Autoimmune Diabetes and Resistance to Xenograft Transplantation Tolerance in NOD Mice


Costimulation blockade induces prolonged rat islet and skin xenograft survival in C57BL/6 mice. Nonobese diabetic (NOD) mice, which are used to model human autoimmune diabetes, are resistant to costimulation blockade–induced allograft tolerance. We tested the hypothesis that NOD mice would also be resistant to costimulation blockade–induced rat xenograft tolerance. We report that rat islet xenograft survival is short in spontaneously diabetic NOD mice treated with a tolerizing regimen of donor-specific transfusion and anti-CD154 antibody. Rat islet xenograft survival is only marginally longer in chemically diabetic NOD mice treated with costimulation blockade but is prolonged further in NOD Idd congenic mice bearing C57-derived chromosome 3 loci. Reciprocally, the presence of NOD-derived chromosome 3 loci shortens islet xenograft survival in tolerized C57BL/6 mice. Islet xenograft survival is longer in tolerized NOD.CD4ζ−/− and (NOD × C57BL/6)F1 mice than in NOD mice but still much shorter than in C57BL/6 mice. Skin xenograft survival in (NOD × C57BL/6)F1 mice treated with costimulation blockade is short, suggesting a strong genetic resistance to skin xenograft tolerance induction. We conclude that the resistance of NOD mice to xenograft tolerance induction involves some mechanisms that also participate in the expression of autoimmunity and other mechanisms that are distinct. Diabetes 54:107–115, 2005

The NOD mouse is an excellent model of human type 1 diabetes (1,2). It develops insulitis and diabetes that are T-cell dependent, with both the CD4+ and CD8+ T-cell subsets contributing to disease (1,2). In addition to targeted destruction of pancreatic β-cells (3,4), NOD mice also exhibit numerous defects in their immune system (2). Their CD8+ T-cells have been reported to be resistant to the induction of antigen-induced apoptosis (5), and they are susceptible to other intercurrent autoimmune diseases including thyroiditis and sialadenitis (2).

Susceptibility to diabetes in both humans and NOD mice is genetically complex (6,7). In NOD mice, as many as 27 Idd loci on 11 different chromosomes have been implicated in disease susceptibility or resistance (8,9). To study the role of these loci on the development of diabetes, investigators have used congenic introgression to replace loci in NOD mice with loci derived from disease-resistant strains. Congenic NOD mice bearing Idd resistance loci exhibit varying degrees of protection from insulitis and diabetes (10–13).

We have previously documented that in addition to autoimmunity, NOD mice have an inherent genetic resistance to allograft tolerance induced by a costimulation blockade protocol consisting of a donor-specific transfusion (DST) and anti-CD154 monoclonal antibody (mAb) (14). The resistance of NOD mice to skin allograft tolerance was determined to be a dominant genetic trait because it also characterizes (NOD × C57BL/6)F1 mice (15). In contrast, the resistance of NOD mice to islet allograft tolerance induced by costimulation blockade is a genetically recessive trait that appears to be controlled in part by loci on chromosome 3 (16).

Because the availability of allogeneic islets for human transplantation is limited, there is a need to investigate the potential of tolerance induction procedures to prolong islet xenograft survival. We have previously shown that treatment of chemically diabetic C57BL/6 mice with DST and anti-CD154 mAb leads to prolonged survival of rat islet and skin xenografts (17,18) and xenogeneic neonatal porcine islet cell clusters (19). Similarly, anti-CD45RB mAb (20), anti-CD4 mAb (21), or coadministration of CTLA4-Fc and anti-CD154 mAb (22,23) leads to prolonged islet xenograft survival in mice. Islet xenograft tolerance has also been reported in monkeys given anti-CD3 immunotoxin, cyclosporine, and steroids (24).

Here, we tested the efficacy of our xenograft tolerance induction protocol based on DST and anti-CD154 mAb in NOD, NOD congenic, knockout, and (NOD × C57BL/6)F1 mice. We tested the hypothesis that genetic elements that contribute to diabetes susceptibility in NOD mice also participate in the control of islet and skin xenograft survival induced by costimulation blockade.

RESEARCH DESIGN AND METHODS
C57BL/6 (H2b) mice were obtained from the National Cancer Institute (Frederick, MD) or from The Jackson Laboratory (Bar Harbor, ME). NOD/MrktacBR (H2c), NOD.B6 Idd3/Idd10/Idd18 R233 (H2c), line 1538 at
Taconic), and NOD.B10 Idd9 (H2z7, line 1104 at Taconic) mice were obtained from Taconic Farms (Germantown, NY). Breeding pairs of B6.NOD.C3 (H2^hp) were obtained from Dr. Ed Leiter at The Jackson Laboratory and bred in our facility (25). NOD.CDSa~~/~ (H2^hp) mice (official designation: NOD.102982[B6]-Cdsa~13048/Dvs) and NOD.CDVa~~/~ (H2^hp) mice (official designation: NOD.12892[B6]-Cdva~13048/Dvs) were obtained from a breeding colony maintained by one of the authors (D.V.S.) at The Jackson Laboratory. The latter two congenic stocks of NOD mice are homozygous for linkage markers delineating all known Idd loci of NOD origin (26–28). NOD.CDSa~~/~ mice (1/17 females diabetic by age 30 weeks) (15) and NOD.CDVa~~/~ mice (0 of 18 females diabetic by age 30 weeks) (28) are both strongly diabetes resistant. LEW rats (RT1”) were obtained from Harlan Sprague Dawley (Indianapolis, IN).

All animals were housed under specific pathogen-free conditions in sterile cages with autoclaved bedding and given autoclaved food and water ad libitum. Rats and mice used in these studies were certified to be serologically free of Sendai virus, pneumonia virus of mice, sialodacryoadenitis virus, rat corona virus, Kilham rat virus, I (Toolan’s virus), GD7, Reo-3, lymphocytic choriomeningitis virus, mouse adenovirus, Hantaan virus, Mycoplasma pulmonis, and encephalitozoon cuniculi. All animals were maintained in accordance with recommendations in the U.S. Government Principles for the Utilization and Care of Vertebrate Animals used in Testing, Research, and Training, the Guide for the Care and Use of Laboratory Animals (29) and local institutional guidelines.

Pancreatic islet transplantation. Spontaneously diabetic NOD mice were ~4–5 months old at the time of grafting. C57BL/6, NOD, and congenic strain recipient mice at 8–10 weeks of age were rendered hyperglycemic by a single intraperitoneal injection of 150 or 160 mg/kg of streptozotocin. Plasma glucose was measured using a Beckman II glucose analyzer (Beckman, Fullerton, CA). The diagnosis of diabetes in graft recipients was established by the observation of a plasma glucose concentration >250 mg/dl on at least two different days before transplantation. Pancreatic islets were harvested from donor rats by collagenase digestion (30) and transplanted at a dose of 15–20/kg body wt into the renal subcapsular space of the recipient. Islet donors were retired breeder male LEW rats. Grafts were judged to have been rejected after the plasma glucose concentration rose to >250 mg/dl on 2 successive days after a minimum of 2 days of normoglycemia. Only one animal, an NOD (~C57/LEW)F1 recipient, never became normoglycemic and was excluded from the analysis as a technical failure. The rate of primary islet xenograft nonfunction among all transplanted streptozotocin-induced diabetic mice (Figs. 1 and 2) was 0.7% (1 of 149). To confirm graft function in recipients, all islet graft specimens for the presence of inflammatory cells and insulin. Because no skin grafts survived indefinitely, none were studied histologically.

RESULTS

Rat islet xenograft survival in NOD mice

Rat islet xenograft survival in spontaneously diabetic NOD mice. We first transplanted LEW rat islets into spontaneously diabetic NOD mice. As expected, in otherwise untreated diabetic NOD mice, graft rejection was rapid; the median survival time (MST) was 8 days (group 1) (Table 1). Similarly, islet xenograft survival was short in spontaneously diabetic NOD recipients treated with anti-CD154 mAb monotherapy (group 2, MST = 8 days). We next treated diabetic NOD mice with a donor-DST plus anti-CD154 mAb, a protocol that induces prolonged islet xenograft survival in chemically diabetic C57BL/6 mice (17). Treatment with DST plus anti-CD154 mAb at doses of 0.25 mg (group 3, MST = 10 days) or 0.5 mg (group 4, MST = 13 days) failed to prolong islet graft survival.

Islet xenograft survival in chemically diabetic NOD mice. NOD mice have recently been shown to resist allotransplantation tolerance based on costimulation blockade; this resistance was observed to apply to both skin and islet allografts and could be separated genetically from the phenotype of autoimmunity (14,15). To begin to discriminate among potential mechanisms underlying the short islet xenograft survival in autoimmune diabetic NOD mice, we next transplanted rat islet xenografts into chemically diabetic NOD recipients.

LEW rat islet xenograft survival, as expected, was short in otherwise untreated chemically diabetic NOD (MST = 12 days) and C57BL/6 (MST = 10 days; Fig. 1C) recipients. Treatment with anti-CD154 mAb monotherapy significantly prolonged rat islet xenograft survival in NOD mice (MST = 22 days; P < 0.025 vs. untreated controls) and to an even greater extent in C57BL/6 mice (MST = 32 days; Fig. 1B; P < 0.001 vs. untreated C57BL/6 controls and P < 0.05 vs. treated NOD mice) mice. Treatment with both DST and anti-CD154 mAb monotherapy further prolonged rat islet xenograft survival in both NOD (MST = 32 days; P < 0.01) and C57BL/6 (MST = 116 days; Fig. 1A; P < 0.001) mice. Again, graft survival in these C57BL/6 recipients was significantly longer than that observed in the similarly treated NOD mice (P < 0.001).

Rat islet survival in Idd congenic mice

NOD-Idd congenic mice. To investigate the genetic basis for the poor survival of islet xenografts in NOD mice following costimulation blockade, we studied Idd congenic NOD mice. We first asked if C57-derived Idd diabetes-resistant loci that reduce the frequency of insulinitis and diabetes in NOD mice would restore susceptibility to the induction of islet xenograft tolerance. Two groups of NOD congenic mice were tested. The first was an NOD congenic stock (NOD.Idd3/10/18) carrying C57BL/6-derived Idd3, Idd10, and Idd18 resistance loci. The incidence of diabetes at 7 months of age in NOD.Idd3/10/18 congenic mice ranges from 3 to 9% (10,35,36). Duration of rat islet xenograft survival was significantly longer in NOD.Idd3/10/18 congenic mice (MST = 58 days; Fig. 1A) treated with
costimulation blockade than in similarly treated NOD mice ($P < 0.001$). However, graft survival in the congenic mice was again significantly shorter than that achieved in C57BL/6 mice ($P < 0.001$). As expected, rat islet xenografts were rapidly rejected in otherwise untreated diabetic NOD. Idd3/10/18 congenic mouse (MST = 11 days; Fig. 1C).

FIG. 1. Eight-week-old mice of various strains (indicated in the figure) of either sex were injected with streptozotocin to induce diabetes as described in RESEARCH DESIGN AND METHODS. All mice received a LEW rat islet xenograft. A: Mice received a single transfusion consisting of $1 \times 10^7$ LEW rat spleen cells intravenously on day 7 and four intraperitoneal injections of 0.25 mg anti-CD154 mAb as indicated on days 7, 4, 0, and 4 relative to islet transplantation on day 0. B: Mice received anti-CD154 mAb on the same schedule but no DST. C: Mice received only the islet xenograft. A: *$P < 0.001$ vs. all other groups and **$P < 0.001$ vs. NOD. B: *$P < 0.05$ vs. NOD. No other paired comparisons are significant. Small vertical bars indicate censored data, i.e., mice that were found dead or were removed from the study for other analyses.
We next studied a NOD stock congenic for the C57BL/10-derived Idd9 locus (37). The incidence of diabetes in these mice is only 3%. The median survival time of rat islet xenografts in NOD.B10 Idd9 mice treated with DST and anti-CD154 mAb was only 31 days (Fig. 1A) and was statistically similar to that observed in NOD mice (P = NS). Rat islet xenografts, as expected, were rapidly rejected in otherwise untreated diabetic NOD.B10 Idd9 mice (MST = 13 days; Fig. 1C).

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C57BL/6.Idd congeneric mice. Rat islet xenograft survival is prolonged in NOD mice bearing segments of chromosome 3 derived from diabetes-resistant C57BL/10 mice. To confirm the contribution of loci on chromosome 3 to prolonged islet xenograft survival, we next studied C57BL/6.NOD.C3 mice. These mice carry the diabetes-susceptible NOD-derived Idd3, Idd17, Idd10, and Idd18 alleles on chromosome 3 (25). The median survival time of rat islet xenografts in C57BL/6.NOD.C3 mice treated with DST and anti-CD154 mAb is prolonged compared to NOD mice (Fig. 1B).

**TABLE 1**

Duration of rat islet xenograft survival in spontaneously diabetic nod mice

<table>
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<tr>
<th>Group</th>
<th>Dose of anti-CD154 mAb</th>
<th>DST</th>
<th>Graft survival (days)</th>
<th>MST (days)</th>
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<td>10</td>
</tr>
<tr>
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<td>0.5 mg</td>
<td>Yes</td>
<td>6, 8, 13, 13, 13, 16</td>
<td>13</td>
</tr>
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</table>

Spontaneously diabetic NOD mice were untreated (group 1), treated with anti-CD154 mAb monotherapy (0.25 mg/injection) on days −7, −4, 0, and 4 relative to grafting (group 2), or injected with a DST consisting of 1 × 10⁷ LEW spleen cells on day −7 and given 0.25 mg/injection anti-CD154 mAb (group 3) or 0.5 mg/injection anti-CD154 mAb (group 4) on the same schedule as used for group 2. There are no statistically significant differences in islet xenograft survival among any of the treated groups.
anti-CD154 mAb was 63 days (Fig. 2A). This was significantly shorter than that achieved in similarly treated diabetic C57BL/6 (MST >168 days; Fig. 2A; P < 0.001). As expected, islet xenograft survival was brief in otherwise untreated diabetic C57BL/6.NOD.C3 mice (MST = 9 days; Fig. 2B). These data support our finding of an important role of congenic intervals on chromosome 3 in the control of the induction of tolerance to rat islet xenografts in NOD mice.

**Cellular mechanisms underlying NOD resistance to rat xenograft survival in NOD mice following co-stimulation blockade**

**Islet xenograft survival in NOD.CD4a−/− mice.** Survival of rat xenografts in C57BL/6 mice treated with DST and anti-CD154 mAb can be further prolonged by concurrent treatment with a depleting anti-CD4 mAb (18). We next hypothesized that genetic deletion of CD4+ T-cells would facilitate islet xenograft survival in NOD recipients. Islet xenograft survival in NOD.CD4a−/− mice treated with DST and anti-CD154 mAb was significantly longer (MST = 73 days) than in similarly treated wild-type NOD mice (Fig. 2A; P < 0.01). Islet xenograft survival in otherwise untreated NOD.CD4a−/− mice (MST = 32 days; Fig. 2B) was also longer than in untreated NOD recipients (P < 0.025).

**Islet xenograft survival in NOD.CD8a−/− mice.** Allograft survival in mice treated with co-stimulation blockade depends in part on the deletion of CD8+ T-cells (38–42), and CD8+ T-cells in NOD mice reportedly resist the induction of peripheral tolerance (5). To determine if the resistance of NOD mice to xenograft tolerance based on co-stimulation blockade involves the failure to delete CD8+ T-cells, we next studied NOD.CD8a−/− mice. Rat islet xenograft survival in NOD.CD8a−/− mice treated with DST plus anti-CD154 mAb (MST = 49; Fig. 2A) was not significantly different than that achieved in similarly treated NOD mice (P = NS).

**Islet xenograft survival in (NOD × C57BL/6)F1 mice.** These data suggest that resistance to islet xenograft tolerance is partially overcome in NOD congenic mice that bear specific Idd resistance loci on chromosome 3 or lack CD4+ T-cells. However, the presence of this group of Idd resistance loci does not prolong islet xenograft survival to the extent that can be achieved in C57BL/6 mice. Most NOD Idd susceptibility loci are recessive (7). (NOD × C57BL/6)F1 mice are heterozygous at all Idd loci and thus bear a large number of C57BL/6-derived diabetes resistance alleles. (NOD × C57BL/6)F1 mice are completely protected from autoimmune diabetes (43), but surprisingly they remain resistant to the induction of skin allograft tolerance by costimulation blockade (15). To determine if they would similarly resist xenograft tolerance induction, chemically diabetic (NOD × C57BL/6)F1 mice were treated with DST and anti-CD154 mAb and transplanted with rat islets. Median duration of graft survival was 83 days (Fig. 2A), an outcome significantly superior to that achieved in NOD (P < 0.001) mice but inferior to that achieved in C57BL/6 (P < 0.001) mice.

**Rad skin xenograft survival in (NOD × C57BL/6)F1 mice.** The dominant resistance to allograft tolerance induction in (NOD × C57BL/6)F1 mice was documented in studies using skin not islet grafts (15). We therefore tested the hypothesis that skin xenograft survival (NOD × C57BL/6)F1 mice treated with DST and anti-CD154 mAb would be short.

As expected, rat skin xenografts were rapidly rejected by untreated C57BL/6 (MST = 7 days), NOD (MST = 10 days), and (NOD × C57BL/6)F1 (MST = 7 days; Fig. 3B) mice. Again, as expected, rat skin xenograft survival was prolonged in C57BL/6 mice treated with DST plus anti-CD154 mAb (MST = 59 days; Fig. 3A). Duration of skin xenograft survival in similarly treated (NOD × C57BL/6)F1 mice was significantly shorter (MST = 18 days; P < 0.001) and statistically similar to that observed in similarly treated NOD mice (MST = 14 days; Fig. 3A; P = NS).

**Discussion**

These data document that co-stimulation blockade is ineffective in prolonging islet xenograft survival in NOD mice. This was true of both spontaneously diabetic NOD mice and NOD mice with chemically induced hyperglycemia. To determine if the ineffectiveness of co-stimulation blockade was due to recurrent autoimmunity or to a generalized resistance of NOD mice to tolerance induction (14,15,44), we used congenic NOD mice and (NOD × C57BL/6)F1 mice. Our data revealed that islet xenograft tolerance induction is regulated in part by Idd loci on chromosome 3 and involves CD4+ T-cells. We observed that islet xenograft survival is prolonged in (NOD × C57BL/6)F1 mice but remains much shorter than that observed in C57BL/6 mice. These observations were unexpected given the fact that islet allograft survival in normal mice requires the presence of CD4+ T-cells (31) and is permanent in (NOD × C57BL/6)F1 mice treated with costimulation blockade (16). These data in a xenograft model document that important differences exist in mechanisms that control the resistance of NOD mice to the induction of allograft versus xenograft tolerance.

In contrast to the survival of islet allografts in (NOD × C57BL/6)F1 mice treated with costimulation blockade (16), which is permanent, islet xenograft survival in these animals is prolonged but not permanent. These results suggest that in contrast to islet allograft tolerance induction (15), resistance to islet xenograft tolerance induction in NOD mice is a genetically dominant trait. When applied to skin xenografts, costimulation blockade is not effective in prolonging graft survival in either NOD or (NOD × C57BL/6)F1 mice. These results suggest that, as is true of their resistance to skin allograft tolerance induction (15),
resistance to skin xenograft tolerance induction in NOD mice is also a genetically dominant trait.

Our data document that islet xenograft survival in autoimmune diabetic NOD mice treated with costimulation blockade is short. This could result from either autoimmune recurrence or a generalized resistance of NOD mice to xenotolerance induction by costimulation blockade. We have previously shown that in chemically diabetic NOD congenic mice and (NOD x C57BL/6)F1 mice, the phenotypes of autoimmune diabetes and transplantation tolerance are not under completely overlapping genetic control (14–16, 45). In those studies, we observed no association of Idd loci with skin allograft tolerance induction (14), and, surprisingly, we found that skin allograft survival was short even in (NOD x C57BL/6)F1 mice (15). However, when islet allografts were tested, permanent allograft survival was observed in (NOD x C57BL/6)F1 mice (16). That study further documented that Idd loci on chromosome 3 were, at least in part, responsible for regulation of islet allograft survival in NOD mice treated with costimulation blockade (16).

To determine if Idd loci control rat islet xenograft survival in chemically diabetic NOD mice treated with costimulation blockade, we tested three congenic strains. In NOD.B6 Idd3/10/18 congenic mice, the cumulative frequency of diabetes at 7 months of age is 3–9% (35, 36), yet these congenic mice exhibited only slightly prolonged xenograft survival compared with NOD mice. More strikingly, NOD.B10 Idd9 mice, in which the cumulative frequency of diabetes at 7 months of age is only 3% (37), did not exhibit prolonged islet xenograft survival compared with NOD mice. These data suggest that genetic control of autoimmunity and tolerance to islet xenografts are partially distinct.

To confirm the importance of chromosome 3 loci as a determinant of islet xenograft survival, which could not be assumed a priori, we tested C57BL/6.NOD.c3 congenic mice. These mice bear a large segment of NOD chromosome 3 that includes Idd3, Idd17, Idd10, and Idd18 (25). Of particular interest is Idd3, which has been narrowed to a small interval containing eight genes. Three of these genes, Il2, Il21, and Fgf2, have known functions that could...
contribute to the establishment of immunological tolerance (46 and L.S.W., L.B.P., unpublished observations). Because Idd3 has been shown to be important in the induction of islet allograft tolerance by costimulation blockade (16), we are currently investigating if genes within the Idd3 region are responsible for the effect of chromosome 3 on islet xenograft tolerance induction.

The results of our studies using congenic mice document that genetic reduction of autoimmunity in NOD mice does not invariably lead to prolonged islet xenograft survival after costimulation blockade. Even more striking was our discovery that islet xenograft survival in (NOD × C57BL/6)F1 mice treated with costimulatory blockade, although prolonged relative to that observed in NOD mice, was much shorter than that achieved in C57BL/6 mice. In (NOD × C57BL/6)F1 mice treated with DST and anti-CD154 mAb, islet allograft is permanent (16). In their aggregate, these data imply that multiple genes must control islet xenograft survival in mice treated with costimulation blockade. The data further suggest that the genetic mechanisms that control islet allograft and xenograft survival are only partially overlapping. This interpretation is supported by our analysis of the cell subsets important in islet xenograft survival. NOD.CD4a−/− mice treated with costimulation blockade exhibit prolonged islet xenograft survival whereas anti-CD4 mAb treatment completely abrogates islet allograft survival, even in normal mice (47).

Skin allograft tolerance, however, appears to be controlled by mechanisms different from those important for islet allograft tolerance. In a complete CD40 knockout model system, islet allograft survival was prolonged whereas skin allograft survival was short (48). More recently, we have found that depletion of CD25+ cells shortens skin allograft survival whereas it has minimal effects on islet allograft survival (47). We speculate that skin allograft survival is more dependent on the presence of regulatory T-cells than is islet allograft survival, a hypothesis currently under investigation.

Based on the published experience with allografts, it was not surprising that skin xenograft survival in NOD mice treated with costimulation blockade appears to be controlled at least in part by mechanisms different from those that control islet xenograft survival. First, we observed that the duration of islet xenograft survival is somewhat prolonged in otherwise untreated NOD.CD4a−/− mice, whereas skin xenografts are promptly rejected. Second, we observed the duration of islet xenograft survival in (NOD × C57BL/6)F1 mice is relatively prolonged, whereas skin xenograft survival is very short. These data suggest that skin and islet xenograft survival are controlled by different cellular mechanisms and that resistance to skin xenograft tolerance induction is a dominant trait in NOD mice.

We have previously shown that some NOD-like defects are corrected in (NOD × C57BL/6)F1 mice, whereas others are genetically dominant cellular defects (15). For example, NOD-like defects in natural killer cell function and macrophage development are corrected in (NOD × C57BL/6)F1 mice, whereas NOD-like abnormalities in dendritic cell maturation are genetically dominant. Of particular interest is the observation that natural killer cells appear to be normal in (NOD × C57BL/6)F1 mice. NK cells have an important role in xenograft rejection (49–51), and although defective in NOD mice (2), this defect does not appear to be the cause of their resistance to xenograft tolerance induction. It remains to be determined if other cellular defects, such as abnormal maturation of dendritic cells, contribute to the relative resistance of (NOD × C57BL/6)F1 mice to xenograft tolerance induction.

In summary, NOD mice are resistant to not only allograft (14,15) but also xenograft tolerance induction. This resistance is largely independent of the degree of protection from diabetes that is conferred by Idd resistance loci. The data highlight the genetically controlled differences between skin and islet xenograft tolerance induced by costimulation blockade, and they lend support to the “genetic threshold” hypothesis for resistance of NOD mice to transplantation tolerance.

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