Islet Allograft Survival Induced by Costimulation Blockade in NOD Mice Is Controlled by Allelic Variants of \textit{Idd3}

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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 \((\text{NOD} \times \text{C57BL/6})\text{F1}\) mice and in NOD-related strains made diabetes-resistant by congenic introduction of protective major histocompatibility complex (MHC) or non-MHC \textit{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 \((\text{NOD} \times \text{C57BL/6})\text{F1}\) mice but not in NOD mice. After costimulation blockade, islet allograft survival was prolonged in diabetes-resistant \text{NOD.B6}\text{Idd3} mice and shortened in diabetes-free \text{C57BL/6}\text{Idd3} congenic for the NOD \textit{Idd3} variant. Islet allograft tolerance could not be induced in diabetes-resistant \text{NOD.B10 Idd5} and NOD.B10 \text{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 \textit{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 \textit{Idd3} resistance locus improves self-tolerance and the induction of allotolerance. \textit{Diabetes} \textbf{53}:1972–1978, 2004

Replacement of insulin-producing islets of Langerehns 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 (\textit{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 \((\text{NOD} \times \text{C57BL/6})\text{F1}\) mice treated with costimulation blockade exhibit short skin allograft survival (5). The short survival of skin allografts in \((\text{NOD} \times \text{C57BL/6})\text{F1}\) 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 \((\text{NOD} \times \text{C57BL/6})\text{F1}\) mice that are treated with costimulation blockade. This contrasts sharply with the dominant resistance of \((\text{NOD} \times \text{C57BL/6})\text{F1}\) mice to skin allograft tolerance induction (5).

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Received for publication 26 December 2003 and accepted in revised form 29 April 2004.

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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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RESEARCH DESIGN AND METHODS

C57BL/6 (H2b) and C3H/HeJ (H2e) mice were obtained from the National Cancer Institute (Frederick, MD), the Jackson Laboratory (Bar Harbor, ME), or Taconic Farms (Germantown, NY). NOD/Mrk-TacR1R (line 1,094), NOD.B6.10dR444 (line 1,094), and NOD.B6.10dR445 (line 1,104) (all H2en) were obtained from Taconic Farms. NOD/Lt (H2e) mice were purchased from The Jackson Laboratory and maintained in our breeding colony at the University of Massachusetts Medical School. C57BL/6.NODc3 (H2b) and C57BL/6.NODc17 (H2e); hereafter called C57BL/6.H2en) 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 × C57BL/6)F1 mice were generated by a single intercross of the appropriate parental strains and are described by the standard nomenclature (female parent × 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 A, mouse polyomavirus, mouse hepatitis virus, lymphocytic choriomeningitis virus, polyoma, Mycoplasma pulmonis, and Encephalitozoon cuniculi. They were housed in a specific-pathogen-free facility in microisolator 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, 107 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 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 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 μg of anti-CD141 and immediately homogenized and RNA extracted in Ultraspec RNA (Biotecx, Houston, TX) using a high-speed Polytron. PolyA RNA was prepared using a Geneute 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 × 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 × 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 (H2e) islet allografts into fully major histocompatibility complex (MHC)-mismatched, chemically diabetic young (NOD × C57BL/6)F1 (H2e/n × H2e) 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] = 181 days; range, 41–218 days; Fig. 1A). The duration of islet allograft survival in (NOD × C57BL/6)F1 mice was statistically similar to that achieved in chemically diabetic C57BL/6 mice that were treated in the same way (MST = 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 × C57BL/6)F1 or the C57BL/6 recipients (P < 0.001). As expected, islet graft survival in control, nontolerized NOD, (NOD × 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 × 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 = 90 days; Fig. 1B). Islet allograft survival in (NOD × C57BL/6)F1 mice that were treated with anti-CD154 mAb monotherapy was also prolonged (MST = 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 × 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, insulitis, and insulin autoantibodies (33). Skin allograft survival on all NOD Idd2 congenic mice that were treated with costimulation blockade is known to be short (12). We hypothesized that Idd loci control the differential survival
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 costimulation 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 11148 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 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 × 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, tolerated 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 × C57BL/6)F1 mice.** Why (NOD × 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 × 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 × C57BL/6)F1 mice. Because NOD mice are NK1.1null, 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 × 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.

**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 × C57BL/6)F1 mouse 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 × 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 × 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 × 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 β2 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 × 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 × C57BL/6)F1 mice. In contrast, NOD-like abnormalities in dendritic cell maturation and in the response of CD4+ T-cells to costimulation blockade are expressed in a genetically dominant manner in (NOD × 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 × 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 × 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 × 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.

ACKNOWLEDGMENTS

This study was supported in part by grants AR35506 and AI42669 and institutional Diabetes Endocrinology Research Center grant DK52530 from the National Institutes
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1978

DIABETES, VOL. 53, AUGUST 2004