Long-Term Islet Graft Survival in NOD Mice by Abrogation of Recurrent Autoimmunity

Qixin Shi, Donghua Wang, Gregg A. Hadley, Adam W. Bingaman, Stephen T. Bartlett, and Donna L. Farber

Islet transplantation has great potential for curing type 1 diabetes; however, long-term islet survival using conventional immunosuppression remains elusive. We present a novel strategy for inducing long-lasting islet graft survival in diabetic NOD mice in the absence of post-transplant immunosuppression by initial treatment with antilymphocyte serum (ALS) followed by coadministration of donor pancreatic lymph node cells (PLNCs). When treated with ALS/PLNC, diabetic NOD mice become normoglycemic and tolerated minor antigen-disparate islet grafts for >100 days and syngeneic islet grafts indefinitely. Donor T-cells are required for graft prolongation, and tolerant hosts have long-term donor T-cell chimerism. Strikingly, host autoreactive T-cells from mice with long-surviving islet grafts predominantly produce interleukin-4, whereas autoreactive T-cells from mice that rejected their islet grafts predominantly produce interferon-γ. We thus demonstrate a clinically relevant approach for ablation of recurrent autoimmunity in islet transplantation, involving donor lymphocyte-driven alteration of pathogenic autoreactive T-cells. *Diabetes* 53:2338–2345, 2004

Type 1 diabetes is a T-cell–mediated autoimmune disease that causes the destruction of pancreatic β-cells, leading to complete insulin dependence in affected individuals. Islet transplantation has great potential to cure diabetes by restoring both β-cell mass and endogenous glycemic control. Although long-term survival of solid organ transplants such as kidneys (>10 years) and pancreata (>5 years) is now routinely accomplished in 55 and 70% of recipients, respectively (data from U.S. Organ Procurement and Transplantation Network/Scientific Registry of Transplant Recipients), the long-term results for islet transplantation have not been as promising. The Edmonton corticosteroid-free immunosuppression protocol for transplantation of pancreatic islets into patients with type 1 diabetes has resulted in insulin independence for 80% of patients in Edmonton after the first year (1,2), with variable success rates of 23–90% in other centers (3,4). Maintaining long-term islet function has proved more elusive; of the six Edmonton recipients with measurable islet graft function after 4 years, four have experienced partial graft loss and only two remain insulin free (J. Shapiro, personal communication). Whether this loss of islet function is due to cellular attrition, autoimmunity, and/or chronic rejection is not known; however, the future success of islet transplantation in curing type 1 diabetes critically depends on the design of alternative, clinically relevant strategies for achieving long-term islet graft survival.

The NOD mouse model for type 1 diabetes has been invaluable in understanding the pathogenesis of autoimmune diabetes. The Edmonton protocol has been used to promote islet graft survival in NOD mice with mean survival times of 34–90 days (5,6), establishing the utility and clinical relevance of using the NOD mouse to develop new strategies for promoting islet graft survival. Thus far, numerous approaches for rendering the immune system tolerant toward organ allografts have proved ineffective in promoting islet graft survival in NOD mice (7). For example, in vivo costimulation blockade with or without donor-specific transfusion promotes long-term acceptance of cardiac, skin, and islet allografts (in chemically induced diabetic mice) (8–11), yet these same strategies do not promote acceptance of syngeneic or allogeneic islet grafts in spontaneously diabetic NOD mice (12–14).

One distinguishing feature of syngeneic islet graft rejection in diabetic NOD mice is its rapid kinetics (15), characteristic of a second-set rejection response mediated by primed effector and/or memory T-cells (16,17). This phenomenon, known as recurrent autoimmunity, is a major barrier to islet graft survival in NOD mice for major histocompatibility complex (MHC)-matched islets (14) and is also associated with graft loss of allogeneic islets in patients with type 1 diabetes (1). Numerous strategies that prevent primary disease in NOD mice are wholly ineffective against recurrent disease (18), suggesting that the presence of preprimed autoreactive T-cells in diabetic individuals may evade and/or hamper the success of known immunosuppression and tolerance induction strategies. Therefore, functional tolerization and/or modulation

From the Division of Transplantation, Department of Surgery, University of Maryland School of Medicine, Baltimore, Maryland.

Address correspondence and reprint requests to Stephen T. Bartlett, MD, Division of Transplantation, Department of Surgery, University of Maryland School of Medicine, 29 South Greene St., Baltimore, MD 21201. E-mail: sbartlett@smail.umaryland.edu.

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Additional information for this article can be found in an online appendix at http://diabetes.diabetesjournals.org.

ALS, antilymphocyte serum; APC, allophycocyanin-conjugated streptavidin; ELISPOT, enzyme-linked immunospot; IFN, interferon; IL, interleukin; mHA, minor histocompatibility antigen; MHC, major histocompatibility complex; PLNC, pancreatic lymph node cell.

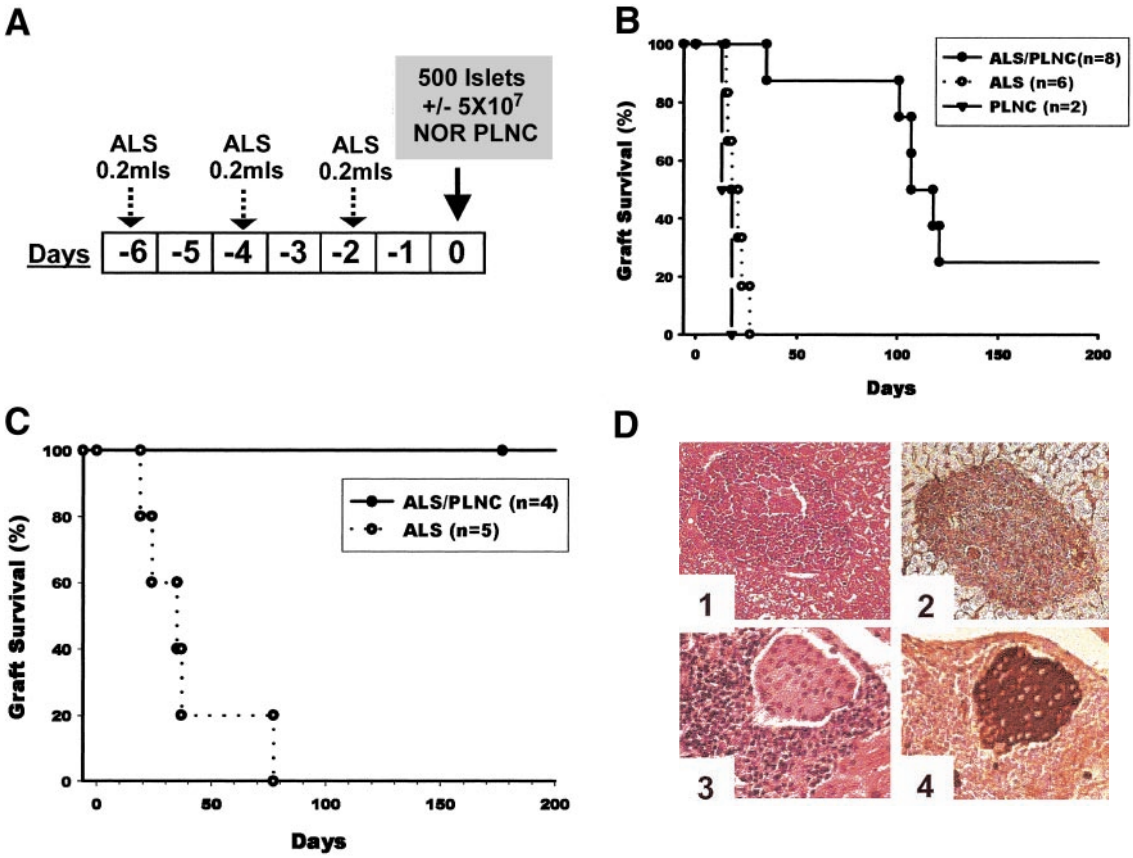

FIG. 1. ALS/PLNC treatment promotes long-term islet graft survival in diabetic NOD mice. *A*: Protocol for treatment of diabetic NOD mice with ALS before administration of pancreatic islets with or without NOR PLNCs. *B*: Islet graft survival in mice that received NOR islets plus ALS and PLNCs (ALS/PLNC) as in *panel A*, ALS alone (ALS), or PLNC without prior ALS treatment (PLNC). Mean survival time for each individual group was 130.5 ± 66.9 days for ALS/PLNC (n = 8), 20 ± 4.56 days for ALS (n = 6), and 15.5 ± 3.5 days for PLNC (n = 2). *C*: Syngeneic islet graft survival in diabetic NOD mice treated with ALS/PLNC or ALS alone. Mean survival times were >226.8 ± 47.9 days for ALS/PLNC (n = 4) and 38.4 ± 22.8 days for ALS (n = 5). *D*: Islet graft histology from liver sections of ALS-treated diabetic NOD mice that received NOR islets 18 days previously (*panels 1* and *2*, 200× magnification) or that received NOR islets and NOR PLNCs 61 days previously (*panels 3* and *4*, 400× magnification). Panels represent hematoxylin and eosin (*panels 1* and *3*) or anti-insulin (*2* and *4*) staining.

of the primed autoreactive T-cell pool has great potential to ameliorate islet graft survival in vivo.

We have developed a clinically relevant model of islet transplantation in NOD mice through portal vein injection (15). Here, we demonstrate long-term survival (>130 days) of minor antigen-disparate and permanent survival (>250 days) of syngeneic pancreatic islet grafts into actively diabetic NOD mice by treatment of these hosts with antilymphocyte serum (ALS) and donor pancreatic lymph node cells (PLNCs) from diabetes-resistant NOR mice. In this model, host NOD T-cells are replenished, and donor T-cells persist with >10% donor T-cell chimerism. Most strikingly, this treatment results in a switch in cytokine profile of the host autoreactive GAD65- and insulin-specific T-cells from predominantly producing interferon (IFN)-γ to a preponderance of interleukin (IL)-4 producers. Our results present a clinically relevant model for enabling islet graft acceptance without posttransplant immunosuppression by alteration of the resident host autoreactive T-cells.

RESEARCH DESIGN AND METHODS

Female NOD/LtJ, NOR/LtJ, NOD.NON-Thy1^a/J (NOD.Thy1^a), and NOD.scid mice were purchased from The Jackson Laboratory (Bar Harbor, ME), and NOD.Thy1^a X NOR F1 mice were bred and maintained under specific pathogen-free conditions in the animal facility at the University of Maryland School of Medicine (Baltimore, MD). Insulin-dependent diabetes (type 1 diabetes) develops in 71% of female NOD mice by 24 weeks of age in our colony.

Antibodies and reagents. The following antibodies were purified from hybridoma culture supernatants: RL172.4 (anti-CD4), 3.115 (anti-CD8), 212.A1 (anti-IA^d), J11D (anti-CD24), M1.70.15 (anti-CD11b), and RA3–3A1/6.1(anti-B220). Fluorochrome-conjugated monoclonal antibodies to CD3ε (145-2C11), CD4 (GK1.5), CD8 (53-6.7), CD25 (7D4), CD44 (IM7), CD45RB (16A), biotinylated anti-Thy1.1 (OX-7) and anti-CD11c (clone HL3), and allophycocyanin-conjugated streptavidin (APC) were purchased from BD PharMingen (San Diego, CA). Rabbit anti-mouse lymphocyte serum (ALS) and rabbit complement were obtained from Accurate Chemical & Scientific (Westbury, NY). GAD and insulin peptides were synthesized by the Biopolymer facility (University of Maryland, Baltimore).

Pancreatic islet transplantation. Diabetic NOD female mice with blood glucose levels >500 mg/dl for 2 consecutive days were used as recipients for islet transplantation and treated three times with 0.2 ml ALS (+1 unit insulin for maintenance) 6 days before islet transplantation (Fig. 1*A*). Pancreatic islets were isolated as previously described (15,19) from NOR females (8–10 weeks old) or pre-diabetic female NOD mice (3–4 weeks old). Freshly isolated islets (n = 500) were injected into the portal vein of each diabetic NOD recipient as described (15), with or without 50 × 10^6 NOR or NOD.Thy1^a X NOR F1 PLNC prepared by isolating peripancreatic and mesenteric lymph node cells, crushing the tissue between glass slides, straining the cell suspension through a Falcon 100-μm mesh filter, and resuspending in PBS. Transplantation was considered successful if the nonfasting blood glucose concentration returned to normal (<200 mg/dl) within 24 h after surgery. Blood glucose was subsequently monitored thrice weekly with a Glucometer Elite instrument (Bayer, Pittsburgh, Pennsylvania), and islet grafts were designated as rejected when the blood glucose exceeded 250 mg/dl for 2 consecutive days.

T-cell depletion, purification, and adoptive transfers. For T-cell depletion studies, NOR splenocytes were treated with anti-CD4 (RL172.) alone or

together with anti-CD8 (3.155) plus complement, resulting in >99% depletion before coadministration with NOR islets by portal vein injection. NOR CD4 T-cells (98% pure) were isolated from PLNCs by negative selection using the autoMACS and CD4 T-cell isolation kit (Miltenyi Biotec, Auburn, CA) plus the addition of anti-CD11c in the depletion cocktail. For adoptive transfers, splenocytes (10^7) from spontaneously diabetic or ALS/PLNC-treated NOD mice with long-surviving islets were transferred intravenously into NOD.scid recipient mice, and blood glucose was measured to monitor diabetes occurrence.

Flow cytometric analysis. For phenotypic monitoring of donor and host T-cells in transplant recipients, peripheral blood was collected from orbital plexus and lymphocytes were counterstained with biotinylated anti-Thy1.1/streptavidin-APC and fluorescein isothiocyanate–, phycoerythrin, and peridinin chlorophyll protein–conjugated antibodies and were analyzed by four-color flow cytometry using the FACSCalibur and CellQuest software (Becton Dickinson, San Jose, CA).

Immunohistochemistry. Graft-bearing livers were fixed in 10% buffered formalin and embedded in paraffin, and 4- to 6-μm sections were stained with hematoxylin and eosin or with anti-insulin (Dako, Carpinteria, CA) followed by biotinylated rabbit anti–guinea pig (Zymed, South San Francisco, CA), streptavidin–horseradish peroxidase system (Zymed), and developed with aminoethylcarbazole.

Enzyme-linked immunospot analysis. Splenic CD4 T-cells (2.0×10^5 cells/well) isolated from islet transplant recipient mice were plated in triplicate with NOR splenic APC (4.0×10^5 cells/well) prepared as described (20) with or without the peptides corresponding to GAD65(524-543), GAD65(539-558), or insulin(15-23) at 25 μg/ml on immunospot plates precoated with anti–IFN-γ or anti–IL-4 antibodies. Plates were incubated for 24 h (IFN-γ) or 48 h (IL-4) at 37°C, and biotinylated anti–IFN-γ (XMG1.2, BD PharMingen) or anti–IL-4 (BVD6–24G2, BD PharMingen) was added overnight at 4°C, followed by streptavidin-horseradish peroxidase (BD PharMingen), and developed with 3-amino-9-ethyl-carbazole (Sigma, St. Louis, MO). Spots were counted with an ImmunoSpot Series 1 analyzer (Cellular Technology Limited, Cleveland, OH).

RESULTS

Long-term islet graft survival using ALS/PLNC treatment. We previously reported the establishment of a clinically relevant model for islet transplantation into NOD mice in which intraportally administered islets from diabetes-resistant NOR mice were rapidly rejected by NOD recipients 7–10 days posttransplantation (15). To develop a strategy to prolong islet graft survival in NOD mice, we drew from our previous experience with the lymphopenic BB rat model for diabetes, in which coadministration of PLNCs with islets prolonged graft survival (21). In the NOD mouse, however, we found that cotransfer of NOR PLNCs with NOR islets did not prolong graft survival (Fig. 1*B*), and we reasoned that it may be necessary to induce a transient lymphopenic state in NOD mice, similar to the BB rat. We thus optimized a protocol in which NOD mice were rendered lymphopenic by the administration of three consecutive doses of ALS. This treatment regimen resulted in an immediate reduction of peripheral blood T-cells, followed by their replenishment after 2–4 weeks (online appendix Fig. 1 [available at http://diabetes.diabetesjournals.org]) and was associated with low-to-undetectable levels of residual circulating anti-lymphocyte antibody present 2–4 days post-ALS treatment (online appendix Fig. 2).

To determine whether the administration of donor PLNCs into ALS-treated diabetic NOD hosts resulted in long-term islet graft survival, we transplanted ALS-treated NOD mice intraportally with islets derived from diabetes-resistant, MHC-matched NOR mice that are mismatched for multiple minor alloantigens (minor histocompatibility antigens [mHAs]) (22), with or without NOR PLNCs (Fig. 1*A*), and monitored graft survival over time by measuring blood glucose. While ALS treatment of NOD hosts before islet transplantation resulted in mean islet survival of 20 days (Fig. 1*B*), representing a modest prolongation of the 7-day mean graft survival time in non-ALS–treated mice (15), coadministration of NOR PLNCs resulted in remarkable long-term survival (mean survival time: 130 days) of mHA-disparate islets, with several of the mice (20%) enjoying indefinite survival of islet grafts beyond 200 days (Fig. 1*B*). These results demonstrate that coadministration of PLNCs to ALS-treated NOD mice results in islet graft acceptance in the absence of posttransplant immunosuppression.

To determine whether the ALS/PLNC treatment was also effective in prolonging survival of syngeneic islets, we transplanted islets derived from pre-diabetic NOD mice into ALS-treated diabetic NOD hosts with or without NOR PLNCs. Strikingly, we observed permanent acceptance of syngeneic islet grafts in NOD hosts using ALS/PLNC treatment (Fig. 1*C*). Interestingly, syngeneic islet graft survival was prolonged compared with mHA-disparate islet survival in NOD mice treated with ALS alone, consistent with an earlier report (23). These data demonstrate that treatment with ALS/PLNC abrogates recurrent autoimmune destruction of syngeneic islets and is also highly effective with mHA-disparate islet grafts.

We examined the integrity of these long-surviving islets in liver sections by histology (Fig. 1*D*). The ALS-treated mice that received islets alone mediated rapid rejection characterized by massive mononuclear infiltrates (Fig. 1*D*, *panel 1*); however, ALS/PLNC-treated mice had intact islets without lymphocytic infiltrate into the islet space (*panel 3*), although peri-islet mononuclear cells were observed in long-surviving islet graft recipients. Insulin staining revealed confluent insulin production from intact islets in ALS/PLNC-treated, but not in ALS-treated, mice (Fig. 1*D*, *panels 2* and *4*). These results demonstrate a lack of islet destruction in ALS/PLNC-treated NOD recipients of mHA-disparate islets, consistent with the long-term maintenance of normoglycemia in these mice (Fig. 1*B*).

ALS/PLNC treatment induces islet tolerance. To establish whether the ALS/PLNC-treated NOD mice were rendered tolerant to islet antigens compared with diabetic NOD mice, we asked whether splenocytes from ALS/PLNS-treated NOD mice with long-surviving islet grafts could induce diabetes when transferred into nondiabetic NOD.scid hosts lacking endogenous lymphocytes. Transfer of splenocytes from diabetic NOD mice into NOD.scid hosts resulted in diabetes occurrence by 20–24 days (Fig. 2*A*), consistent other reports (24). By contrast, transfer of splenocytes from ALS/PLNC-treated mice with long-surviving islet allografts into NOD.scid hosts did not lead to diabetes occurrence for up to 70 days posttransfer (Fig. 2). Between 75 and 80 days, several of the NOD.scid recipients developed diabetes (Fig. 2), indicating the presence of diabetogenic precursor cells in the ALS/PLNC recipient. These results demonstrate that the capacity to mediate autoimmune destruction of islets was dramatically curtailed in ALS/PLNC-treated NOD mice that tolerated their islet grafts.

Donor CD4 T-cells are required for inducing long-term islet graft survival. We asked whether the presence of T-cells within the donor lymphocyte infusion was required for long-term graft survival. Because these experiments require high cell numbers, we used splenocytes as a source of donor cells and compared islet survival in ALS-treated mice that received whole donor splenocytes or splenocytes in which total T-cells or CD4 T-cells were

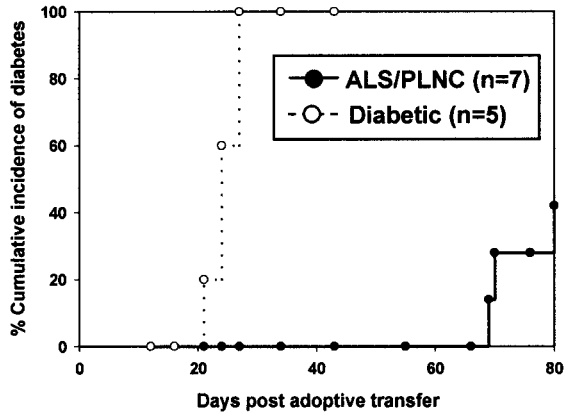

FIG. 2. ALS/PLNC treatment induces islet cell tolerance. Equivalent numbers of splenocytes from spontaneously diabetic NOD mice or ALS/PLNC-treated NOD recipients of long-surviving islet allografts were transferred intravenously into NOD.scid mice. Mean time for diabetes onset was 24 ± 1 days for recipients of diabetic NOD splenocytes (n = 5) and 108 ± 16 days for recipients of splenocytes from ALS/PLNC-treated recipients of long-surviving NOR islet grafts (n = 7).

depleted before coadministration with islets. We found that coadministration of total NOR splenocytes also resulted in impressive prolongation of islet graft survival to >75 days (Fig. 3*A*), comparable with that observed with NOR PLNCs (Fig. 1*A*). However, when either total T-cells or CD4 T-cells were removed from the donor cell innoculum, islet graft survival was reduced to <20 days (Fig. 3*A*), similar to survival of islets in mice treated with ALS alone (Fig. 1*B*). These results suggested that the presence of donor T-cells, particularly the CD4 T-cell subset, was required for inducing long-term islet graft survival following ALS treatment.

To establish whether donor CD4 T-cells were sufficient to establish long-term islet graft survival in the absence of CD8 T-cells and dendritic cells, we rigorously purified CD4 T-cells from NOR PLNCs and assayed their ability to induce long-term islet graft survival in ALS-treated diabetic NOD hosts. The donor CD4 T-cells were >98% pure and had no contaminating CD8 T-cells or CD11b- or CD11c-expressing monocytic or dendritic cells (Fig. 3*B*, *top*). When coadministered with NOR islets to ALS-treated diabetic NOD recipients, these PLNC-derived CD4 T-cells induced long-term normoglycemia (Fig. 3*B*, *bottom*), establishing that donor CD4 T-cells are necessary and sufficient for inducing long-term, mHA-disparate islet graft survival in NOD mice.

Establishment and persistence of donor T-cell chimerism. Because donor T-cells were required to induce long-term islet graft survival, and donor cell chimerism is known to play a role in tolerance induction in other transplant models (25), we modified our in vivo system to analyze donor and host T-cells by transferring PLNCs isolated from (NOR × NOD.Thy1^a)F1(Thy1.1$^+$) mice into ALS-treated diabetic NOD (Thy1.1$^-$) recipient mice. In these hosts, we consistently found a high level of donor T-cell chimerism, ranging from 2 to 20%, that peaked between 30 and 50 days posttransplant and gradually declined from 50 to 200 days posttransplant, although the mice remained normoglycemic (Fig. 4*A* and data not shown). These results demonstrate that ALS treatment can provide space for long-term persistence of donor peripheral T-cells.

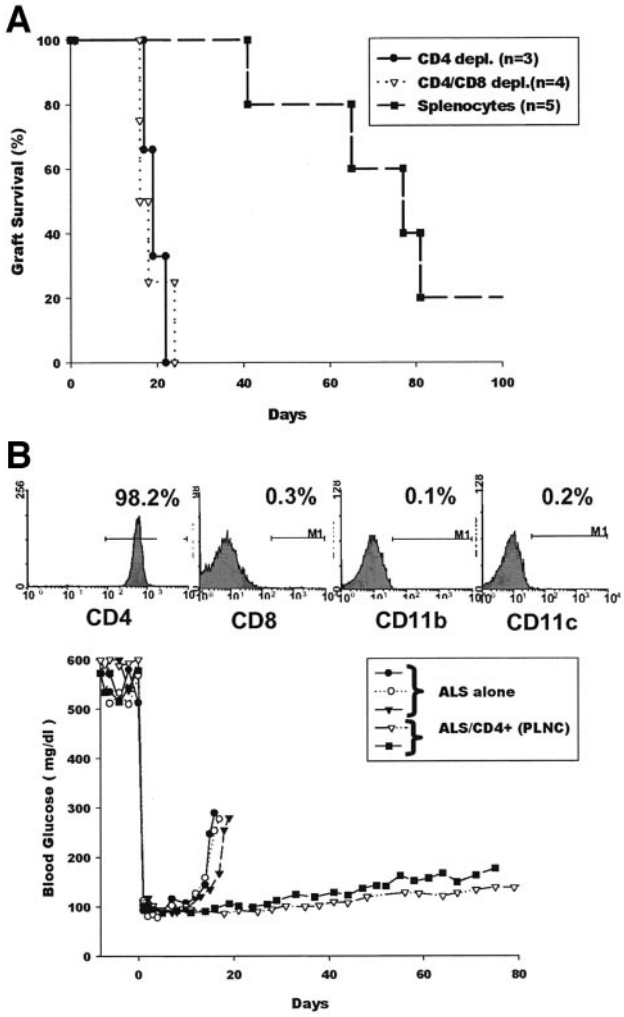

FIG. 3. CD4 T-cells are necessary and sufficient for inducing long-term islet graft survival in ALS-treated NOD mice. *A*: Islet graft survival of diabetic ALS-treated NOD mice that received NOR islets plus 5 × 10^7 NOR splenocytes (n = 7), splenocytes depleted of total T-cells ([CD4/CD8-depl.], n = 4), or splenocytes or PLNCs depleted of CD4 T-cells ([CD4-depl.], n = 3). Mean graft survival times were 73.8 ± 23.4 days (n = 5) for splenocytes alone, 18.5 ± 3.8 (n = 4) for CD4/CD8-depleted splenocytes, and 19.5 ± 3.5 (n = 3) for CD4-depleted splenocytes or PLNCs. *B*: PLNC-derived CD4 T-cells induce normoglycemia in ALS-treated NOD recipients of NOR islet grafts. *Top*: CD4, CD8, CD11b, and CD11c expression profile of purified PLNC CD4 T-cells. *Bottom*: Blood glucose levels for individual mice before and after islet transplantation into diabetic mice treated with ALS (n = 3) or with ALS and 5 × 10^7 PLNC-derived CD4 T-cells (n = 2).

We asked whether the donor and/or host T-cells were undergoing dynamic changes in their activation or differentiation state during the persistence of islet grafts in these mice. To address this question, we analyzed the host and donor-cell–specific expression of T-cell activation and differentiation markers, such as CD45RB and CD25, over time in chimeric mice with long-surviving islet grafts. We found that before transfer, the donor T-cells exhibited a predominant naive phenotype (CD25lo/CD45RBhi/CD44lo) with 30% CD45RBlo cells, indicative of activated or memory T-cells (Fig. 4*B* and data not shown). In the presence of a long-surviving islet graft, the proportion of donor-derived memory or activated (CD45RBlo/CD25$^+$) phenotype cells decreased over time, with a corresponding increase in naive-phenotype cells, so that by day 90, donor T-cells were primarily CD45RBhi/CD25$^-$ (Fig. 4*B*). These results

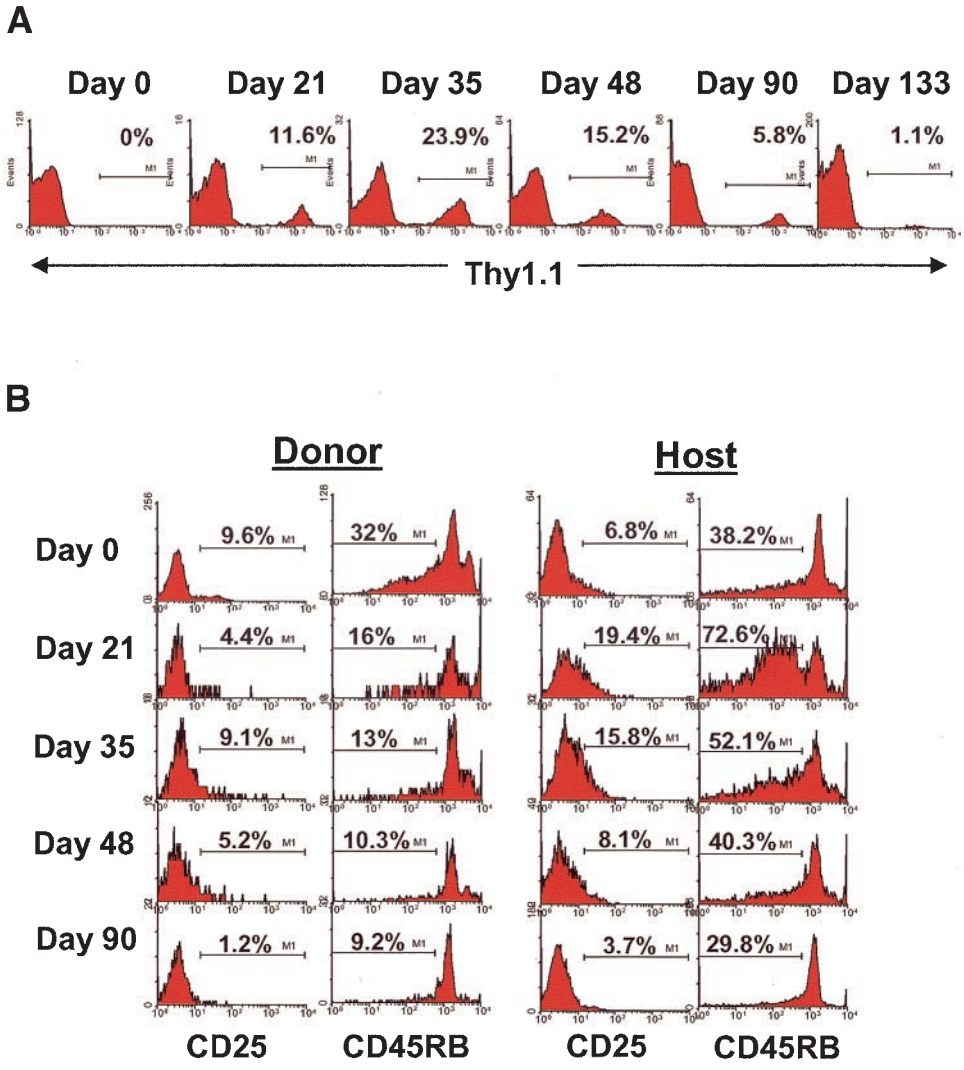

FIG. 4. Donor T-cell chimerism and donor and host T-cell phenotype in ALS/PLNC-treated graft recipients. *A*: Donor T-cell chimerism as measured by the percentage of Thy1.1$^+$ cells in NOD recipients of long-surviving islets that were administered ALS and PLNCs derived from (NOR × NOD.Thy1^a)F1 mice. Results are representative of six chimeric mice monitored over time. *B*: Cell surface phenotype of donor (Thy1.1$^+$) and host (Thy1.2$^+$) T-cells showing expression of CD25 and CD45RB for gated Thy1.1$^+$ or Thy1.2$^+$ CD4$^+$ T-cells from peripheral blood. Representative of three mice analyzed.

indicate a loss of donor-derived activated or memory T-cells during their persistence in NOD hosts.

In contrast to the loss of the activated/memory subset in the donor T-cell pool, there was a striking yet transient increase in the proportion of host-derived, memory-phenotype T-cells. Before ALS/PLNC treatment, the host T-cells comprised 62% naive-phenotype (CD45RBhi) and 38% memory-phenotype (CD45RBlo/CD44hi) T-cells (Fig. 4*B* and data not shown). Following ALS/PLNC treatment and islet transplantation, there was a dramatic increase in the proportion of memory-phenotype host T-cells (72% CD45RBlo) that was apparent 3–5 weeks posttransplantation and decreased to the starting composition (<40%) by 50–90 days posttransplantation (Fig. 4*B*). This transient upregulation of activation/memory-phenotype T-cells is a hallmark of T-cells undergoing homeostatic expansion in lymphopenic hosts (26–28) and correlates with the kinetics of host T-cell replenishment after ALS administration (online appendix Fig. 1).

Islet-reactive host T-cells in ALS/PLNC-treated mice display altered cytokine production. Although host T-cells in both ALS- and ALS/PLNC-treated NOD mice were replenished, islet graft rejection did not occur in ALS/PLNC-treated mice. We hypothesized that ALS/PLNC treatment either subverted the return of autoreactive T-cells after ALS treatment and/or altered the autoreactive T-cell compartment, rendering it nonpathogenic. We used enzyme-linked immunospot (ELISPOT) to quantitatively evaluate the proportion of islet-reactive T-cells in ALS-treated NOD mice that rejected their islets compared with ALS/PLNC-treated mice with long-surviving islet allografts. For islet reactivity, we monitored T-cell responses to the islet autoantigens GAD65 (29) and insulin (30) and analyzed both IFN-γ and IL-4 production. Consistent with previous reports on the cytokine profile of islet-reactive T-cells in diabetic NOD mice (31), T-cells from ALS-treated mice produced primarily IFN-γ, with low amounts of IL-4 in response to islet-specific antigens. By contrast, GAD65- and insulin-reactive T-cells derived from ALS/PLNC-treated islet graft recipients produced predominantly IL-4, with lower numbers of autoreactive IFN-γ producers (Fig. 5*A*). This cytokine shift in ALS versus ALS/PLNC recipient mice was highly reproducible (Table 1) and is reflected in a change in the ratio of autoantigen-specific IFN-γ to IL-4 producers from >2 in ALS-treated NOD recipients that rejected their islet grafts to <0.5 from ALS/PLNC-treated mice that tolerated their islet grafts (Table 1). These results demonstrate a shift from IFN-γ to IL-4 predominance in ALS/PLNC-treated NOD recipient mice rendered tolerant of their islet grafts.

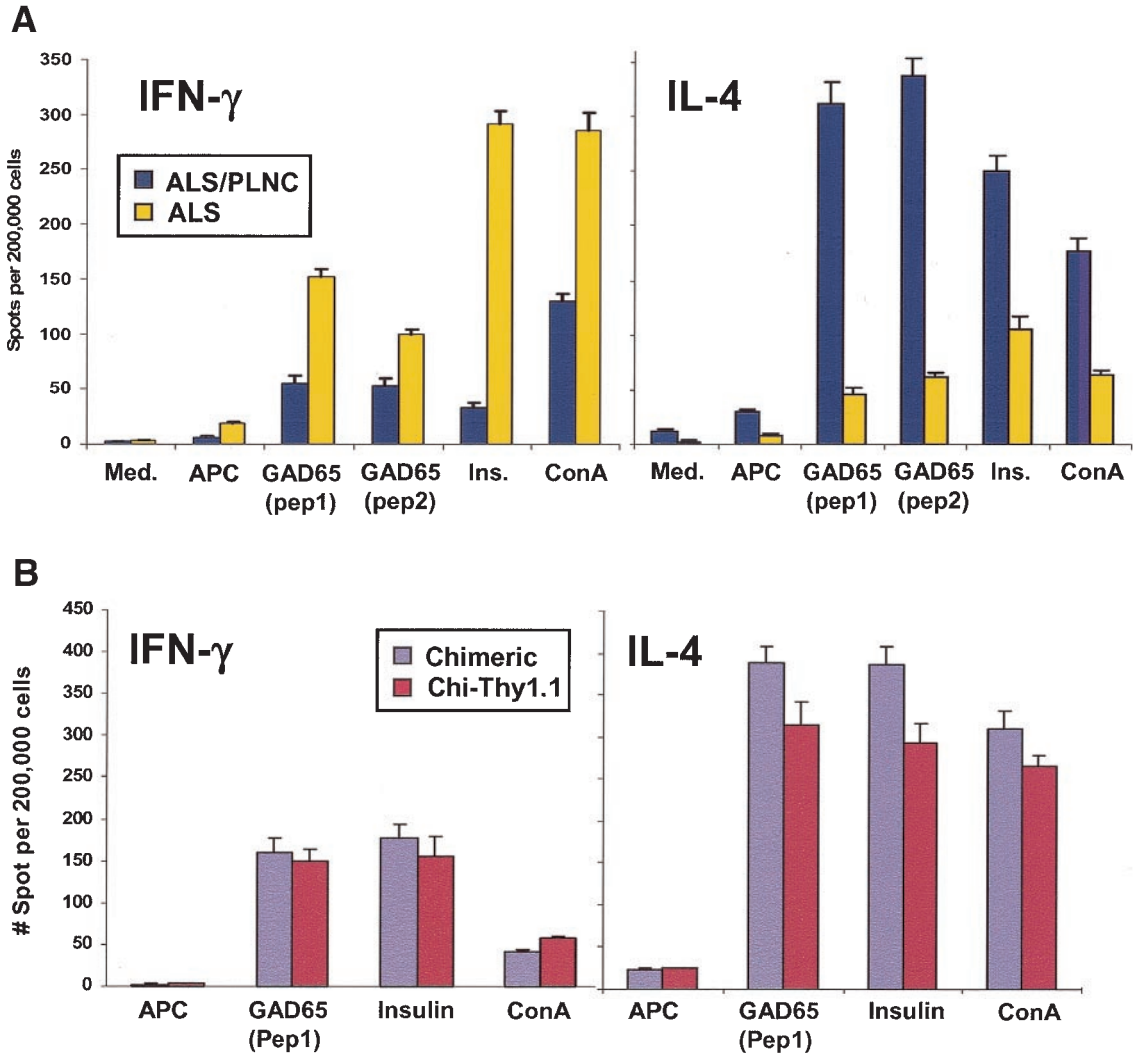

FIG. 5. Altered cytokine profile of islet-reactive T-cells in ALS/PLNC-treated islet graft recipients. *A*: Cytokine profile of islet autoantigen-specific CD4 T-cells derived from ALS/PLNC-treated NOD mice with long-surviving grafts versus ALS-treated NOD mice that rejected their islet grafts. Splenic CD4 T-cells were cultured with NOR APC with or without GAD65- or insulin-specific peptides, and IFN-γ and IL-4 production was monitored by ELISPOT (see RESEARCH DESIGN AND METHODS). Results are presented as the mean ± SD of triplicate wells, and results are representative of data obtained from five mice with long-surviving grafts and four mice that rejected their islets. GAD65 (pep1), residues 524–543; GAD65(pep2), residues 539–558; ins, insulin residues 15–23. *B*: CD4 T-cells from ALS/PLNC-treated NOD mice with long-surviving islets that were chimeric for Thy1.1 PLNCs were either treated with complement alone (Chimeric) or anti-Thy1.1 and complement to deplete donor-derived T-cells (Chi-Thy1.1), and ELISPOT analysis of IFN-γ and IL-4 was performed as above. Results are the average of triplicate wells and representative of two experiments.

We asked whether this shift in autoantigen-specific cytokine profile in ALS/PLNC NOD recipients was due to the endogenous host NOD T-cells or the donor NOR lymphocytes. To address this question, we performed ELISPOT analysis on purified T-cells derived from ALS/PLNC recipients of islet transplants containing Thy1.1 donor PLNC as demonstrated in Fig. 4. We assessed the cytokine profile of intact T-cells (host + donor) or T-cells treated with anti-Thy1.1 and complement to deplete donor cells (host alone). We found a similar proportion and preponderance of autoreactive IL-4–producing cells regardless of the presence of Thy1.1 donor T-cells (Fig. 5*B*). These results demonstrate that the IL-4–producing autoreactive cells in the ALS/PLNC-treated mice are host derived.

DISCUSSION

Islet transplantation has great promise to cure diabetes, although consistent long-term survival of islet grafts under potent immunosuppression has proved elusive. In this study, we demonstrate a novel, clinically relevant protocol that enables long-term survival of mHA-disparate islets and indefinite survival of syngeneic islets in the NOD mouse model for human type 1 diabetes. We show that initial lymphocyte depletion with ALS followed by intraportal administration of islets and PLNCs derived from diabetes-resistant donor NOR mice results in abrogation of recurrent autoimmunity in diabetic NOD recipients. This ALS/PLNC treatment results in significant donor T-cell chimerism and a striking switch in cytokine profile of the host islet-reactive T-cells from predominantly IFN-γ producers to IL-4 producers. Our results indicate that ALS/PLNC treatment results in functional conversion of the host autoreactive T-cell pool from pathogenic to nonpathogenic.

It has been notoriously difficult to induce tolerance to islet grafts in diabetic NOD mice. To date, the most successful experimental strategy enabling long-term survival of islet

TABLE 1
Summary of ELISPOT results of autoantigen-specific cytokine production from NOD islet recipients treated with ALS/PLNC or ALS alone

Experiment/ treatment	IFN-γ producers*	IL-4 producers*	Ratio of IFN-γ to IL-4 producers
1			
ALS/PLNC	89 ± 7.1	542 ± 27.9	0.16
ALS	363 ± 16.7	154 ± 21.5	2.36
2			
ALS/PLNC	54 ± 6.4	313 ± 18.8	0.17
ALS	398 ± 17.3	192 ± 15.2	2.07
3			
ALS/PLNC	16 ± 1.8	362 ± 26.5	0.04
Diabetic NOD†	174 ± 11.3	23 ± 2.1	7.56
4			
ALS/PLNC	328 ± 14.7	777 ± 34.6	0.42

Data are means ±SD for triplicate wells. *Total number of GAD65- and insulin-specific, cytokine-producing T-cells per 200,000 cells; †spontaneously diabetic NOD mice.

allografts in NOD mice uses myeloablative irradiation or potent cytotoxic drugs followed by administration of donor bone marrow (32,33). In our system, we generate donor lymphocyte chimerism in NOD recipient mice in the absence of irradiation, cytotoxic conditioning, and bone marrow transplantation, as used by others (25), by implementing short-term lymphocyte depletion by ALS treatment and administration of donor PLNCs. Following ALS treatment, host T-cells are replenished, likely due to both thymic output and homeostatic expansion of the peripheral T-cells, as our results showing transient expansion of a host-derived, memory-phenotype subset are consistent with T-cells undergoing homeostatic proliferation (26).

As shown here, the islet-reactive T-cells from ALS/PLNC-treated NOD recipients with long-surviving grafts exhibited a preponderance of IL-4 producers, whereas IFN-γ producers prevailed among autoreactive T-cells derived from ALS-treated mice that rejected their islet grafts. It has previously been established (31,34) that the autoreactive T-cell compartment in NOD mice is primarily of the Th1-type producing IFN-γ. Protection from the development of spontaneous diabetes in NOD mice has been associated with a cytokine switch by the autoreactive T-cells to Th2-like IL-4 producers (35,36). Here, we demonstrate protection from recurrent destruction of islets that correlates with a Th1-to-Th2 cytokine shift. Similarly, Bluestone and colleagues have found a reduction in Th1 cytokine production and an increase in Th2-promoting cytokines IL-5 and -10 in diabetic patients treated with an FcR nonbinding anti-CD3 monoclonal antibody (37) that was recently shown to dampen the severity of ongoing type 1 diabetes (38).

The cytokine switch in ALS/PLNC mice was completely due to host T-cells, as depletion of the chimeric donor T-cells did not alter the magnitude or profile of the autoreactive T-cell response, arguing against the presence of a donor-derived regulatory T-cell that mediates the cytokine shift. The maintenance of a $CD25^-$ phenotype in the donor T-cells further suggests that a $CD25^+$ regulatory T ("Treg")-cell (39) is probably not involved. Whether the presence of the donor T-cells in vivo is necessary for the host cells to maintain their nonpathogenic state or whether a type of "infectious tolerance" (40) occurred in which host cells were irreversibly altered by initial contact with donor T-cells remains to be established. It should be noted that the level of donor cell chimerism gradually declined to <1.0%, although the mice remained normoglycemic, suggesting that long-term maintenance of a chimeric state is not essential for islet survival and/or alteration of the host autoreactive T-cell cytokine profile.

Whether ALS/PLNC treatment will prolong survival of MHC-mismatched islet allografts has yet to be established, although previous evidence from our laboratory indicate that blocking autoimmunity may have indirect or direct effects on alloreactivity. We previously showed that recurrent autoimmunity hastens the allogeneic response, as evidenced by the destruction of NOR pancreatic α-cells in NOR islets grafted into NOD recipient mice (15). We also found that NOR islets are eventually rejected by 89 days in nonautoimmune streptozocin-treated NOD recipients (Q.S., D.W., G.A.H., S.T.B., D.L.F., unpublished data), and therefore the prolonged survival (130–200 days) of NOR islets in ALS/PLNC-treated NOD mice represents a dampening of the alloimmune response as well.

Can the creation of a donor cell chimeric state in the context of T-cell depletion strategies as presented here be a viable strategy to induce long-term islet graft survival in patients with type 1 diabetes? We believe that the clinical utility of this protocol is suggested by considering the long-term outcomes of patients with type 1 diabetes who undergo whole-organ pancreas transplants. These recipients tolerate their pancreas allografts and remain insulin free under conventional immunosuppression (41) and do not exhibit evidence of recurrent autoimmunity (42). Importantly, pancreas transplantation includes administration of associated pancreatic-draining lymph node tissue, similar to the PLNCs, which have been shown here to abrogate recurrent autoimmunity. We therefore propose that a simultaneous infusion of donor lymphocytes along with islets into diabetic recipients (following an initial treatment with a T-cell–depleting antibody) may likewise abrogate recurrent autoimmunity and enable long-term islet graft survival in diabetic patients similar to that observed with pancreas allografts.

In conclusion, we present a novel strategy for enabling long-term survival of islet allografts and permanent acceptance of syngeneic islets in diabetic NOD mice involving initial host lymphocyte depletion and administration of donor lymphocytes, resulting in the replenishment of host T-cells and a switch in their cytokine profile from pathogenic to nonpathogenic. Our results demonstrate that long-term survival of islet grafts in diabetic recipients appears possible through donor lymphocyte-driven manipulation of the host immune system.

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