Mixed Hematopoietic Chimerism Allows Cure of Autoimmune Diabetes Through Allogeneic Tolerance and Reversal of Autoimmunity

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Bone marrow transplantation from diabetes-resistant strains with complete replacement of the recipient immune system by the allogeneic donor has led to tolerance to donor islets and cure of diabetes in a mouse model of type 1 diabetes. However, the ability to tolerize host T-cells of diabetic NOD mice is unknown. We demonstrate that nonmyeloablative conditioning achieves mixed hematopoietic chimerism across major histocompatibility complex (MHC) barriers in spontaneously diabetic NOD mice. This conditioning preserves alloreactive and autoreactive diabetogenic host NOD T-cells, but when mixed chimerism was established, diabetic NOD mice accepted donor-type allogeneic islet grafts and were cured of diabetes, despite a significant recipient T-cell contribution. Furthermore, induction of mixed chimerism permitted acceptance of NOD islet grafts, demonstrating reversal of autoimmunity. Allogeneic bone marrow transplantation was critical for tolerization of diabetogenic and alloreactive host T-cells. Thus, mixed hematopoietic chimerism induces tolerance to donor islets and reverses established autoimmunity in diabetic NOD mice. *Diabetes* 53:376–383, 2004

Islet transplantation can overcome insulin requirements and prevent diabetic complications (1). In addition to islet rejection, destruction of transplanted islets may also reflect recurrence of autoimmunity, as reported in animal models and type 1 diabetic patients (2–5). Recently, the immunosuppressive "Edmonton Protocol" has provided a major step forward in islet transplantation (6–8). Nevertheless, life-long immunosuppressive therapy is hard to justify in young diabetic patients. Tolerance is desirable, as it permits graft survival without continuous immunosuppressive therapy (6). Tolerance must overcome alloresponses to donor islets and prevent recurrent autoimmunity in the graft.

Bone marrow transplantation (BMT) is a potential therapy for autoimmune diseases. Genetic resistance to autoimmune diseases can be transferred by BMT. The immunomodulatory effects of pre-BMT conditioning may also affect autoimmunity. In animal models of autoimmunity, including diabetes, ablation and hematopoietic rescue prevent disease (9–12). Mixed lymphohematopoietic chimerism prevents overt diabetes and reverses isletitis in myeloablated prediabetic mice (13,14). Clinical attempts to ameliorate malignant autoimmune diseases have included high-dose chemotherapy with rescue by autologous hematopoietic cell transplantation (15).

Engraftment of allogeneic pluripotent hematopoietic stem cells in nonmyeloablated animals results in tolerance to the donor (16), permitting acceptance of donor tissue without immunosuppression. Thus, combined islet and hematopoietic cell transplantation could potentially cure diabetes. However, the clinical application of BMT for tolerance induction has been previously precluded by toxicity of host conditioning and by the formidable complications of graft-versus-host disease (GVHD) and failure of engraftment, especially when major histocompatibility barriers are transgressed (17,18). Therefore, a reliable, nontoxic method of inducing mixed chimerism and tolerance is needed for clinical application of this approach.

Mixed hematopoietic chimerism can be achieved with relatively mild, nonmyeloablative conditioning regimens in nonautoimmune mice (19–21), nonhuman primates (22,23) and humans (24,25) and leads to donor islet allograft acceptance in nonautoimmune diabetes models (26,27). In nondiabetic NOD mice, major histocompatibility complex (MHC)-mismatched mixed chimerism has been achieved using myeloablative total body irradiation (TBI) and allogeneic plus syngeneic BMT, which prevented the development of diabetes (9,13,28). A nonmyeloablative regimen using costimulatory blockade with sublethal TBI cured diabetes by donor islet transplantation in diabetic NOD mice (28). However, nearly all hematopoiesis in these animals was donor-derived (28). Thus, it remains to be determined whether sustained mixed chimerism can be induced across MHC barriers with nonmyeloablative conditioning in diabetic NOD mice and whether, in the presence of host T-cells, cure of diabetes can be achieved with donor islet transplantation. In this study, we have addressed these questions.

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BMC, bone marrow cell; BMT, bone marrow transplantation; FCM, flow cytometric; FITC, fluorescein isothiocyanate; GVHD, graft-versus-host disease; H&E, hematoxylin and eosin; MHC, major histocompatibility complex; MST, median survival time; TBI, total body irradiation; WBC, white blood cell.

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TABLE 1
T-cell reconstitution in B6→diabetic NOD mice

Weeks post BMT	$CD4^+$ T-cells			$CD8^+$ T-cells		
	Donor*	Host†	% Host‡	Donor*	Host†	% Host‡
3	2.6 ± 0.3	21.9 ± 3.8	89.3	NEC	NEC	NEC
7	13.5	8.3	38.1	4.3	1.4	24.6
10	15.4 ± 0.9	10 ± 1.2	39.2	4.5 ± 1.0	0.8 ± 0.2	15.1
13	20.5 ± 0.3	9.4 ± 2.1	31.4	14.1 ± 1.3	3.3 ± 1.0	18.8
20	16.8	2.1	11.1	12.6	0.1	0.8
34	11.1 ± 2.6	1 ± 0.3	8.5	16.6 ± 1.6	0.2 ± 0.1	1

For 3, 10, 13, and 34 week post-BMT, data are collected from 2–8 mice. For 7 and 20 week post-BMT, data are collected from one mouse. All data are obtained from peripheral WBC, of mixed chimeric NOD mice at different times post-BMT. *Average percentage ± SEM of donor Kb^+ T-cells in WBCs; †average percentage ± SEM of host Kb^- T-cells in WBCs; ‡host Kb^- T-cells/(Donor Kb^+ T-cells + Host Kb^- T-cells × 100. NEC, not enough cells.

RESEARCH DESIGN AND METHODS

Animals. Female C57BL/6 (B6: H-2^b) and Balb/c (H-2^d) mice were purchased from Frederick Cancer Research Facility/Harley-Sprague Dawley (Frederick, MD). Female NOD (H-2^{g7}) and NOD-SCID mice were purchased from The Jackson Laboratory (Bar Harbor, ME).

Conditioning and BMT. Two weeks to 3 months following diabetes onset, diabetic NOD mice received combinations of anti-CD4 (days −6 and −1, 3.5 mg per injection of mAb GK1.5 [29]), anti-CD8 (days −1, 0, 1, 6, 7, and 8, 0.33 mg per injection of mAb 116-13-1), anti-Thy1.2 (days −6 and −1, 0.56 mg per injection of mAb 30-H12 [30]), and anti-CD40L mAb (day 0, 2 mg of mAb MR1 [31]). Some animals also received additional anti-CD8 (anti-Ly2.2) (1.4 mg of mAb 2.43 on days −6 and −1). Similar outcomes were achieved with and without 2.43 treatment, so recipients of both regimens are discussed together. On day 0, mAb-treated animals received 4 Gy TBI, as described (19). Four to 6 hours later, 3.0×10^7 B6 bone marrow cells (BMCs) were administered intravenously.

Flow cytometric analysis of chimerism. The level of allogeneic donor T- and non–T-cell reconstitution was evaluated by two-color flow cytometric (FCM) analysis on a FACScan (Becton Dickinson, Mountain View, CA) as described (32). Biotinylated or fluorescein isothiocyanate (FITC)-conjugated mAbs included anti-H-$2K^b$, anti-CD45.1, anti-CD45.2, anti-CD4, anti-CD8α, anti-GR-1, anti-CD19, anti-Mac-1, and anti-B220 (Pharmingen, San Diego, CA).

Islet transplantation. Islets were prepared from NOD-SCID or C57BL/6 mice as described (33). Six hundred to 800 C57BL/6 or 800–1,000 NOD-SCID islets were transplanted beneath the kidney capsule of diabetic NOD mice 3–5 weeks after conditioning. Before islet transplantation, diabetic NOD mice were treated with daily insulin injections or with time-release insulin pellets (Linplant, Linshin, Scarborough, Canada) implanted subcutaneously. Pellets were removed 1 day before islet transplantation. Daily monitoring of blood glucose was used to assess islet graft function. Rejection was defined as the return of hyperglycemia (>200 mg/dl on three consecutive measurements). Hyperglycemia after removal of the graft (nephrectomy) was used to confirm long-term allograft function (>100 days).

Immunohistochemical staining. Islet grafts and pancreata were fixed in 2.5% buffered formalin. Hematoxylin and eosin (H&E) and immunohistochemical staining were performed on paraffin-embedded tissue section as described (33).

Skin grafting. Tail skin grafting was performed as previously described (19). Grafts were inspected on day 7, then daily for the first month and two to three times per week thereafter. Grafts were considered rejected at the time of complete sloughing or formation of a dry scab.

Statistical analysis. Statistical significance was determined using Student's *t* test. A P value of <0.05 was considered to be statistically significant.

RESULTS

Mixed hematopoietic chimerism in NOD mice treated with nonmyeloablative conditioning. Spontaneously diabetic 17- to 21-week-old female NOD mice were conditioned with anti-CD4 (days −6 and –1), anti-CD8.1α (days −1, 0, 1, 6, 7, and 8), anti-Thy1.2 (days −6 and −1), and anti-CD40L (day 0) mAbs, and 4 Gy TBI (day 0). Transplantation of 30×10^6 allogeneic C57BL/6 BMC led to high levels of multilineage mixed chimerism (up to 20 weeks post-BMT, 40–80% of donor white blood cells [WBCs] in all lineages; data not shown) in 50 of 54 diabetic mice. At early time points, the level of donor T-cell reconstitution was different from donor reconstitution in the other hematopoietic lineages. At 3 weeks post-BMT, the majority of T-cells were NOD host CD4 T-cells, which constituted an average of 21.9% of all WBCs and 89.3% of all CD4 T-cells in peripheral blood of chimeric mice (Table 1). CD8 T-cells comprised <1% of all WBCs. By 10 weeks post-BMT (mean) 39.2% of CD4 T-cells and 15.1% of CD8 T-cells were host-derived. Additional studies revealed that the relatively low level of donor T-cell chimerism at early times reflected resistance of NOD host T-cells (especially $CD4^+$ T-cells) to depletion by the conditioning regimen (Y.T., B.N., I.L., M.S., manuscript in preparation). The majority of mice at late time points (>20 weeks post-BMT) developed increasing donor chimerism.

Animals receiving this conditioning regimen with or without B6 BMT showed no mortality, demonstrating that the conditioning regimen was nonmyeloablative and relatively nontoxic. Throughout the period of observation (>350 days post-BMT), there was no clinical evidence of GVHD in diabetic NOD recipients of B6 BMT (data not shown).

To determine whether, as in normal mice, mixed chimerism might induce tolerance to donor tissue grafts in NOD mice, mixed chimeras were grafted with donor-derived (B6) or third-party (Balb/c) skin grafts. Tolerance was demonstrated in B6→NOD mixed chimeras by specific acceptance of donor skin (median survival time [MST] >100 days) and rapid rejection of third-party skin (MST = 18 days, $n = 5$) grafted 14 weeks after BMT. Thus, mixed chimerism induced with nonmyeloablative conditioning results in donor-specific tolerance in NOD mice.

Conditioned diabetic mice destroy NOD-SCID and B6 islet grafts. To determine whether nonmyeloablative conditioning without BMT reversed autoimmunity, we performed syngeneic (NOD-SCID) islet transplantation in spontaneously diabetic mice conditioned with the above nonmyeloablative regimen. Approximately 3–5 weeks postconditioning, the mice were transplanted with NOD-SCID islets. Four of five of these conditioned diabetic animals developed recurrent autoimmunity that destroyed the islets, and hyperglycemia recurred at various rates (7–160 days following transplantation) (Fig. 1*A*). The fifth

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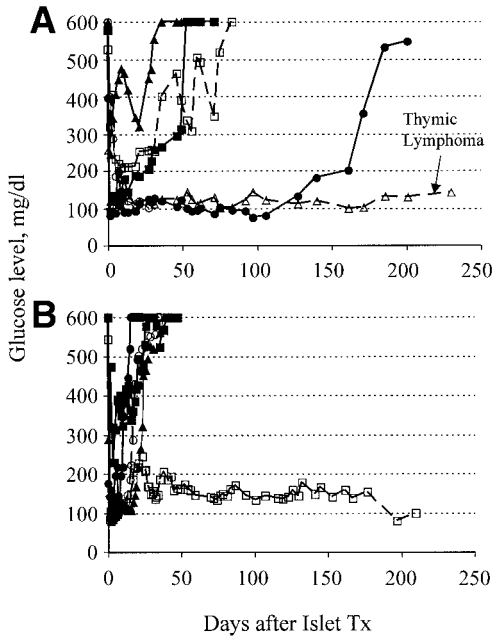

FIG. 1. Conditioned non-BMT recipients destroy syngeneic and allogeneic islet grafts. *A*: Recurrence of diabetes in diabetic NOD mice receiving nonmyeloablative conditioning and syngeneic (NOD-SCID) islet transplantation. *B*: Rejection of allogeneic (B6) islets by diabetic NOD mice receiving nonmyeloablative conditioning without BMT. Blood glucose levels were used to assess islet graft function.

animal developed an apparent lymphoproliferative disease, with evidence of a thymic lymphoma on autopsy and a heavy lymphocytic infiltrate in its islet graft (Fig. 1*A*). Although conditioning alone did not alter the immune response of diabetic NOD mice sufficiently to prevent the recurrence of autoimmune diabetes in transplanted islets, the recurrence was delayed relative to published results in untreated diabetic NOD mice (34), suggesting that the overall immune response was partially suppressed due to conditioning in some of the animals.

Another group of conditioned diabetic NOD mice received allogeneic (B6) islet grafts. Four of five animals rapidly (within 25 days) rejected allogeneic B6 islets, demonstrating that either alloresponses were intact in these mice and/or that autoimmunity affected allogeneic grafts (Fig. 1*B*). Thus, nonmyeloablative conditioning without BMT failed to induce tolerance in diabetic NOD mice toward syngeneic or allogeneic islets.

Tolerance to donor islet grafts in overtly diabetic mixed chimeric NOD mice. Another group of spontaneously diabetic NOD mice received nonmyeloablative conditioning before allogeneic C57BL/6 BMT. At the time of donor islet transplantation (4–8 weeks post-BMT), T-cells were detectable in peripheral blood of these diabetic mixed chimeras (Fig. 2*A*). Most T-cells were host NOD CD4 cells (mean 82.1 ± 11%, $n = 9$, of CD4 T-cells in peripheral WBCs at the time of islet transplantation were host-derived).

Diabetic mixed chimeras (10 of 10 in four experiments) demonstrated sustained normoglycemia after transplantation with donor islets (Fig. 2*B*). To confirm graft function in B6→diabetic NOD mixed chimeras that were normoglycemic for longer than 100 days after grafting B6 islets, nephrectomy of the kidney hosting the islet graft was performed in some animals. Graft removal resulted in hyperglycemia in all tested animals ($n = 4$), confirming that the transplanted islets had been responsible for maintaining normoglycemia (Fig. 2*B*). Thus, induction of mixed chimerism with nonmyeloablative conditioning allows acceptance of donor islet allografts and reversal of diabetes in diabetic NOD mice. This tolerance induction occurred despite the persistence of NOD T-cells in mixed chimeras.

Some mixed chimeras were sacrificed for analysis of chimerism and immunohistochemical analysis of their B6 islet grafts and pancreata at 103 ($n = 1$), 130 ($n = 2$), 230 ($n = 3$), and 316 ($n = 1$) days postislet transplantation. These mice demonstrated high levels of donor chimerism in their WBCs (46–80% donor cells), splenocytes (27–80% donor cells), and thymocytes (30–90% donor cells). A mean ± SD of 11 ± 6% of all WBCs and 22 ± 9% of splenocytes were NOD host CD4 T-cells, and 37 ± 5% of all thymocytes were host-derived.

The islet grafts were visible under the kidney capsule in five of six mice. Immunohistochemical and histological examination demonstrated a lack of lymphocyte infiltrates and the presence of healthy insulin- and glucagon-producing cells in the allogeneic grafts (Fig. 3). Various degrees of lymphocyte infiltration and the absence of insulin secretion were observed in the native NOD pancreata in 9 of 12 animals (Fig. 3). In three animals, we observed occasional periductal insulin staining, suggesting possible islet regeneration. Nevertheless, as graft removal via nephrectomy resulted in recurrence of hyperglycemia in all mice (Fig. 2*B*), significant regeneration of islets had not occurred in the native pancreata.

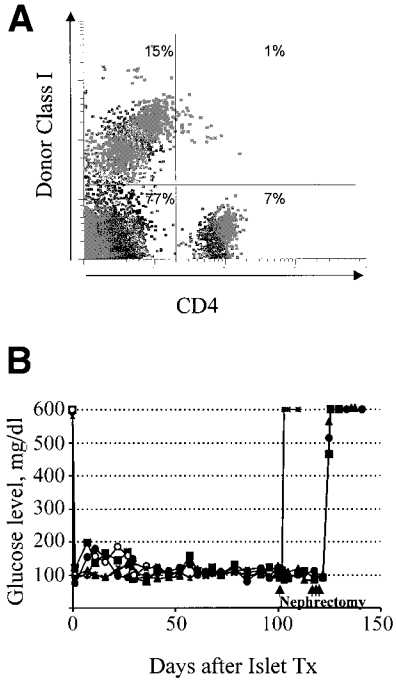

FIG. 2. Reversal of diabetes by induction of mixed allogeneic chimerism and allogeneic islet transplantation in diabetic NOD mice. *A*: Mixed chimerism among CD4 cells in WBCs of a diabetic NOD mouse at the time of B6 donor islet transplantation (day 32 post-BMT). *B*: Reversal of diabetes by induction of mixed allogeneic chimerism and allogeneic islet transplantation in diabetic NOD mice. B6 islets were transplanted beneath the kidney capsule ($n = 4$). The arrows indicate nephrectomy (B6 graft removal).

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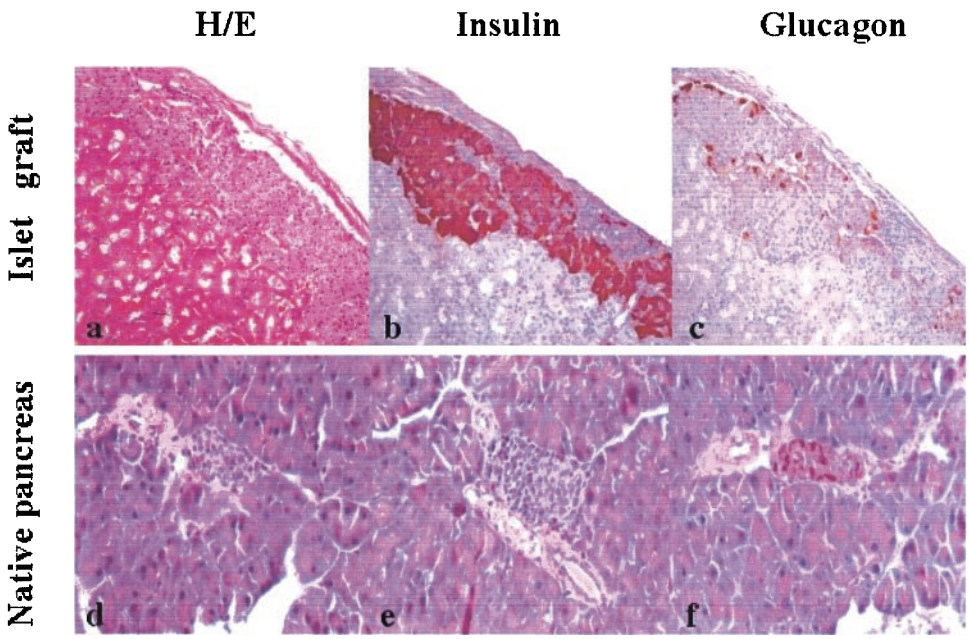

FIG. 3. Absence of infiltrates in donor islet graft 316 days postislet transplantation in mixed chimeric NOD mouse cured of diabetes by islet transplantation. *A*: H/E staining of the allogeneic islet graft (B6) implanted under the NOD kidney capsule. *B*: Insulin staining of the allogeneic islet graft. *C*: Glucagon staining of the allogeneic islet graft. *D*: H&E staining of the native pancreas. *E*: Insulin staining of the native pancreas. *F*: Glucagon staining of the native pancreas.

Reversal of autoimmunity in overtly diabetic mixed chimeric NOD mice. To determine whether induction of mixed chimerism reversed the autoimmune state in addition to inducing tolerance toward donor islet allografts, we evaluated syngeneic (NOD-SCID) islet grafts. Chimeric diabetic mice received "double" syngeneic (NOD-SCID) and allogeneic (B6) islet transplants. Syngeneic (NOD-SCID) islets were grafted under the left kidney capsule, and allogeneic (B6) islets were grafted under the right kidney capsule. This experimental design allowed us to compare histology of allogeneic donor-type and syngeneic grafts in the same chimeras and to thereby assess tolerance to the donor and autoimmunity simultaneously. Control animals received similar conditioning and islet transplantation without BMT. The conditioned control mice and mixed chimeras contained significant numbers of NOD T-cells in their peripheral blood.

Eight of 10 diabetic animals that received conditioning without BMT, followed by double islet grafting, rejected both syngeneic and allogeneic islet transplants and resumed hyperglycemia 14–107 days following transplantation (Fig. 4*A*). Scarring, without any islets (or insulin staining) ($n = 7$) or with a severe lymphocytic infiltration and acute rejection ($n = 1$), was evident upon histological evaluation of allogeneic and syngeneic grafts (not shown). One animal rejected its NOD-SCID islet graft and became hyperglycemic 1 week after right (B6 islets) nephrectomy (day 127 post–islet transplantation), and the other animal died 1 day post–right nephrectomy (B6) while still normoglycemic. Severe inflammation and lymphocytic infiltration was observed in both grafts of these animals, indicating ongoing rejection (not shown). Thus, nonmyeloablative conditioning without BMT failed to induce tolerance in diabetic NOD mice toward syngeneic or allogeneic islets. Nevertheless, the time required to reject the double amount of islet tissue varied from being quite rapid in some animals to being markedly delayed in others, presumably due to conditioning-induced immunosuppression.

In striking contrast, all mixed chimeric animals ($n = 8$) demonstrated sustained normoglycemia after double B6 and NOD-SCID islet transplantation (Fig. 4*B*). To determine whether syngeneic grafts were functioning in mixed chimeras that were normoglycemic >100 days, the right kidney containing the allogeneic (B6) islet graft was removed, and the animals were followed for 2–12 weeks after nephrectomy. Allogeneic graft removal did not lead to hyperglycemia in any of eight tested animals, demonstrating that the transplanted syngeneic (NOD-SCID) islets under the left kidney capsule were also functional (Fig. 4*B*). The allogeneic islet grafts were visible under the excised kidney capsule in all mice ($n = 8$) and confirmed by insulin/glucagon staining. Importantly, the syngeneic grafts examined at the time of sacrifice were also visible under the kidney capsule and intact islets were visible, with insulin and glucagon staining ($n = 8$). No lymphocyte infiltrates were seen in either the allogeneic or the syngeneic grafts (Fig. 4*C*). Various degrees of lymphocyte infiltration and the absence of insulin secretion were observed in the native NOD pancreata (not shown). All examined animals were mixed chimeras, with significant numbers of host NOD T-cells in their WBCs, spleens, and thymi (not shown).

Thus, induction of mixed chimerism with the nonmyeloablative regimen allows acceptance of donor islet allografts and reversal of autoimmunity in diabetic NOD mice, despite the persistence of NOD T-cells in mixed chimeras.

DISCUSSION

We demonstrate here that diabetic NOD mice in which mixed hematopoietic chimerism was established with nonmyeloablative conditioning accepted donor islets and were thereby cured of diabetes, despite significant T-cell reconstitution by the NOD recipients. Mixed hematopoietic chimerism also reversed destructive autoimmunity. Without BMT, nonmyeloablative conditioning did not induce tolerance in NOD mice to either syngeneic or allogeneic islets.

Previous studies have demonstrated that *1*) MHC-mismatched allogeneic BMT with mixed or full chimerism

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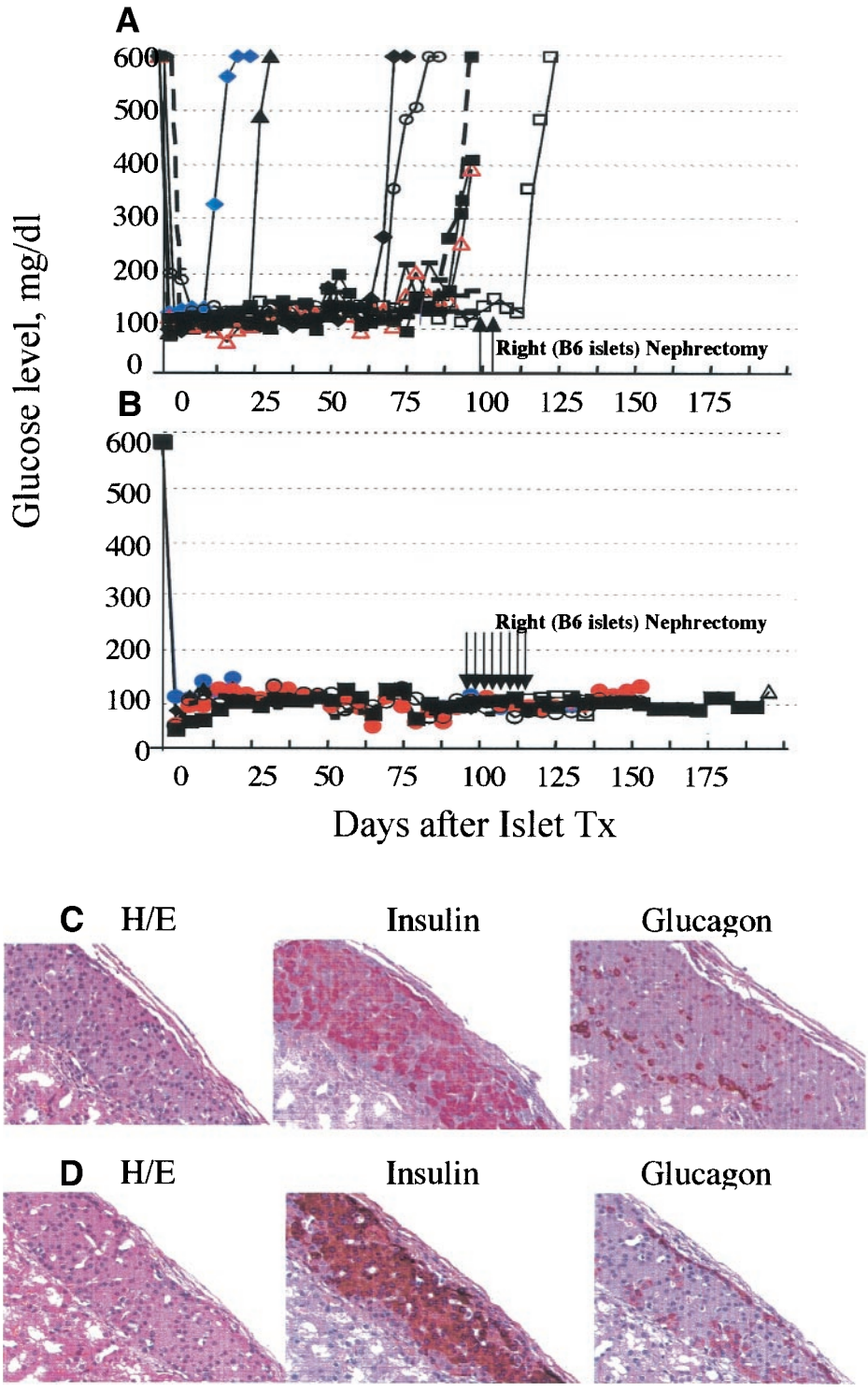

FIG. 4. Reversal of autoimmunity and induction of allotolerance. Allogeneic BMT is required for acceptance of both allogeneic donor (B6) and syngeneic (NOD-SCID) islets by diabetic NOD mice. *A*: Survival of allogeneic (B6) and syngeneic (NOD-SCID) islets in conditioned (no BMT) diabetic NOD mice (n = 10). Each line and symbol represents an individual animal. *B*: Survival of allogeneic (B6) and syngeneic (NOD-SCID) islets in mixed chimeric diabetic NOD mice (n = 8). Each line and symbol represents an individual animal. *C*: H&E staining (left), insulin staining (middle), and glucagon staining (right) of the syngeneic islet graft (NOD-SCID) implanted under the NOD kidney capsule (left kidney). *D*: H&E staining (left), insulin staining (middle), and glucagon staining (right) of the allogeneic islet graft (B6) implanted under the NOD kidney capsule (right kidney)

prevents diabetes in young NOD mice (9,13,28) and *2*) MHC-mismatched BMT leading to full chimerism in diabetic NOD mice induces tolerance toward donor islets (28,35,36). These studies utilized lethal or sublethal TBI conditioning that resulted in complete or near-complete donor T-cell repopulation (14,28,35). Thus, the resulting tolerance toward islet grafts may have reflected total T-cell replacement by the donor. We now demonstrate that diabetic NOD mice in which mixed hematopoietic (including T-cell) chimerism was established with nonmyeloablative conditioning accepted donor islets and were cured of diabetes. The persistence of NOD T-cells in mixed chimeras despite conditioning is consistent with the reported resistance of NOD CD4 T-cells to mAb depletion (37) and irradiation (38). NOD mice have also been reported to be resistant to tolerance induction via costimulatory blockade (39). The increased resistance of NOD T-cells to such treatments is likely responsible for the requirement of large amounts of antibody in our regimen. We initially evaluated several protocols utilizing various doses of mAbs, irradiation, and BMC (unpublished data), and the conditioning regimen described in this manuscript was the

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first in our hands that reliably achieved sustained mixed chimerism. However, human type 1 diabetic subjects might not have similar abnormalities, so this aspect of our regimen does not necessarily preclude application of the approach. GVHD has been avoided in our nonmyeloablative BMT models because the few donor T-cells administered are destroyed and/or inactivated by mAbs used in the conditioning regimen.

A recent study demonstrated that treatment of mice with a nonmyeloablative regimen including antilymphocyte serum, fludarabine, cyclophosphamide and rapamycin allowed MHC-matched marrow engraftment and acceptance of MHC-matched NOR islet grafts in NOD mice (34). As semiquantitative PCR assays were used to detect donor chimerism, the level of donor T-cell chimerism was not determined (34). These chimeras showed robust tolerance, as both primary and secondary donor islet grafts persisted long term (34). However, islet antigen-specific autoimmune lymphocytes may have persisted, as mononuclear cell infiltrates surrounded the donor islet grafts (34). The autoimmune T-cells might have infiltrated the donor islets without destroying them, due to the establishment of a regulatory process. A recent study demonstrated that a small number ($n = 2$) of overtly diabetic mice were cured by lethal irradiation plus anti-CD4 and anti-NK cell antibodies followed by cotransplantation of allogeneic hematopoietic stem cells and donor-matched islets (36). Although recipient T-cells were present in these animals, these may have been preexisting cells that were severely incapacitated by the high-dose conditioning treatment used. The induction of mixed chimerism with sublethal irradiation did not allow long-term islet survival in this model, suggesting that preexisting T-cells were not fully tolerized by BMT in this setting (36).

Our studies, to our knowledge, provide the first demonstration of tolerance induction to allogeneic islets in diabetic NOD mice via induction of mixed chimerism across MHC barriers using nonmyeloablative conditioning that preserves host T-cells. The induction of systemic tolerance to allogeneic donors via mixed chimerism induction with related nonmyeloablative conditioning regimens has been documented in nonautoimmune mice (19,21,40). In these models, preexisting CD4 cells present at the time of BMT are rendered tolerant to the donor by BMT in the presence of anti-CD40L (40). Thus, in addition to demonstrating that preexisting NOD CD4 T-cells do not destroy donor islets, our studies demonstrate that NOD CD4 T-cells are susceptible to tolerance induction to alloantigens with an anti-CD40L–containing regimen.

The complete absence of isletitis in donor islets >300 days following implantation to diabetic mixed allogeneic chimeras, despite the presence of recipient T-cells that are capable of destroying syngeneic islets in conditioned (non-BMT) controls, is consistent with two hypotheses: First, the extensive MHC disparity between donor (BMT) and recipient might render donor islets resistant to destruction by autoreactive T-cells. While the donors and recipients in our studies shared the class I MHC allele D^b, Makhlouf et al. (33) showed a greater role for class II MHC than class I sharing in rendering islet allografts susceptible to autoimmune destruction. Second, in addition to being tolerant to donor alloantigens, mixed chimerism may reverse the autoimmune state. Our data indicate that destructive autoimmunity to islet grafts is indeed reversed in mixed chimeras. This is indicated by the lack of infiltrates and the intact insulin production by syngeneic NOD islets grafted to mixed chimeras, despite the fact that recipient T-cells were not fully depleted by the conditioning protocol. Although it led to delayed isograft rejection in some animals, conditioning alone did not reverse the destructive autoimmunity, ruling out lymphoablation as the cause of its reversal in mixed chimeras.

When donor islets were transplanted (3–5 weeks post-BMT), many of the T-cells present in mixed chimeras were residual nondepleted NOD CD4 T-cells. At later times, these residual T-cells were presumably diluted by new tolerant donor and host-derived T-cells produced in the chimeric host thymus. Despite a gradual increase in donor T-cell chimerism, we detected a significant number of host CD4 T-cells in the WBCs, spleens, and thymi of all long-term mixed chimeric tolerant animals. In view of the long-lasting mixed chimerism detected among thymocytes, recipient T-cells probably also included T-cells that developed de novo following BMT. We have previously shown that preexisting peripheral alloreactive CD4 T-cells are rendered tolerant by a deletional mechanism in mice receiving allogeneic BMT with anti-CD40L, and that the establishment of mixed chimerism with this approach also tolerizes newly developing thymocytes via deletion (21,41). Similar mechanisms are likely to explain the tolerance to alloantigens demonstrated here in mixed chimeric NOD mice. Additional mechanisms must be implicated to explain the tolerance to autoantigens induced among NOD T-cells, including both preexisting peripheral T-cells and those developing in the thymus following establishment of mixed chimerism. Several mechanisms of protection from diabetes by "protective" MHC class II molecules have been described, including intrathymic deletion of diabetogenic T-cells, induction of peripheral anergy, a change in the Th1/Th2 profile of diabetogenic T-cells, and positive selection in the thymus of regulatory T-cells (42–45). An "active" mechanism, such as that mediated by regulatory T-cells, or peripheral deletion induced by donor MHC-expressing cells plus anti-CD40L, as described (21), might induce tolerance in preexisting NOD CD4 cells. Further studies are needed to elucidate the mechanisms of tolerance of NOD T-cells to autoantigens and alloantigens in these mice.

In summary, our studies show that nonmyeloablative BMT using anti-CD40L has the potential to cure type 1 diabetes. Engraftment of allogeneic hematopoietic stem cells in nonmyeloablated recipients tolerizes host and donor T-cells to donor-derived tissue, including pancreatic islets. Reversal of autoimmunity also results from induction of mixed chimerism and overcomes the obstacle of recurrent autoimmunity that has limited success in other islet transplantation protocols. These data also suggest that HLA sharing between donor and recipient might not be deleterious with respect to donor islet survival in the context of mixed chimerism. The extension of these approaches from mice to large animal models and humans will support the development of conditioning regimens that will be acceptable for use in diabetic patients in need of islet and possibly kidney transplantation as well.

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