BIOLOGY

Combined Effects of Interleukin-7 and Stem Cell Factor Administration on Lymphopoiesis after Murine Bone Marrow Transplantation

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The decreased ability of the thymus to generate T cells after bone marrow transplantation (BMT) is a clinically significant problem. Interleukin (IL)-7 and stem cell factor (SCF) induce proliferation, differentiation, and survival of thymocytes. Although previous studies have shown that administration of recombinant human IL-7 (rhIL-7) after murine and human BMT improves thymopoiesis and immune function, whether administration of SCF exerts similar effects is unclear. To evaluate independent or combinatorial effects of IL-7 and SCF in post-BMT thymopoiesis, bone marrow (BM)-derived mesenchymal stem cells transduced ex vivo with the rhIL-7 or murine SCF (mSCF) genes were cotransplanted with T cell–depleted BM cells into lethally irradiated mice. Although rhIL-7 and mSCF each improved immune reconstitution, the combination treatment had a significantly greater effect than either cytokine alone. Moreover, the combination treatment significantly increased donor-derived common lymphoid progenitors (CLPs) in BM, suggesting that transplanted CLPs expand more rapidly in response to IL-7 and SCF and may promote immune reconstitution. Our findings demonstrate that IL-7 and SCF might be therapeutically useful for enhancing de novo T cell development. Furthermore, combination therapy may allow the administration of lower doses of IL-7, thereby decreasing the likelihood of IL-7–mediated expansion of mature T cells.

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INTRODUCTION

The loss of postnatal thymic function due to chemotherapy, radiation, aging, human immunodeficiency virus (HIV) infection, and graft-versus-host disease (GVHD) is a major problem contributing to the increased risk of infection in certain clinical settings, notably cancer chemotherapy, hematopoietic stem cell transplantation (HSCT), HIV infection, and aging [1-10]. The loss of thymopoietic capacity appears to result from injury or loss of the thymic micronenvironment, particularly of thymic epithelial cells (TECs) [5,8,11]. The ability of adolescents and adults to generate thymic-derived new T lymphocytes is significantly diminished after receiving preparative chemotherapy or radiotherapy for HSCT [12,13]. Further complicating allogeneic HSCT is the targeting and killing of TECs by alloreactive T cells [14]. Thymic insufficiency limits or delays the capacity of HSCT recipients to reconstitute their T cell compartment, contributing to an increased susceptibility to opportunistic infections and viral-associated malignancies [15].

Interleukin (IL)-7 and stem cell factor (SCF; a Kit ligand) are the major lymphopoietic cytokines produced in the thymus and bone marrow compartment [16-18]. IL-7 induces proliferation, differentiation, and survival of immature T lymphocytes throughout development. During normal T cell development in the thymus, IL-7 and SCF, produced by TECs, bind to their cognate IL-7R and Kit receptors expressed on the surface of immature T lymphoid progenitor cells. IL-7 stimulates the differentiation of immature $CD3^{-}CD4^{-}CD8^{-}$ (triple-negative [TN]) thymocytes to later stages, ultimately resulting in the development of mature

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$CD4^+CD8^-$ or $CD4^-CD8^+$ T cells. The importance of IL-7 for thymopoiesis is demonstrated by mice with targeted mutations of the IL-7, IL-7 receptor α, or common γ chain (γc) gene; dogs with X severe combined immunodeficiency (SCID) (γc mutations); and humans with IL-7R–deficient SCID or X-SCID—all of which have defective thymopoiesis and impaired ability to produce T lymphocytes [18-21]. We and others have previously shown that administration of recombinant human IL-7 (rhIL-7) corrects the thymopoietic defects observed after histocompatible BMT, suggesting that post-BMT IL-7 administration might be a potential therapy for posttransplantation immunodeficiency [22-25].

The role of SCF in T cell development has been described in mice with mutations of SCF (SL/SL) or its receptor, Kit (receptor tyrosine kinase), which have significantly lower numbers of thymocytes compared with their wild-type counterparts [16,26]. Analyses of Kit- or SCF-deficient mice by bromodeoxyuridine administration in vivo have shown that approximately 50% of the proliferation of TN thymocytes is dependent on the Kit-signaling pathway. Furthermore, the addition of SCF has been reported to increase TN thymocytes in vitro, and the administration of anti-Kit antibody blocks the ability of TN thymocytes to develop into mature T cells, demonstrating the importance of SCF in the early stage of T lymphocyte development [27,28]. Interestingly, the Kit pathway interacts directly with other cytokine receptors of hematopoietic progenitors, notably IL-3R, erythropoietin receptor, and IL-7R [29-32]. In addition, mice that are doubly mutant for Kit and for γc, IL-7, or IL-7Rα exhibit complete failure of T cell development, profoundly more severe than that observed in either parental strain [26,33]. Thus, the IL-7 and SCF signaling pathways are essential, redundant, and partially synergistic during T cell generation.

Here we hypothesize that BM-derived mesenchymal stem cells (MSCs) transduced with a retroviral vector containing either IL-7 or SCF cDNA would be a safe and efficient delivery vehicle for the administration of either cytokine alone or both cytokines together. The transduced MSCs would produce IL-7 or SCF continuously, which is more physiological than the high intermittent doses administered in other systemic cytokine treatment studies, including our previous study of IL-7 injections [22,34,35]. In the present study, we demonstrate that cotransplantation of BM and BM-derived MSCs stably transduced with either the IL-7 or SCF genes resulted in enhanced thymopoiesis and immune reconstitution. Moreover, combined IL-7 and SCF therapy had a greater effect on the expansion of common lymphoid progenitors (CLPs), enhancement of donor-derived thymopoiesis, and peripheral T cell recovery compared with either cytokine treatment alone, suggesting that combined IL-7 and SCF therapy can promote thymopoiesis and T cell reconstitution after BMT.

MATERIALS AND METHODS

Mice

Eight- to 10-week-old female recipient mice (C57BL/6J [$CD45.2^+$]) and male donor mice (congenic B6.SJL [$CD45.1^+$]) were purchased from Jackson Laboratory (Bar Harbor, ME). The mice were kept in laminar flow cages with autoclaved food and acidified water. The protocol for maintaining the animals before and after BMT was approved by Stanford University's Animal Care Committee.

Generation and Transduction of BM-Derived MSCs

Three retroviral vectors—MND-SCF-IRES-EGFP, MND-IL-7-IRES-EGFP, and MND-EGFP—were used to transduce MSCs. These retroviral vectors were generated at the Vector Core Laboratory under the direction of Dr. Donald Kohn at Children's Hospital Los Angeles, and contain either the rhIL-7 or murine SCF (mSCF) cDNA under transcriptional control of the Moloney murine leukemia virus (M-MuLV) long terminal repeat (LTR). Transcription of enhanced green fluorescent protein (EGFP) is controlled by an internal ribosomal entry site (IRES). Supernatants containing each vector were prepared by infection of the PA317 amphotropic packaging cell line with titers of 1×10^6. BM-derived MSC cultures were generated from 8- to 10-week-old C57BL/6J ($CD45.2^+$) mice. After sacrifice, the femurs were dissected and the medullary cavity was flushed and then crushed. Both BM fragments and the BM cavity were cultured on the tissue culture dishes in DOM medium to produce an adherent cell layer. At approximately 80% confluence, $CD45^+$ cells were removed using rat anti-CD45 monoclonal antibody (BD Pharmingen, San Diego, CA) and immunomagnetic beads (Dynal, Great Neck, NY). After generation of MSCs, the cells were transduced by the addition of MND-EGFP only, MND-IL-7 IRES EGFP, or MND-SCF IRES EGFP retroviral supernatants in the presence of 4 μg/mL of protamine sulfate. A total of 4 exposures to retroviral supernatant over 2 days was used to transduce the cells. After transduction, isolation of $EGFP^+$ cells was performed using fluorescence-activated cell sorting (FACS). MSC production of IL-7 and SCF was measured after plating 1×10^6 transduced MSCs in 1 mL of medium in individual wells of 6-well plates. After 24 hours, the medium was collected and serial dilutions were tested using an enzyme-linked immunosorbent assay (ELISA) kit for detection of rhIL-7 and SCF (R&D Systems, Minneapolis, MN). The mean of duplicate samples was expressed as pg of IL-7 or SCF produced per 10^6 cells in 24 hours.

BMT Procedure

Female recipient C57BL/6J (CD45.2) mice were given two separate doses of radiation (700 cGy on day −1 and 600 cGy on day 0). The BM cells from B6.SJL (CD45.1) donor mice were obtained by perfusion of the femur. The donor BM cells were depleted for mature T lymphocytes by immmunomagnetic depletion, using rat anti-mouse Thy 1.2, CD4, and CD8 monoclonal antibodies (BD Pharmingen) and sheep anti-rat antibodies conjugated to beads (Dynal). Each recipient mouse received <1000 T (Thy 1.2^+) cells, as determined by FACS of the infused marrow (<0.1% contamination). After irradiation of recipient mice, 1×10^6 T cell–depleted (TCD) BM and different dosages of transduced MSCs were cotransplanted into recipients via tail vein injection. In each experiment, a group of mice received irradiation but no transplanted TCD BM cells, to confirm delivery of a lethal dose of radiation.

Immunophenotyping

BMT recipients were sacrificed by CO_2 narcosis, after which the thymus, spleen, and BM cells were removed. Single-cell suspensions of each organ were prepared, and the total cell numbers were determined. Then 1×10^5 cells were stained with conjugated antibodies directed against Thy-1.2, CD3, CD4, CD8, CD45.1, CD45.2, B220, CD11b, GR1, CD25, CD44, Ter119, or isotype control monoclonal antibodies (BD Pharmingen). To identify hematopoietic stem cells (HSCs) or CLPs, BM cells also were stained with conjugated antibodies against Kit, Sca-1, and IL-7Rα. After staining, cells were washed and analyzed on a FACSCalibur analyzer (BD Biosciences, San Jose, CA).

ELISA for Detection of rhIL-7 and mSCF

The rhIL-7 and mSCF concentrations in the serum samples were measured using a commercially available high-sensitivity ELISA kit (R&D Systems) in accordance with the manufacturer's instructions. A standard curve was prepared for each plate, plotting optical density versus various concentrations of rhIL-7 and mSCF. All standards and samples were tested in duplicate.

Histology

Spleen, liver, and thymic tissues were fixed in 4% paraformaldehyde for 30 minutes and then embedded in 1.5% gelatin containing 0.12 M phosphate buffer (Sigma-Aldrich, St Louis, MO). The gelatin-embedded tissue samples were dried for 15 minutes and then carefully sectioned using a Leica VT1000 S vibrotome (Leica Microsystems, Bannockburn, IL). Then 30-μm sections of each tissue sample were examined for EGFP expression using a Leica TCS SP1 confocal microscope. Tissue sections from the recipients of nontransduced MSCs were used to establish the lowest threshold of EGFP intensity.

Sheep Red Blood Cell Immunization

Normal and recipient mice were immunized with sheep red blood cells (SRBCs; Colorado Serum, Denver, CO) via i.p. injection at day 30 post-BMT. After 2 weeks, peripheral blood was drawn for assessment of primary antibody responses against SRBCs, and the mice were boosted with SRBCs for secondary responses, which were measured 1 week later. The agglutination antibody titers in the serum were determined by serial dilution and incubation with SRBCs in 96-well V-bottomed microplates (Corning, Corning, NY).

Statistical Analyses

Different immunophenotypic populations of cells after transplantation were analyzed using the two-tailed t test with unequal distributions. A P value $\leq .05$ was considered statistically significant.

RESULTS

Generation and Characterization of IL-7– and SCF-Transduced MSCs

We took an IL-7 and SCF gene therapy approach using retrovirally mediated transduction of BM-derived MSCs. We hypothesized that continuous production of either IL-7 or SCF by MSCs would be more physiological than boluses of cytokine injections, and that the transduced MSCs could be transplanted into the lymphoid organs. We chose BM-derived MSCs as an efficient delivery vehicle for the administration of cytokine, because MSCs are derived from the hematopoietic niche and readily expand in vitro. In addition, BM MSCs have been reported to support hematopoiesis after cotransplantation of BM MSCs with HSCs in several animal experiments [36,37]. To generate BM-derived MSCs, BM stromal cells from 8- to 10-week-old C57BL/6J mice were collected and cultured in vitro based on their ability to bind to tissue culture plates, followed by depletion of $CD45^+$ cells to exclude all hematopoietic lineage–derived cells. After enrichment, the cells expressed the BM-derived MSC markers CD90, CD105, Sca-1, and CD44 (Figure 1A). In addition, the BM-derived MSCs were able to differentiate into different mesenchymal lineages (adipogenesis, osteogenesis, and myogenesis), as described previously (data not shown) [38].

To transduce MSCs, we generated the retroviral vectors expressing EGFP only (MND-EGFP), rhIL-7 cDNA (MND-hIL-7-IRES-EGFP), or mSCF (secreted form) cDNA (MND-mSCF-IRES-EGFP) (Figure 1B). After retroviral supernatants were added to the cultured MSCs, the transduced MSCs were sorted

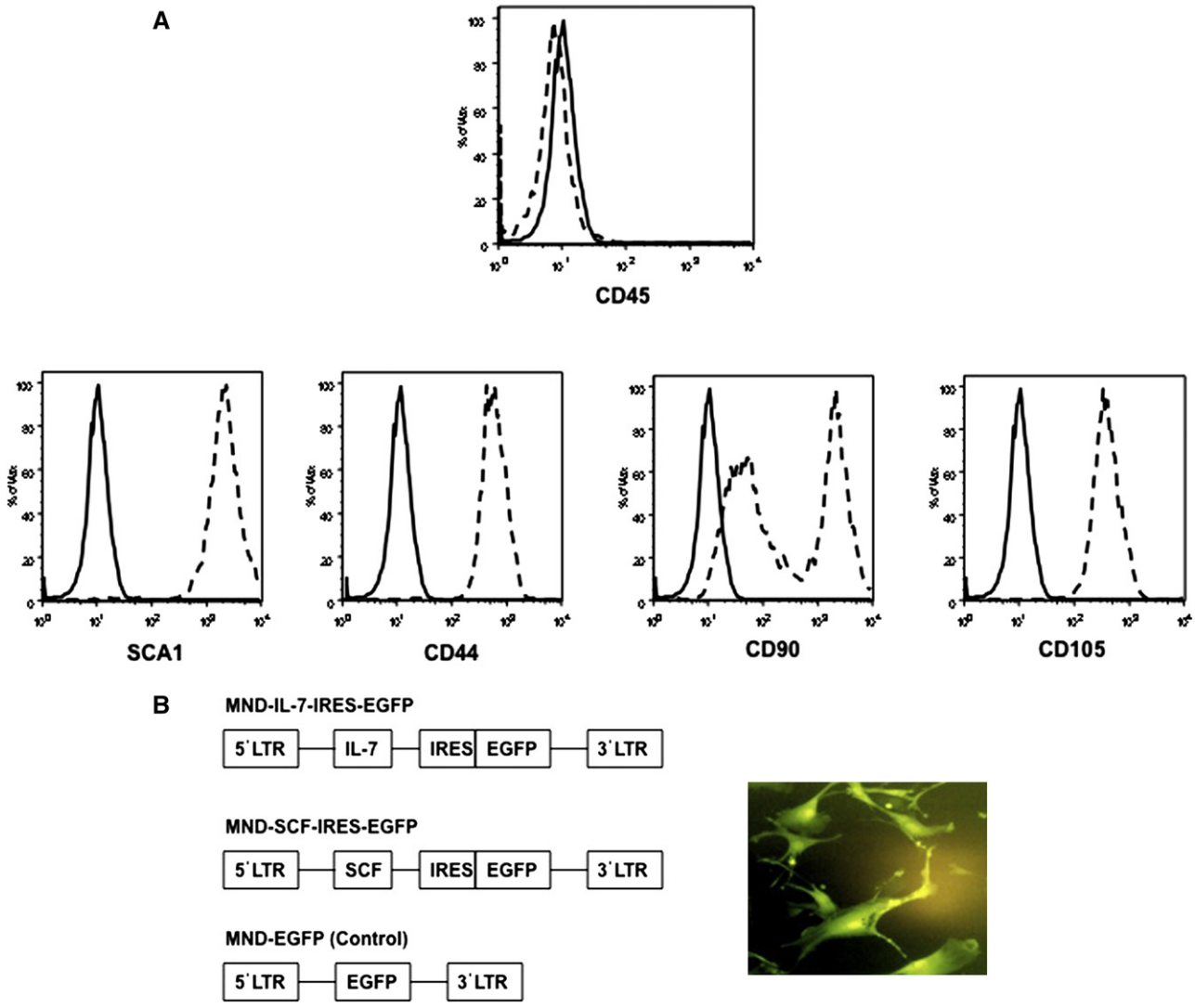

Figure 1. Phenotypic characterization of BM-derived MSCs and schematic diagrams of of MND-IL-7-IRES-EGFP, MND-SCF-IRES-EGFP, and MND-EGFP vectors. In an IL-7 and SCF gene therapy approach using retrovirally mediated transduction, murine BM–derived MSCs were generated and used as a delivery vehicle for the administration of cytokine. (A) Characterization of cell surface markers expressed on BM-derived MSCs. The majority of BM-derived MSCs were positive for CD105, CD90, CD44, and Sca-1 and negative for CD45. (B) The MND LTR is based on the M-MuLV LTR, and the vectors were constructed so that the LTR drives expression of the EGFP reporter gene, rhIL-7 cDNA, or mSCF cDNA. Both IL-7 and SCF vectors contain an IRES in which the EGFP marker gene is expressed downstream of the IRES. The imunofluorescence image of MSC tranduced with the MND vector is shown on the right.

based on EGFP expression and analyzed by ELISA for production of rhIL-7 and mSCF. In several experiments, small amounts of both IL-7 (5 pg/L $\times$ 10^6 cells/24 hours) and SCF (20 pg/L $\times$ 10^6 cells/24 hours) were produced by the MSCs transduced with the MND-EGFP vector. In contrast, the MSCs transduced with MND-hIL-7-IRES-EGFP or MND-mSCF-IRES-EGFP vector produced large amounts of IL-7 (1600 pg/L $\times$ 10^6 cells/24 hours) and SCF (1200 pg/L $\times$ 10^6 cells/24 hours) (data not shown).

Engraftment of Transduced MSCs and Circulating Levels of rhIL-7 and mSCF in BMT Recipients

Because the IL-7 and SCF MSCs were injected i.v., MSC engraftment in multiple sites was expected. At the time of sacrifice, organs were separated, sectioned, and visualized under fluorescent microscopy. The level of fluorescence was sufficient to allow tracking of transduced cells after transplantation, and we detected EGFP-positive MSCs in lymphohematopoietic organs (eg, marrow, spleen, liver) (Figure 2A). We also measured the circulating levels of both rhIL-7 and mSCF in peripheral blood of recipients of transduced MSCs at day 30 to evaluate whether engrafted MSCs were capable of secreting the cytokines. The serum samples from recipients of IL-7 or/and SCF MSCs displayed increased levels of circulating rhIL-7 or/and mSCF compared with EGFP MSC control animals, and mice receiving 0.3 $\times$ 10^6 transduced MSCs had significantly higher rhIL-7 levels than those receiving 0.1 $\times$ 10^6 MSCs. The SCF assays were complicated by

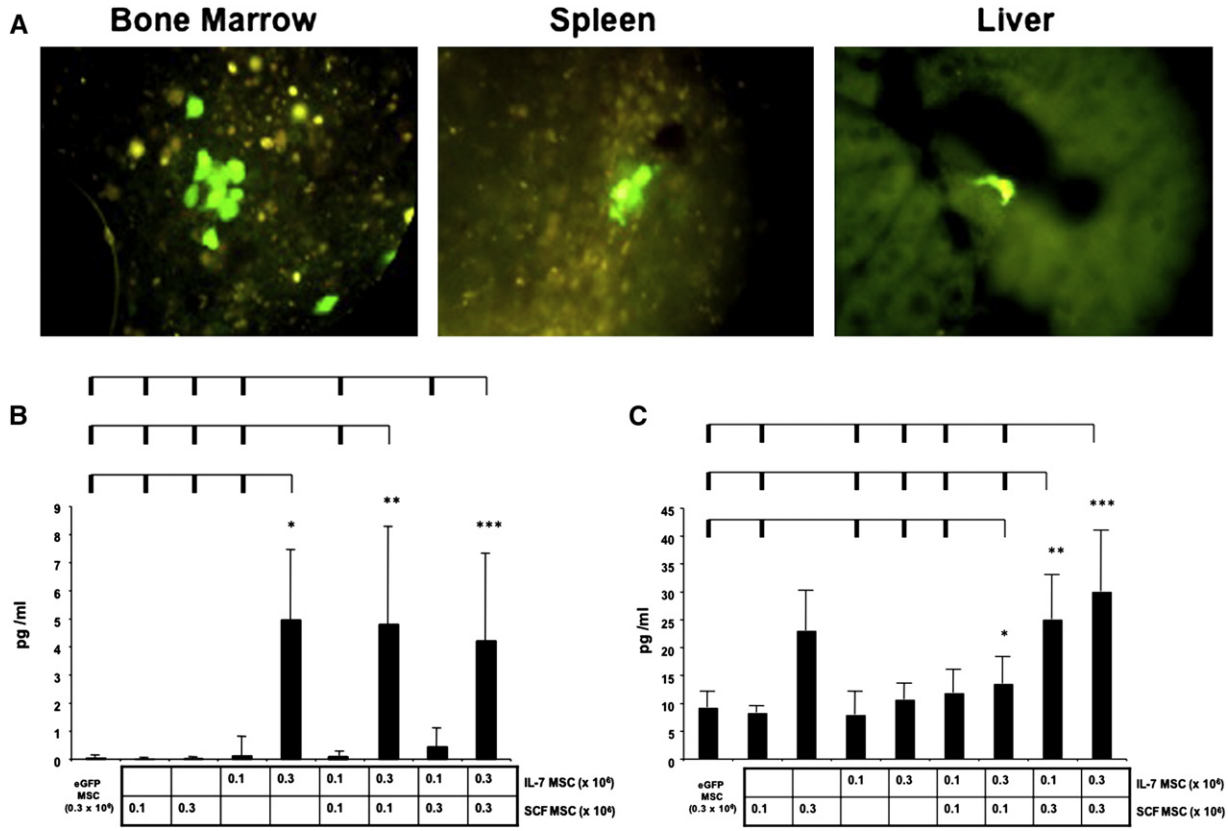

Figure 2. In vivo detection of transduced MSCs at day 30 post-BMT and serum levels of rhIL-7 and mSCF. After cotransplantation of TCD BM and transduced MSCs, EGFP-expressing cells were detected in the tissue sections of BM, spleen, and liver by fluorescence microscopy at day 30. (Original magnification, 200×.) (A) After cotransplantation of TCD BM and retrovirally transduced MSCs, serum levels of rhIL-7 and mSCF were measured in recipient mice at day 30. (B and C) Increased serum levels of rhIL-7 (B) and mSCF (C) were in mice that underwent transplantation with rIL-7 and/or mSCF MSCs. All values are mean ± standard deviation; an asterisk indicates significant differences. Each vertical bold line branching off from the top of the horizontal lines represent statistically significant *P* values when the 0.3×10^6 IL-7 + 0.3×10^6 SCF, 0.1×10^6 IL-7 + 0.3×10^6 SCF, and 0.3×10^6 IL-7 + 0.1×10^6 SCF groups were individually compared with all other groups of animals. *P* values for serum rhIL-7 levels: **P* ≤.0005; ***P* ≤.01; ****P* ≤.006. *P* values for serum mSCF levels: **P* ≤.02; ***P* ≤.005; ****P* ≤.002). Each value represents the mean of two independent experiments with a total of 3-5 animals per group in each experiment. Consistent results were obtained in repeated experiments throughout.

the need to use mSCF in the vectors, because human SCF does not efficiently transduce signals through the murine Kit receptor. As a result, the assay detected both MSC-derived SCF and endogenously generated SCF. Plasma levels of mSCF were related to the dose of transplanted SCF MSCs infused, but with greater intergroup variability (Figure 2B and C).

Cotransplantation of IL-7 and SCF MSCs Increased Donor Thymocytes

At day 30 post-BMT, the amount of donor-derived thymocytes was measured in the mice that received transduced MSCs (Figure 3A). Based on previous published results by our group and others [5,8,9,22,23], full donor-derived thymocyte engraftment was observed after experimental murine TCD BMT at days 28 and 30. No increment in thymocyte numbers was found in mice receiving EGFP MSCs compared with those not receiving MSCs. In contrast, the number of donor-derived thymocytes was greater in the mice that received IL-7 and/or SCF MSCs. An incremental increase in thymic cellularity was related to the number of transduced IL-7 or SCF MSCs given. In general, transplantation of IL-7 MSCs resulted in higher donor thymocyte recovery compared with transplantation of SCF MSCs alone. Moreover, the combined effect of IL-7 and SCF resulted in significantly greater number of donor-derived thymocytes compared with the effect of either cytokine alone.

Based on our finding that cotransplantation of IL-7 and SCF resulted in greater enhancement of donor-derived thymopoiesis, we next analyzed the effect of these cytokines on subpopulations of donor thymocytes. The donor-derived thymocytes were divided into 4 sequential developmental stages: immature $Thy1^+CD4^-CD8^-$ double-negative (DN), intermediate $Thy1^+CD4^+CD8^+$ double-positive (DP), mature $Thy1^+CD4^+CD8^-$ single-positive CD4 (SP CD4), and $Thy1^+$ $CD4^-CD8^+$ single-positive CD8 (SP CD8) (Supplementary Figure 1). We found no significant effects of either IL-7 or SCF along on the

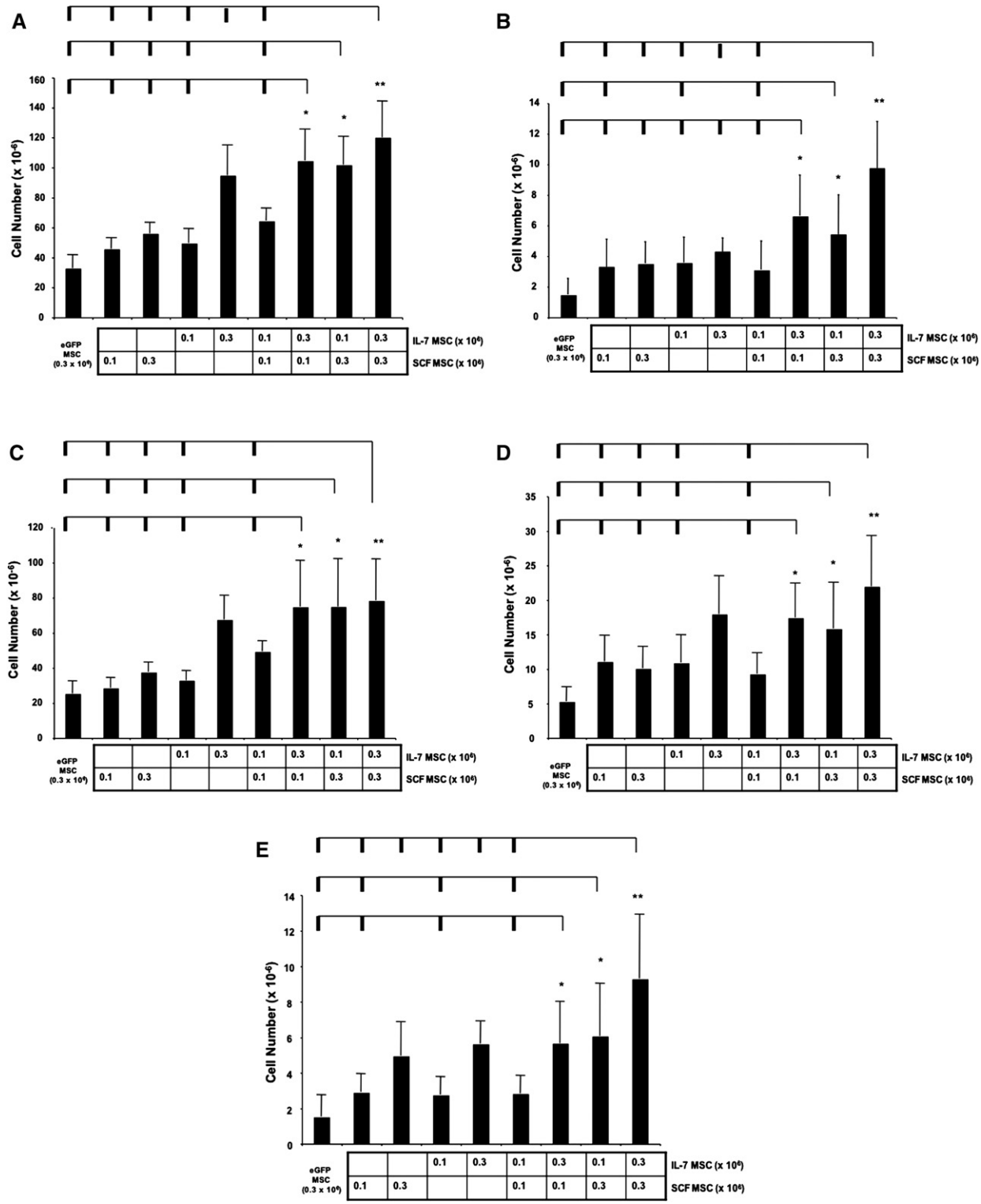

Figure 3. Effects of SCF and IL-7 BM-derived MSCs on the numbers of donor-derived thymocytes and thymic subpopulations at day 30 post-BMT. The number of donor-derived thymocytes ($CD45.1^+Thy1^+$) from each group of transplanted recipient mice was analyzed at day 30 post-BMT. Shown are (A) numbers of overall donor thymocytes (*$P \leq .0002$; **$P \leq .03$]), (B) donor DN ($Thy1^+CD4^-CD8^-$) thymocytes (*$P \leq .05$; **$P \leq .03$), (C) donor DP ($Thy1^+CD4^+CD8^+$) thymocytes (*$P \leq .008$; **$P \leq .004$), (D) donor SP CD4 ($Thy1^+CD4^+CD8^-$) thymocytes (*$P \leq .05$; **$P \leq .001$), and (E) donor SP CD8 ($Thy1^+CD4^-CD8^+$) thymocytes (*$P \leq .05$; **$P \leq .001$). An asterisk indicates significant differences between each group of animals. Each figure represents the mean of 3 independent experiments, each with a total of 3-5 animals per group. Consistent results were obtained in repeated experiments throughout. Each vertical bold line branching off from the top of the horizontal lines represent statistically significant *P* values when the 0.3×10^6 IL-7 + 0.3×10^6 SCF, 0.1×10^6 IL-7 + 0.3×10^6 SCF, and 0.3×10^6 IL-7 + 0.1×10^6 SCF groups were individually compared with all other groups of animals.

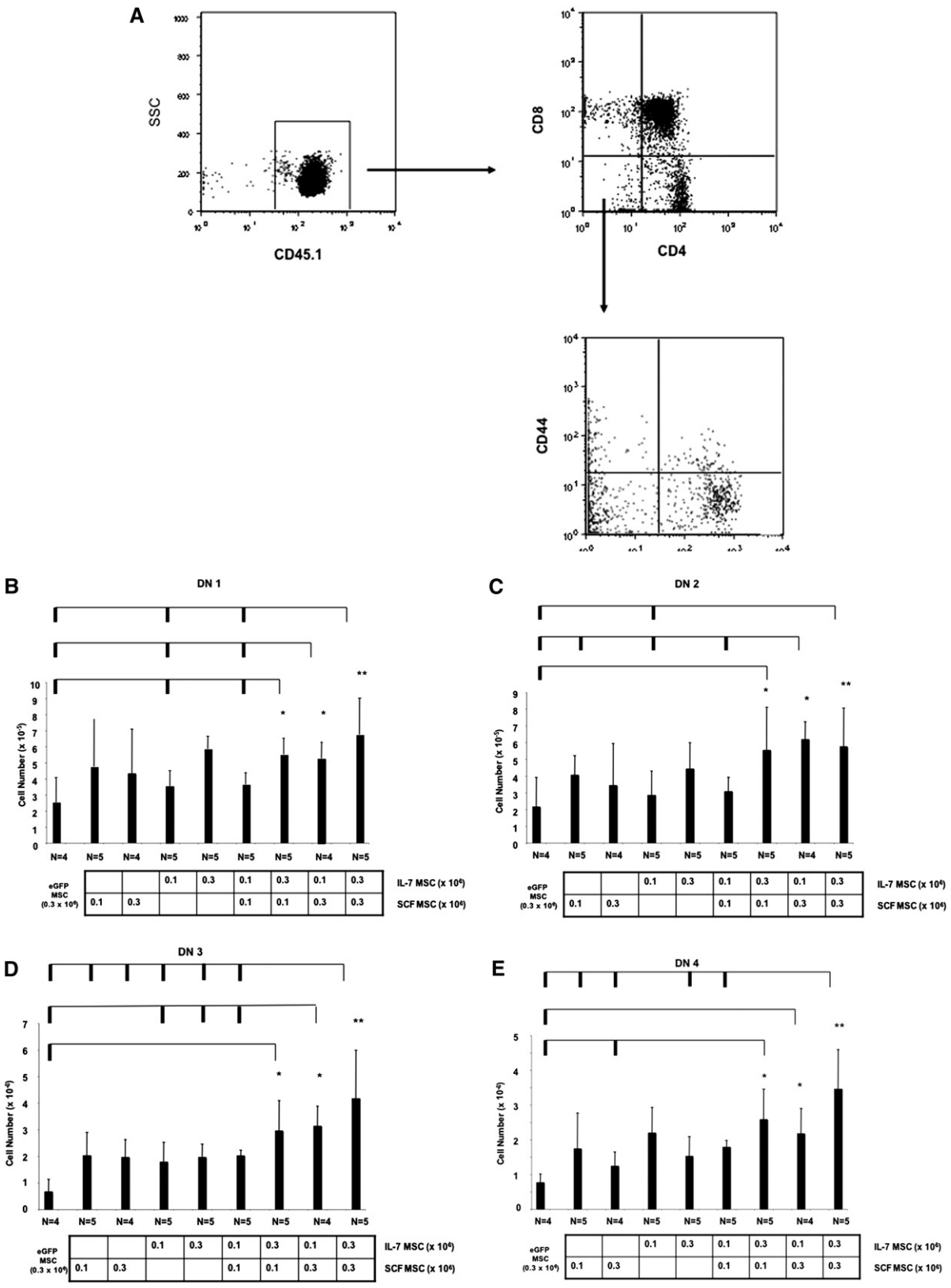

Figure 4. Effects of SCF and IL-7 BM-derived MSCs on the number of donor-derived DN subpopulations at day 30 post-BMT. To analyze and count the number of donor-derived DN subpopulations, $CD45.1^{+}CD4^{-}CD8^{-}$ donor thymocytes were gated and analyzed for the expression of CD44 and CD25. (A) FACS analysis of donor-derived DN cells expressing $CD44^{+}CD25^{-}$ (DN1), $CD44^{+}CD25^{+}$ (DN2), $CD44^{-}CD25^{+}$ (DN3), and $CD44^{-}CD25^{-}$ (DN4). (B-D) Graphs showing the numbers of (B) donor-derived DN1 (*$P \leq .004$; **$P \leq .035$), (C) DN2 (*$P \leq .05$; **$P \leq .05$), (D) DN3 (*$P \leq .05$; **$P \leq .048$), and (E) DN4 (*$P \leq .025$; **$P \leq .036$) subpopulations. An asterisk indicates significant differences between each group of animals.

frequency and differentiation of donor thymocytes (data not shown); however, mice treated with both cytokines displayed significantly higher numbers of all 4 subpopulations, with the highest increase in the DN population (Figure 3B-E). We investigated which subset of donor-derived DN cell populations was most affected by IL-7 and SCF. Based on CD44 and CD25 expression of DN cells, 4 sequential developmental stages ($CD4^-CD8^-CD44^+CD25^-$ [DN1], $CD4^-CD8^-CD44^+CD25^+$ [DN2], $CD4^-CD8^-CD44^-CD25^+$ [DN3], and $CD4^-CD8^-CD44^-CD25^-$ [DN4]) were analyzed (Figure 4A). Overall, IL-7 and SCF resulted in increased levels of all 4 subpopulations for donor DN cells, with the donor DN3 population most affected by the combination of IL-7 and SCF (Figure 4B-E). The combined effects of IL-7 and SCF acted in a dose-dependent manner, resulting in an increased number of thymocytes in our current observation, similar to previous studies reporting that a combination of IL-7 and SCF enhanced the proliferation and survival of murine $CD3^-CD4^-CD8^-$ thymocytes, human $CD34^+CD7^+$ thymocytes, human cord blood T cells, and a human immature T cell line in vitro [39-42]. Thus, our data suggest that proliferation and expansion were affected by the combined treatment with IL-7 and SCF, but differentiation was not.

Increased Numbers of BM-Derived CLPs in Recipients of SCF and IL-7 MSCs

Having observed significant differences in the number of donor-derived thymocytes among recipients of high and low numbers of IL-7 and/or SCF MSCs, we further investigated the effects of IL-7 and SCF in an earlier stage of lymphoid differentiation by examining the number of donor CLPs in BMT recipients. Murine CLPs are progenitors for T, B, and NK lymphocytes and express both IL-7Rα and Kit [43-45]. Recent data from Krueger et al. [46] suggest that CLPs also play a crucial role in donor-derived thymic reconstitution after experimental BMT. Because CLPs coexpress IL-7R and c-Kit, we were interested in examining whether the combining effects of IL-7 and SCF increase the number of donor-derived CLPs in transduced MSC recipients. Figure 5A shows phenotypic analyses of murine CLPs in bone marrow in which CLPs are characterized as lineage marker negative (Lin^-), Sca-1^{low}, Thy1.2^-, Kit^{low}, and IL-$7R^+$ [47]. At day 30 posttransplantation, the number of CLPs in the BM was significantly higher in IL-7 and SCF MSC recipients than in recipients of MSCs producing either cytokine alone (Figure 5B). Similar to earlier thymus and spleen data, the increased number of donor CLPs was related to the dose of IL-7 and SCF MSCs. The combination of IL-7 and SCF MSCs demonstrated potentiation of the individual effects; the addition of IL-7 to suboptimal SCF doses and vice versa resulted in increased numbers of CLPs. The combination of higher doses (0.3×10^6 MSCs of each) resulted in significantly greater numbers of CLPs. Thus, combined IL-7 and SCF therapy expanded the population of BM-derived CLPs to a greater extent than did either cytokine alone.

Because HSCs residing in the BM compartment give rise to CLPs, we also analyzed the number of donor-derived HSCs (Lin^-, Sca-1^+, Thy1^{low}, Kit^{l+}, and IL-$7R^-$) in the BM from mice that underwent HSCT to determine whether the enhanced immune reconstitution in these animals is related to an overall increase in donor HSCs [47]. Our analysis showed that the combined effects of IL-7 and SCF did not significantly increase the numbers of donor-derived HSCs in recipients of both IL-7 and SCF MSCs compared with those mice that underwent transplantation with large amounts of either SCF MSCs (0.3×10^6) or IL-7 MSCs (0.3×10^6). Furthermore, there was no significant difference in the numbers of donor HSCs between the recipients of IL-7 (0.1×10^6)/SCF (0.3×10^6) and other mice that underwent HSCT despite the greater donor immune reconstitution seen in the former group (Supplementary Figure 2). Thus, our data suggest that the combined effects of IL-7 and SCF have a significant impact on donor CLP numbers.

Increased Peripheral (Splenic) Lymphocytes in the IL-7 and SCF MSC Group

Similar to donor thymocyte recovery, the number of donor-derived cells present in the spleens of mice that underwent HSCT differed significantly between treatment groups at day 30 after cotransplantation of transduced MSCs and TCD BM cells (Figure 6A). The cell recovery from the spleens of animals receiving IL-7 or SCF alone again showed an association between the number of donor-derived CD4 T cells, CD8 T cells, and B cells and the number of cotransplanted IL-7 or/and SCF MSCs (Figure 6B and C). Transplantation of IL-7 MSCs resulted in more donor T cell recovery than that of SCF MSCs, although the difference was not statistically significant. Again, there was evidence of potentiated effects of each cytokine by the other cytokine; mice receiving both IL-7 and SCF had greater numbers of donor-derived T and B lymphocytes than those receiving individual suboptimal doses, and the maximal effects were observed from higher doses of cotransplanted IL-7 and SCF MSCs. The number of donor splenic T cells recovered from the animals after cotransplantation of 0.1×10^6 IL-7 MSCs and 0.3×10^6 SCF MSCs was similar to that recovered from animals that received 0.3×10^6 IL-7 MSCs alone, demonstrating that the reduced proliferative signals produced by a low dose of IL-7 MSCs can be compensated for by cotransplantation of SCF MSCs.

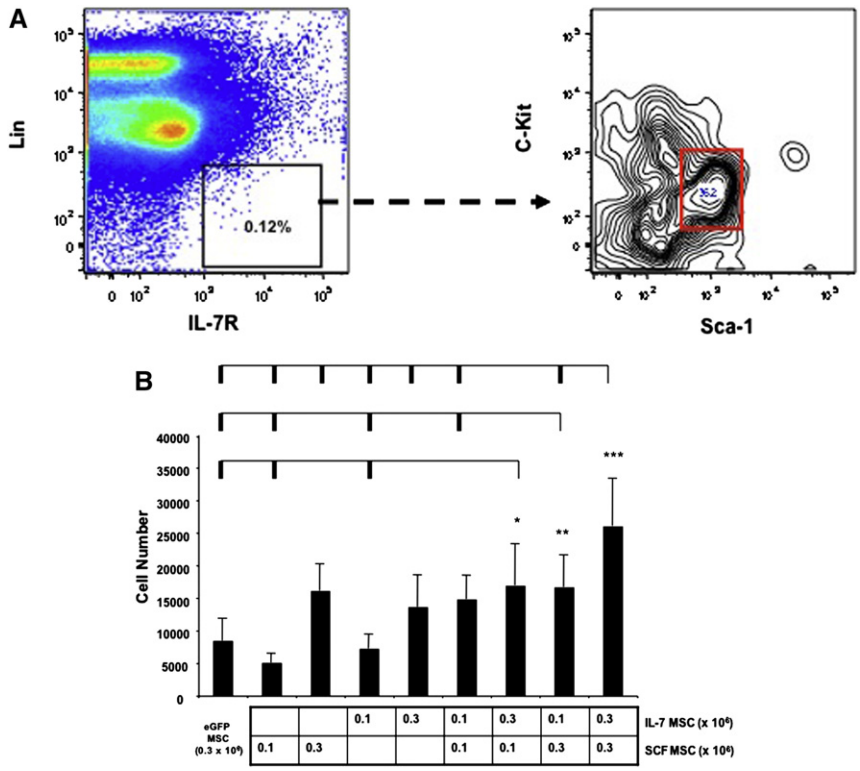

Figure 5. Increased numbers of CLPs are seen in mice that underwent cotransplantation with IL-7 and SCF MSCs. To analyze and count the number of donor-derived CLPs, the BM cells obtained from the femurs of recipient animals were stained with a cocktail of monoclonal antibodies (anti-CD117, −CD127, –Sca-1, and −CD45.1) and antilineage cell surface markers (CD3, CD4, CD5, CD8, CD11b, Gr-1, B220, Thy1.2, and Ter119) at day 30 post-BMT. The donor-positive ($CD45.1^+$) samples were analyzed for Lin^- $IL\text{-}7R\alpha^+$ Kit^{low} $Sca\text{-}1^{low}$ CLP cells by FACS. (A) FACS analysis of donor-derived CLPs in the BM of recipient animals. (B) Donor-derived CLPs from each group of mice that underwent cotransplantation with TCD BM and transduced MSCs. An asterisk indicates significant differences between groups of animals (*$P \leq .006$; **$P \leq .003$; ***$P \leq .05$). The figure represents the mean of 2 or 3 independent experiments each with a total of 3-5 animals per group. Consistent results were obtained in repeated experiments throughout. The vertical bold lines branching off from the top of the horizontal lines represent statistically significant P values when the 0.3×10^6 IL-7 + 0.3×10^6 SCF, 0.1×10^6 IL-7 + 0.3×10^6 SCF, and 0.3×10^6 IL-7 + 0.1×10^6 SCF groups were individually compared with all other groups of animals.

Cotransplantation of IL-7 MSCs and SCF MSCs Improved Development of T Cell–Dependent Antibody Responsiveness after BMT

Because cotransplantation of IL-7 MSC and SCF MSCs resulted in improvement of donor-derived thymopoiesis at day 30, we examined functional T cell–dependent immunity by measuring the antibody response to SRBCs. The mice were immunized with SRBCs on day 30 posttransplantation, with a secondary boost given 2 weeks after primary immunization (day 44). After immunization, we measured primary and secondary antibody responses directed against SRBCs. As expected, the recipients of EGFP MSCs displayed the lowest antibody titer against SRBCs. SCF MSCs alone had no effect on anti-SRBC titers, whereas both low and higher doses of IL-7 improved titers, consistent with previous results [22]. Combinations of SCF and IL-7 generally yielded greater antibody titers, with the greatest responses seen in the mice that received maximal doses of SCF and IL-7 MSCs (Figure 7). Thus, our data suggest that IL-7 and SCF MSCs together promote the generation of functional antigen-specific responsive donor lymphocytes.

DISCUSSION

The present study demonstrates that transplantation of BM-derived MSCs transduced with either the IL-7 or SCF genes can effectively enhance donor-derived immune reconstitution after TCD BMT. The observed effects include increased thymic and splenic cellularity, increased donor peripheral T and B lymphocyte levels, and increased numbers of donor CLPs.

The importance of IL-7 and SCF in thymopoiesis has been well documented. For example, mice with mutations of IL-7, γ_c, SCF, and Kit have decreased numbers of thymocytes and mature T cells, and administration of IL-7 or SCF after BMT has been shown to increase the number of HSC and promote lymphocyte development in experimental murine models [16,22,36,48]. However, unlike in $IL\text{-}7^{-/-}$ and $IL\text{-}7R^{-/-}$ mice, the smaller reduction of thymocyte numbers in SCF/Kit mutant mice suggests that the Kit pathway appears to interact with multiple other cytokine receptors in hematopoietic progenitors, including IL-7R in lymphoid progenitors [30,32]. Mice that are doubly mutated for IL-7 and Kit or for IL-7R and Kit exhibit a complete loss of thymopoietic

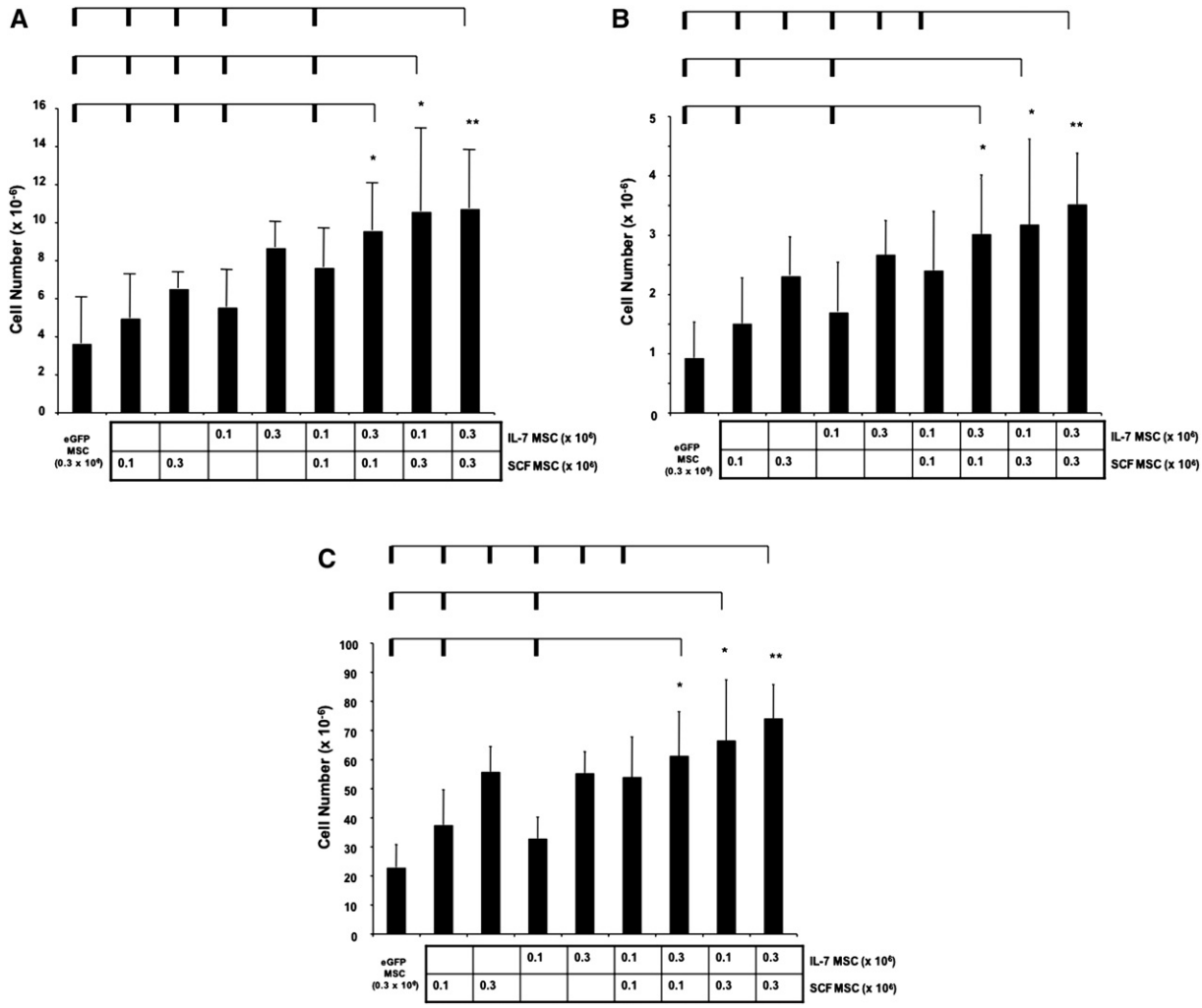

Figure 6. Cotransplantation of IL-7 and SCF MSCs results in increased numbers of donor T and B cells in the spleen at day 30 post-BMT. We analyzed the numbers of donor-derived splenic $CD4^+$ T cells, $CD8^+$ T cells, and $B220^+$ B cells in each group of animals that underwent cotransplantation with TCD BM and retrovirally transduced MSCs. Shown are numbers of (A) donor $Thy1^+CD4^+$ T lymphocytes (*$P \leq .05$; **$P \leq .02$), (B) donor $Thy1^+$ $CD8^+$ T lymphocytes (*$P \leq .007$; **$P \leq .03$), and (C) donor $B220^+$ B cells (*$P \leq .0007$; **$P \leq .04$) at day 30 in spleen. An asterisk indicates significant differences between groups of mice. Each value represents the mean of 3 independent experiments each with a total of 3-5 animals per group. Consistent results were obtained in repeated experiments throughout. The vertical bold lines branching off from the top of the horizontal lines represent statistically significant *P* values when the 0.3×10^6 IL-7 + 0.3×10^6 SCF, 0.1×10^6 IL-7 + 0.3×10^6 SCF, and 0.3×10^6 IL-7 + 0.1×10^6 SCF groups were individually compared with all other groups of animals.

capacity, demonstrating that IL-7 and SCF signaling have a synergistic affect on the survival, maintenance, and proliferation of thymocytes [33].

The greater effects of IL-7 and SCF together on the development of CLPs and lymphoid progenitors compared with either cytokine alone suggest that IL-7 and SCF are complementary signals for the proliferation, survival, and differentiation of these cells. The partially redundant effects of IL-7 and SCF on the proliferation of murine thymocytes and human immature T cell lines in vitro have been reported previously. The increase in the number of DN thymocytes was approximately 10-fold greater in murine fetal thymic lobes cultured in the presence of IL-7 and SCF than in thymic lobes cultured with IL-7 or SCF alone [39]. Similar to murine studies, the proliferative response of the immature human T cell line expressing CD34 and CD7 was significantly greater in the presence of both IL-7 and SCF than in the presence of either cytokine alone [40]. Because only the DN thymocyte population expresses both IL-7R and Kit in the thymus, the number of donor-derived DNs was most profoundly affected by the combined IL-7 and SCF MSC treatment in our data as well (Figure 3B). Evidence supporting redundant roles for IL-7R and Kit was provided in a recent study where the interaction of IL-7R and Kit after Kit stimulation resulted in amplification signals in the absence of IL-7 [32].

The finding of increased donor-derived CLPs and enhanced donor T cell development in SCF/IL-7 MSC recipients suggests that stimulation of the IL-7R– and Kit-expressing BM-derived CLPs and DN thymocytes enhances donor lymphocyte

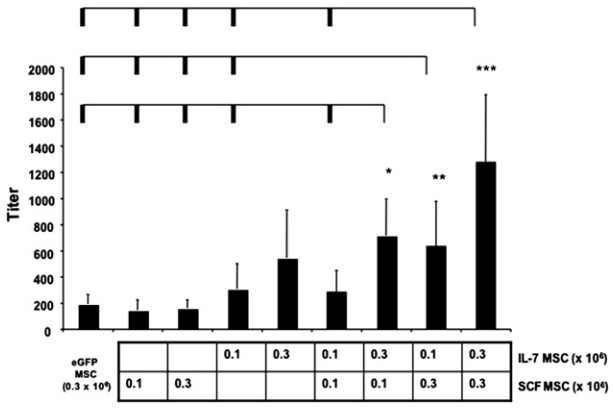

Figure 7. Cotransplantation of IL-7 and SCF MSCs results in increased anti-SRBC titer after immunization. Anti-SRBC agglutinating titers were measured 2 weeks after the primary immunization (at day 44) and the secondary immunization (at day 51) post-BMT. An asterisk indicates significant differences between groups of animals that underwent transplantation with the transduced MSCs (*$P \leq .03$; **$P \leq .03$; ***$P \leq .04$). Each value represents the mean of 2 or 3 independent experiments each with a total of 3-5 animals per group. Consistent results were obtained in repeated experiments throughout. The vertical bold lines branching off from the top of the horizontal lines represent statistically significant P values when the 0.3×10^6 IL-7 + 0.3×10^6 SCF, 0.1×10^6 IL-7 + 0.3×10^6 SCF, and 0.3×10^6 IL-7 + 0.1×10^6 SCF groups were individually compared with all other groups of animals.

engraftment. Although the functions of IL-7R and Kit in the biology of CLP are not clear, our laboratory has demonstrated that analyses of mice doubly mutated in the γc subunit of IL-7R and Kit, in IL-7 and Kit, or in IL-7R and Kit have decreased frequency and numbers of CLPs, indicating that the regulation of the proliferation and survival of CLPs is dependent on IL-7 and SCF signaling [49]. Moreover, Fahlman et al. [50] reported that IL-7 and SCF synergize to promote the proliferation of freshly isolated Lin^-Sca1^+ murine BM progenitor cells. A strategy that promotes the expansion of CLPs to restore immunity after BMT might have important therapeutic applications. Arber et al. [47] demonstrated that transplantation of CLPs along with purified HSCs significantly enhanced early lymphoid reconstitution and improved the survival rate after an experimental challenge with murine cytomegalovirus compared with HSCT alone. Given the very low frequency of CLPs in the BM, therapeutic application of CLPs for transplantation likely would require ex vivo expansion to be clinically relevant. The therapeutic use of IL-7 and SCF to promote proliferation of transplanted CLPs would accelerate restoration of immunity without the need to transplant high numbers of CLPs. Given that multipotent progenitors (MMPs; $Lin^-Sca\text{-}1^+IL\text{-}7R\alpha^-Kit^{hi}$) and more differentiated lymphoid-primed multipotent progenitors (LMMPs; $Lin^-Sca\text{-}1^+IL\text{-}7R\alpha^-Kit^{hi}$) residing in the BM compartment give rise to CLPs, it would be interesting to analyze the effects of IL-7 and SCF on both MMP and LMMP populations and their relations to overall immune reconstitution [45,51].

Our IL-7/SCF gene therapy BMT model offers several advantages for correction of immune deficiency. We have previously shown that thymic microenvironmental defects are related to radiation damage to the TECs and also to decreased intrathymic IL-7 transcript levels [5,8]. As a result, the ability of the irradiated thymus to support donor-derived T lymphocyte development is severely impaired. With the aim of restoring thymopoietic capacity after BMT, several groups have demonstrated that s.c. injection of rhIL-7 into BMT recipient animals results in normalization of thymopoiesis and immune reconstitution. Although s.c. injection of hematopoietic cytokines can enhance immune reconstitution in murine BMT recipients, the engineering of BM-derived MSCs to produce IL-7 or SCF might provide several advantages over injection treatment [34,35,37]. Administration of cytokines has been associated with toxicity due to the high levels of short-lived agents needed to achieve therapeutic effects. On the other hand, the continuous production of IL-7 or SCF from the transduced MSCs might be more physiologically effective and less toxic. The efficacy of cytokine secretion by the transduced MSCs likely was increased by higher local concentrations after their engraftment in lymphohematopoietic organs (Figure 2A). Furthermore, studies have demonstrated that cotransplantation of MSCs and HSCs enhances hematopoietic engraftment, suggesting the ability of MSCs to promote immune reconstitution [52-55]. The mechanisms behind the enhanced hematopoietic engraftment by MSC transplantation remain unclear, however. It is possible that the release of various factors by the BM-derived MSCs, including hematopoietic cytokines and chemokines, combined with IL-7 and SCF in the circulation further enhanced either engraftment or expansion of donor-derived T lymphocytes in our congeneric murine recipients. Although in the present study, circulating levels of rhIL-7 and mSCF were significantly higher in the engrafted mice compared with control animals with no deleterious side effects, a strategy to permit regulation of MSC expression (eg, via an inducible vector or introduction of a suicide gene in the vector to permit ablation) might be useful for clinical studies as a means to abet potential toxicities.

Finally, recent reports demonstrating that IL-7 can cause expansion of mature T cells and that IL-7 treatment after experimental allogeneic BMT increases the frequency of GVHD suggest that IL-7 treatment after BMT to enhance immune reconstitution in the allogeneic setting may have adverse effects [56]. We previously demonstrated that treatment of allogeneic BMT recipients with anti–IL-7Rα antibody prevented GVHD by blocking IL-7 signaling in donor mature T cells, further suggesting the potential danger of using IL-7 in an allogeneic setting [9]. The amount of IL-7 needed to develop GVHD is unknown, however. Shinha et al. [10] reported that exacerbation of

GVHD is directly related to the amount of IL-7 injected into allogeneic BMT murine recipients. Our current finding of greater enhancement of donor-derived immune reconstitution in the recipients of combined IL-7 and SCF MSCs compared with recipients of IL-7 MSCs alone provide a potential new model for engineering of the immune system.

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AUTHORSHIP STATEMENT

Brile Chung designed research, performed research, analyzed data, and wrote the manuscript. Dullei Min performed research. Mark Krampf performed research. Lukas W. Joo, Jing Huang, Yujun Yang, Sumana Shashidhar, and Eric Dudl assisted with research. Janice Brown designed research. Kenneth Weinberg designed research, analyzed data, and wrote the manuscript.

SUPPLEMENTARY DATA

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bbmt.2010.07.027.

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