantation is sufficient to allow for productive short-term stem cell engraftment without the need for the standard toxic cytoreductive drugs commonly used for B(-) non-SCID patients before bone marrow transplant (37). However, by 12 wk after HSC transplantation, donor B cell, T cell, and myeloid chimerism was lost in all recipient mice (not depicted). These data suggest that either the  $\alpha$ -CD4-mediated depletion of mature T cells was incomplete, or that sufficient numbers of donor-derived dendritic cells were not generated to mediate lasting tolerance through negative selection in the thymus (38).

**Short-term reconstitution in unconditioned aged recipients**  
Aged individuals show marked reductions in thymus size and T cell function (39). To determine if the reduced lymphoid function in aged mice would allow acceptance of transplants without conditioning, we repetitively transplanted HSCs from GFP-transgenic mice into old (22 mo) and young (2 mo) recipients. As seen in Fig. 10, short-term low-level myeloid chimerism was observed in all old recipients in contrast to the young recipients that showed no engraftment. However, donor-derived cells declined to undetectable levels in all but one of the old recipients with time, suggesting that rejection of the transplants did occur, but with significantly reduced kinetics relative to the younger animals (not depicted). The level of granulocyte chimerism in this experiment was similar to the low levels seen in transplants of younger, genetically unreactive or immunodeficient mice, suggesting that aged HSCs cannot be displaced from their endogenous niches. This is in contrast to previous studies performed with unfractionated bone marrow transplants (40) and suggests that reduced



**Figure 9. Transient CD4 depletion allows productive engraftment of HSCs with minor histocompatibility mismatches.** (A) Treatment of  $C\mu^{-/-}$  mice with a depleting antibody leads to efficient CD4<sup>+</sup> T cell removal. Mice were treated consecutively for 3 d with anti-CD4 antibody, and peripheral blood was analyzed for TCR $\beta$ <sup>+</sup> CD8<sup>+</sup> cells to assess depletion 1 d after the final treatment. The plots are gated on Ter119<sup>+</sup> TCR $\beta$ <sup>+</sup> cells. (B) HSC engraftment occurs in anti-CD4-treated animals. Three  $C\mu^{-/-}$  mice that were treated with anti-CD4 antibody and two  $C\mu^{-/-}$  mice that were left untreated were transplanted with 800 GFP<sup>+</sup> HSCs. Peripheral blood was analyzed at 8 wk after transplan-

tation for granulocyte, B cell, and T cell chimerism. Granulocytes were pre-gated as side scatter<sup>high</sup> B220<sup>+</sup> CD3<sup>+</sup> cells, B cells were pre-gated as Mac-1<sup>+</sup> CD3<sup>+</sup> cells, and T cells were pre-gated as B220<sup>+</sup> Mac-1<sup>+</sup> cells. Representative plots are shown. (C) Significant short-term multilineage engraftment in all anti-CD4-treated recipients. Donor contribution to granulocytes, T cells, and B cells at 8 wk after HSC transplant is shown. B cells are quantified as number of cells contained within 100  $\mu$ l of peripheral blood.  $\diamond$ , anti-CD4-treated animals;  $\bullet$ , untreated animals. Data points that were undetectable are shown below the dotted line.

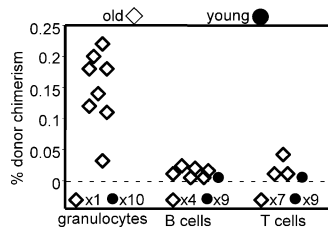
immune capacity is responsible for short-term engraftment of donor HSCs.

## DISCUSSION

The remarkable ability of HSCs to sustain multilineage hematopoiesis for the lifetime of an individual constitutes the foundation for their routine use in a range of clinical applications, including the treatment of primary immunodeficiencies (41, 42), malignancies (43–45), as conditioners for transplantation tolerance of tissue or organ grafts from the donors (46), and as a method to reverse some types of autoimmunity (47). The success of such therapies relies on the ability of HSCs to home to unique niches leading to sustained multilineage hematopoiesis. The studies presented

here have quantified the number of these HSC niches that are available for engraftment at any given point in unconditioned animals as  $\sim 0.1$ – $1.0\%$  of all HSC niches. Assuming a total adult murine bone marrow cellularity of  $5 \times 10^8$  cells (48) and an endogenous HSC frequency of  $0.01\%$  (Fig. 4), the number of open HSC niches can be estimated to be 50–500. This is strikingly similar to the number of HSCs estimated to be in circulation at any given point (16). The data suggest that HSCs that circulate normally have exited and left vacant their previous HSC niche. Thus, a constant exchange may be occurring between endogenous HSCs under normal circumstances, perhaps to maintain hematopoietic balance between and within each bone marrow compartment. In support of this, we have found little difference in the granulocyte





**Figure 10. Transplanted HSC grafts are accepted more readily in aged recipients than in young mice.** Purified eGFP<sup>+</sup> HSCs were sorted and 1,750–4,000 cells were repetitively transplanted into 2-mo-old C57Bl6/Ka and 24-mo-old C57Bl6/Harland mice. Donor granulocyte, B cell, and T cell chimerism are shown from the first peripheral bleed taken at 6.5 wk after the first transplant (3 d after the final transplant). ●, young recipients; ◇, aged recipients. The numbers of animals with no detectable chimerism in each group are listed below the dotted line.

chimerism rates between experiments when a single transplant of HSCs is provided in doses ranging from 800 to 4,000 cells. Although previous reports have suggested that the cell doses of transplanted bone marrow correlate with total chimerism linearly, such data at most show replacement of bone marrow and mature cells in bulk and do not reflect replacement of HSCs, which represent only 0.01% of unfractionated marrow (9). When HSCs are repetitively transplanted, however, we have observed increases in granulocyte chimerism (Fig. 2 B vs. Fig. 7 B). Thus, occupation of available HSC niches after transplantation of an excess of exogenous HSCs, which remain in circulation for ~1–5 min after transplantation (16), does not preclude additional niches from becoming available subsequently. Conceivably, continuous transfusion of low numbers of HSCs would be superior to singly administered boluses, as the rate of niche emptying and filling is high. Because there does not appear to be an obvious increase in granulocyte chimerism with time or cell dose above a threshold level, the data also suggest that transplanted HSCs must find their way rapidly to an appropriate niche and cannot recirculate indefinitely in search of empty niches without the loss of hematopoietic potential.

The ability of transplanted HSCs to self-renew for the lifetime of the organism ensures a constant production of normal lymphoid cells through each developmental stage. In the genetic mutants used in our work, host lymphocyte development is blocked or perturbed at defined developmental stages. At each developmental stage or thereafter, wild-type donor cells have a competitive advantage and can opportunistically expand or accumulate to ultimately give rise to large numbers of normal mature lymphocytes. Several factors have been implicated in the expansion of the early B cell and thymocyte lineages, including IL-7 (24, 49), stem cell factor (50), Flt3 ligand (22, 51), and recently, various Wnt/Frizzled pathways (52). In the case of  $\gamma c^{-/-}$  animals, the pro-B-B population appears to have defects in IL-7-dependent expansion, providing a proliferative advantage to wild-type donor cells at these stages (24, 53–56). RAG2<sup>-/-</sup> mice likely reconstitute more slowly because their lymphocytes can develop

normally through the pro-B cell as well as DN3 thymocyte stages and occupy the appropriate stromal microenvironments (31). However, because RAG2<sup>-/-</sup> lymphocytes cannot advance past these stages (57), small numbers of developing donor-derived cells can expand and accumulate without competition at the pre-B as well as DN4 thymocyte cell stages and all subsequent developmental steps. Although it is possible that the donor LT-HSCs will only persist for finite periods of time because of the considerable demand imposed by the ~250,000-fold expansion to the lymphocyte stage, we have observed no meaningful decline in granulocyte or lymphocyte chimerism at any time point up to 30 wk after transplantation of primary recipients. Nonetheless, because we have observed some declines in HSC potency after secondary transplantation (Fig. 3 B vs. Fig. 2 B), in clinical settings it would be advisable to keep donor LT-HSCs stored in the event that grafts do not persist indefinitely. Additionally, the low levels of granulocyte chimerism achieved from a single transplant are unlikely to be clinically useful for patients suffering from myeloid deficiencies.

We also show conclusively that stable engraftment within these rare niches by minor histocompatibility-mismatched HSCs is tightly regulated by host CD4<sup>+</sup> T cells. HSCs from CD45.1 mice cannot productively engraft unirradiated congenic CD45.2 mice, yet they routinely engraft the genetically unreactive F1 strain (CD45.1 × CD45.2). To our knowledge, the only antigenic difference between these strains is the CD45 allele, which is normally considered to be a relatively innocuous congenic marker. Similarly, HSCs isolated from GFP-transgenic mice backcrossed to the C57BL/Ka genetic background cannot productively engraft wild-type C57BL/Ka mice. The only antigenic difference between these strains to our knowledge is the GFP gene product. These experiments prove that very small antigenic differences lead to a complete rejection of donor HSC grafts in the absence of cytoreductive conditioning.

Encouragingly, however, the elimination of CD4<sup>+</sup> T cell function allows for the functional and sustained engraftment of HSCs with minor histocompatibility mismatches in our system. Xu et al. (17) have shown that transient antibody-mediated depletion of host  $\alpha\beta$  T cells in mice enhances engraftment of donor bone marrow with minor histocompatibility mismatches. Spitzer et al. (58) have shown that conditioning haploidentical patients with a depleting  $\alpha$ -CD2 antibody along with low-dose cytoreductive treatments before bone marrow transplantation allows, at the minimum, transient multilineage engraftment. Consistent with this, we demonstrate that transient antibody-mediated CD4<sup>+</sup> T cell depletion alone is sufficient to allow short-term engraftment of wild-type donor HSCs and restoration of B cells in a mouse model of non-SCID. More complete CD4<sup>+</sup> T cell depletions and/or better methods to increase donor-derived thymic dendritic cell contribution might allow for lasting donor hematopoiesis.

Even in the absence of inherited genetic mutations, both mice and humans develop diminished immune capacity with age. This progressive loss of immune function has recently

been attributed to HSC-intrinsic defects in differentiation to lymphoid-primed progenitors (29). Because we have demonstrated that a very small number of properly functioning HSCs can mask the defects in a much larger pool of HSCs, it is tempting to speculate that age-related immune defects don't become readily apparent until nearly all fully "young" HSCs are exhausted. The reintroduction of fully multipotent HSCs, perhaps obtained as an autologous sample earlier in life, might significantly delay age-related immune decline.

Numerous studies have shown how common conditioning treatments used before bone marrow transplantation, such as irradiation, cyclophosphamide, and busulfan, can cause serious side effects, including lowered platelet counts, infertility, and secondary malignancies (59). When these cytotoxic therapies are used to treat hematologic malignancies, the side effects must unfortunately be tolerated as a byproduct of necessary chemotherapy. The necessity of such conditioning treatments for hematopoietic deficiencies before HSC transplantation, however, should be reconsidered. Although it is true that available niche space is low under normal conditions, we show that transplantation of modest numbers of highly purified HSCs can engraft the few niches that are available and correct lymphoid deficiencies. Thus, niche space is not an absolute limiting factor to HSC-mediated correction of B, T, or NK cell deficiencies.

Unlike the myeloablative regimens almost always performed on non-SCID immunodeficient patients, SCID patients who receive MHC-matched CD34-enriched or T cell-depleted bone marrow grafts generally do not receive cytoreductive conditioning before transplantation (37, 60). However, it has been suggested that HSC engraftment does not occur in these patients (61). Because many of these patients show poor B cell lymphopoiesis and lose T cell counts with time, it has been proposed that the lymphoid correction occurs as a result of engraftment of short-lived progenitor cells, which along with mature cells constitute the vast majority of transplanted cells, rather than HSCs with full hematopoietic and self-renewal potential (62, 63). A careful examination of the data, however, shows that ~0.8% of CD34<sup>+</sup> cells in an unconditioned SCID patient who received a bone marrow transplant are not of host origin (61). Although the authors, understandably, did not consider this level of engraftment meaningful, our results suggest that small numbers of HSCs have engrafted in these patients and that eventual T cell loss may be a reflection of HSC exhaustion rather than an initial failure to engraft. This hypothesis is reinforced by suggestions that the process of physiological HSC circulation seems to be conserved between mice and humans (16, 64, 65). Alternatively, the lack of B and T lymphopoiesis has correlated well with graft-versus-host disease (GVHD) in previous studies (66, 67). In MHC-matched settings, bone marrow grafts are often transplanted without manipulation, whereas in HLA-mismatched settings, T cell depletions or CD34 enrichments of donor marrow can still leave up to 10<sup>4</sup> T cells/kg (37). Our results show that even in MHC-matched congenic mouse model systems, immune responses can recognize and reject very slightly mismatched cells, suggesting that it is very likely that GVH responses occur in all patients that receive any mature T cells as part of their nonautologous graft. Although GVHD may not be classified as clinically significant or obviously symptomatic in all cases, subtle GVH effects on B and T lymphopoiesis might still occur. Thus, the use of purified HSC transplants, which do not cause GVHD (30), may potentially avoid poor B lymphopoiesis. In our mouse model system, we have observed sustained B and T lymphopoiesis for the duration of our experiments after purified HSC transplantation.

The mechanism by which transplanted HSCs correct hematopoietic deficiencies in our unconditioned recipients is applicable to the correction of many types of both SCID and non-SCID immunodeficiencies, but these studies at the same time clearly demonstrate that very subtle minor histocompatibility differences can mediate the rejection of HSC grafts when host T lymphocytes are present. Our data suggest that transplantation of purified HSCs, in combination with highly specific lymphoablative treatments when necessary, can correct lymphoid deficiencies in immunodeficient patients without the undesired side effects, such as toxic conditioning and GVHD, often associated with current conditioning and transplantation regimens. Future experiments will determine if the same strategy can be applied to the correction of myeloid deficiencies.

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## MATERIALS AND METHODS

**Animals.** All animal procedures were approved by the International Animal Care and Use Committee. C57BL/Ka-Thy1.1 CD45.2<sup>+</sup> (HZ) and C57BL/Ka-Thy1.1 CD45.1 (BA) strains were derived and maintained in our laboratory. eGFP transgenic mice used in these studies were backcrossed at least 20 generations to either the BA or HZ strain. C57Bl6/Harland mice used for the aging studies were obtained from the National Institute of Aging. The RAG2<sup>-/-</sup>, RAG2<sup>-/-</sup>γc<sup>-/-</sup>, I-A<sup>-/-</sup>, and β<sub>2</sub>m<sup>-/-</sup> mice have been described previously (19, 32, 36, 57) and were bred at least 20 generations onto the C57BL/Ka-Thy1.2 CD45.1<sup>+</sup>, C57BL/Ka-Thy1.2 CD45.2<sup>+</sup>, and C57BL/Ka-Thy1.1 CD45.2 backgrounds. TCRα<sup>-/-</sup>β<sup>-/-</sup> mice were provided by J. Campbell and M. Davis (Stanford University, Stanford, CA). Cμ<sup>-/-</sup> mice were provided by J. Tung and L. Herzenberg (Stanford University). Peripheral blood was sampled from the tail vein, and all HSC transplants were performed by injection into the retroorbital sinus of isoflurane-anesthetized mice. For repetitive transplants, between 1,750 and 4,000 HSCs were transplanted weekly for 6 wk. Donor mice were 4–6-wk old, and recipient mice ranged from 4–12 wk of age unless otherwise noted.

**Antibodies.** The following monoclonal antibodies were purified and conjugated using hybridomas maintained in our laboratory: 19XE5 (anti-Thy1.1), 2C11 (anti-CD3), GK1.5 (anti-CD4), 53-7.3 (anti-CD5), 53-6.7 (anti-CD8), 6B2 (anti-B220), 8C5 (anti-Gr-1), M1/70 (anti-Mac-1), TER119 (anti-Ter119), A20.1.7 (anti-CD45.1), AL1-4A2 (anti-CD45.2), 2B8 (anti-c-kit), E13-161-7 (anti-Sca-1), anti-CD16/CD32 (2.4G2), and A7R34 (anti-IL-7 receptor α). Antibodies were conjugated to biotin, PE, allophycocyanin (APC), Alexa 405, Alexa 430, or Alexa 488 (Invitrogen), according to the manufacturer's instructions. The following were purchased from eBiosciences: antibodies against CD3, CD4, CD8, B220, Mac-1, Ter119, and Gr-1 conjugated to PE-Cy5; anti-c-kit and anti-Mac-1 conjugated to PE-Cy7; anti-Sca-1 conjugated to PE-Cy5.5; anti-CD45.1 and anti-CD45.2 (104) conjugated to APC-Cy5.5; anti-B220 conjugated to APC-Cy7; anti-Flk2 (A2F10) conjugated to PE or biotin; and streptavidin conjugated to APC. Anti-CD34 (RAM34) conjugated to FITC or biotin,

anti-TCR $\beta$  (H57-597) conjugated to APC, anti-CD43 (1B11) conjugated to PE, anti-I-A/I-E (M5.114.15.2) conjugated to PE, and anti-Ly51 (6C3) conjugated to biotin were purchased from BD Biosciences. Streptavidin conjugated to APC-Cy7 was purchased from Caltag.

**FACS and analysis.** All cells were sorted on a FACS Vantage or a FACS Aria (Becton Dickinson). All peripheral blood analysis was performed on an LSR-Scan (Becton Dickinson). Peripheral blood was obtained from the tail vein, red blood cells were sedimented with 2% dextran, and the remaining red blood cells were lysed with an ammonium chloride solution. The remaining white blood cells were stained with anti-CD45.2-Alexa 488, anti-CD45.1-PE, anti-Ter119-PE-Cy5, anti-Mac-1-PE-Cy7, anti-TCR $\beta$ -APC, and anti-B220-APC-Cy7. When peripheral blood containing eGFP<sup>+</sup> cells was analyzed, anti-CD45.1-Alexa-488 was omitted and anti-Gr-1-PE was used instead of anti-CD45.2-PE if warranted by the experiment. For HSC isolation, bone marrow was first enriched using anti-c-kit beads and immunomagnetic selection was performed on an AutoMACS machine (Miltenyi Biotec). Enriched cells were stained with anti-CD34-FITC, anti-Flk2-PE, anti-lineage (CD3, CD4, CD8, B220, Ter119, Mac-1, Gr-1)-PE-Cy5, anti-Sca-1-PE-Cy5.5, and anti-c-kit-PE-Cy7, and cells were double sorted before transplantation. For analysis of HSC chimerism, CD45.1-APC-Cy5.5 was included, and CD34 staining was achieved with anti-CD34 biotin followed by streptavidin APC. For isolation of HSCs from eGFP-transgenic mice, cells were stained with anti-lineage-Cy5-PE, anti-Sca-1-PE-Cy5.5, anti-c-kit-PE-Cy7, anti-Flk2-PE, and anti-CD34 biotin followed by streptavidin APC. For analysis of eGFP HSC chimerism, anti-CD45.1-APC-Cy5.5 was included.

**Immunizations.** Mice were immunized intraperitoneally with 100  $\mu$ g NP conjugated to chicken  $\gamma$  globulin (Biosearch Technologies) precipitated in 10% aluminum potassium sulfate (Sigma-Aldrich). NP-specific enzyme-linked immunosorbent assays were performed with serum obtained 1 wk after immunization on high-protein binding 96-well plates coated with 5  $\mu$ g NP-BSA (Biosearch Technologies). Wells were developed with anti-mouse IgG-horseradish peroxidase (Southern Biotechnology Associates, Inc.) followed by 1 mg/ml ABTS reagent (Sigma-Aldrich), and the reactions were stopped by the addition of 0.1% sodium azide (Sigma-Aldrich). Absorbance was read at a wavelength of 405 nm.

**In vivo depletion of CD4<sup>+</sup> T cells.** Mice were treated consecutively for 3 d with 500  $\mu$ g of intravenously injected purified anti-CD4 antibody (GK1.5). Peripheral blood was analyzed for TCR $\beta$  and CD8 expression to quantitatively assess CD4<sup>+</sup> T cell depletion relative to untreated animals. These mice were then transplanted with 800 GFP<sup>+</sup> HSCs 1 d after the third injection. The mice were then given weekly injections of anti-CD4 for the first 3 wk after HSC transplantation and left untreated afterward.

We thank L. Jerabek for laboratory management, C. Richter for antibody production, L. Hidalgo for animal care, and M. Davis and L. Herzenberg for providing mice.

This work was supported by National Institutes of Health grants 5R01HL058770 and 2R01AI047457 (to I.L. Weissman). D. Bhattacharya is supported by a fellowship from the Cancer Research Institute, D.J. Rossi is supported by a fellowship from the Damon Runyon Cancer Foundation, and D. Bryder is supported by a scholarship from the Swedish Medical Research Council.

Affiliations that might be perceived to have biased this work are as follows: I.L. Weissman was a member of the scientific advisory board of Amgen and owns significant Amgen stock, cofounded and consulted for Systemix, is a cofounder and director of Stem Cells, Inc., and recently cofounded Cellerant, Inc. All other authors have no conflicting financial interests.

Submitted: 24 August 2005

Accepted: 21 November 2005

## REFERENCES

- Morrison, S.J., A.M. Wandycz, K. Akashi, A. Globerson, and I.L. Weissman. 1996. The aging of hematopoietic stem cells. *Nat. Med.* 2: 1011–1016.
- Schofield, R. 1978. The relationship between the spleen colony-forming cell and the haemopoietic stem cell. *Blood Cells.* 4:7–25.
- Hackney, J.A., P. Charbord, B.P. Brunk, C.J. Stoeckert, I.R. Lemischka, and K.A. Moore. 2002. A molecular profile of a hematopoietic stem cell niche. *Proc. Natl. Acad. Sci. USA.* 99:13061–13066.
- Zhang, C.C., and H.F. Lodish. 2004. Insulin-like growth factor 2 expressed in a novel fetal liver cell population is a growth factor for hematopoietic stem cells. *Blood.* 103:2513–2521.
- Calvi, L.M., G.B. Adams, K.W. Weibrecht, J.M. Weber, D.P. Olson, M.C. Knight, R.P. Martin, E. Schipani, P. Divieti, F.R. Bringhurst, et al. 2003. Osteoblastic cells regulate the haematopoietic stem cell niche. *Nature.* 425:841–846.
- Zhang, J., C. Niu, L. Ye, H. Huang, X. He, W.G. Tong, J. Ross, J. Haug, T. Johnson, J.Q. Feng, et al. 2003. Identification of the haematopoietic stem cell niche and control of the niche size. *Nature.* 425: 836–841.
- Osawa, M., K. Hanada, H. Hamada, and H. Nakauchi. 1996. Long-term lymphohematopoietic reconstitution by a single CD34-low/negative hematopoietic stem cell. *Science.* 273:242–245.
- Wagers, A.J., R.I. Sherwood, J.L. Christensen, and I.L. Weissman. 2002. Little evidence for developmental plasticity of adult hematopoietic stem cells. *Science.* 297:2256–2259.
- Rao, S.S., S.O. Peters, R.B. Crittenden, F.M. Stewart, H.S. Ramshaw, and P.J. Quesenberry. 1997. Stem cell transplantation in the normal nonmyeloablative host: relationship between cell dose, schedule, and engraftment. *Exp. Hematol.* 25:114–121.
- Porritt, H.E., K. Gordon, and H.T. Petrie. 2003. Kinetics of steady-state differentiation and mapping of intrathymic-signaling environments by stem cell transplantation in nonirradiated mice. *J. Exp. Med.* 198: 957–962.
- Ron, Y., and J. Sprent. 1985. Prolonged survival in vivo of unprimed B cells responsive to a T-independent antigen. *J. Exp. Med.* 161: 1581–1586.
- Adolfsson, J., O.J. Borge, D. Bryder, K. Theilgaard-Monch, I. Astrand-Grundstrom, E. Sitnicka, Y. Sasaki, and S.E. Jacobsen. 2001. Upregulation of Flt3 expression within the bone marrow Lin(−)Sca1(+)c-kit(+) stem cell compartment is accompanied by loss of self-renewal capacity. *Immunity.* 15:659–669.
- Forster, I., and K. Rajewsky. 1990. The bulk of the peripheral B-cell pool in mice is stable and not rapidly renewed from the bone marrow. *Proc. Natl. Acad. Sci. USA.* 87:4781–4784.
- Tarbell, N.J., D.A. Amato, J.D. Down, P. Mauch, and S. Hellman. 1987. Fractionation and dose rate effects in mice: a model for bone marrow transplantation in man. *Int. J. Radiat. Oncol. Biol. Phys.* 13: 1065–1069.
- Tomita, Y., D.H. Sachs, and M. Sykes. 1994. Myelosuppressive conditioning is required to achieve engraftment of pluripotent stem cells contained in moderate doses of syngeneic bone marrow. *Blood.* 83: 939–948.
- Wright, D.E., A.J. Wagers, A.P. Gulati, F.L. Johnson, and I.L. Weissman. 2001. Physiological migration of hematopoietic stem and progenitor cells. *Science.* 294:1933–1936.
- Xu, H., B.G. Exner, P.M. Chilton, C. Schanie, and S.T. Ildstad. 2004. CD45 congenic bone marrow transplantation: evidence for T cell-mediated immunity. *Stem Cells.* 22:1039–1048.
- van Os, R., T.M. Sheridan, S. Robinson, D. Drukeins, J.L. Ferrara, and P.M. Mauch. 2001. Immunogenicity of Ly5 (CD45)-antigens hampers long-term engraftment following minimal conditioning in a murine bone marrow transplantation model. *Stem Cells.* 19:80–87.
- Goldman, J.P., M.P. Blundell, L. Lopes, C. Kinnon, J.P. Di Santo, and A.J. Thrasher. 1998. Enhanced human cell engraftment in mice deficient in RAG2 and the common cytokine receptor gamma chain. *Br. J. Haematol.* 103:335–342.
- Akashi, K., D. Traver, T. Miyamoto, and I.L. Weissman. 2000. A clonogenic common myeloid progenitor that gives rise to all myeloid lineages. *Nature.* 404:193–197.
- Kondo, M., I.L. Weissman, and K. Akashi. 1997. Identification of clonogenic common lymphoid progenitors in mouse bone marrow. *Cell.* 91:661–672.

22. Sitnicka, E., D. Bryder, K. Theilgaard-Monch, N. Buza-Vidas, J. Adolfsson, and S.E. Jacobsen. 2002. Key role of flt3 ligand in regulation of the common lymphoid progenitor but not in maintenance of the hematopoietic stem cell pool. *Immunity*. 17:463–472.
23. Karsunky, H., M. Merad, A. Cozzio, I.L. Weissman, and M.G. Manz. 2003. Flt3 ligand regulates dendritic cell development from Flt3+ lymphoid and myeloid-committed progenitors to Flt3+ dendritic cells in vivo. *J. Exp. Med.* 198:305–313.
24. Hardy, R.R., C.E. Carmack, S.A. Shinton, J.D. Kemp, and K. Hayakawa. 1991. Resolution and characterization of pro-B and pre-pro-B cell stages in normal mouse bone marrow. *J. Exp. Med.* 173:1213–1225.
25. Li, Y.S., R. Wasserman, K. Hayakawa, and R.R. Hardy. 1996. Identification of the earliest B lineage stage in mouse bone marrow. *Immunity*. 5:527–535.
26. Kondo, M., and I.L. Weissman. 2000. Function of cytokines in lymphocyte development. *Curr. Top. Microbiol. Immunol.* 251:59–65.
27. Tesch, H., F.I. Smith, W.J. Muller-Hermes, and K. Rajewsky. 1984. Heterogeneous and monoclonal helper T cells induce similar anti-(4-hydroxy-3-nitrophenyl)acetyl (NP) antibody populations in the primary adoptive response. I. Isotype distribution. *Eur. J. Immunol.* 14:188–194.
28. Smith, F.I., H. Tesch, and K. Rajewsky. 1984. Heterogeneous and monoclonal helper T cells induce similar anti-(4-hydroxy-3-nitrophenyl)acetyl (NP) antibody populations in the primary adoptive response. II. Lambda light chain dominance and idiotope expression. *Eur. J. Immunol.* 14:195–200.
29. Rossi, D.J., D. Bryder, J.M. Zahn, H. Ahlenius, R. Sonu, A.J. Wagers, and I.L. Weissman. 2005. Cell intrinsic alterations underlie hematopoietic stem cell aging. *Proc. Natl. Acad. Sci. USA*. 102:9194–9199.
30. Shizuru, J.A., L. Jerabek, C.T. Edwards, and I.L. Weissman. 1996. Transplantation of purified hematopoietic stem cells: requirements for overcoming the barriers of allogeneic engraftment. *Biol. Blood Marrow Transplant.* 2:3–14.
31. Prockop, S.E., and H.T. Petrie. 2004. Regulation of thymus size by competition for stromal niches among early T cell progenitors. *J. Immunol.* 173:1604–1611.
32. Grusby, M.J., R.S. Johnson, V.E. Papaioannou, and L.H. Glimcher. 1991. Depletion of CD4+ T cells in major histocompatibility complex class II-deficient mice. *Science*. 253:1417–1420.
33. Zijlstra, M., M. Bix, N.E. Simister, J.M. Loring, D.H. Raulet, and R. Jaenisch. 1990. Beta 2-microglobulin deficient mice lack CD4+8+ cytolytic T cells. *Nature*. 344:742–746.
34. Lamouse-Smith, E., V.K. Clements, and S. Ostrand-Rosenberg. 1993. Beta 2M-/- knockout mice contain low levels of CD8+ cytotoxic T lymphocyte that mediate specific tumor rejection. *J. Immunol.* 151:6283–6290.
35. Apasov, S., and M. Sitkovsky. 1993. Highly lytic CD8+, alpha beta T-cell receptor cytotoxic T cells with major histocompatibility complex (MHC) class I antigen-directed cytotoxicity in beta 2-microglobulin, MHC class I-deficient mice. *Proc. Natl. Acad. Sci. USA*. 90:2837–2841.
36. Bix, M., N.S. Liao, M. Zijlstra, J. Loring, R. Jaenisch, and D. Raulet. 1991. Rejection of class I MHC-deficient haemopoietic cells by irradiated MHC-matched mice. *Nature*. 349:329–331.
37. Antoine, C., S. Muller, A. Cant, M. Cavazzana-Calvo, P. Veys, J. Vossen, A. Fasth, C. Heilmann, N. Wulffraat, R. Seger, et al. 2003. Long-term survival and transplantation of haemopoietic stem cells for immunodeficiencies: report of the European experience 1968–99. *Lancet*. 361:553–560.
38. Taniguchi, H., M. Abe, T. Shirai, K. Fukao, and H. Nakauchi. 1995. Reconstitution ratio is critical for alloreactive T cell deletion and skin graft survival in mixed bone marrow chimeras. *J. Immunol.* 155:5631–5636.
39. Kay, M.M., and T. Makinodan. 1981. Relationship between aging and immune system. *Prog. Allergy*. 29:134–181.
40. Kamminga, L.M., R. van Os, A. Ausema, E.J. Noach, E. Weersing, B. Dontje, E. Vellenga, and G. de Haan. 2005. Impaired hematopoietic stem cell functioning after serial transplantation and during normal aging. *Stem Cells*. 23:82–92.
41. Gatti, R.A., H.J. Meuwissen, H.D. Allen, R. Hong, and R.A. Good. 1968. Immunological reconstitution of sex-linked lymphopenic immunological deficiency. *Lancet*. 2:1366–1369.
42. Bach, F.H., R.J. Albertini, P. Joo, J.L. Anderson, and M.M. Bortin. 1968. Bone-marrow transplantation in a patient with the Wiskott-Aldrich syndrome. *Lancet*. 2:1364–1366.
43. Buckner, C.D., R.B. Epstein, R.H. Rudolph, R.A. Clift, R. Storb, and E.D. Thomas. 1970. Allogeneic marrow engraftment following whole body irradiation in a patient with leukemia. *Blood*. 35:741–750.
44. Appelbaum, F.R., G.P. Herzig, J.L. Ziegler, R.G. Graw, A.S. Levine, and A.B. Deisseroth. 1978. Successful engraftment of cryopreserved autologous bone marrow in patients with malignant lymphoma. *Blood*. 52:85–95.
45. Thomas, E.D., H.L. Lochte Jr., W.C. Lu, and J.W. Ferrebee. 1957. Intravenous infusion of bone marrow in patients receiving radiation and chemotherapy. *N. Engl. J. Med.* 257:491–496.
46. Sayegh, M.H., N.A. Fine, J.L. Smith, H.G. Rennke, E.L. Milford, and N.L. Tilney. 1991. Immunologic tolerance to renal allografts after bone marrow transplants from the same donors. *Ann. Intern. Med.* 114:954–955.
47. Nelson, J.L., R. Torrez, F.M. Louie, O.S. Choe, R. Storb, and K.M. Sullivan. 1997. Pre-existing autoimmune disease in patients with long-term survival after allogeneic bone marrow transplantation. *J. Rheumatol. Suppl.* 48:23–29.
48. Boggs, D.R. 1984. The total marrow mass of the mouse: a simplified method of measurement. *Am. J. Hematol.* 16:277–286.
49. Peschon, J.J., P.J. Morrissey, K.H. Grabstein, F.J. Ramsdell, E. Maraskovsky, B.C. Gliniak, L.S. Park, S.F. Ziegler, D.E. Williams, C. B. Ware, et al. 1994. Early lymphocyte expansion is severely impaired in interleukin 7 receptor-deficient mice. *J. Exp. Med.* 180:1955–1960.
50. Rodewald, H.R., K. Kretzschmar, W. Swat, and S. Takeda. 1995. Intrathymically expressed c-kit ligand (stem cell factor) is a major factor driving expansion of very immature thymocytes in vivo. *Immunity*. 3:313–319.
51. Sitnicka, E., C. Brakebusch, I.L. Martensson, M. Svensson, W.W. Agace, M. Sigvardsson, N. Buza-Vidas, D. Bryder, C.M. Cilio, H. Ahlenius, et al. 2003. Complementary signaling through flt3 and interleukin-7 receptor  $\alpha$  is indispensable for fetal and adult B cell genesis. *J. Exp. Med.* 198:1495–1506.
52. Ranzheim, E.A., H.C. Kwan, T. Reya, Y.K. Wang, I.L. Weissman, and U. Francke. 2005. Frizzled 9 knock-out mice have abnormal B-cell development. *Blood*. 105:2487–2494.
53. Balciunaite, G., R. Ceredig, H.J. Fehling, J.C. Zuniga-Pflucker, and A.G. Rolink. 2005. The role of Notch and IL-7 signaling in early thymocyte proliferation and differentiation. *Eur. J. Immunol.* 35:1292–1300.
54. Cao, X., E.W. Shores, J. Hu-Li, M.R. Anver, B.L. Kelsall, S.M. Russell, J. Drago, M. Noguchi, A. Grinberg, E.T. Bloom, et al. 1995. Defective lymphoid development in mice lacking expression of the common cytokine receptor gamma chain. *Immunity*. 2:223–238.
55. DiSanto, J.P., D. Guy-Grand, A. Fisher, and A. Tarakhovsky. 1996. Critical role for the common cytokine receptor  $\gamma$  chain in intrathymic and peripheral T cell selection. *J. Exp. Med.* 183:1111–1118.
56. Kume, A., M. Koremoto, H. Mizukami, T. Okada, Y. Hanazono, K. Sugamura, and K. Ozawa. 2002. Selective growth advantage of wild-type lymphocytes in X-linked SCID recipients. *Bone Marrow Transplant.* 30:113–118.
57. Shinkai, Y., G. Rathbun, K.P. Lam, E.M. Oltz, V. Stewart, M. Mendelsohn, J. Charron, M. Datta, F. Young, A.M. Stall, et al. 1992. RAG-2-deficient mice lack mature lymphocytes owing to inability to initiate V(D)J rearrangement. *Cell*. 68:855–867.
58. Spitzer, T.R., S.L. McAfee, B.R. Dey, C. Colby, J. Hope, H. Grossberg, F. Preffer, J. Shaffer, S.I. Alexander, D.H. Sachs, and M. Sykes. 2003. Nonmyeloablative haploidentical stem-cell transplantation using anti-CD2 monoclonal antibody (MEDI-507)-based conditioning for refractory hematologic malignancies. *Transplantation*. 75:1748–1751.
59. Ferry, C., and G. Socie. 2003. Busulfan-cyclophosphamide versus total body irradiation-cyclophosphamide as preparative regimen before allogeneic hematopoietic stem cell transplantation for acute myeloid leukemia: what have we learned? *Exp. Hematol.* 31:1182–1186.



60. Buckley, R.H., S.E. Schiff, R.I. Schiff, L. Markert, L.W. Williams, J.L. Roberts, L.A. Myers, and F.E. Ward. 1999. Hematopoietic stem-cell transplantation for the treatment of severe combined immunodeficiency. *N. Engl. J. Med.* 340:508–516.
61. Muller, S.M., T. Kohn, A.S. Schulz, K.M. Debatin, and W. Friedrich. 2000. Similar pattern of thymic-dependent T-cell reconstitution in infants with severe combined immunodeficiency after human leukocyte antigen (HLA)-identical and HLA-nonidentical stem cell transplantation. *Blood.* 96:4344–4349.
62. Fischer, A., F. Le Deist, S. Hacein-Bey-Abina, I. Andre-Schmutz, S. Basile Gde, J.P. de Villartay, and M. Cavazzana-Calvo. 2005. Severe combined immunodeficiency. A model disease for molecular immunology and therapy. *Immunol. Rev.* 203:98–109.
63. Cavazzana-Calvo, M., C. Lagresle, S. Hacein-Bey-Abina, and A. Fischer. 2005. Gene therapy for severe combined immunodeficiency. *Annu. Rev. Med.* 56:585–602.
64. McCredie, K.B., E.M. Hersh, and E.J. Freireich. 1971. Cells capable of colony formation in the peripheral blood of man. *Science.* 171:293–294.
65. Goodman, J.W., and G.S. Hodgson. 1962. Evidence for stem cells in the peripheral blood of mice. *Blood.* 19:702–714.
66. Storek, J., R.P. Witherspoon, D. Webb, and R. Storb. 1996. Lack of B cells precursors in marrow transplant recipients with chronic graft-versus-host disease. *Am. J. Hematol.* 52:82–89.
67. Wall, D.A., S.D. Hamberg, D.S. Reynolds, S.J. Burakoff, A.K. Abbas, and J.L. Ferrara. 1988. Immunodeficiency in graft-versus-host disease. I. Mechanism of immune suppression. *J. Immunol.* 140:2970–2976.