

# Islet Allograft Survival Induced by Costimulation Blockade in NOD Mice Is Controlled by Allelic Variants of *Idd3*

Todd Pearson,<sup>1</sup> Peter Weiser,<sup>2</sup> Thomas G. Markees,<sup>2</sup> David V. Serreze,<sup>1,2,3</sup> Linda S. Wicker,<sup>4</sup> Laurence B. Peterson,<sup>5</sup> Anne-Marie Cumisky,<sup>5</sup> Leonard D. Shultz,<sup>1,2,3</sup> John P. Mordes,<sup>2</sup> Aldo A. Rossini,<sup>1,2,6</sup> and Dale L. Greiner<sup>1,2</sup>

NOD mice develop type 1 autoimmune diabetes and exhibit genetically dominant resistance to transplantation tolerance induction. These two phenotypes are genetically separable. Costimulation blockade fails to prolong skin allograft survival in (NOD × C57BL/6)F1 mice and in NOD-related strains made diabetes-resistant by congenic introduction of protective major histocompatibility complex (MHC) or non-MHC *Idd* region genes. Here, we tested the hypothesis that the genetic basis for the resistance of NOD mice to skin allograft tolerance also applies to islet allografts. Surprisingly, costimulation blockade induced permanent islet allograft survival in (NOD × C57BL/6)F1 mice but not in NOD mice. After costimulation blockade, islet allograft survival was prolonged in diabetes-resistant NOD.B6 *Idd3* mice and shortened in diabetes-free C57BL/6 mice congenic for the NOD *Idd3* variant. Islet allograft tolerance could not be induced in diabetes-resistant NOD.B10 *Idd5* and NOD.B10 *Idd9* mice. The data demonstrate that 1) NOD mice resist islet allograft tolerance induction; 2) unlike skin allografts, resistance to islet allograft tolerance is a genetically recessive trait; 3) an *Idd3* region gene(s) is an important determinant of islet allograft tolerance induction; and 4) there may be overlap in the mechanism by which the *Idd3* resistance locus improves self-tolerance and the induction of allotolerance. *Diabetes* 53:1972–1978, 2004

From the <sup>1</sup>Program in Immunology and Virology, The University of Massachusetts Medical School, Worcester, Massachusetts; <sup>2</sup>Department of Medicine, The University of Massachusetts Medical School, Worcester, Massachusetts; <sup>3</sup>The Jackson Laboratory, Bar Harbor, Maine; the <sup>4</sup>Juvenile Diabetes Research Foundation/Wellcome Trust Diabetes and Inflammation Laboratory, University of Cambridge, Cambridge, U.K.; the <sup>5</sup>Department of Pharmacology, Merck Research Laboratories, Rahway, New Jersey; and the <sup>6</sup>Program in Molecular Medicine, The University of Massachusetts Medical School, Worcester, Massachusetts.

Address correspondence and reprint requests to Dale L. Greiner, PhD, University of Massachusetts Medical School, 373 Plantation St., Biotech 2, Suite 218, Worcester, MA 01605. E-mail: dale.greiner@umassmed.edu.

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T.P. and P.W. contributed equally to this work.

DST, donor-specific transfusion; IL, interleukin; mAb, monoclonal antibody; MHC, major histocompatibility complex; MST, median survival time; NKT, natural killer T.

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Replacement of insulin-producing islets of Langerhans by transplantation can cure human type 1 diabetes (1), but recipients require lifelong immunosuppression. Researchers have focused on developing alternatives to immunosuppression, using animal models to evaluate new protocols. The NOD mouse is one of the most widely studied animal models of human type 1 diabetes (2). NOD mice have been used extensively for the evaluation of transplantation tolerance protocols in the setting of autoimmune diabetes (3–11). However, NOD mice are remarkably resistant to the induction of transplantation tolerance not only to islets (the target of the autoimmune attack) but also to a number of different tissues, leading us to hypothesize that NOD mice have a generalized resistance to transplantation tolerance (10).

Recent work by our laboratory (5,12,13) has begun to delineate the genetic basis of resistance to transplantation tolerance in NOD mice. We found that several insulin-dependent diabetes (*Idd*) loci that greatly reduce the expression of autoimmunity do not restore the ability of costimulation blockade to prolong skin allograft survival (12). In addition, diabetes-free (NOD × C57BL/6)F1 mice treated with costimulation blockade exhibit short skin allograft survival (5). The short survival of skin allografts in (NOD × C57BL/6)F1 mice that are treated with costimulation blockade suggests the presence of a genetically dominant NOD-derived trait. The experimental findings also separate the autoimmune phenotype (diabetes) from the tolerance resistance phenotype.

However, the survival of skin versus islet allografts in response to costimulation blockade is different even in normal mice (14–16). In addition, in the case of islet allografts, there could be a role for autoimmunity in graft destruction in NOD mice (9,17). The data suggest that, for islet allografts to survive in the setting of autoimmune diabetes, costimulation blockade must overcome both the genetic resistance of NOD mice to allotolerance induction and ongoing autoimmunity.

In the present study, we document that islet allografts survive permanently in (NOD × C57BL/6)F1 mice that are treated with costimulation blockade. This contrasts sharply with the dominant resistance of (NOD × C57BL/6)F1 mice to skin allograft tolerance induction (5).

However, similar to that observed for skin allograft tolerance (5,12), the parental NOD strain could not be rendered tolerant to islet allografts. Genetic analysis revealed that the resistance of NOD mice to islet allograft tolerance is associated with a gene(s) on chromosome 3.

## RESEARCH DESIGN AND METHODS

C57BL/6 ( $H2^b$ ) and C3H/HeJ ( $H2^k$ ) mice were obtained from the National Cancer Institute (Frederick, MD), The Jackson Laboratory (Bar Harbor, ME), or Taconic Farms (Germantown, NY). NOD/Mrk-TacBR, NOD.B6 *Idd3*R450 (line 1,098), NOD.B10 *Idd5*R444 (line 1,094), and NOD.B10 *Idd9*R28 (line 1,104) (all  $H2^{g^7}$ ) were obtained from Taconic Farms. NOD/Lt ( $H2^{g^7}$ ) mice were purchased from The Jackson Laboratory and maintained in our breeding colony at the University of Massachusetts Medical School. C57BL/6.NODc3 ( $H2^b$ ) and C57BL/6.NODc17 ( $H2^{g^7}$ ; hereafter called C57BL/6. $H2^{g^7}$ ) mice, developed by Edward Wakeland (University of Texas Southwestern Medical Center, Dallas, TX [18]), were the gift of Dr. Edward Leiter (The Jackson Laboratory). (NOD  $\times$  C57BL/6)F1 mice were generated by a single intercross of the appropriate parental strains and are described by the standard nomenclature (female parent  $\times$  male parent)F1.

All animals were certified to be free of Sendai virus, pneumonia virus of mice, murine hepatitis virus, minute virus of mice, ectromelia, lactate dehydrogenase-elevating virus, mouse poliovirus, Reo-3 virus, mouse adenovirus, lymphocytic choriomeningitis virus, polyoma, *Mycoplasma pulmonis*, and *Encephalitozoon cuniculi*. They were housed in a specific pathogen-free facility in microisolation cages and given autoclaved food and acidified water ad libitum. All animal use was in accordance with the guidelines of the Institutional Animal Care and Use Committee of the University of Massachusetts Medical School and recommendations in the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, National Research Council, National Academy of Sciences, 1996).

**Tolerance induction and islet allograft transplantation.** Graft recipient mice were treated with a single donor-specific transfusion (DST) and anti-CD154 monoclonal antibody (mAb) and received an islet allograft as described (19–22). Briefly,  $10^7$  spleen cells obtained from 5- to 10-week-old female C3H/HeJ mice were injected intravenously in a volume of 0.5 ml. DST was given on day  $-7$  relative to transplantation. MR1 hamster anti-mouse CD154 mAb was produced as ascites in C3H/HeJ-*scid* mice and purified by affinity chromatography (23,24). Antibody concentration was determined by measurement of optical density and confirmed by enzyme-linked immunosorbent assay (25). The concentration of contaminating endotoxin was determined commercially (Charles River Endosafe, Charleston, SC) and was uniformly  $<10$  units/mg mAb (23). Islet allograft recipients received an intraperitoneal injection of anti-CD154 mAb (0.5 mg/dose) on days  $-7$ ,  $-4$ , 0, and 4 relative to transplantation.

Islets were isolated from C3H/HeJ donors by collagenase digestion followed by density gradient separation as described (10,26). Handpicked islets (20 islets/g body wt) were transplanted into the renal subcapsular space of recipients.

Diabetes was induced in male mice by a single intraperitoneal injection of streptozotocin (150 mg/kg). Hyperglycemia was verified by 2 consecutive days of plasma glucose levels  $>250$  mg/dl (Glucose Analyzer 2; Beckman Instruments, Fullerton, CA). Diabetes was induced at least 1 week before the tolerance induction and transplantation procedures were initiated. Plasma glucose concentrations were measured twice weekly, and allograft rejection was defined as recurrent hyperglycemia ( $>250$  mg/dl) on at least 2 consecutive days.

**Histology.** Unilateral nephrectomy of the graft-bearing kidney was performed on all islet allograft recipients that were normoglycemic at the conclusion of an experiment. Islet graft function was defined as recurrent hyperglycemia after the nephrectomy. In some cases, islet allograft survival was inferred from histological study. Graft-bearing kidneys were fixed in 10% neutral-buffered formalin. Paraffin-embedded sections were prepared and stained with hematoxylin and eosin; additional sections were stained immunohistochemically for the presence of insulin and glucagon. A qualified pathologist who was unaware of the treatment status of the donors evaluated all islet graft specimens for the presence of inflammatory cells, insulin, and glucagon.

**Splenic natural killer T-cell functional assay.** Natural killer T (NKT) cell function was assessed by methods modified from those described previously (27). NKT cells were activated in vivo by intravenous injection of  $1 \mu\text{g}$  of anti-CD3 (clone 145-2C11; American Type Culture Collection) (27–30). Spleens were removed from mice 90 min after injection of anti-CD3 and immediately homogenized and RNA extracted in Ultraspec RNA (Biotecx,

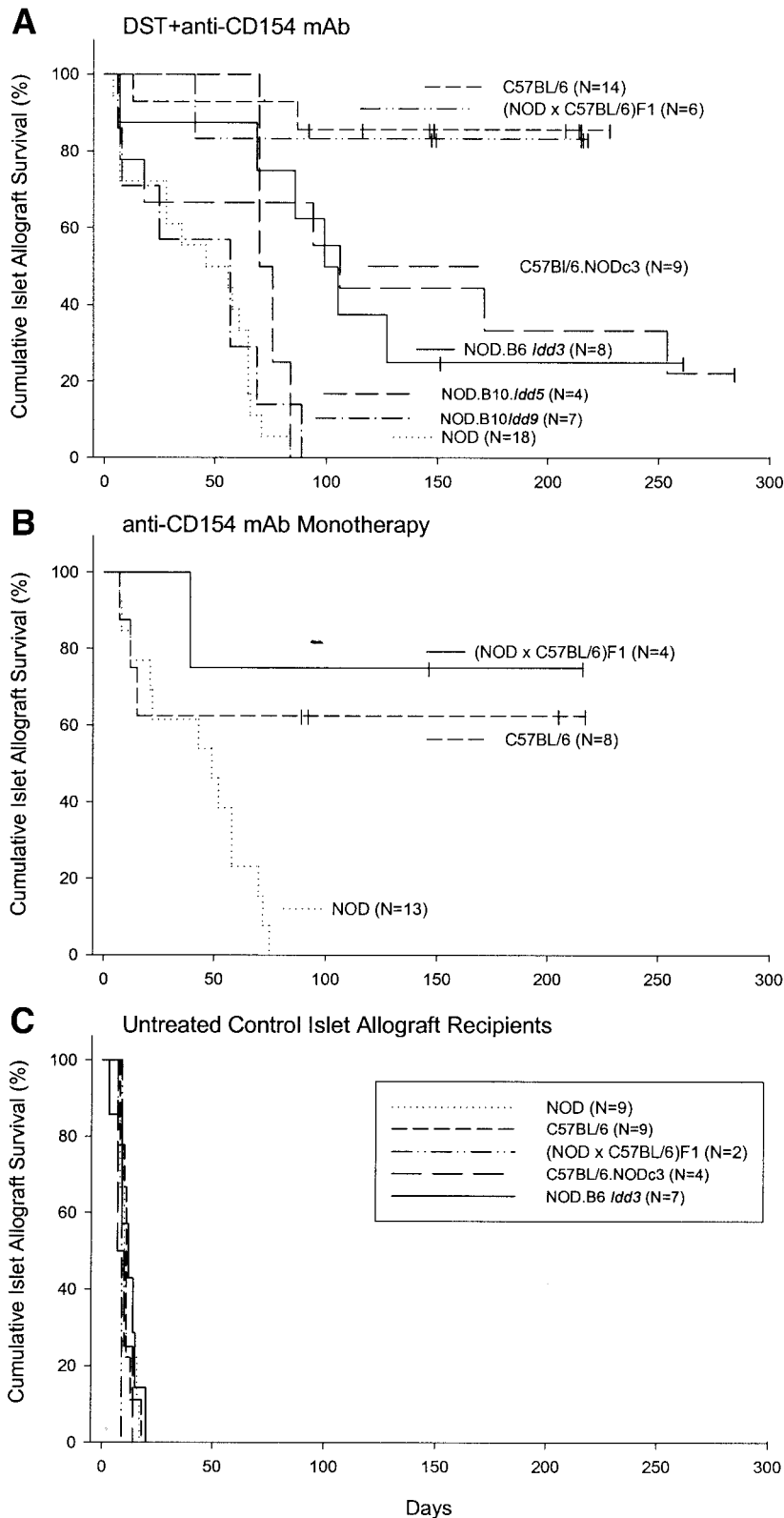
Houston, TX) using a high-speed Polytron. PolyA RNA was prepared using a Genelute mRNA Miniprep kit (Sigma, St. Louis, MO) and quantified with an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). PCRs after reverse transcription of RNA (RT-PCR) were performed with Superscript II (Invitrogen, Carlsbad, CA). Real-time PCR amplification was performed using TaqMan (Applied Biosystems, Foster City, CA), and product detection and analysis was performed on the TaqMan 7700 (Applied Biosystems). Interleukin-4 (IL-4) TaqMan primers and probe were obtained from Applied Biosystems. Results were normalized to glyceraldehyde-3-phosphate dehydrogenase (Applied Biosystems). Data between groups were analyzed for significance using an unpaired *t* test.

## RESULTS

**Islet allograft survival in chemically diabetic (NOD  $\times$  C57BL/6)F1 mice that were treated with DST and anti-CD154 mAb is prolonged.** We first tested the hypothesis that islet allograft survival in (NOD  $\times$  C57BL/6)F1 mice that were treated with costimulation blockade would be brief, as is known to be the case for skin allografts (5). We transplanted C3H/HeJ ( $H2^k$ ) islet allografts into fully major histocompatibility complex (MHC)-mismatched, chemically diabetic young (NOD  $\times$  C57BL/6)F1 ( $H2^{g^7} \times H2^b$ ) mice that were treated with DST and anti-CD154 mAb. Surprisingly, most islet allografts were accepted long term, and five of six of the grafts survived until the conclusion of the experiment (median survival time [MST]  $\geq 181$  days; range, 41–218 days; Fig. 1A). The duration of islet allograft survival in (NOD  $\times$  C57BL/6)F1 mice was statistically similar to that achieved in chemically diabetic C57BL/6 mice that were treated in the same way (MST  $\geq 148$  days; range, 13–228 days; NS). In contrast, islet allograft survival in chemically diabetic NOD mice was significantly shorter (MST = 46 days) than in either the (NOD  $\times$  C57BL/6)F1 or the C57BL/6 recipients ( $P < 0.001$ ). As expected, islet graft survival in control, non-tolerized NOD, (NOD  $\times$  C57BL/6)F1, and C57BL/6 recipients was uniformly brief ( $<18$  days in all cases; Fig. 1C).

Given this observation, we next tested the hypothesis that anti-CD154 mAb monotherapy would extend the survival of islet allografts in (NOD  $\times$  C57BL/6)F1 mice, just as it does in C57BL/6 and other normal mouse strains (22,31,32). We first confirmed that islet allograft survival in C57BL/6 recipients that were given anti-CD154 mAb monotherapy was prolonged and in most cases seemed to be permanent (MST  $\geq 90$  days; Fig. 1B). Islet allograft survival in (NOD  $\times$  C57BL/6)F1 mice that were treated with anti-CD154 mAb monotherapy was also prolonged (MST  $\geq 146$  days) and statistically similar to that achieved in C57BL/6 (NS). In contrast, islet allograft survival in chemically diabetic young NOD recipients that were treated with anti-CD154 mAb monotherapy was uniformly brief (MST = 49 days;  $P < 0.02$  vs. both C57BL/6 and the F1s), and all grafts were rejected by day 75.

**Allelic variants of *Idd3* control prolonged islet allograft survival in NOD mice.** The surprising observation that islet allograft survival in (NOD  $\times$  C57BL/6)F1 mice is similar to that achieved in C57BL/6 mice whereas the same is not true of skin allografts (5) prompted us to test a number of *Idd*-congenic NOD mice that have various degrees of protection from developing type 1 diabetes, insulinitis, and insulin autoantibodies (33). Skin allograft survival on all NOD *Idd* congenic mice that were treated with costimulation blockade is known to be short (12). We hypothesized that *Idd* loci control the differential survival



**FIG. 1.** Life table analysis of islet allograft survival in chemically diabetic mice. Groups of 6- to 8-week old chemically diabetic mice were given C3H/HeJ islet allografts as described in RESEARCH DESIGN AND METHODS. **A:** Mice were also treated with a DST plus anti-CD154 mAb. DST ( $10^7$  C3H/HeJ spleen cells) was given on day -7 relative to transplantation, and anti-CD154 mAb (0.5 mg/dose) was given on days -7, -4, 0, and 4. **B:** Mice were treated with anti-CD154 mAb monotherapy (0.5 mg/dose), which was given on days -7, -4, 0, and 4 relative to transplantation. **C:** Mice received allografts but no other treatment. The experiment was terminated arbitrarily at various time points as mice were used for other experiments. Vertical bars indicate mice that were removed from the study with intact grafts or alive with intact grafts at the conclusion of the period of observation.

of islet versus skin allografts in chemically diabetic NOD mice that are treated with costimulation blockade.

We first tested NOD.B6 *Idd3* mice. The presence of alleles of *Idd3* that are of C57BL/6 origin greatly reduces the frequency of autoimmune diabetes in NOD mice (34,35). Islet allograft survival in chemically diabetic NOD.B6 *Idd3* recipients that were treated with costimula-

tion blockade (MST = 99 days; Fig. 1A) was significantly longer than that observed in chemically diabetic NOD mice (MST = 46 days;  $P < 0.001$ ; Fig. 1A), although somewhat shorter than that achieved in C57BL/6 mice ( $P < 0.01$ ; Fig. 1A).

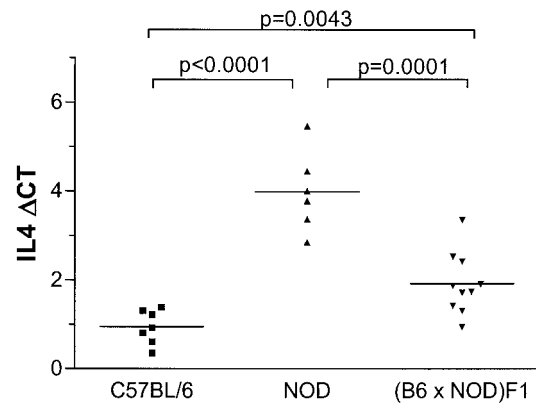
The importance of genes on chromosome 3, which includes genes within the *Idd3* interval, was confirmed by

testing C57BL/6.NODc3 mice, which harbor NOD-origin alleles of *Idd3* as well as *Idd17*, *Idd10*, and *Idd18* region genes (18; L.S.W., unpublished observations). Islet allograft survival in chemically diabetic C57BL/6.NODc3 mice that were treated with DST and anti-CD154 mAb was significantly shorter (MST = 106 days) than in similarly treated C57BL/6 recipients (MST  $\geq$ 148 days;  $P < 0.025$ ; Fig. 1A) and statistically similar to that achieved in NOD.B6 *Idd3* recipients (NS). Although the C57BL/6.NODc3 strain includes a large region of NOD-derived introgressed DNA, including regions encoding *Idd17*, *Idd10*, and *Idd18*, as well as *Idd3*, the observation that a reciprocal effect is seen in NOD.B6 *Idd3* congenic mice suggests that it is the *Idd3* locus that controls islet allograft survival in chemically diabetic NOD mice that are treated with costimulation blockade. The development of C57BL/6.NODc3 congenic mice that have smaller introgressed regions of NOD DNA will be required to confirm this hypothesis. That after DST and anti-CD154 mAb treatment islet allograft survival time in C57BL/6.NODc3 mice remained greater than in NOD mice indicates that genes outside the *Idd3* region also regulate this phenotype, an observation consistent with the indefinite graft survival in (NOD  $\times$  C57BL/6)F1 mice.

To extend this observation, we also tested two other NOD *Idd* congenic mice, NOD.B10 *Idd5* and NOD.B10 *Idd9*, in which the frequency of diabetes is very low (36,37). Islet allograft survival in chemically diabetic, tolerized NOD.B10 *Idd9* mice (MST = 57 days;  $n = 7$ ; Fig. 1A) and in NOD.B10 *Idd9* mice (MST = 70 days;  $n = 4$ ; Fig. 1A) was brief and, in both cases, statistically similar to that observed in tolerized chemically diabetic NOD mice (NS). All grafts in the NOD.B10 *Idd5* and NOD.B10 *Idd9* recipients were rejected by day 89.

**Improved NKT cell function in (NOD  $\times$  C57BL/6)F1 mice.** Why (NOD  $\times$  C57BL/6)F1 and NOD.B6 *Idd3* mice should resist tolerance induction to skin (5) but not islet allografts (Fig. 1) is not immediately clear. To begin to investigate possible mechanisms, we studied NKT cell function. We have previously documented (5) that certain cellular compartments in (NOD  $\times$  C57BL/6)F1 mice exhibit NOD-like abnormalities, whereas others exhibit normal C57BL/6-like function. NKT cell function was not previously examined but has been suggested to be important in regulating both autoimmune diabetes (38–41) and transplantation tolerance (42). We therefore evaluated the function of NKT cells in (NOD  $\times$  C57BL/6)F1 mice. Because NOD mice are NK1.1<sup>null</sup>, we chose a functional assay that does not require phenotypic identification of NKT cells and measured rapid transcription of IL-4 mRNA in splenocytes upon intravenous anti-CD3 mAb administration (27–30).

Production of IL-4 mRNA after injection of anti-CD3 mAb, as expected (43), was low in NOD mice as compared with C57BL/6 mice ( $P < 0.0001$ ; Fig. 2). In contrast, (NOD  $\times$  C57BL/6)F1 mice showed significantly increased production of IL-4 mRNA as compared with the NOD parental ( $P < 0.0001$ ). IL-4 mRNA upregulation in the F1 was inferior ( $P < 0.005$ ) to that observed in the C57BL/6 parental, demonstrating a codominant inheritance of the NKT cell phenotype.



**FIG. 2. Improved NKT cell function in (NOD  $\times$  C57BL/6)F1 mice.** Expression of IL-4 mRNA was measured by real-time quantitative PCR from whole spleen 90 min after anti-CD3 (1  $\mu$ g/mouse) stimulation *in vivo*. Relative levels of mRNA ( $\Delta$ CT) from individual mice are expressed as the IL-4 cycle threshold (CT) detection value minus the glyceraldehyde-3-phosphate dehydrogenase cycle threshold value. Every unit increase in  $\Delta$ CT represents a twofold decrease in mRNA expression. In the data shown, NOD has eightfold less IL-4 mRNA than C57BL/6 and fourfold less IL-4 mRNA than (C57BL/6  $\times$  NOD)F1.

## DISCUSSION

The survival of skin allografts in C57BL/6 mice that are treated with costimulation blockade is greatly prolonged (20), but neither NOD nor (NOD  $\times$  C57BL/6)F1 mice can be tolerized to skin allografts in this manner (5). In this study, we tested the hypothesis that the genetically dominant resistance of NOD mice to allograft tolerance induction would similarly shorten islet allograft survival in NOD and (NOD  $\times$  C57BL/6)F1 mice. Given the brief survival of islet allografts in autoimmune diabetic NOD mice (9,10), our observation that islet allograft survival is short in chemically diabetic NOD mice that are treated with costimulation blockade was not surprising. It was, however, very surprising to observe prolonged and possibly permanent islet graft survival in (NOD  $\times$  C57BL/6)F1 mice that were treated with costimulation blockade.

There are at least two possible interpretations of this observation. The first is that the genes that control skin allograft survival are different from those that control islet allograft survival. Neither islet nor skin allografts survive long term in NOD mice that are treated with costimulation blockade, whereas allografts of both tissues survive long term in tolerized C57BL/6 mice. Our data derived from NOD and (NOD  $\times$  C57BL/6)F1 mice could be interpreted to suggest that mechanisms that control skin allograft survival are defective in the NOD mouse and are genetically dominant, whereas the mechanisms that control islet allograft survival are genetically recessive.

The alternative interpretation, which we have discussed previously in the context of autoimmunity (5,13), is that survival of skin and islet allografts after tolerance induction is controlled by the same set of genes, but the “threshold” for skin transplantation tolerance is higher than for islets. It is widely recognized that the transplantation of islets represents a less stringent test of tolerance induction than does the transplantation of skin (14,15). This difference in graft survival outcomes could be due to differences in the population of antigen-presenting cells in each tissue, but it has recently been suggested that this is not the case (44). Irrespective of the mechanisms under-

lying the differences between skin and islets, the islet allograft data presented here are consistent with the “unmasking” of a genetically determined threshold effect.

To investigate further the “genetic” versus “threshold” interpretations, we used congenic mice. We have previously documented that in NOD congenic mice (e.g., NOD.B6 *Idd3* mice) that are largely protected from autoimmune diabetes, costimulation blockade fails to prolong skin allograft survival (12). The data indicated that single or multiple combinations of non-NOD-origin alleles of *Idd* loci do not correct resistance to skin allograft tolerance after costimulation blockade. In contrast, the present data unexpectedly document that C57BL/6-origin alleles on chromosome 3 that include genes within the *Idd3* interval can enhance islet allograft survival in NOD mice after costimulation blockade. Why this should be the case is not immediately clear, but the *Idd3* locus has been narrowed to a small interval that contains eight genes, three of which—*Il2*, *Il21*, and *Fgf2*—have known functions that could contribute to the establishment of immunological tolerance (45; L.S.W. and L.B.P., unpublished observations). Of particular interest is IL-2, which is required for the induction of allograft tolerance by costimulation blockade (46). We are currently investigating the possibility that abnormalities in IL-2 expression or function in NOD mice are associated with their resistance to islet allograft tolerance induction.

Because islet allograft survival in NOD.B6 *Idd3* congenic mice was intermediate between that observed in NOD and C57BL/6 mice, it is clear that other genes involved in the process of tolerance induction to islets must be defective in NOD mice. We therefore investigated two additional loci, *Idd5* (consisting of at least two *Idd* loci, *Idd5.1* and *Idd5.2* [37]) and *Idd9* (consisting of at least three *Idd* loci, *Idd9.1*, *Idd9.2*, and *Idd9.3* [36]), which contribute to diabetes susceptibility in NOD mice. C57BL/10-origin alleles of *Idd5.1* in NOD mice reduce the incidence of spontaneous diabetes. The protective alleles are associated with variations in CTLA-4 gene splicing (47), and expression of a functional CTLA-4 molecule is important for the induction of tolerance using DST and anti-CD154 mAb (20). However, replacing the NOD-origin allele of both *Idd5.1* and *Idd5.2* with a C57BL/10-derived resistance allele did not improve islet allograft survival. Similarly, NOD.B10 *Idd9* mice develop spontaneous diabetes only rarely, but as was the case for *Idd5*, islet allograft survival after costimulation blockade remained short.

Of course, our dataset is not complete, and there are large numbers of *Idd* loci that could be playing a role in these observations. Two NOD congenic mice that have not yet been tested but are of considerable interest include NOD.NOR *Idd13* and C57BL/6.NOD *Idd4* mice. One of the genes within the NOD.NOR *Idd13* congenic interval controlling diabetes susceptibility has been shown to be *B2m* (48), which encodes  $\beta 2$  microglobulin, a molecule required for MHC class I expression. Variations in the expression of MHC class I clearly could affect islet allograft tolerance induction by costimulation blockade. *Idd4* has been associated with overexpression of IL-12p40 in NOD mice (49). IL-12p40 is associated with Th1-type inflammatory responses that would be expected in recipients that resist transplantation tolerance induction and reject their grafts.

It will be of interest to determine whether C57BL/6.NOD *Idd4* congenic mice have shortened islet allograft survival in the absence of autoimmunity but in the presence of overproduction of IL-12p40.

The very long duration of islet but not skin allograft survival in (NOD  $\times$  C57BL/6)F1 mice now permits us to begin to search for and identify cellular mechanisms that control the survival of these two tissue grafts in tolerized mice. In previous studies (5), we have shown that NOD-like defects in NK cell function and macrophage development are corrected in (NOD  $\times$  C57BL/6)F1 mice. In contrast, NOD-like abnormalities in dendritic cell maturation and in the response of CD4<sup>+</sup> T-cells to costimulation blockade are expressed in a genetically dominant manner in (NOD  $\times$  C57BL/6)F1 mice. Of additional interest is the NKT cell subset that was not studied in our earlier reports (5,12).

NKT cells are a link between the innate and adaptive arms of the immune system (50) and have been hypothesized to be important in NOD autoimmunity (38–41), to suppress the development of graft-versus-host disease (51,52), and to be involved in allograft rejection and tolerance induction (42,53). NKT cell activation of dendritic cells also leads to an enhanced ability to stimulate allogeneic T-cell responses (54). Furthermore, NKT cells regulate the maturation of dendritic cells in the pancreatic draining lymph node, leading to improved islet cell self-tolerance in NOD mice (38). Our data quantifying the rapid upregulation of IL-4 mRNA after injection of anti-CD3, a response associated with NKT cell activation (27–30), show that restoration of NKT cell function in (NOD  $\times$  C57BL/6)F1 mice is associated with prolonged islet allograft survival in tolerized recipients. This finding is consistent with the function of NKT cells in maintaining endogenous islet tolerance (38–41). Gene variants within *Idd3* alone are not responsible for the higher NKT cell phenotype present in (NOD  $\times$  C57BL/6)F1 mice as we did not observe any differences in NKT cell function in NOD and NOD.B6 *Idd3* mice (L.S.W. and L.B.P., unpublished observations). However, it remains possible that the non-*Idd3* gene variants that also contribute to prolonged graft retention in tolerized (NOD  $\times$  C57BL/6)F1 and C57BL/6.NODc3 mice do so by increasing NKT cell function.

In summary, we have identified a non-MHC *Idd* locus that, in part, controls islet allograft tolerance induction in chemically diabetic NOD mice. This was observed to be true in both NOD.B6 *Idd3* and C57BL/6.NODc3 mice. Importantly, this beneficial effect on islet transplantation tolerance is independent of the degree of protection from diabetes. Other *Idd* congenic NOD strains with increased protection from expression of diabetes did not show improved islet allograft survival in response to costimulation blockade. The data highlight the genetically controlled differences between skin (5,12) and islet allograft tolerance induced by costimulation blockade and lend support to the “genetic threshold” hypothesis for resistance of NOD mice to transplantation tolerance.

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## REFERENCES

- Shapiro AMJ, Lakey JRT, Ryan EA, Korbutt GS, Toth E, Warnock GL, Kneteman NM, Rajotte RV: Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. *N Engl J Med* 343:230–238, 2000
- Atkinson M, Leiter EH: The NOD mouse model of insulin dependent diabetes: as good as it gets? *Nat Med* 5:601–604, 1999
- Molano RD, Pileggi A, Berney T, Poggioli R, Zahr E, Oliver R, Malek TR, Ricordi C, Inverardi L: Long-term islet allograft survival in nonobese diabetic mice treated with tacrolimus, rapamycin, and anti-interleukin-2 antibody. *Transplantation* 75:1812–1819, 2003
- Molano RD, Pileggi A, Berney T, Poggioli R, Zahr E, Oliver R, Ricordi C, Rothstein DM, Basadonna GP, Inverardi L: Prolonged islet allograft survival in diabetic NOD mice by targeting CD45RB and CD154. *Diabetes* 52:957–964, 2003
- Pearson T, Markees TG, Serreze DV, Pierce MA, Marron MP, Wicker LS, Peterson LB, Shultz LD, Mordes JP, Rossini AA, Greiner DL: Genetic disassociation of autoimmunity and resistance to costimulation blockade-induced transplantation tolerance in nonobese diabetic mice. *J Immunol* 171:185–195, 2003
- Rabinovitch A, Suarez-Pinzon WL, Shapiro AMJ, Rajotte RV, Power R: Combination therapy with sirolimus and interleukin-2 prevents spontaneous and recurrent autoimmune diabetes in NOD mice. *Diabetes* 51:638–645, 2002
- Wu T, Levay-Young B, Heuss N, Sozen H, Kirchhof N, Sutherland DER, Hering B, Guo ZG: Inducing tolerance to MHC-matched allogeneic islet grafts in diabetic NOD mice by simultaneous islet and bone marrow transplantation under nonirradiative and nonmyeloablative conditioning therapy. *Transplantation* 74:22–27, 2002
- Guo ZG, Wu T, Kirchhof N, Mital D, Williams JW, Azuma M, Sutherland DER, Hering BJ: Immunotherapy with nondepleting anti-CD4 monoclonal antibodies but not CD28 antagonists protects islet graft in spontaneously diabetic NOD mice from autoimmune destruction and allogeneic and xenogeneic graft rejection. *Transplantation* 71:1656–1665, 2001
- Molano RD, Berney T, Li H, Cattani P, Pileggi A, Vizzardelli C, Kenyon NS, Ricordi C, Burkly LC, Inverardi L: Prolonged islet graft survival in NOD mice by blockade of the CD40-CD154 pathway of T-cell costimulation. *Diabetes* 50:270–276, 2001
- Markees TG, Serreze DV, Phillips NE, Sorli CH, Noelle RJ, Woda BA, Greiner DL, Mordes JP, Rossini AA: NOD mice have a generalized defect in their response to transplantation tolerance induction. *Diabetes* 48:967–974, 1999
- Demirci G, Strom TB, Li XC: Islet allograft rejection in nonobese diabetic mice involves the common gamma-chain and CD28/CD154-dependent and -independent mechanisms. *J Immunol* 171:3878–3885, 2003
- Pearson T, Markees TG, Wicker LS, Serreze DV, Peterson LB, Mordes JP, Rossini AA, Greiner DL: NOD congenic mice genetically protected from autoimmune diabetes remain resistant to transplantation tolerance induction. *Diabetes* 52:321–326, 2002
- Pearson T, Markees TG, Serreze DV, Pierce MA, Wicker LS, Peterson LB, Shultz LD, Mordes JP, Rossini AA, Greiner DL: Islet cell autoimmunity and transplantation tolerance: two distinct mechanisms? In *Immunology of Diabetes: Pathogenesis from Mouse to Man*. Sanjeevi CB, Eisenbarth GS, Eds. New York, New York Academy of Sciences, 2003, p. 148–156
- Phillips NE, Markees TG, Mordes JP, Greiner DL, Rossini AA: Blockade of CD40-mediated signaling is sufficient for inducing islet but not skin transplantation tolerance. *J Immunol* 170:3015–3023, 2003
- Karim M, Steger U, Bushell AR, Wood KJ: The role of the graft in establishing tolerance. *Front Biosci* 7:e129–e154, 2002
- Rossini AA, Greiner DL, Mordes JP: Induction of immunological tolerance for transplantation. *Physiol Rev* 79:99–141, 1999
- Makhoul L, Kishimoto K, Smith RN, Abdi R, Koulmanda M, Winn HJ, Auchincloss H Jr, Sayegh MH: The role of autoimmunity in islet allograft destruction: major histocompatibility complex class II matching is necessary for autoimmune destruction of allogeneic islet transplants after T-cell costimulatory blockade. *Diabetes* 51:3202–3210, 2002
- Yui MA, Muralidharan K, Moreno-Altamirano B, Perrin G, Chestnut K, Wakeland EK: Production of congenic mouse strains carrying NOD-derived diabetogenic genetic intervals: an approach for the genetic dissection of complex traits. *Mamm Genome* 7:331–334, 1996
- Iwakoshi NN, Mordes JP, Markees TG, Phillips NE, Greiner DL, Rossini AA: Treatment of allograft recipients with donor specific transfusion and anti-CD154 antibody leads to deletion of alloreactive CD8<sup>+</sup> T cells and prolonged graft survival in a CTLA4-dependent manner. *J Immunol* 164:512–521, 2000
- Markees TG, Phillips NE, Gordon EJ, Noelle RJ, Shultz LD, Mordes JP, Greiner DL, Rossini AA: Long-term survival of skin allografts induced by donor splenocytes and anti-CD154 antibody in thymectomized mice requires CD4<sup>+</sup> T cells, interferon-gamma, and CTLA4. *J Clin Invest* 101:2446–2455, 1998
- Markees TG, Phillips NE, Noelle RJ, Shultz LD, Mordes JP, Greiner DL, Rossini AA: Prolonged survival of mouse skin allografts in recipients treated with donor splenocytes and antibody to CD40 ligand. *Transplantation* 64:329–335, 1997
- Parker DC, Greiner DL, Phillips NE, Appel MC, Steele AW, Durie FH, Noelle RJ, Mordes JP, Rossini AA: Survival of mouse pancreatic islet allografts in recipients treated with allogeneic small lymphocytes and antibody to CD40 ligand. *Proc Natl Acad Sci U S A* 92:9560–9564, 1995
- Foy TM, Shepherd DM, Durie FH, Aruffo A, Ledbetter JA, Noelle RJ: In vivo CD40-gp39 interactions are essential for thymus-dependent humoral immunity. II. Prolonged suppression of the humoral immune response by an antibody to the ligand for CD40, gp39. *J Exp Med* 178:1567–1575, 1993
- Noelle RJ, Roy M, Shepherd DM, Stamenkovic I, Ledbetter JA, Aruffo A: A 39-kDa protein on activated helper T cells binds CD40 and transduces the signal for cognate activation of B cells. *Proc Natl Acad Sci U S A* 89:6550–6554, 1992
- Iwakoshi NN, Markees TG, Turgeon NA, Thornley T, Cuthbert A, Leif JH, Phillips NE, Mordes JP, Greiner DL, Rossini AA: Skin allograft maintenance in a new synchimeric model system of tolerance. *J Immunol* 167:6623–6630, 2001
- Seung E, Iwakoshi N, Woda BA, Markees TG, Mordes JP, Rossini AA, Greiner DL: Allogeneic hematopoietic chimerism in mice treated with sublethal myeloablation and anti-CD154 antibody: absence of graft-versus-host disease, induction of skin allograft tolerance, and prevention of recurrent autoimmunity in islet-allografted NOD/Lt mice. *Blood* 95:2175–2182, 2000
- Bendelac A: Mouse NK1<sup>+</sup> T cells. *Curr Opin Immunol* 7:367–374, 1995
- Yoshimoto T, Paul WE: CD4<sup>pos</sup>, NK1.1<sup>pos</sup> T cells promptly produce interleukin 4 in response to in vivo challenge with anti-CD3. *J Exp Med* 179:1285–1295, 1994
- Chiu YH, Jayawardena J, Weiss A, Lee D, Park SH, Dautry-Varsat A, Bendelac A: Distinct subsets of CD1d-restricted T cells recognize self-antigens loaded in different cellular compartments. *J Exp Med* 189:103–110, 1999
- Harada M, Seino K, Wakao H, Sakata S, Ishizuka Y, Ito T, Kojo S, Nakayama T, Taniguchi M: Down-regulation of the invariant V $\alpha$ 14 antigen receptor in NKT cells upon activation. *Int Immunol* 16:241–247, 2004
- Rossini AA, Mordes JP, Greiner DL, Stoff JS: Islet cell transplantation tolerance. *Transplantation* 72:S43–S46, 2001
- Rossini AA, Mordes JP, Markees TG, Phillips NE, Gordon EJ, Greiner DL: Induction of islet transplantation tolerance using donor specific transfusion and anti-CD154 monoclonal antibody. *Transplant Proc* 31:629–632, 1999
- Robles DT, Eisenbarth GS, Dailey NJM, Peterson LB, Wicker LS: Insulin autoantibodies are associated with islet inflammation but not always related to diabetes progression in NOD congenic mice. *Diabetes* 52:882–886, 2003
- Todd JA, Wicker LS: Genetic protection from the inflammatory disease type 1 diabetes in humans and animal models. *Immunity* 15:387–395, 2001
- Podolin PL, Wilusz MB, Cubbon RM, Pajvani U, Lord CJ, Todd JA, Peterson LB, Wicker LS, Lyons PA: Differential glycosylation of interleukin 2, the

- molecular basis for the NOD Idd3 type 1 diabetes gene? *Cytokine* 12:477–482, 2000
36. Lyons PA, Hancock WW, Denny P, Lord CJ, Hill NJ, Armitage N, Siegmund T, Todd JA, Phillips MS, Hess JF, Chen SL, Fischer PA, Peterson LB, Wicker LS: The NOD Idd9 genetic interval influences the pathogenicity of insulinitis and contains molecular variants of Cd30, Tnfr2, and Cd137. *Immunity* 13:107–115, 2000
  37. Hill NJ, Lyons PA, Armitage N, Todd JA, Wicker LS, Peterson LB: NOD *Idd5* locus controls insulinitis and diabetes and overlaps the orthologous CTLA4/IDDM12 and NRAMP1 loci in humans. *Diabetes* 49:1744–1747, 2000
  38. Naumov YN, Bahjat KS, Gausling R, Abraham R, Exley MA, Koezuka Y, Balk SB, Strominger JL, Clare-Salzler M, Wilson SB: Activation of CD1d-restricted T cells protects NOD mice from developing diabetes by regulating dendritic cell subsets. *Proc Natl Acad Sci U S A* 98:13838–13843, 2001
  39. Sharif S, Arreaza GA, Zucker P, Mi QS, Sharif S, Arreaza GA, Zucker P, Mi QS, Sondhi J, Naidenko OV, Kronenberg M, Koezuka Y, Delovitch TL, Gombert JM, Leite-de-Moraes M, Gouarin C, Zhu R, Hameg A, Nakayama T, Taniguchi M, Lepault F, Lehuen A, Bach JF, Herbelin A: Activation of natural killer T cells by  $\alpha$ -galactosylceramide treatment prevents the onset and recurrence of autoimmune type 1 diabetes. *Nat Med* 7:1057–1062, 2001
  40. Wang B, Geng YB, Wang CR: CD1-restricted NK T cells protect nonobese diabetic mice from developing diabetes. *J Exp Med* 194:313–319, 2001
  41. Sharif S, Arreaza GA, Zucker P, Mi QS, Delovitch TL: Regulation of autoimmune disease by natural killer T cells. *J Mol Med* 80:290–300, 2002
  42. Seino K, Fukao K, Muramoto K, Yanagisawa K, Takada Y, Kakuta S, Iwakura Y, Van Kaer L, Takeda K, Nakayama T, Taniguchi M, Bashuda H, Yagita H, Okumura K: Requirement for natural killer T (NKT) cells in the induction of allograft tolerance. *Proc Natl Acad Sci U S A* 98:2577–2581, 2001
  43. Gombert JM, Tancredè-Bohin E, Hameg A, Leite-de-Moraes MD, Vicari A, Bach JF, Herbelin A: IL-7 reverses NK1<sup>+</sup> T cell-defective IL-4 production in the non-obese diabetic mouse. *Int Immunol* 8:1751–1758, 1996
  44. Jones ND, Turvey SE, Van Maurik A, Hara M, Kingsley CI, Smith CH, Mellor AL, Morris PJ, Wood KJ: Differential susceptibility of heart, skin, and islet allografts to T cell-mediated rejection. *J Immunol* 166:2824–2830, 2001
  45. Lyons PA, Armitage N, Argentina F, Denny P, Hill NJ, Lord CJ, Wilusz MB, Peterson LB, Wicker LS, Todd JA: Congenic mapping of the type 1 diabetes locus, *idd3*, to a 780-kb region of mouse chromosome 3: identification of a candidate segment of ancestral DNA by haplotype mapping. *Genome Res* 10:446–453, 2000
  46. Dai ZH, Konieczny BT, Baddoura FK, Lakkis FG: Impaired alloantigen-mediated T cell apoptosis and failure to induce long-term allograft survival in IL-2-deficient mice. *J Immunol* 161:1659–1663, 1998
  47. Ueda H, Howson JM, Esposito L, Heward J, Snook H, Chamberlain G, Rainbow DB, Hunter KM, Smith AN, Di Genova G, Herr MH, Dahlman I, Payne F, Smyth D, Lowe C, Twells RC, Howlett S, Healy B, Nutland S, Rance HE, Everett V, Smink LJ, Lam AC, Cordell HJ, Walker NM, Bordin C, Hulme J, Motzo C, Cucca F, Hess JF, Metzker ML, Rogers J, Gregory S, Allahabadi A, Nithyananthan R, Tuomilehto-Wolf E, Tuomilehto J, Bingley P, Gillespie KM, Undlien DE, Ronningen KS, Guja C, Ionescu-Tirgoviste C, Savage DA, Maxwell AP, Carson DJ, Patterson CC, Franklyn JA, Clayton DG, Peterson LB, Wicker LS, Todd JA, Gough SC: Association of the T-cell regulatory gene CTLA4 with susceptibility to autoimmune disease. *Nature* 423:506–511, 2003
  48. Hamilton-Williams EE, Serreze DV, Charlton B, Johnson EA, Marron MP, Müllbacher A, Slattery RM: Transgenic rescue implicates  $\beta_2$ -microglobulin as a diabetes susceptibility gene in nonobese diabetic (NOD) mice. *Proc Natl Acad Sci U S A* 98:11533–11538, 2001
  49. Simpson PB, Mistry MS, Maki RA, Yang W, Schwarz DA, Johnson EB, Lio FM, Alleva DG: Cutoff edge: diabetes-associated quantitative trait locus, *Idd4*, is responsible for the IL-12p40 overexpression defect in nonobese diabetic (NOD) mice. *J Immunol* 171:3333–3337, 2003
  50. Godfrey DI, Hammond KJ, Poulton LD, Smyth MJ, Baxter AG: NKT cells: facts, functions and fallacies. *Immunol Today* 21:573–583, 2000
  51. Weerasinghe A, Kawamura T, Moroda T, Seki S, Watanabe H, Abo T: Intermediate TCR cells can induce graft-versus-host disease after allogeneic bone marrow transplantation. *Cell Immunol* 185:14–29, 1998
  52. Onoe Y, Harada M, Tamada K, Abe K, Li T, Tada H, Nomoto K: Involvement of both donor cytotoxic T lymphocytes and host NK1.1<sup>+</sup> T cells in the thymic atrophy of mice suffering from acute graft-versus-host disease. *Immunology* 95:248–256, 1998
  53. Tsukahara A, Kawamura H, Iiai T, Moroda T, Suzuki S, Tada T, Minagawa M, Musha N, Hatakeyama K, Abo T: Participation of NK1.1<sup>+</sup> T cells in the rejection of *lpr*  $\alpha\beta$ T cells when bone marrow cells of *lpr* mice are transplanted into B6 mice. *Microbiol Immunol* 42:447–456, 1998
  54. Fujii S, Shimizu K, Smith C, Bonifaz L, Steinman RM: Activation of natural killer T cells by  $\alpha$ -galactosylceramide rapidly induces the full maturation of dendritic cells in vivo and thereby acts as an adjuvant for combined CD4 and CD8 T cell immunity to a coadministered protein. *J Exp Med* 198:267–279, 2003