Prevention of Type 1 Diabetes with Major Histocompatibility Complex–Compatible and Nonmarrow Ablative Hematopoietic Stem Cell Transplants

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Progression to hyperglycemia in young nonobese diabetic (NOD) mice is blocked by the transplantation of hematopoietic cells mismatched at the major histocompatibility complex (MHC). Because the NOD MHC class II allele, I-A^{g7}, is the primary disease susceptibility gene, it is logical to conclude that MHC-mismatched hematopoietic grafts prevent diabetes by replacement of this susceptibility allele on critical hematolymphoid populations. In this report, transplantation of MHC-matched purified hematopoietic stem cells (HSCs) prevented diabetes development in NOD mice, demonstrating that alleles of non-MHC background genes expressed on hematopoietic cells are sufficient to disrupt the autoaggressive process. Nonmarrow ablative conditioning was 100% protective, further showing that elimination of NOD hematopoiesis, including T-cells, was not required for the graft to block diabetes pathogenesis. The current standard clinical practice of hematopoietic cell transplantation uses donor/recipient pairs that are matched at the MHC. In our view, the principles established here using an MHC-matched engineered hematopoietic graft in conjunction with nonmarrow ablative conditioning to successfully block autoimmune diabetes sufficiently reduces the morbidity of the allogeneic transplantation procedure such that a similar approach can be translated to the treatment of human autoimmune disorders. *Diabetes* 54:1770–1779, 2005

Islet destruction in type 1 diabetes results from aberrant T-cell activation in the setting of a permissive genetic background (1). In both humans and nonobese diabetic (NOD) mice, type 1 diabetes arises as a complex polygenic trait, and the strongest genetic link with disease susceptibility are certain major histocompatibility complex (MHC) class II alleles (2). NOD mice express only a single unique MHC class II molecule (3) designated I-A^{g7}, which is the primary gene conferring diabetes susceptibility, and the I-E molecule is not expressed (4). The way that MHC class II molecules mediate diabetes susceptibility is not yet determined (5). However, based on the central role of MHC class II molecules in T-cell selection and in modulating T-cell responses, it has been hypothesized that impaired peptide/MHC class II interactions alter the mechanisms mediating T-cell tolerance. Introduction of a non-NOD I-A or I-E molecule into NOD mice as transgenes (6–9) or by genetic crossing (10) provides disease protection.

Reports beginning in the 1980s showed that transplantation of allogeneic hematopoietic cells from nonautoimmune donors prevents diabetes in NOD recipients (11–14). Because all of the transplantation studies that resulted in disease protection were performed using MHC-disparate donors, it might be concluded that allografts mediate protection through the actions of nonsusceptibility MHC class II molecules expressed on donor hematolymphoid cells. Further evidence that non-I-A^{g7} allele expression on hematopoietic cells is sufficient to abrogate diabetes comes from studies in which diabetes protection was observed by using transgenic NOD mice that constitutively expressed non-NOD class II molecules (I-A^{k} or I-E) (15,16) as bone marrow donors or by transplanting NOD bone marrow made to express either I-A^{k} or I-A^{d} by retroviral transduction (17).

Given the wealth of preclinical data demonstrating the curative potential of allogeneic hematopoietic cell transplantation (AHCT) in NOD mice (11–14,18) and other autoimmune rodent models (19), there has been great interest in applying this approach to patients afflicted with severe autoimmune diseases (20,21). AHCT is routinely performed for patients with life-threatening hematologic malignancies or bone marrow failure states. However, the clinical practice of AHCT strongly favors the use of donors who are matched at the MHC to limit the complications of the procedure.

In this study, we asked whether expression of non-I-A^{g7} molecules on hematopoietic cells is required to prevent diabetes in NOD mice after AHCT. To address this question, C57BL/6 mice congenic for the NOD MHC region (designated B6.H-2^{g7}) were used as hematopoietic donors.

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AHCT, allogeneic hematopoietic cell transplantation; APC, antigen-presenting cell; FACS, fluorescence-activated cell sorter; GVHD, graft-versus-host disease; HSC, hematopoietic stem cell; MHC, major histocompatibility complex; NK, natural killer.

B6.H-2^{g7} mice do not develop islet inflammation or hyperglycemia. However, it is unclear whether genes expressed by their hematopoietic cells will transfer diabetes protection. The genetic mismatch between NOD and B6.H-2^{g7} is analogous to the common clinical scenario in which patients undergo transplantation from unrelated donors matched at the human MHC but that differ at other background loci.

We further sought to establish ways that minimize AHCT-related complications by using purified hematopoietic stem cell (HSC) grafts in conjunction with nonmarrow ablative regimens. By virtue of their unique ability to self-renew and regenerate all blood lineages, HSCs are the only cells that can permanently engraft in an AHCT procedure. Hematopoietic graft content directly affects the outcomes of clinical transplantation because grafts that contain mature immune cells cause a complication called graft-versus-host disease (GVHD). GVHD is mediated by allogeneic T-cells that recognize and respond against recipient tissues. Although this complication occurs more frequently and with greater severity when MHC disparities exist between donor and recipient, GVHD can still develop in a significant percentage of patients with MHC-matched grafts. Purified HSCs do not contain T-cells, and thus they do not induce GVHD.

Here, we observed 100% protection of pre-diabetic NOD mice engrafted with B6.H-2^{g7} HSC grafts. Our results demonstrate that non-MHC background genes expressed on transplanted hematopoietic cells abrogate NOD diabetes and that it is possible to achieve protection from this autoimmune disease using nonmorbid conditioning with engraftment of purified HSCs.

RESEARCH DESIGN AND METHODS

Founders for our current NOD colony (H-2^{g7}, Thy-1.2, and CD45.1) were the gift of M. Hattori (Boston, MA). B6.NOD-(D17Mit21-D17Mit10)/LtJ mice (B6.H-2^{g7}, Thy-1.2, and CD45.2) were purchased from The Jackson Laboratories (Bar Harbor, ME). We established a line of congenic B6.H-2^{g7}.Thy1.1 mice by crossing B6.H2^{g7} with C57BL/Ka (Thy1.1, H-2^{b}, and CD45.1) mice. (NOD.Thy1.1×B6.H-2^{g7})F$_1$ mice were generated by crossing B6.H-2^{g7}.Thy1.1 with Thy-1.1 congenic NOD mice (NOD.NON-Thy1^{a} or NOD.NON-Thy1^{a}/1Lt, the latter a gift from E. Leiter [Bar Harbor, ME]). A B-cell–deficient B6.H-2^{g7} line (B6.H-2^{g7}.μMT$^{-/-}$) was generated by crossing B6.129S2-Igh-6^{tm1Cgn}/J (The Jackson Laboratories) with B6.H-2^{g7}.Thy1.1, and F3 mice homozygotes for H-2^{d}, CD45.2, and μMT$^{-/-}$ were intercrossed and maintained by brother-sister mating to generation of F7. Lymphocyte-deficient B6.H-2^{g7} mice (B6.H-2^{g7}.SCID) were generated by crossing B6.CB17-*Prkdc*^{scid}/SzJ (The Jackson Laboratories) and B6.H-2^{g7}.Thy1.1 mice. The lymphocyte-deficient F3 mice homozygotes for H-2K^{d} were intercrossed and maintained by brother-sister mating. All mice were bred in our colony and kept under specific pathogen-free conditions.

HSC purification. Purified HSCs were obtained by modification of the methods described by Spangrude et al. (22) and later Randall et al. (23). Briefly, B6. H-2^{g7} (Thy1.2) bone marrow cells were positively selected for c-kit over magnetic beads and sorted on the basis of positive staining for c-Kit, CD38, and Sca-1 and negative staining for lineage-specific markers (B-cells, T-cells, macrophage, granulocyte, and early red cells) (22). Propidium iodide staining (1 mg/ml) was used to exclude dead cells. Cells were sorted on a dual laser fluorescence-activated cell sorter (FACS) (Becton Dickinson, Mountain View, CA). After sorting for the CD38-FITC^{hi}, Sca-1-TR^{hi}, c-Kit-APC^{hi}, and lineage-PE$^{-/lo}$, the resultant CD38^{hi}Lin$^{-/lo}$Sca-1^{hi}c-Kit^{hi} population was noted on reanalysis by FACS to be >95% pure.

Hematopoietic cell transplantation. Eight- to 9-week-old NOD mice were prepared for transplantation with radiation, using a Phillips Unit Irradiator (250 kV, 15 mA) to deliver radiation on day 0 as two split doses 3–4 h apart (950 or 700 cGy) or as a single 500-cGy fraction. In most experiments, 4,000 B6.H-2^{g7} HSCs or 4 × 10^{6} bone marrow cells were delivered via intravenous route. Syngeneic NOD recipients received either 2,000 HSCs or 4 × 10^{6} bone marrow. Each HSC inoculum was tested by day 12 spleen colony-formation unit-assay. Posttransplantation glucose levels were measured semimonthly from tail vein blood (Freestyle; TheraSense, Alameda, CA; and OneTouch; LifeScan, Milpitas, CA). NOD mice with blood glucose concentrations of ≥250 mg/dl on two separate measurements were deemed diabetic.

Chimerism analysis. Blood chimerism was assessed by FACS analysis at 6–8 weeks posttransplant and 3 months later using methods previously described (14). Donor versus host cells were differentiated by monoclonal antibodies specific for the two allelic forms of CD45 expressed on all blood lineages. NOD mice are CD45.1, whereas B6.H-2^{g7} donors are CD45.2. Double staining for lineage-specific markers included T-cells, B-cells, monocytes, granulocytes, and natural killer (NK) cells. FACS analysis was performed using a modified FACS Scanford and Vantage machine (Becton Dickinson). Dendritic cells from the spleen, draining pancreatic lymph nodes, and mesenteric lymph nodes were prepared by teasing the organs followed by digestion with Collagenase IV (Worthington, 500 Mandl units/ml) at 37°C for 30 min. Digested material was dispersed and filtered. Cells were washed, and erythrocytes were lysed. After blocking with purified α-FcγR (2.4G2), cell suspensions were stained for multicolor flow cytometry using a cocktail of monoclonal antibodies (CD3, CD45.1, CD45.2, and CD11c).

Histology evaluation. Frozen tissue samples, stored at −80°C were brought to room temperature and fixed in acetone. All primary antibodies were biotinylated and included: α-CD45.1 (clone A20) and α-CD45.2 (clone 104), both from Pharmingen; α-CD4, α-CD8, α-B220, and α-Mac-1 (gifts from Dr. I. Weissman); and α-insulin from Linco Research (St. Charles, MO). Sections were blocked using a biotin/avidin kit from Vector Laboratories (Burlingame, CA) before primary antibody staining. The secondary antibody for the lineage-specific panel was goat-α-rat-horseradish peroxidase (BioSource International, Camarillo, CA). The secondary antibody for the insulin was goat-α-guinea pig-horseradish peroxidase (Southern Biotechnology, Birmingham, AL). Slides were incubated with 3-amino-9-ethyl-carbazole solution and then counterstained with Gills III hematoxylin.

Statistical analysis. Time to diabetes onset among groups was compared using the log-rank test with the program GraphPad Prism Version 3.1 for PC and 4.0b for Mac (GraphPad Software, San Diego, CA). Differences in donor cell chimerism, white blood cell, and CD4^{+} subsets were calculated using the nonparametric Mann-Whitney or Kruskal-Wallis (multigroup comparison) test. $P < 0.05$ were considered statistically significant.

RESULTS

Transplantation of B6.H-2^{g7} HSCs. In prior studies, we demonstrated that transplantation of purified MHC-mismatched HSCs blocked diabetes in NOD mice and that such grafts modified the function of residual NOD T-cells (14). Here, we tested whether AHCT from donors sharing the main diabetes susceptibility allele, I-Aβ^{g7}, could disrupt NOD disease. MHC-congenic B6.H-2^{g7} mice were used as donors of HSCs or bone marrow. Recipients were pre-diabetic NOD mice transplanted with established islet infiltrates (8 weeks old). In the initial studies, mice were conditioned with lethal irradiation and infused with either purified B6.H-2^{g7} HSCs or bone marrow. Figure 1*A* shows that recipients of either B6.H-2^{g7} HSCs or bone marrow were protected from diabetes. To control for the immunosuppressive effects of radiation conditioning, control-irradiated mice received syngeneic NOD bone marrow or NOD HSCs. The majority of such control mice succumbed to disease, although hyperglycemia was delayed by ~8 weeks compared with unmanipulated NOD mice from our colony ($P < 0.001$).

Hematopoietic chimerism. Blood chimerism was assessed in NOD recipients at different time intervals posttransplantation. Figure 1*B* shows that consistent with our prior observations, donor T-cell levels differed between recipients of HSCs versus bone marrow. Early after transplantation (~day 50) HSC-engrafted mice demonstrated significant levels of persistent NOD-derived T-cells compared with bone marrow recipients ($P < 0.001$). Donor T-cell chimerism steadily increased in HSC recipients. However, as long as 4 months post-HSC transplant, 10–

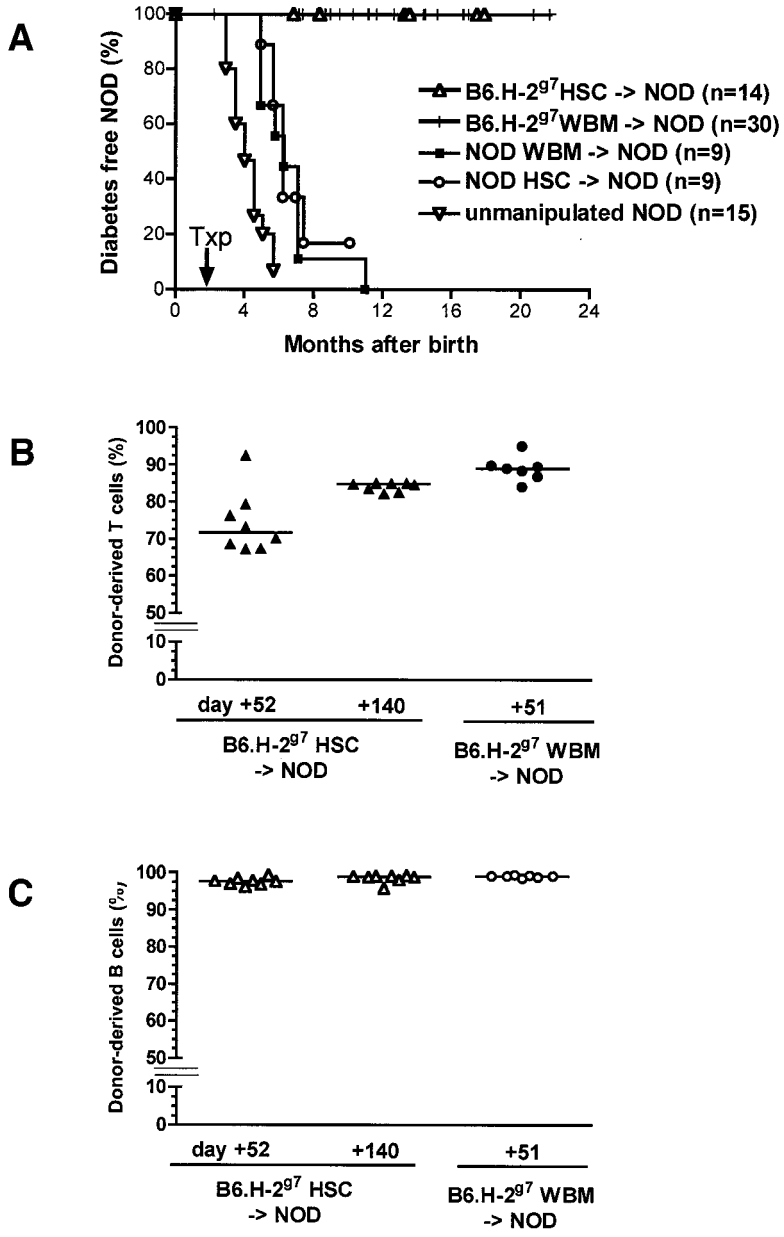

FIG. 1. Diabetes-free survival and donor chimerism of NOD mice engrafted with hematopoietic cells. Eight-week-old pre-diabetic NOD mice were prepared for transplantation with 950 cGy of lethal irradiation followed by infusion of purified HSCs or unmanipulated bone marrow (WBM). *A*: All recipients engrafted with HSC (△) or bone marrow (vertical ticks) derived from MHC-matched B6.H-2^{g7} donors were protected from diabetes development. In contrast, nearly all mice that received NOD HSCs (○) or bone marrow (■) progressed to hyperglycemia ($P < 0.001$), although the time to overt disease was delayed compared with untreated NOD mice (▽) ($P < 0.001$). *B*: Significant differences in the development of T-cell chimerism were noted after B6.H-2^{g7} HSC (▲) versus bone marrow (●) transplantation (each symbol represents one mouse). NOD mice reconstituted with purified HSCs showed lower levels of donor-derived T-cells and retained more host T-cells. In contrast, NOD mice reconstituted with bone marrow demonstrated high levels of donor-derived T-cells within 2 months posttransplant ($P < 0.001$). Over time, donor-derived T-cells slowly increased in HSC-transplanted NOD mice. *C*: Non–T-cell lineages (B-cells shown here) were predominantly donor derived both in HSC (△)- and bone marrow (○)-transplanted NOD mice early after transplantation.

15% NOD T-cells still remained (Fig. 1*B*). We previously showed in studies using NOD.SCID mice as HSC donors that NOD T-cells that persist after lethal radiation are capable of mediating islet destruction (14). This result taken together with the present data leads us to conclude that engraftment of B6.H-2^{g7} hematopoietic cells block ongoing islet destruction and that allograft alters the autoreactivity of remaining NOD cells. In contrast to the T-cell chimerism results, variations in chimerism levels among the other blood lineages were not observed—a finding that also concurs with our earlier reports using MHC-mismatched HSCs (14,24). Donor B-cell and myeloid chimerism were high in recipients of B6.H-2^{g7} HSC or bone marrow by day 52 posttransplant (Fig. 1*C*; Table 1). Chimerism evaluations were also performed on lymphoid organs including the spleen, draining pancreatic and mesenteric lymph nodes at 6 months' posttransplantation. FACS staining for CD45 alleles revealed that donor-type cells comprised >98% of the hematopoietic cell content of these organs. Staining for donor dendritic cells using CD11c^{+}, CD3^{-}, and CD45 allelic markers also showed that the majority (>97%) of dendritic cells were donor derived (Fig. 2).

One gene dose from B6.H-2^{g7} confers diabetes protection. We next tested whether the diabetes protective effect conferred by the non-MHC background genes expressed on B6.H-2^{g7} hematopoietic cells acts as a dominant trait. (B6.H-2^{g7}×NOD)F$_1$ offspring were generated that were homozygous for the NOD susceptibility MHC allele but heterozygous for the remaining genes. As shown in Fig. 3, transplantation of bone marrow from F$_1$ donors into pre-diabetic NOD mice resulted in 100% disease protection. Control mice received either congenic NOD or allogeneic B6.H-2^{g7} bone marrow. As expected, the congenic recipients progressed to diabetes, whereas recipients of B6.H-2^{g7} bone marrow did not. Thus, these data show that a single dose of a gene or genes expressed on hematopoietic cells derived from the C57BL/6 background was sufficient to block diabetes in NOD recipients.

TABLE 1
Hematopoietic cell chimerism posttransplantation

Recipient	Preconditioning treatment (rad)	Graft	Transplanted cells	Engrafted mice/transplanted mice	Donor-derived PBMCs		
					$CD3^+$ (%)	$B220^+$ (%)	$Mac\text{-}1^+/Gr\text{-}1^+$ (%)
NOD (n = 16)	950	C57BL/6.H-2^{g7} HSC	4,000	16/16	69.6 ± 8.0	96.9 ± 1.2	89.1 ± 16.7
NOD (n = 9)	700	C57BL/6.H-2^{g7} HSC	4,000	9/9	64.5 ± 5.6	90.6 ± 2.2	80.4 ± 12.1
NOD (n = 7)	950	C57BL/6.H-2^{g7} bone marrow	6×10^6	7/7	88.9 ± 3.3	98.9 ± 0.2	99.1 ± 0.6
NOD.SCID (n = 10)	300	C57BL/6.H-2^{g7} bone marrow	6×10^6	5/10*	99.8 ± 0.1	99.7 ± 0.2	97.8 ± 3.4

Data are means ± SD. NOD or NOD.SCID mice were 8–9 weeks old at the time they were conditioned for AHCT with radiation treatment. Recipients received either C57BL/6.H-2^{g7} HSC or bone marrow grafts. Donor blood chimerism was evaluated at ~50 days' posttransplant by FACS analysis with double staining using CD45.2 and lineage-specific labeled monoclonal antibodies. *Five mice died before chimerism evaluation. PBMCs, peripheral blood mononuclear cells.

Nonmarrow ablative conditioning. To reduce the toxicity of the preparative regimen, we tested whether nonmyeloablative radiation (700 or 500 cGy) could permit stable HSC engraftment and prevent diabetes. As shown in Table 1 and Fig. 4*A*, mice in both sublethal radiation groups engrafted with B6.H-2^{g7} hematopoietic cells and did not develop hyperglycemia. Control mice that received 500 or 700 cGy survived without hematopoietic cell rescue (data not shown), confirming that these radiation doses were nonmyeloablative. Interestingly, blood chimerism measurements at 2 months revealed that HSC-engrafted NOD mice conditioned with 700 cGy had high donor chimerism levels similar to recipients more aggressively prepared with 950 cGy (Table 1).

Donor chimerism in mice conditioned with 500 cGy differed significantly from mice treated with the higher radiation doses. Figure 4*B* shows that the 500 cGy recipients of HSC or bone marrow developed long-term multilineage partial chimerism, including in the macrophage/granulocytes cell lineages. Interestingly, the chimerism level in bone marrow–engrafted mice uniformly increased over time, whereas the trends in HSC-engrafted mice were more variable. Also of note, B-cell lineage was the most vulnerable to radiation because high levels of donor B-cells were observed early posttransplantation, and conversion to >95% donor B-cells was noted by day 140. Thus, nonmarrow ablative conditioning, and establishment of multilineage partial donor chimerism was sufficient to prevent NOD diabetes.

Phenotype of islet infiltrating cells. To determine the effect of B6.H-2^{g7} AHCT on insulitis, selected chimeras were killed, and their pancreata were evaluated by immunohistochemistry. Table 2 shows the insulitis score of pancreata from HSC- or bone marrow–engrafted NOD mice. Pancreata were stained with antibodies that bind donor and host CD45 alleles and with lineage-specific antibodies including CD4, CD8, B220 (B-cells), macrophage, and granulocytes. Most islets in the chimeras showed none or minimal insulitis. Interestingly, in islets with significant cellular infiltrates, an unexpected predominance of donor-type $B220^+$ cells was noted (Fig. 5*A*). Staining with a second marker ($CD19^+$) confirmed that these infiltrating cells were B-cells (data not shown). In contrast to the chimeras, islet infiltrates in 8-week-old control NOD mice were predominantly T-cells (data not shown). Transplantation studies of B6.H-2^{g7} bone marrow into NOD-.SCID recipients showed no evidence of B donor cells trafficking into the islets (Table 2). These latter studies suggest that the B-cell infiltrates resulted from turnover of donor cells trafficking to preexisting inflamed islets rather than by de novo development after transfer of B6.H-2^{g7} cells.

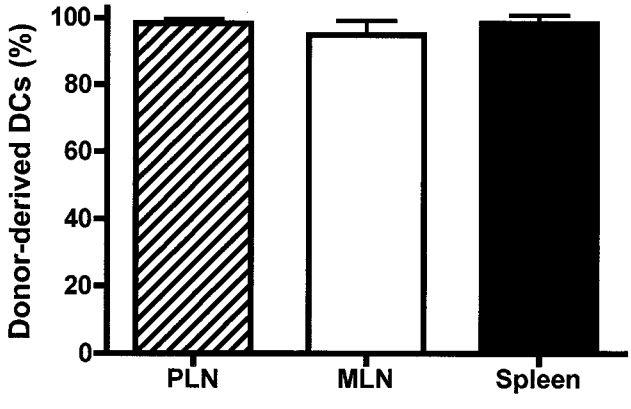

FIG. 2. Dendritic cell chimerism of transplanted NOD mice. Splenocytes and lymphocytes of chimeric NOD mice (925 cGy, 4×10^6 B6.H-2^{g7} bone marrow cells) were analyzed at 6 months' posttransplantation by FACS. Dendritic cells ($CD11c^+CD3^-$) were stained for the expression of the two $CD45^+$ isoforms, $CD45.1^+$ (host) and $CD45.2^+$ (donor). More than 97% of dendritic cells in pancreatic draining lymph nodes (PLN), mesenteric lymph nodes (MLN), and spleen are donor derived. Means ± SD, n = 3.

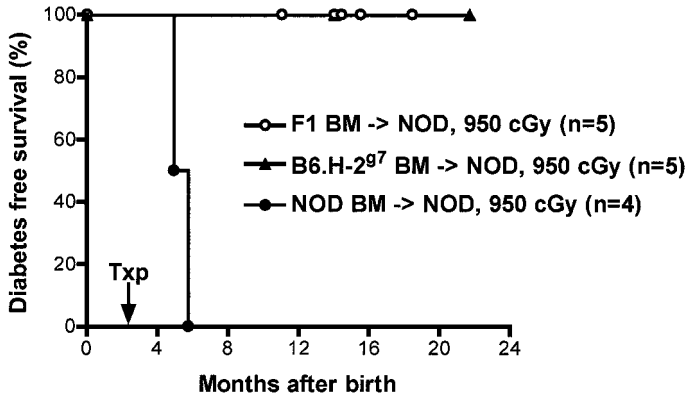

FIG. 3. Diabetes-free survival of lethally irradiated NOD mice transplanted with (NOD×B6.H-2^{g7})F_1 bone marrow. Recipients of 6×10^6 (NOD×B6.H-2^{g7})F_1 bone marrow cells (○) were protected from diabetes as were control NOD mice that received B6.H-2^{g7} bone marrow (▲). Control recipients of syngeneic NOD bone marrow (●) all developed hyperglycemia.

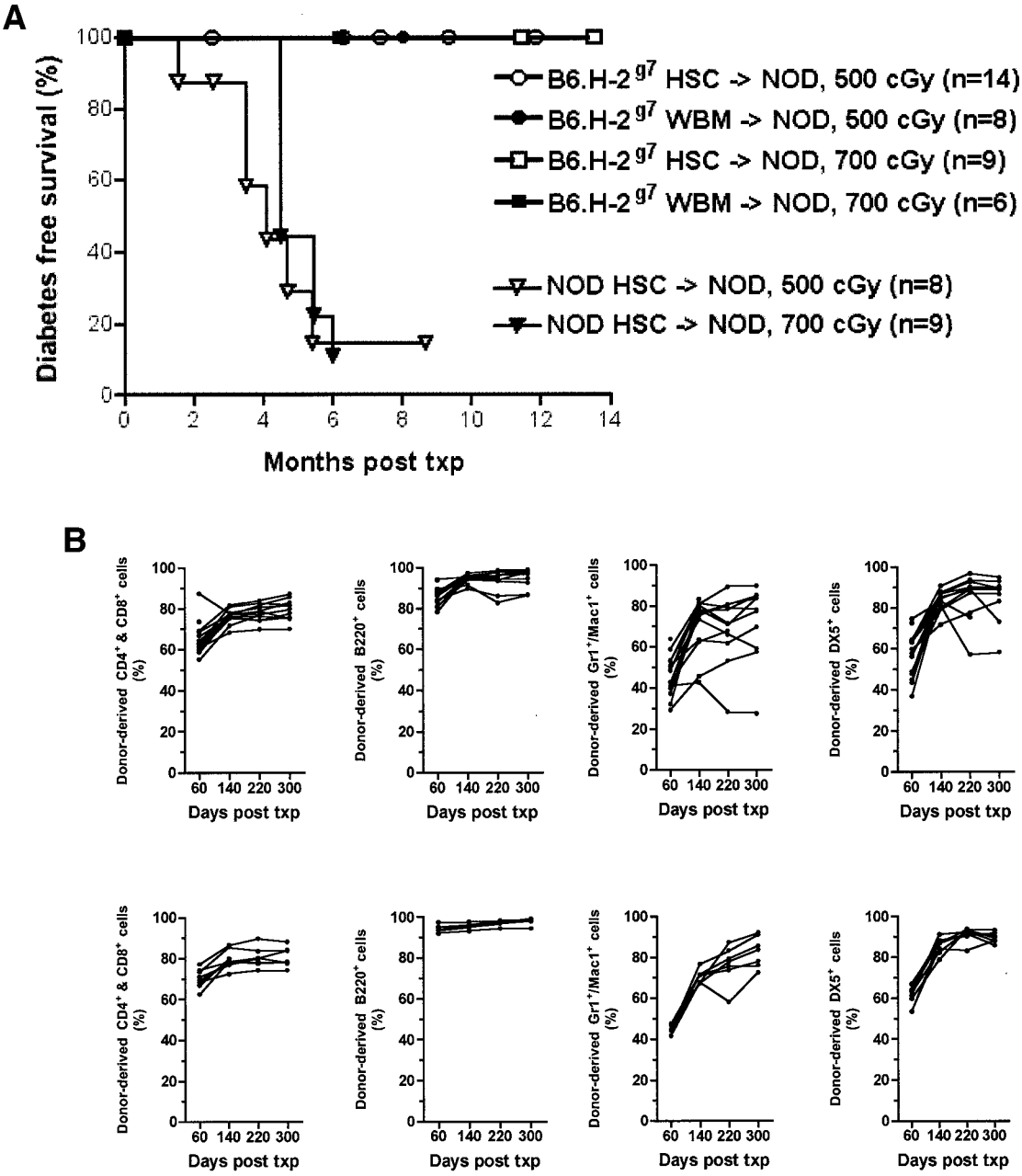

FIG. 4. Diabetes-free survival and donor chimerism in NOD mice conditioned with nonmyeloablative (sublethal) radiation. *A*: Transplantation of 8-week-old NOD mice with B6.H-2^{g7} bone marrow (8×10^6 cells) or HSCs (4,000 cells) blocks diabetes development after sublethal irradiation with 500 cGy (HSC, ○; bone marrow, ●) or 700 cGy (HSC, □; bone marrow, ■). Control NOD mice conditioned with 500 cGy (▽) or 700 cGy (▼) and infused with NOD HSCs developed diabetes ($P < 0.001$). *B*: Donor cell chimerism at four different time points posttransplantation for NOD mice conditioned with 500 cGy and transplantation of B6.H-2^{g7} HSCs (*top panels*; $n = 14$) or bone marrow (*bottom panels*; $n = 8$). Multilineage mixed chimerism in peripheral blood was observed in recipients of both graft types in the T-cell, granulocyte/macrophage, and NK cell lineages. In contrast, host B-cells appear more radiosensitive, resulting in near complete conversion to donor B-cell type. Chimerism data for recipients conditioned with 700 cGy and transplantation of B6.H-2^{g7} HSCs are shown in Table 1. Data for the 700 cGy group transplanted with B6.H-2^{g7} bone marrow are not shown.

Donor lymphocytes and diabetes protection. The predominance of donor B-cells in the islet infiltrates together with the high level of donor B-cell chimerism observed in all allografted NODs led us to ask whether donor B-cells were required to confer diabetes protection after AHCT. Thus, B-cell–deficient B6.H-2^{g7} donors were generated by breeding B6.H-2^{g7} mice with a line (C57BL/6.μMT$^{-/-}$) that lacks B-cells because of a targeted disruption of the IgM heavy chain (25) (see RESEARCH DESIGN AND METHODS). As shown in Fig. 5*B*, transplantation of bone marrow from these B-cell–deficient B6.H-2^{g7}.μMT$^{-/-}$ donors was as protective as bone marrow from B-cell–replete B6.H-2^{g7} mice. Chimerism studies performed at 3 and 6 months' posttransplantation revealed that B6.H-2^{g7}.μMT$^{-/-}$ into NOD recipients were devoid of B-cells in the peripheral blood (data not shown).

We also examined the requirement for donor T-cells to be present in chimeric mice to achieve diabetes protection. Hematopoietic grafts from B6.H-2^{g7}.SCID mice cannot give rise to B- or T-cells, including the putative

TABLE 2
Prevalence of diabetes and insulitis in transplanted NOD mice

Transplanted mice	Recipient sex	Pancreas histology					Hyperglycemia
		No. of islets observed	Histology score (%)				
			0	1	2	3	
C57BL/6.H-2^{g7} WBM → NOD 1	F	11	46	18	9	27	No
C57BL/6.H-2^{g7} WBM → NOD 2	F	14	43	22	21	14	No
C57BL/6.H-2^{g7} HSC → NOD 1	F	7	86	0	14	0	No
C57BL/6.H-2^{g7} HSC → NOD 2	F	8	87	13	0	0	No
NOD control 1	F	13	0	0	15	85	No
NOD control 2	F	22	23	9	36	32	No
C57BL/6.H-2^{g7} control 1	F	13	100	0	0	0	No
C57BL/6.H-2^{g7} control 2	F	16	100	0	0	0	No
C57BL/6.H-2^{g7} WBM → NOD.SCID	M	5	100	0	0	0	No

Islet cell infiltration: NOD mice were conditioned with 950 cGy and transplanted with HSCs or bone marrow from B6.H-2^{g7} mice were killed 6 months posttransplantation. Eight-week-old unmanipulated B6.H-2^{g7} and NOD mice are included in the analysis. Adjacent sections of pancreata were analyzed, and islet infiltrates were scored according to a grading system described by Wicker et al. (54). Histology score: grade 0, no infiltration; grade 1, perivascular/periductular or perinsular infiltration, but not penetrating; grade 2, mild leukocytic islet mass infiltration; grade 3, severe infiltration, islet mass destruction. WBM, whole bone marrow.

regulatory populations CD4$^+$CD25$^+$ or NK–T-cells. Blood chimerism analysis of B6.H-2^{g7}.SCID HSC recipients revealed high levels of NOD T-cells relative to other allografted groups and a small percentage of residual B-cells (data not shown). As expected, granulocytes and macrophages were all primarily donor derived. Despite the absence of donor T-cells and persistence of significant numbers of NOD T-cells, only 1 of 11 mice developed diabetes at 6 months' posttransplantation (Fig. 5*C*).

CD4$^+$CD25$^+$ T-cells in chimeric NOD mice. To further investigate a role for regulatory CD4$^+$CD25$^+$ cells in conferring diabetes protection, the absolute and relative numbers of these cells were measured in the blood of chimeras (Fig. 6*A–D*). Total white blood cell counts (data not shown) and the relative and absolute numbers of blood CD4$^+$ cells were also assessed. As controls, analyses were performed on age-matched B6.H-2^{g7} mice, C57BL/6 mice that do not express the H-2^{g7} allele, NOD mice, and NOD mice transplanted with NOD congenic (NOD.Thy1.1) HSCs. Figure 6 shows that direct relationships exist between the measured parameters and strain type. Among the three strains, NOD mice had the lowest absolute white blood cell count ($P < 0.001$, data not shown); however, they had the highest relative numbers of CD4$^+$ cells ($P < 0.001$). Of note, the percent and absolute numbers of blood CD4$^+$CD25$^+$ cells were higher in NOD compared with B6.H-2^{g7} mice ($P < 0.024$ and $P < 0.018$, respectively), but not different from C57BL/6 mice ($P < 0.4$ and $P < 0.3$, respectively). Measurements taken 2 months posttransplantation revealed that the relative and absolute levels of CD4$^+$CD25$^+$ cells were similar between NOD chimeras engrafted with either B6.H-2^{g7} or congenic NOD HSCs ($P < 0.9$ and $P < 0.6$, respectively). These levels of CD4$^+$CD25$^+$ cells in transplanted mice were comparable with those observed in wild-type NOD ($P < 0.14$ and $P < 0.18$, respectively) and significantly higher than B6.H-2^{g7} donors ($P < 0.002$ and $P < 0.008$, respectively). Thus, from analysis of blood, we found no evidence that diabetes-resistant B6.H-2^{g7} into NOD chimeras had quantitative differences in CD4$^+$CD25$^+$ cells compared with standard NOD mice. Furthermore, these data taken together with the B6.H-2^{g7}.SCID into NOD transplantations do not support a significant role for donor regulatory T-cells in protecting NOD mice from diabetes postallogeneic HSC transplantation.

DISCUSSION

AHCT in NOD mice. The long-standing observation that AHCT prevents NOD diabetes proved that genes expressed on donor hematopoietic cells can block the autoimmune pathogenesis (11–14). While inheritance of NOD disease is complex, the main susceptibility allele, I-A^{g7}, is expressed on marrow-derived cells, suggesting that donated cells exert their influence through the actions of class II molecules other than I-A^{g7}. Indeed, AHCT from a variety of MHC-mismatched donor strains uniformly protects pre-diabetic NOD mice from hyperglycemia (11–14), whereas experiments using allogeneic donors that share the NOD MHC have not shown the same effect (26). In this report, we demonstrate that HSC and bone marrow transplantations from MHC-matched B6.H-2^{g7} mice successfully prevented NOD diabetes, proving that non-MHC genes expressed on hematopoietic cells and derived from the C57BL/6 background can confer disease protection. Furthermore, complete elimination of NOD hematopoietic cells was not required because nonmyeloabative transplantations were also 100% protective.

Other studies of MHC-matched AHCT in NOD mice. In an earlier study, Serreze et al. (26) investigated the effect of bone marrow transplantations from MHC-matched NOR (H-2^{g7}) mice on pre-diabetic NODs. The NOR strain is a recombinant congenic stock that does not develop diabetes despite sharing of a large portion of its genome (~88%) with NOD (27–29). Transplantation of 4-week-old NOD mice with NOR bone marrow still resulted in a significant diabetes incidence. In a separate report, these same investigators carried out transplantations using NOD bone marrow mixed with bone marrow from another related diabetes-resistant MHC-matched strain designated NON.H-2^{g7} (30–32) into (NOD×NON)F$_1$ offspring. The NON.H-2^{g7} bone marrow blocked disease transfer by NOD bone marrow in (NODxNON)F$_1$ recipients (30); however, the effect of NON.H-2^{g7} bone marrow engraftment on

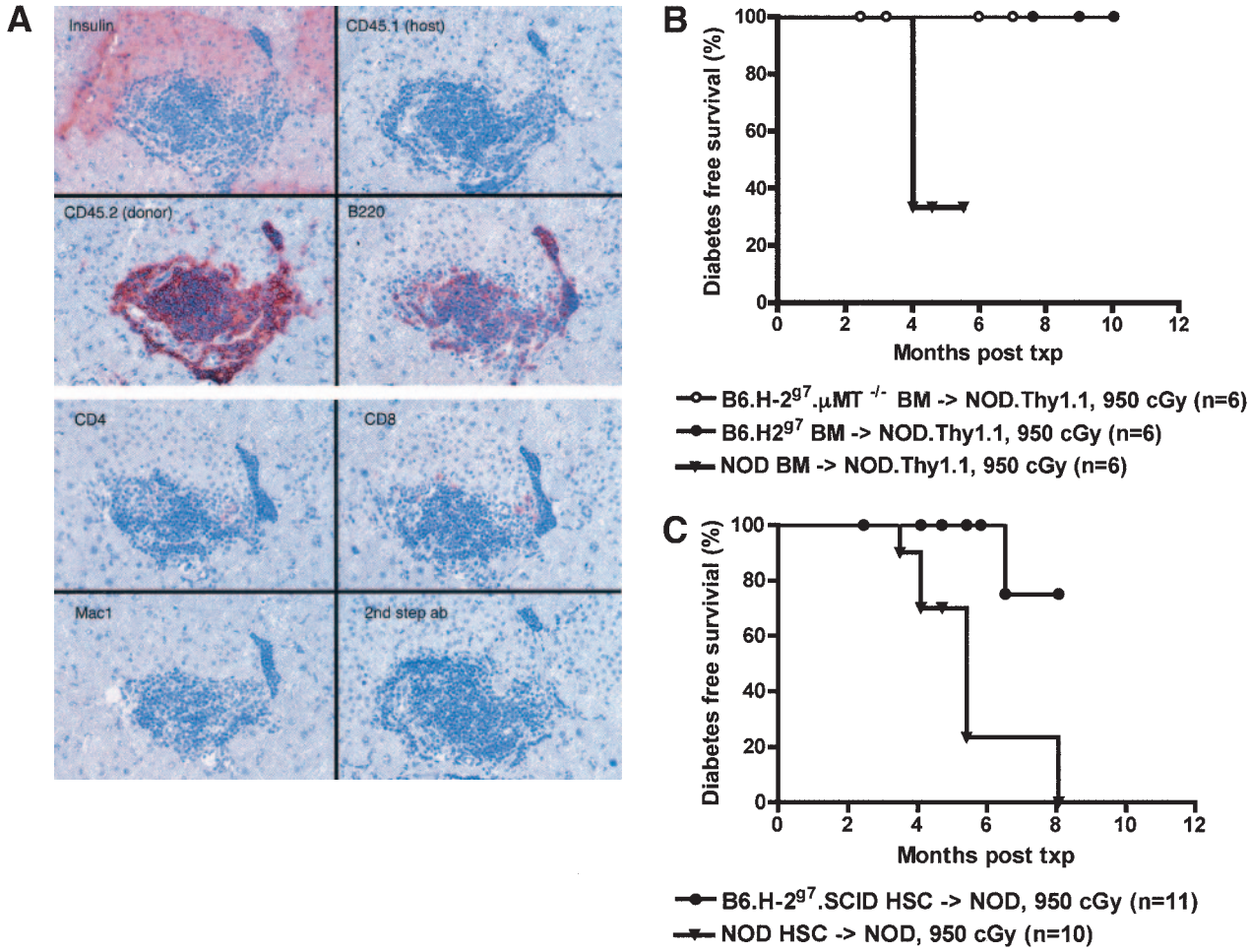

FIG. 5. Donor B-cells predominate in islet infiltrates of chimeric mice but are not required for diabetes protection. *A*: Immunohistochemistry of an islet from a B6.H-2^{g7} bone marrow into NOD chimera 6 months after transplantation. Shown are stains of serial sections of the same islet. The findings are representative of immunohistochemical analyses performed on islets from diabetes-free chimeric mice. Most islets in the chimeras were minimally infiltrated (Table 2); however, the few islets that showed severe infiltrates were strongly positive for B220^{+} and CD45.2^{+} (donor derived). Infiltration with CD4^{+} and CD8^{+} T-cells and Mac-1^{+} cells were minimal. *B*: Diabetes-free survival of NOD mice engrafted with B-cell–deficient B6.H-2^{g7} bone marrow. Lethally irradiated (950 cGy) NOD mice that were transplanted with bone marrow from B-cell–deficient B6.H-2^{g7}.μMT$^{-/-}$ donors (○) were 100% protected from diabetes as were control NOD mice reconstituted with B6.H2^{g7} bone marrow (●). Control NOD mice reconstituted with syngeneic NOD bone marrow (▼) developed diabetes ($P < 0.036$). *C*: Lethally irradiated NOD mice transplanted with HSCs from T- and B-cell–deficient B6.H-2^{g7}.SCID mice (●) demonstrated significant protection from diabetes compared with syngeneic recipients (▼) ($P < 0.002$).

disease in wild-type NODs was not reported. Studies by Wu et al. (33) examined cotransplantations of NOR bone marrow with NOR islets into hyperglycemic NOD mice to induce donor-specific tolerance to islets. By using an aggressive combination of lymphoablative and lymphosuppressive agents, long-lasting normoglycemia and islet graft survival was achieved. Because prolonged NOR islet graft survival was also observed in recipients who received the preparative regimen without NOR bone marrow cells, the conclusion that NOR bone marrow mediated tolerance induction was somewhat obscured by the potential effects of the regimen on NOD immune responses.

Diabetes protection does not require complete donor chimerism. We noted that complete elimination of NOD hematopoietic elements, including T-cells and antigen presenting cells (APCs), was not required for B6.H-2^{g7} cells to block diabetes. Disease blockade was achieved using nonablative radiation with establishment of partial donor chimerism. Thus, the presence of B6.H-2^{g7} grafts was sufficient to modify the activity of regimen-resistant autoreactive NOD cells. Others have reported that T-cells in NOD mice are more radiation resistant compared with other strains (34) and we previously showed using NOD.SCID mice as donors that lethal radiation alone does not completely destroy NOD cells capable of mediating islet destruction (14). More recently, in an attempt to better track the effect of AHCT on islet-specific clones, we performed B6.H-2^{g7} HSC transplantations into NOD T-cell receptor transgenic mice (NOD.BDC2.5). Surprisingly, accelerated diabetes development rather than protection was observed (G.F.B., R.R.L., J.A.S., unpublished data), again showing that complete deletion of autoreactive cells does not occur by the combination lethal radiation and engraftment of allogeneic hematopoietic cells. Although deletion of anti-islet T-cells is certainly an important factor in HCT-mediated diabetes protection, we have noted in wild-type NOD, but not NOD.BDC2.5 transgenic mice (which have an overwhelming burden of an anti-islet clone), that B6.H-2^{g7} donor cells modulate the remaining host immune elements.

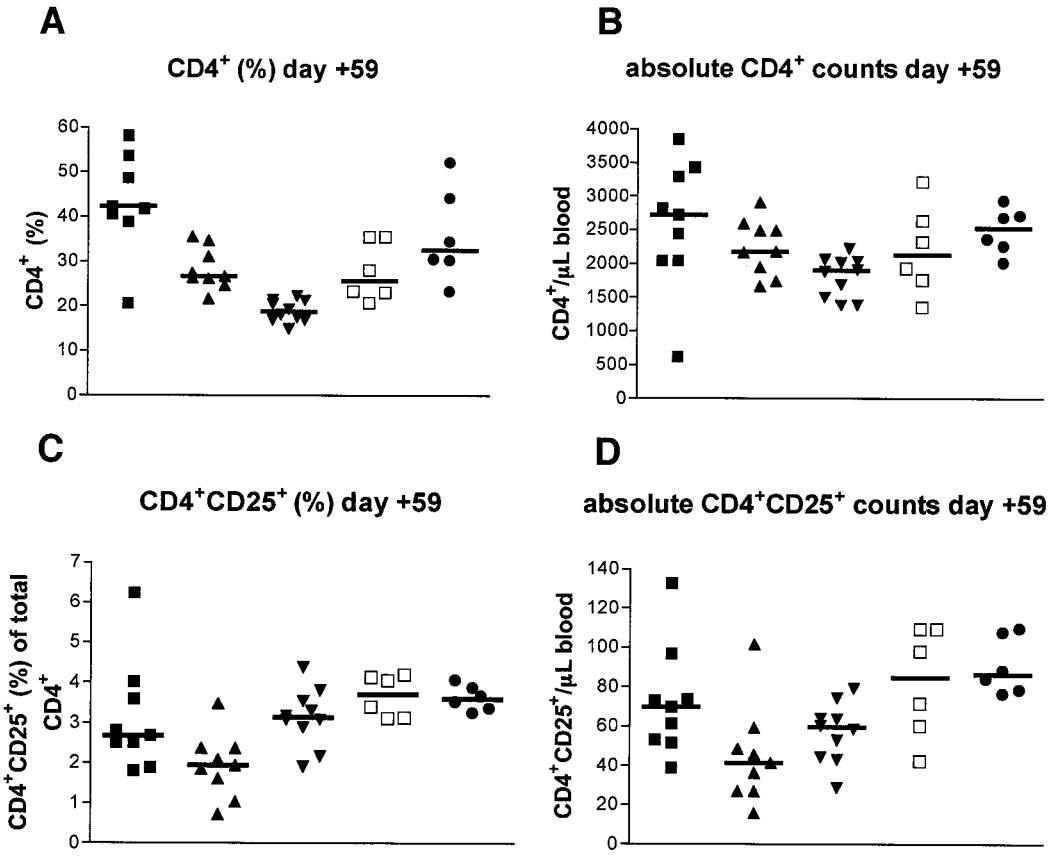

FIG. 6. Analysis of peripheral blood $CD4^+CD25^+$ T-cells after B6.H-2^{g7} HSC transplantation. Blood subsets levels from transplanted NOD mice were compared with three strains of age- and sex-matched unmanipulated mice. *A* and *B*: Relative and absolute numbers of blood $CD4^+$ cells. *C* and *D*: Relative and absolute blood $CD4^+CD25^+$ cells. Data are presented for individual NOD mice that had been transplanted 2 months previously with either B6.H-2^{g7} HSC (□; $n = 6$) or syngeneic NOD HSC (●; $n = 6$). The unmanipulated reference control strains were NOD (■; $n = 9$), B6.H-2^{g7} (▲; $n = 9$), and C57BL/6 mice (▼; $n = 10$).

Mechanisms of protection. The diabetes protection observed in mixed chimeric NOD mice suggests that one or more B6.H-2^{g7} blood cell–derived populations replace or modulate the activity of defective NOD cells. Several blood lineages have been proposed to be responsible for diabetes development in NOD mice, including defects in APCs (macrophages, dendritic cells, B-cells) (35), NK cells (36,37), and regulatory subpopulations of T- and NK–T-cells (38–40). Our prior studies using MHC-mismatched HSCs supported a central role for donor APCs in altering the NOD T-cell repertoire by influencing negative T-cell selection (14,41). Other reports in the NOD cell transplantation literature similarly suggest that diabetes protection can be mediated by transferred macrophages (30) and dendritic cells (42–44). Here, we further examined the possible role of transferred donor B- and T-cells on blocking NOD disease.

In islets of nondiabetic NOD chimeras, we observed an unexpected prominence of donor B-cells, suggesting that B6.H-2^{g7} B-cells might be required to block islet destruction. B-cell–deficient NOD mice (NOD.$\mu MT^{-/-}$) rarely become diabetic, which provides further evidence that NOD B-cells might in fact promote diabetes development (45–47). Thus, we transplanted grafts from B-cell–deficient B6.H-2^{g7}.$\mu MT^{-/-}$ donors and observed that none of the recipients progressed to diabetes, demonstrating that donor B-cells were not necessary for disease protection. Although replacement of the NOD B-cell pool with donor cells was not required per se, it can be argued that given the radiosensitivity of the B lineage, ablation of NOD B-cells alone was sufficient to block disease. However, our prior HCT studies using NOD.SCID grafts in lethally irradiated NOD recipients showed that after congenic transplantation and prolonged B-cell lymphopenia, recipients still developed hyperglycemia (14).

We also examined the potential contribution of regulatory T-cells to disease amelioration. Prior reports by some groups (38,48), but not all (49), suggest that NOD mice have reduced numbers of $CD4^+CD25^+$ regulatory cells compared with other strains. We found that the percentage and absolute numbers of $CD4^+CD25^+$ cells in the blood of unmanipulated NOD mice were higher compared with B6.H-2^{g7} mice and equivalent to C57BL/6 mice, and transplant recipients demonstrated $CD4^+CD25^+$ values comparable with the wild-type NODs. Of note, we analyzed blood $CD4^+CD25^+$ cells, whereas other investigators analyzed the frequency of this T-cell subset in other lymphoid organs. A role for donor-derived regulatory T-cells was also assessed by using T- and B-cell–deficient B6.H-2^{g7}.SCID mice as donors. B6.H-2^{g7}.SCID mice lack both $CD4^+CD25^+$ and another subset of regulatory T-cells, NK–T-cells, and yet these donors provided nearly 100% disease protection. Thus, our studies do not support a significant immunoregulatory role for donor T-cells in the abrogation of NOD disease after AHCT. We have not yet ruled out a possible role for host-derived regulatory T-cells

or for host/donor NK cells. To address the latter issue, B6.H-2^{g7} donors that lack all lymphoid lineages, including NK cells, are being produced.

The observations that B- and T-cell–deficient B6.H-2^{g7} grafts protect NOD mice from diabetes strengthens the already existing evidence that donor APCs act as central mediators of tolerance post-HCT (35,50–52). Defects in NOD APCs have been widely reported including reduced expression of molecules involved in activation and maturation, impaired signaling (35), and skewing of dendritic cell subsets (51–53). Conflicting data have been published as to whether the dendritic cell defects are cell intrinsic (52) or, as recently proposed, can be corrected by exogenous factors such as could be provided by bone marrow cells derived from a hematopoietic donor (50). Our data show that donor dendritic cells engraft in the lymphoid organs that are in the vicinity of the pancreas and are the dominant dendritic cell population after myeloablation.

To further identify the diabetes protective elements expressed by hematopoietic cells, we are using as hematopoietic donors diabetes-resistant NOD.*Idd* congenic mice that carry limited segments of the C57BL background. Identification of the genes expressed on hematopoietic cells that confer protection from autoimmunity is an important step in the implementation of cell-based therapies for the treatment of human autoimmune disease.

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