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Hematopoietic Stem Cell Mobilization Is Necessary but Not Sufficient for Tolerance in Islet Transplantation

Diabetes 2017;66:127–133 | DOI: 10.2337/db16-0444

Overcoming the immune response to establish durable immune tolerance in type 1 diabetes remains a substantial challenge. The ongoing effector immune response involves numerous immune cell types but is ultimately orchestrated and sustained by the hematopoietic stem cell (HSC) niche. We therefore hypothesized that tolerance induction also requires these pluripotent precursors. In this study, we determined that the tolerance-inducing agent anti-CD45RB induces HSC mobilization in nonautoimmune B6 mice but not in diabetes-prone NOD mice. Ablation of HSCs impaired tolerance to allogeneic islet transplants in B6 recipients. Mobilization of HSCs resulted in part from decreasing osteoblast expression of HSC retention factors. Furthermore, HSC mobilization required a functioning sympathetic nervous system; sympathectomy prevented HSC mobilization and completely abrogated tolerance induction. NOD HSCs were held in their niche by excess expression of CXCR4, which, when blocked, led to HSC mobilization and prolonged islet allograft survival. Overall, these findings indicate that the HSC compartment plays an underrecognized role in the establishment and maintenance of immune tolerance, and this role is disrupted in diabetes-prone NOD mice. Understanding the stem cell response to immune therapies in ongoing human clinical studies may help identify and maximize the effect of immune interventions for type 1 diabetes.

Less than half of patients with type 1 diabetes (T1D) undergoing islet transplantation maintain insulin independence after 3 years (1). The induction of permanent immune tolerance would enhance the outcome of β-cell replacement strategies by protecting the restored islet mass from immune destruction. Like the experience in human recipients, animal models of T1D are extraordinarily resistant to transplantation tolerance. Even though transplantation tolerance is readily induced in nonautoimmune mice, neither islet nor other organ allografts have consistently shown long-term acceptance when transplanted into T1D-prone NOD recipients even prior to spontaneous diabetes onset (2–4).

The most successful attempts to induce transplant tolerance in animal models of T1D have included bone marrow transplantation (5,6); similar regimens have suggested some potential in maintaining islet tolerance in patients with T1D (7). Addition of allogeneic bone marrow likely creates a state of microchimerism that facilitates engraftment of matched organs. In addition to the presentation of novel antigens, transplanted bone marrow also contributes to formation of new cells from hematopoietic stem cells (HSCs). The degree to which HSC activity contributes to tolerance induction is not well defined.

Bone marrow–resident HSCs are not quiescent but rather are the master control center of the immune response. In response to infectious stimuli and cytokines, HSCs generate a robust effector cell response while maintaining immune homeostasis (8,9). We hypothesized that HSCs play a critical role during the establishment of allograft tolerance by regulating the immune response to the organ transplant.

In this report, we observe that HSCs are mobilized during tolerance induction with anti-CD45RB in healthy, nonautoimmune B6 mice but not during treatment of prediabetic, T1D-prone NOD mice. Depleting HSCs or inhibiting their mobilization prevented establishment

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Received 6 April 2016 and accepted 21 October 2016.
This article contains Supplementary Data online at http://diabetes.diabetesjournals.org/lookup/suppl/doi:10.2337/db16-0444/-/DC1.

of tolerance to islet allografts. We observed that poor mobilization in prediabetic NOD mice related to HSC overexpression of CXCR4, which maintains HSCs in their bone marrow niche. Targeted HSC mobilization improved graft outcomes in NOD mice, suggesting that clinical therapies should measure the HSC response and attempt to enhance it to maximize immune tolerance in T1D.

RESEARCH DESIGN AND METHODS

Animals

The Institutional Animal Care and Use Committee at Vanderbilt University approved all procedures. Mice were housed in a specific pathogen-free facility. All mice (C57BL6/J [B6], NOD/ShiLtJ [NOD], and C3H/HeJ [C3H]) were purchased from The Jackson Laboratory (Bar Harbor, ME).

Flow Cytometry

Splenocytes or bone marrow cells (BMCs) were stained with fluorophore-conjugated antibodies as described in Supplementary Fig. 1*A*. Samples were acquired on a BD LSRFortessa Flow Cytometer (BD Biosciences) and analyzed by FlowJo software (Tree Star, Ashland, OR).

Colony-Forming Cell Assay

Methylcellulose colony-forming cell (CFC) assay was performed as described (R&D Systems, Minneapolis, MN) (10). A total of 5,000 BMCs in Iscove's modified Dulbecco's medium/2% FBS was combined 1:10 with methylcellulose complete media containing human Epo (5 IU/mL) and mouse interleukin-3 (10 ng/mL), interleukin-6 (10 ng/mL), and stem cell factor (50 ng/mL). Cells were cultured for 8 days in sextuplicate, and colonies were counted by a blinded observer.

Osteoblast Isolation, Culture, and Functional Analysis

BMCs were differentiated to osteoblasts using 50 μg/mL ascorbic acid and 5 mmol β-glycerophosphate (Sigma-Aldrich, St. Louis, MO) (11). Osteoblasts were cultured with 100 ng/mL recombinant murine granulocyte colony-stimulating factor (G-CSF) (R&D Systems), 50 μg/mL anti-CD45RB (BioXCell, West Lebanon, NH), or media alone (control) for 24 h. Cells were analyzed by flow cytometry or RT-PCR (primers described in Supplementary Fig. 1*B*). Individual gene expression was normalized to GAPDH, and relative gene expression changes were calculated using the $2^{-\Delta\Delta Ct}$ method.

Islet Allografts

Subcapsular renal islet transplantation was carried out as described (12). B6 and NOD mice were made diabetic by a single injection of streptozotocin (STZ) (200 mg/kg) and transplanted with 400 allogeneic C3H islets. Anti-CD45RB monoclonal antibody was injected on days 0, 1, 3, 5, and 7 after transplantation to induce tolerance. Two consecutive blood glucose readings >250 mg/dL defined rejection.

Statistical Analysis

Statistical analysis was performed with GraphPad Prism V6 (GraphPad Software, La Jolla, CA) using the Student *t* test or one-way or two-way ANOVA followed by a Tukey or Sidak posttest to compare multiple groups. Graft rejection was graphed as a Kaplan-Meier curve and compared by log-rank statistical analysis. Statistical comparisons with P values <0.05 were deemed significant.

RESULTS

The Tolerance-Inducing Agent Anti-CD45RB Promotes HSC Mobilization That Is Necessary to Establish Transplant Tolerance

Transplant tolerance–inducing agents are partially immune depleting. Thus, we hypothesized that the HSC compartment would respond to this loss of lymphocytes following anti-CD45RB therapy; mobilization in the marrow leads HSCs to leave their quiescent niche and begin to proliferate in situ. Nondiabetic B6 and prediabetic NOD mice were untreated or received a standard 7-day course of anti-CD45RB. On day 8, marrow LSK cells were identified by flow cytometry; these cells were negative for lineage markers and positive for Sca-1 and anti-mouse CD117 (c-Kit), as illustrated in Fig. 1*A*. Anti-CD45RB enhanced B6 stem cell frequency by 30% (Fig. 1*A*) ($P < 0.005$). In contrast, anti-CD45RB treatment had no effect on HSC frequency in prediabetic NOD mice (Fig. 1*A*). Anti-CD45RB treatment did not increase total BMC number in either strain (not shown; P was NS).

Because there is no absolute standard for identification of HSCs by cell-surface markers, we confirmed the suspected increase in HSC mobilization with a functional assay. HSC activation was assessed by a CFC assay in which bone marrow from anti-CD45RB–treated, nondiabetic B6 mice developed nearly twice as many colonies per 5,000 plated BMCs as compared with control (Fig. 1*B*). Thus, the tolerogenic agent anti-CD45RB activates bone marrow stem cells in tolerance-susceptible B6 mice.

To determine whether this change in stem cell activity affected anti-CD45RB tolerance induction, we depleted HSCs during tolerance induction with ACK2 treatment (c-Kit and rat IgG2b) (13); because ACK2 depletion is short-lived, we performed both a short course and prolonged treatment. STZ-treated, diabetic B6 mice that were depleted of HSCs by either approach demonstrated a decrease in graft survival (Fig. 1*C*, white circles and squares), although this graft loss did not affect all animals. Because the HSC response may contribute to both effector and regulatory immunity, we next determined how HSCs are induced during immune therapy so that we could modulate this response to study its effect on impairing or improving the transplant outcome.

The Effect of Anti-CD45RB Is Not Directly on HSCs but Rather on Osteoblasts

We considered whether differences in the response of the two strains related to differential expression of CD45RB on HSCs. We therefore analyzed CD45RB expression on LSK marrow cells by flow cytometry. Neither nondiabetic B6 nor prediabetic NOD HSCs demonstrated CD45RB expression (Fig. 2*A*). In comparison, a significant portion

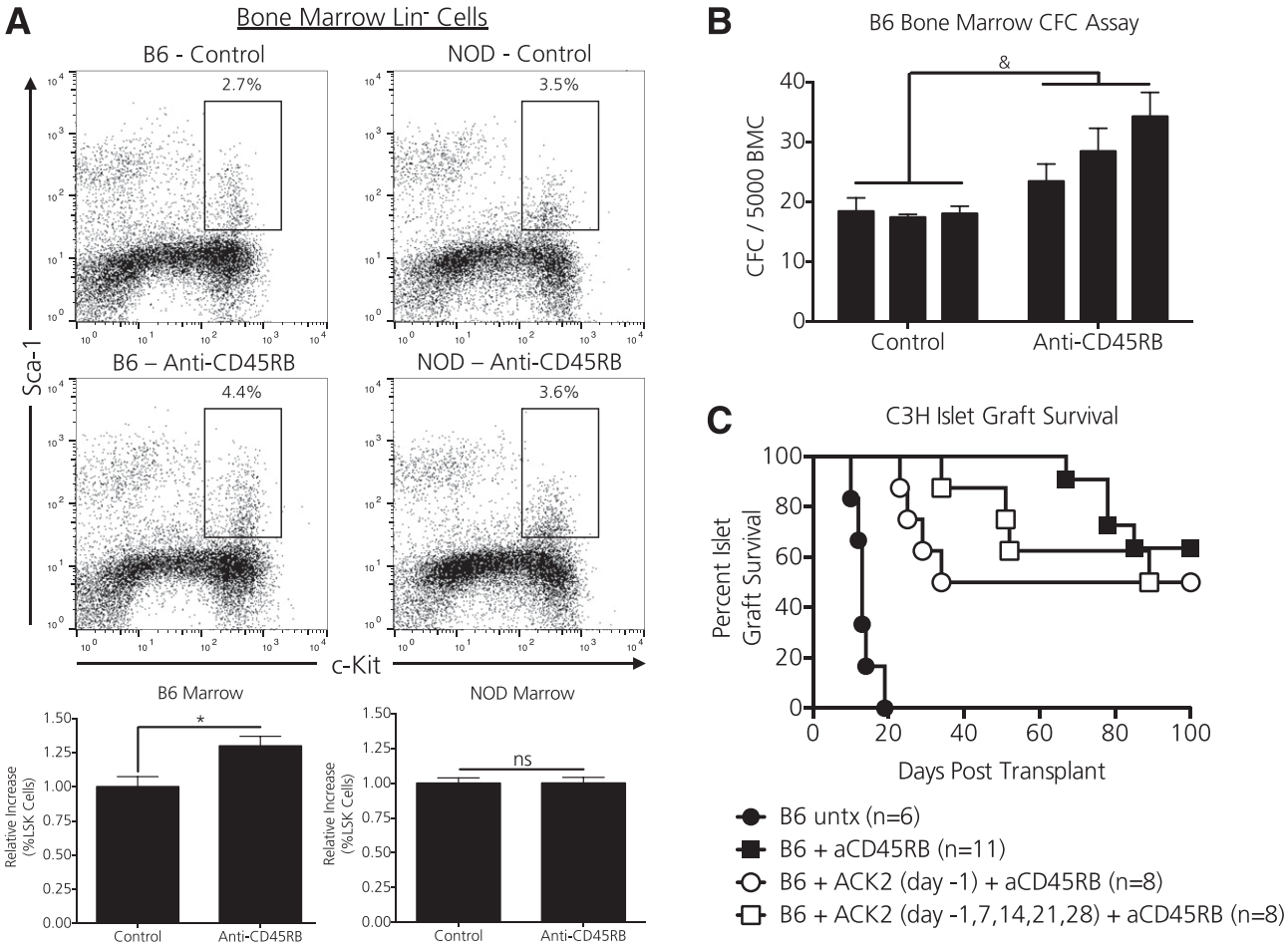

Figure 1—The tolerance-inducing agent anti-CD45RB promotes HSC mobilization that is necessary to establish transplant tolerance. *A*: HSCs were identified in 8-week-old female nondiabetic B6 and prediabetic NOD mice as LSK cells by staining with a pan-lineage kit to identify lineage-negative (Lin^-) cells that were then analyzed for expression of c-Kit and Sca-1 (Lin^- cells shown in plots). Treatment with anti-CD45RB (100 μg/day on days 0, 1, 3, 5, and 7) led to an increase in LSK cells in B6 marrow relative to untreated mice. The frequency of stem cells in the control groups was set to 1 in each experimental repetition (*$P < 0.005$ by *t* test; five independent experiments; $n = 14$ for control and anti-CD45RB). Treatment of prediabetic NOD mice with the same regimen of anti-CD45RB led to no change in LSK frequency (P = NS by *t* test; three independent experiments; $n = 6$ for control and anti-CD45RB). *B*: To verify increased HSC activity in B6 mice, a CFC assay was performed. A total of 5,000 BMCs from anti-CD45RB–treated or untreated (untx) B6 mice (nondiabetic) was plated for 7 days in methylcellulose culture medium, and colonies were counted. Marrow from anti-CD45RB–treated mice produced more than double the colonies (&$P < 0.0001$ by one-way ANOVA; three independent samples per condition in six replicates each, repeated twice). *C*: HSCs were depleted by either a single 500-μg injection of monoclonal antibody ACK2 (c-Kit; Tocris Bioscience, Bristol, U.K.) 1 day prior to transplantation (administered on day −1) or weekly via 500-μg injections of ACK2 for 5 weeks (administered on days −1, 7, 14, 21, 28). On day 0, STZ-treated, chemically diabetic, B6 mice were transplanted with allogeneic C3H islets and treated with a standard 7-day course of anti-CD45RB (aCD45RB). Both transient and prolonged depletion of HSCs prior to anti-CD45RB therapy led to more rapid rejection in a portion of some islet allograft recipients.

of lineage-positive cells in both strains expressed CD45RB (Fig. 2*A*). Thus, we concluded that anti-CD45RB mobilizes HSCs indirectly.

The HSC niche is partly governed by osteoblasts, which derive from the macrophage lineage and should express CD45RB (14). As expected, osteoblasts cultured from nondiabetic B6 mice expressed Rank-L and osteocalcin along with the CD45RB isoform (Fig. 2*B*). To demonstrate that osteoblast expression of CD45RB was functionally relevant, we compared the response of cultured B6 osteoblasts to G-CSF, which mobilizes HSCs through an osteoblast-dependent mechanism (10), and anti-CD45RB. Gene expression changes over untreated control cells revealed that both agents downregulated osteoblast CXCL12 expression, which facilitates HSC mobilization. Despite downregulating several other osteoblast-specific functional genes, these cells maintained expression of osteopontin, indicating viability (Fig. 2*C*).

Sympathectomy Prevents HSC Mobilization by Anti-CD45RB and Abrogates Transplantation Tolerance

Osteoblast-dependent mobilization of HSCs following G-CSF is prevented by sympathectomy (10), which may be relevant to nervous system dysfunction in transplant

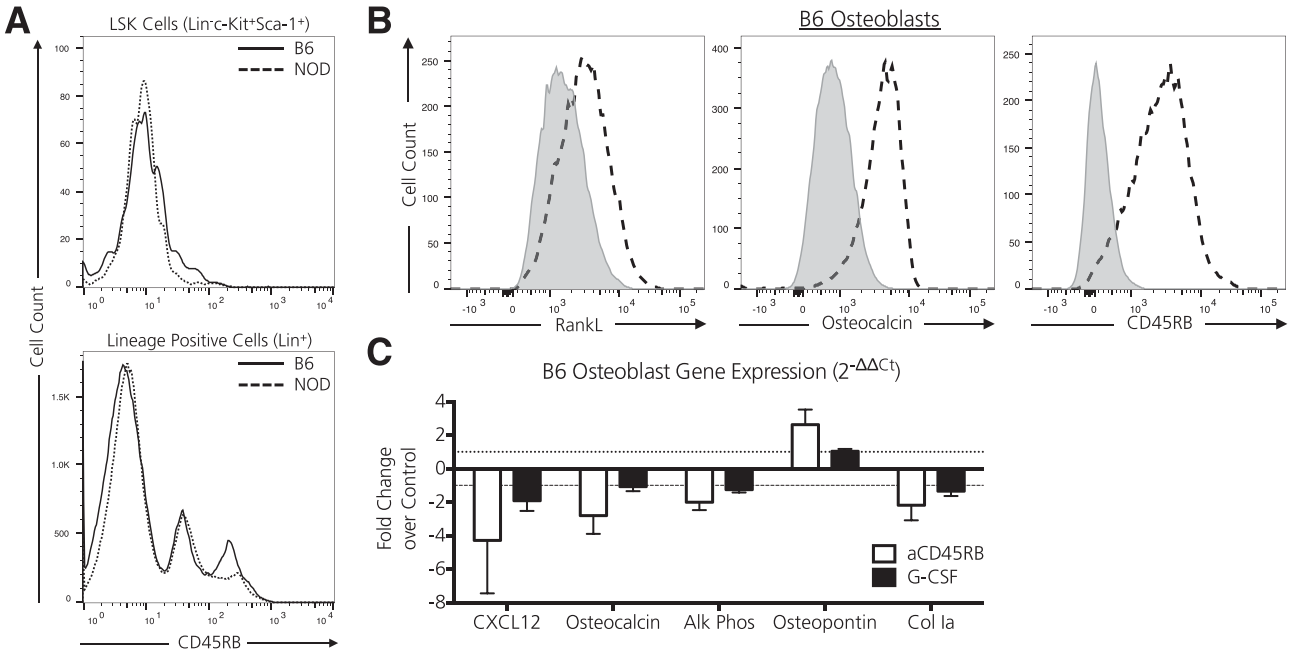

Figure 2—The effect of anti-CD45RB is not directly on HSCs but rather on osteoblasts. *A*: Analysis of bone marrow–resident HSCs (top) from both nondiabetic B6 and prediabetic NOD mice showed that neither expressed significant levels of the CD45RB isoform. In comparison, a significant portion of lineage-positive (Lin^+) BMCs in both strains expressed CD45RB (bottom). *B*: Cytometric analysis of cultured osteoblasts from nondiabetic B6 mice demonstrate expression of Rank-L and osteocalcin as expected. Moreover, these osteoblasts express CD45RB (gray is isotype control). *C*: Cultured nondiabetic B6 osteoblasts were treated with G-CSF (R&D Systems) or anti-CD45RB (aCD45RB) or left untreated (control) to determine whether the expressed CD45RB was functional (n = 4–6 mice/group, repeated in triplicate). As compared with untreated osteoblasts, G-CSF and anti-CD45RB–treated osteoblasts downregulated the expression of CXCL12, which normally retains HSCs in their niche. Other osteoblast activation markers were also slightly downregulated, but osteopontin was maintained, indicating viable cells. Alk Phos, alkaline phosphatase. Col Ia, Collagen Ia; Lin−, lineage negative.

candidates with T1D (15,16). To determine whether anti-CD45RB–induced HSC mobilization was similarly inhibitable, nondiabetic B6 mice were chemically sympathectomized with 6-hydroxydopamine (6-OHD) before initiation of anti-CD45RB therapy. Consistent with Fig. 1, anti-CD45RB treatment alone activated HSC mobilization, which was ablated by pretreatment with 6-OHD (Fig. 3*A*).

Because sympathectomy prevented HSC mobilization, we determined whether it also prevented tolerance induction. STZ-treated, chemically diabetic B6 mice were sympathectomized with 6-OHD, transplanted with C3H islets, and administered anti-CD45RB. Sympathectomy significantly diminished anti-CD45RB–mediated tolerance induction, with only one recipient demonstrating long-term transplant survival (Fig. 3*B*).

Because an intact sympathetic nervous system (SNS) is required for long-term transplant tolerance in B6 mice, we questioned whether prediabetic NOD mice were deficient in SNS activity. Although spontaneously diabetic NOD mice demonstrate impaired SNS activity (17), whether such failures in SNS activity are present before diabetes onset remains unknown. Overall, prediabetic NOD mice demonstrated impaired global SNS activity as revealed by their cold tolerance (18); in response to housing at 4°C for 1 h, NOD mice demonstrated a greater absolute loss of body temperature, lost more specific heat, and failed to increase circulating norepinephrine levels as compared with nondiabetic B6 mice (Supplementary Fig. 2). As a control, 6-OHD–treated, chemically sympathectomized mice lost the greatest amount of body temperature and failed to increase circulating norepinephrine levels. Despite reduced SNS activity, we found no observable difference in bone marrow SNS nerve fibers (Supplementary Fig. 3, tyrosine hydroxylase staining) (19).

Blockade of Excess CXCR4 Enhances NOD HSC Mobilization and Extends Islet Transplant Survival

Finally, we investigated whether enhanced HSC mobilization would augment islet transplantation in the setting of T1D. Because prediabetic NOD mice possessed impaired global SNS function (compared with Supplementary Fig. 2), we next investigated whether G-CSF, which acts with the marrow sympathetic nervous innervation to reduce osteoblast-mediated HSC retention (10), would enhance HSC mobilization in NOD mice. Although G-CSF treatment was highly effective in mobilizing HSCs in nondiabetic B6 mice, this agent exhibited a modest, nonsignificant effect on prediabetic NOD HSC mobilization (Fig. 4*A*). In addition to the role of the SNS–marrow axis, HSCs are retained in their quiescent niche by their expression of CXCR4. Flow cytometry analysis demonstrated significantly higher CXCR4 expression on prediabetic NOD than nondiabetic B6 LSK

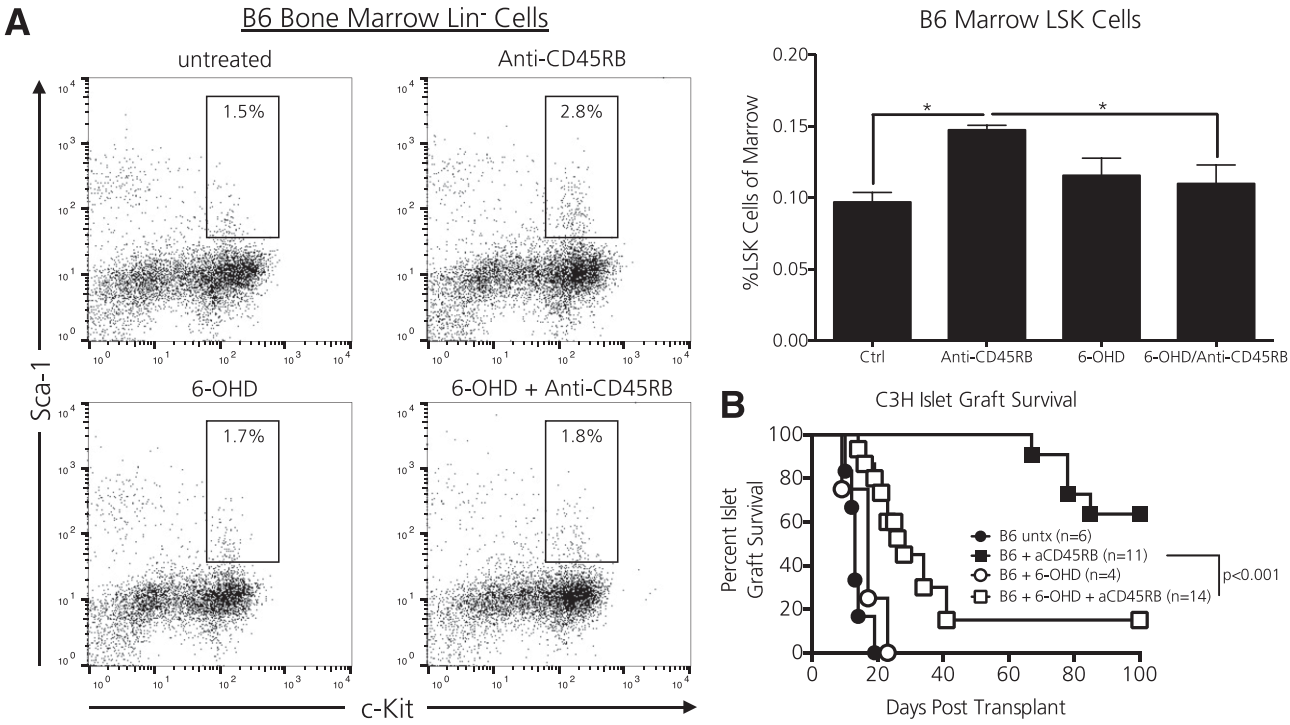

Figure 3—Sympathectomy prevents HSC mobilization by anti-CD45RB and abrogates transplantation tolerance. *A*: Nondiabetic B6 mice were chemically sympathectomized with 6-OHD (Sigma-Aldrich) on days −4 (100 mg/kg) and −2 (250 mg/kg) relative to the initiation of a standard 7-day course of anti-CD45RB. The marrow LSK cell response was then measured on day 8. As previously shown, anti-CD45RB induced a significant increase in the percent of LSK cells. Sympathectomy itself had no effect, whereas it completely abolished anti-CD45RB–mediated LSK response ($P < 0.01$ by one-way ANOVA followed by Tukey multiple-comparisons posttest: $^*P < 0.05$ anti-CD45RB vs. 6-OHD/CD45, $^*P < 0.05$ anti-CD45RB vs. control; $N = 6$ mice/group). *B*: 6-OHD–sympathectomized or control STZ-treated, chemically diabetic B6 mice received allogeneic C3H islet transplants and were left untreated (untx) or treated with anti-CD45RB (aCD45RB). Sympathectomy itself had no effect on rejection kinetics. Tolerance induction by anti-CD45RB was abrogated by prior sympathectomy ($P < 0.001$ by log-rank test). Lin−, lineage negative.

cells (Fig. 4*B*). Targeting CXCR4 with AMD3100 resulted in HSC mobilization in prediabetic NOD mice that exceeded the B6 response (Fig. 4*C*).

We next determined whether HSC mobilization improved islet allogeneic transplant survival in STZ-treated, chemically diabetic 8-week-old NOD mice. STZ-treated NOD mice, in lieu of naturally diabetic NOD mice, were studied to assess whether enhanced HSC retention represented a barrier to tolerance prior to development of spontaneous diabetes, as failed transplantation tolerance has commonly been reported in NOD prior to diabetes onset (4). Treatment with AMD3100 alone led to a nearly sevenfold prolongation over anti-CD45RB NOD recipients receiving C3H islet allografts (Fig. 4*D*). Addition of anti-CD45RB to the AMD3100 pretreatment led to a further, albeit slight, increase in allograft survival. However, permanent tolerance induction was not observed, suggesting that NOD immune cells resist tolerance induction at multiple steps.

DISCUSSION

Our data suggest that proper HSC mobilization and sympathetic innervation are prerequisites for tolerance induction during islet transplantation. Persons with established T1D have disturbed sympathetic function (15,16,20). These disruptions have been linked to inadequate HSC mobilization in patients with T1D, which others have connected to increased risk for cardiovascular disease (15). Our data indicate that this disruption will also impede their responses to immune therapies. Whether persons at risk for T1D have sympathetic dysfunction is less well studied; however, some patients close to disease onset exhibit autonomic dysfunction, suggesting that decrements in the SNS–immune axis could contribute to diabetes progression. In the NOD mouse, diabetes has also been correlated with autonomic dysfunction (17), and multiple nervous system antigens are known targets of the autoimmune attack (21–23).

Our study adds to these barriers by demonstrating that T1D-prone NOD mice resist SNS-dependent, G-CSF–mediated mobilization of HSCs. Furthermore, we determined that excess CXCR4 on NOD HSCs may contribute to failures in bone marrow responsiveness. Similar to work published by Fiorina et al. (24) in healthy B6 mice, our data confirm that targeting CXCR4 with AMD3100 prolongs islet allograft survival. In both studies, administration of AMD3100 at transplantation failed to induce permanent allograft tolerance. Our work is unique,

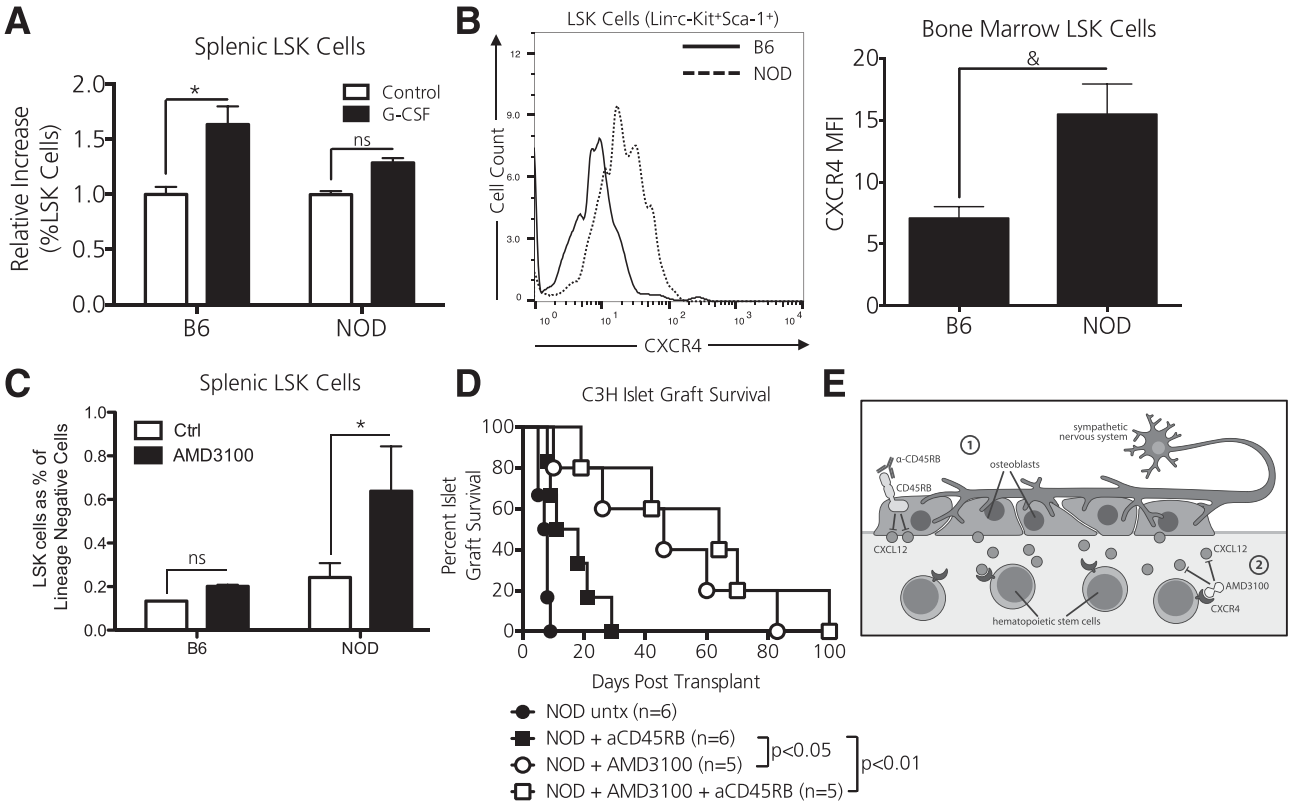

Figure 4—Blockade of excess CXCR4 enhances NOD HSC mobilization and extends islet transplant survival. *A*: Nondiabetic B6 and prediabetic NOD mice were left untreated or treated with G-CSF (250 μg/kg/day for 5 days in eight divided doses). Spleens were harvested 2 to 3 h after the last dose. Whereas B6 mice demonstrated a robust increased in LSK cells following G-CSF treatment, little change was seen in NOD mice (*$P < 0.001$ by two-way ANOVA followed by Sidak multiple-comparisons posttest). *B*: Prediabetic NOD bone marrow–resident LSK cells demonstrated significantly greater CXCR4 expression than B6 LSK cells as measured by flow cytometry (&$P < 0.05$ by Student *t* test). *C*: Blockade of CXCR4 with AMD3100 (100 μg/day for 5 days, subcutaneous injections; Sigma-Aldrich) led to significantly increased LSK cell frequencies in the spleens of prediabetic NOD mice (*$P < 0.05$ by two-way ANOVA followed by Sidak multiple-comparisons posttest). *D*: Islet transplantation following pretreatment with AMD3100 (100 μg/day on days −4, −3, −2, −1, and 0 relative to the day of transplant) in STZ-treated, chemically diabetic NOD mice led to a significant prolongation in islet survival, which was further extended by addition of anti-CD45RB (aCD45RB), although permanent tolerance was not achieved (*P* values determined by log-rank test). *E*: A schematic of the bone marrow HSC niche indicating the presumed action of anti-CD45RB on osteoblasts and the contribution of the SNS (1), as well as the ability of CXCR4-CXCL12 blockade to bypass these steps and promote mobilization in NOD mice (2). Lin−, lineage negative; MFI, mean fluorescence intensity; untx, untreated.

however, in that we connect the HSC mobilization to tolerance induction. Also in line with our findings, Fadini et al. (25) demonstrated that G-CSF fails to significantly mobilize HSCs in patients with T1D. Rather, treatment with plerixafor (AMD3100) was the optimal stimulus for HSC mobilization in these patients (25).

As SNS-mediated HSC mobilization may underlie tolerance induction, measuring the HSC response could represent a new opportunity to identify responders and nonresponders to T1D immune therapy. HSC enhancement could also explain the success of bone marrow transplantation in the NOD model for diabetes prevention and islet allograft potentiation (5,6) in addition to the promising preliminary clinical results of autologous marrow transplantation in human T1D (7). However, the contribution of HSC mobilization has not been directly examined in these settings. Our data suggest that: *1*) enhancing HSC activity could foster the success of other immune therapies and *2*) tolerance induction is a multistep process beginning with the HSC. Further investigation of the hematopoietic progenitor response during T1D prevention and reversal trials may yield additional insight into fundamental aspects of the immune response that lead to β-cell loss.

Funding. This study was supported by National Institutes of Health grants K08-DK-090146 (to D.J.M.), R03-DK-097410 (to D.J.M.), and F31-DK-107321 (to C.S.W.); a JDRF Career Development Award (to D.J.M.); the Hazinski Scholars of the Turner Fund for Pediatric Research Award (to D.J.M.); the Alice and Henry Hooker Diabetes Scholar Award (to D.J.M.); the Price Family Discovery Award (to D.J.M.); National Institute of General Medical Sciences grant T32-GM-007347 (Vanderbilt Medical Science Training Program support to B.T.S. and Y.Z.); the Merz Family Scholar Award in Transplantation (to B.T.S.); Cellular, Biochemical, and Molecular Sciences training grant 5T32-GM-008554-17 (to C.S.W.); and institutional funds provided by the Vanderbilt Department of Pediatrics and Vanderbilt Diabetes Center (to D.J.M.).

Duality of Interest. No potential conflicts of interest relevant to this article were reported.

Author Contributions. B.T.S., A.B.T., and D.J.M. wrote the manuscript. B.T.S., A.B.T., S.K.E., Y.Z., A.F.M., C.S.W., and D.J.M. designed, performed, and analyzed experiments. B.T.S. performed transplants. B.T.S., A.B.T., S.K.E., A.F.M., C.S.W., and D.J.M. performed flow cytometry. B.T.S. and Y.Z. analyzed osteoblasts. A.B.T., S.K.E., and D.J.M. analyzed HSC mobilization and colony formation. D.J.M. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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