Idd loci synergize to prolong islet allograft survival induced by costimulation blockade in NOD mice

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Et al.
Idd Loci Synergize to Prolong Islet Allograft Survival Induced by Costimulation Blockade in NOD Mice

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OBJECTIVE—NOD mice model human type 1 diabetes and are used to investigate tolerance induction protocols for islet transplantation in a setting of autoimmunity. However, costimulation blockade–based tolerance protocols have failed in prolonging islet allograft survival in NOD mice.

RESEARCH DESIGN AND METHODS—To investigate the underlying mechanisms, we studied the ability of costimulation blockade to prolong islet allograft survival in congenic NOD mice bearing insulin-dependent diabetes (Idd) loci that reduce the frequency of diabetes.

RESULTS—The frequency of diabetes is reduced in NOD.B6 Idd3 mice and is virtually absent in NOD.B6/B10 Idd3 Idd5 mice. Islet allograft survival in NOD.B6 Idd3 mice treated with costimulation blockade is prolonged compared with NOD mice, and in NOD.B6/B10 Idd3 Idd5 mice, islet allograft survival is similar to that achieved in C57BL/6 mice. Conversely, some Idd loci were not beneficial for the induction of transplantation tolerance. Alloreactive CD8 T-cell depletion in (NOD × CBA)F1 mice treated with costimulation blockade was impaired compared with similarly treated (C57BL/6.H2b17 × CBA)F1 mice. Injection of exogenous interleukin (IL)-2 into NOD mice treated with costimulation prolonged islet allograft survival. NOD.B6 Idd3 mice treated with costimulation blockade deleted alloreactive CD8 T-cells and exhibited prolonged islet allograft survival.

CONCLUSIONS—IId2 is the Idd3 diabetes susceptibility gene and can influence the outcome of T-cell deletion and islet allograft survival in mice treated with costimulation blockade. These data suggest that Idd loci can facilitate induction of transplantation tolerance by costimulation blockade and that IL-2/Idd3 is a critical component in this process. Diabetes 58: 165–173, 2009

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Received 25 February 2008 and accepted 17 October 2008.
Published ahead of print at http://diabetes.diabetesjournals.org on 4 November 2008. DOI: 10.2337/db08-0275.

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T he NOD mouse is a model of type 1–like autoimmune diabetes and is used to study costimulation blockade–based transplantation tolerance within the context of autoimmunity (1–4). However, costimulation blockade protocols fail in NOD mice. To investigate further the cellular and genetic control of costimulation blockade–induced transplantation tolerance, we used NOD Idd congenic mice that have small introgressed regions of genetic intervals derived from diabetes-resistant C57 stocks. These mice exhibit varying degrees of protection from autoantibodies, insulitis, and diabetes (5). Using Idd congenic NOD mice, we have observed that islet allograft survival is improved by the addition of the diabetes-protective Idd3 locus (6,7).

Idd3 modulates infiltration of autoreactive lymphocytes into the islets (8), and there is compelling evidence that Idd3 is the interleukin (IL)-2 gene (9). In vivo stimulated NOD T-cells produce twofold less IL-2 mRNA than cells from NOD congenic mice having protective alleles at Idd3 (9,10). Neutralizing antibodies to IL-2 lead to accelerated disease in NOD mice (11), and targeted genetic disruption of IL-2 accelerates type 1–like autoimmune diabetes (9). Treatment with exogenous IL-2 inhibits diabetes development in NOD mice and improves T regulatory (Treg) function (12). IL-2 is also known to have a nonredundant role in CD8 T-cell activation–induced cell death via the CD95 (Fas) pathway (13), is required for the development of self-tolerance (14), and is essential for the induction of allograft tolerance by costimulation blockade (15). However, IL-2 is a double-edged sword, since administration of IL-2 in vivo can either enhance or depress a cytotoxic T lymphocyte (CTL) response (16).

In this study, we show that costimulation blockade fails to delete alloreactive CD8 T-cells in NOD mice. Genetic replacement of IL-2 in NOD.B6 Idd3 mice enhances alloreactive CD8 T-cell deletion and improves islet allograft survival. Finally, we show that Idd3 synergizes with genes within the Idd5 interval, leading to permanent islet allograft survival in a majority of NOD.B6/B10 Idd3 Idd5 mice treated with costimulation blockade.
were comparable (9), these groups have been combined for presentation and are referred to in the text as NOD.B6 Idd3 mice. A schematic of the congenc intervals on mouse chromosomes is shown in Fig. 1. C57BL/6.NODc17 (H2g7, C57BL/6.H2b) mice were developed by Edward Wakeland, University of Texas Southwest Medical Center, Dallas, Texas (17). (K5B CBA × C57BL/6.H2b) F1 mice (K5B CBA × NOD) F1 mice were generated by a single intercross of the appropriate parental strains and were bred in our facility. The KB5 TCR transgene is expressed in CBA (H2r) mice by CD8+ T-cells and is specific for native H2-K^r (18).

Animals were certified to be free of infectious pathogens, housed in microisolator cages within a specific pathogen-free facility, and given autoclaved food and acidified water ad libitum. All animal use was in accordance with the guidelines of the 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).

Generation of KB5 synchimeras. KB5 synchimeric mice were generated using a previously described procedure (18). Briefly, (CBA/J × NOD) F1 mice carrying a single copy of a B6-like allele of the IL-2 gene (19) and (CBA/J × C57BL/6.H2b) F1 mice carrying two copies of a B6 or B6-like IL-2 gene were irradiated with 400 cGy from a 137Cs source (Gammaccell 40; Atomic Energy of Canada, Ltd.) and given intravenous injections of 0.5 x 10^6 (KBS CBA × NOD) F1 or (KBS CBA × C57BL/6.H2b) F1 transgenic bone marrow, respectively. Mice were entered into experiments 8–12 weeks after bone marrow transplantation. Antibodies and flow cytometry. The KB5-specific clonotypic Desire` (DES) antibody was the gift of Dr. John Iacomini (Harvard Medical School, Boston, MA). Fluorescein isothiocyanate– conjugated anti-mouse IgG2a (clone R19-15) and PerCP-conjugated anti-mouse CD8 (clone 53-6.7) were obtained from BD PharMingen (San Diego, CA). Isotype control mAbs including rat PerCP-conjugated IgG2a (clone R35-95) and mouse IgG2a κ anti-TNP (clone G155-178) were purchased from BD PharMingen.

Two-color flow cytometry analyses of lymph node and spleen cells were performed (20). Briefly, cells were incubated with anti-FC-γRIII/II mAb (clone 2.4G2) to eliminate nonspecific Fc binding. Cells were washed, incubated with fluorescein isothiocyanate– conjugated anti-mouse IgG2a mAb plus a mixture of conjugated mAbs. Whole marrow cells, respectively. Mice were entered into experiments 8–12 weeks after bone marrow transplantation.

RESULTS

Idd loci in NOD mice. Idd loci in NOD mice are referred to in the text as NOD.B6 Idd3 mice. A schematic of the congenc intervals on mouse chromosomes is shown in Fig. 1. C57BL/6.NODc17 (H2g7, C57BL/6.H2b) mice were developed by Edward Wakeland, University of Texas Southwest Medical Center, Dallas, Texas (17). (K5B CBA × C57BL/6.H2b) F1 mice (K5B CBA × NOD) F1 mice were generated by a single intercross of the appropriate parental strains and were bred in our facility. The KB5 TCR transgene is expressed in CBA (H2r) mice by CD8+ T-cells and is specific for native H2-K^r (18).

In vivo cytotoxicity assay. The in vivo cytotoxicity assay was performed as previously described (23). Briefly, 6- to 8-week-old male NOD, C57BL/6, or NOD.B6 Idd3 mice were treated on day −7 with DST and on days −7 and −4 with anti-CD154 mAb relative to depletion of natural killer (NK) cells on day −8 by injection of 1 mg anti-CD122 mAb (24). On day 0 (the normal day of islet transplantation), carboxyfluorescein diacetate succinimidyl ester–labeled splenocytes were adoptively transferred intravenously into naive recipient mice or into the indicated recipient mice. Spleens from recipient mice were harvested 20 h later, and the survival of each transferred population was assessed by flow cytometry at this time. Specific lysis was calculated as described (23).

In vitro NK cell cytotoxicity assay. In the in vitro NK cell cytotoxicity assay was performed as previously described (25) using spleen cells from mice injected 24 h previously with 100 μg polynosinic:polycytidylic (poly E) as effectors and γ−δ T-cell–labeled YAC-1 cells as targets. In some cohorts, NK cells were depleted by injection of 1 mg anti-CD122 mAb 1 day before poly E administration. Percent specific lysis was calculated as follows: % specific lysis = ([experimental lysis – spontaneous lysis]/[maximal lysis – spontaneous lysis]) x 100.

Statistical analysis. Median duration of allograft survival is presented. Graft survival among groups was compared using the method of Kaplan and Meier. The equality of allograft survival distributions for animals in different treatment groups was tested using the log rank statistic. P values <0.05 were considered statistically significant. Data are presented as the mean ± 1 SD. Comparisons of two means used Student’s t test with separate variance estimates. Comparisons of three or more means used one-way ANOVA and the least significant difference procedure for a posteriori contrasts.

RESULTS

Islet allograft rejection in chemically diabetic male NOD mice is not due to islet autoimmunity. Islet allograft survival in chemically diabetic male NOD mice treated with our costimulation blockade protocol is relatively short (6). However, in those experiments, islet graft rejection could have resulted from islet autoimmunity or the failure to induce allograft tolerance. To investigate this, chemically diabetic NOD mice were transplanted with syngeneic NOD-scid islets. As reported previously (26), we observed through 150 days after islet transplantation that all mice (5/5) remained normoglycemic. Analysis of the islet-bearing kidney revealed an insulin-producing islet graft present at the time of necropsy with only a small amount of leukocytic infiltrate. These data suggest that islet allograft rejection in our model system results from failure to induce tolerance and not to the development of islet autoimmunity.

Islet allograft survival in NOD.B6 Idd10 Idd18 and NOD.B6 Idd3 Idd10 Idd18 congenic mice after treatment with DST and anti-CD154 mAb. Islet allograft survival is prolonged in NOD mice bearing the diabetes-resistant Idd3 congenic interval (6). However, Idd3 is only partially protective but, when combined with certain other Idd loci, almost completely protects NOD mice from diabetes (Fig. 1). We hypothesized that combinations of Idd loci that are strongly protective against diabetes would enhance islet allograft survival. NOD mice congenic for the Idd10 Idd18 intervals have reduced incidence of diabetes (27,28) and, when combined with Idd3, have a very low frequency of diabetes (8,27,29). Confirming our previous report (6), islet allograft survival in NOD mice treated with costimulation blockade is short (median survival time [MST] was 74 days), whereas permanent islet allograft survival (MST >240 days) is observed in the majority of similarly treated C57BL/6 mice (Fig. 2A). We also confirmed that NOD mice bearing the Idd3 congenic interval exhibit islet allograft survival that is prolonged (MST = 140 days, [6]) compared with NOD mice but significantly shorter than that achieved in
FIG. 1. Schematic representation of candidate gene interval and chromosomal location. The filled bars represent B10-derived or B6-derived congenic segments on chromosomes 1 and 3, respectively. The arrows represent the size of each Idd interval as previously defined using additional congenic strains of mice: Idd3 (650 kb) (ref. 9), Idd10 (950 kb) (ref. 48), Idd18 (4.0 Mb) (ref. 29), Idd5.1 (2.1 Mb) (ref. 30), Idd5.2 (1.52 Mb) (ref. 30), and Idd5.3 (3.6 Mb) (ref. 31). The “diabetes” column indicates the percentage of females developing diabetes by 7 months of age. Where a range is indicated, this summarizes the results of a number of frequency studies performed over many years.
C57BL/6 mice (Fig. 2B). Surprisingly, we observed that NOD Idd10 Idd18 congenic mice exhibited shorter islet allograft survival (MST = 63 days) than that achieved in NOD mice (Fig. 2). Combination of the Idd10 Idd18 genetic intervals with the beneficial effects of Idd3 did not alter islet allograft survival compared with that achieved in NOD.B6 Idd3 congenic mice (NS, Fig. 2A and B).

**Protective Idd5 and Idd3 alleles synergize to prolong islet allograft survival in chemically diabetic NOD mice treated with DST and anti-CD154 mAb.** We next studied the effects of Idd5 alone or in combination with Idd3. NOD mice bearing the Idd5 disease-resistant loci are partially protected from diabetes (30,31), and the addition of Idd3 protective alleles results in nearly complete disease suppression (31,32).

NOD.B10 Idd5 congenic mice treated with DST and anti-CD154 did not exhibit prolonged islet allograft survival (MST = 96 days) compared with that achieved in NOD mice (NS, Fig. 2B) and was significantly shorter than that achieved in NOD.B6 Idd3 congenic mice (P < 0.005, Fig. 2B). These data, combined with the NOD.B6 Idd10 Idd18 results, demonstrate that enhancement of islet allograft survival by Idd loci does not strictly correlate with the extent to which they suppress diabetes.

Strikingly, NOD mice bearing the diabetes-protective Idd3 and Idd5 congenic intervals exhibited prolonged islet allograft survival (MST > 250 days), which was similar to that achieved in C57BL/6 mice (NS) and significantly greater than that achieved in NOD (P < 0.005) or NOD.B10 Idd5 mice (P < 0.01, Fig. 2B). In long-term surviving islet allografts, we routinely observed minimal to no mononuclear infiltration.

**Islet allograft survival in Idd3 congenic NOD mice bearing different Idd5 subregions treated with costimulation blockade.** The Idd5 interval congenic strain used in this study contains at least three diabetes-resistant loci: Idd5.1, Idd5.2, and Idd5.3 (30,31). To begin to identify the congenic Idd5 interval that synergizes with Idd3 to prolong islet allograft survival in NOD mice treated with costimulation blockade, we tested two newly developed NOD congenic lines that carry the B6-derived Idd3 congenic interval as well as the B10-derived Idd5.1 or Idd5.3 intervals (Fig. 1).

NOD.B6 Idd3 B10 Idd5.1 congenic mice treated with costimulation blockade exhibited islet allograft survival shorter (MST = 69 days) than that achieved in C57BL/6 mice (P < 0.0001) and not different from that achieved in NOD mice (NS, Fig. 3). Islet allograft survival was also not enhanced in NOD.B6 Idd3 B10 Idd5.1 Idd5.3 mice (MST = 13 days) over that achieved in NOD.B6 Idd3 B10 Idd5.1 congenic mice (NS, Fig. 3). These data suggest that expression of Idd5.2 encoding a nonfunctional protein is important for the beneficial effects of Idd5 in conjunction with Idd3 and that all three subregions are needed—or that Idd5.2 alone or in combo with Idd5.1 or Idd5.3 is beneficial. Interestingly, we observed that a proportion of animals maintained long-term graft function.

**Failure of costimulation blockade treatment to delete alloreactive CD8⁺ T-cells in (NOD × KB5)F1 synchimeric mice is reversed by IL-2.** Idd3-mediated diabetes susceptibility in NOD mice is caused by an IL-2 allele that is transcribed at lower levels than variants contributing to disease resistance (9) and is important for
tolerance induction (33). Therefore, we hypothesized that a deficiency in IL-2 production in NOD mice impairs host alloreactive CD8\(^+\) T-cell deletion.

To test this hypothesis, we modified our synchimera model system based on KB5 TCR transgenic alloreactive CD8\(^+\) T-cells (18). KB5 CBA mice were mated with NOD mice or with C57BL/6, H\(^2\)g7 mice bearing the NOD major histocompatibility complex and were used to generate synchimeric mice (18). Synchimeric mice were treated with C57BL/6 DST and anti-CD154, and the circulating levels of KB5 transgenic alloreactive CD8\(^+\) T-cells were analyzed on day 0. Two groups of synchimeric mice were also given five daily injections of mouse rIL-2 beginning on the day of DST.

KB5 CBA (NODxKB5)F1 synchimeric mice treated with costimulation blockade exhibited marked deletion of their alloreactive CD8\(^+\) T-cells (63 ± 30%, Fig. 4). Similar results were seen in the (KB5 CBA (NODxKB5)F1 synchimeric mice that received exogenous IL-2 in addition to the DST and anti-CD154 (70 ± 6%). In contrast, (KB5 CBA × NOD)F1 mice exhibited significantly less deletion of their alloreactive CD8\(^+\) T-cell population (34 ± 24%), which was restored by IL-2 treatment (63 ± 21%) to a level similar to that observed in (KB5 CBA × C57BL/6, H\(^2\)g7)F1 mice (Fig. 4).

IL-2 improves islet allograft survival in NOD mice treated with costimulation blockade. We next hypothesized that increased deletion of alloreactive CD8\(^+\) T-cells in (KB5 CBA × NOD)F1 mice treated with costimulation blockade plus IL-2 would lead to a difference in islet allograft survival. To test this, chemically diabetic NOD mice were treated with costimulation blockade and trans-

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**FIG. 3.** Life table analysis of islet allograft survival in chemically diabetic Idd3 congenic NOD mice bearing different Idd5 congenic intervals. Groups of 6- to 8-week-old chemically diabetic male mice were treated with a DST plus anti-CD154 mAb. A DST (10\(^7\) C3H/He spleen cells) was given on day −7, and anti-CD154 mAb (0.5 mg/dose) was given on days −7, −4, 0, and +4 relative to transplantation with C3H/He islets on day 0. Vertical bars indicate mice removed from the study with intact grafts or alive with intact grafts at the conclusion of the period of observation. Islet allograft survival in C57BL/6, NOD, and NOD Idd3 Idd5 congenic mice shown in Fig. 2 is reproduced here for ease of comparison with other strains. Comparative \(P\) values of islet allograft survival in the groups are shown.

**FIG. 4.** Scatter plot of alloreactive CD8\(^+\) T-cell deletion in synchimeric mice. (KB5 CBA × C57BL/6, H\(^2\)g7)F1 and (KB5 CBA × NOD)F1 synchimeric mice were treated with a C57BL/6 DST on day −7 and anti-CD154 mAb on days −7 and −4 relative to analysis of their circulating levels of KB5 transgenic CD8\(^+\) T-cells on day 0 as described in RESEARCH DESIGN AND METHODS. \(P\) values are indicated by horizontal bars.
planted with C3H/He islets with or without peri-transplant injection of IL-2.

As expected (6), islet allograft survival in NOD mice treated with costimulation blockade was short (MST = 46 days, Fig. 5). In contrast, NOD mice treated with costimulation blockade and IL-2 exhibited slightly but significantly prolonged islet allograft survival (MST = 83 days), although all islet allografts were eventually rejected (Fig. 5).

**NOD.B6 Idd3 congenic mice exhibit a restored ability to delete alloreactive CD8 T-cells after costimulation blockade.** Having observed that the Idd3 locus improved islet allograft survival (Fig. 5) and that exogenous administration of IL-2 improved alloreactive CD8 T-cell deletion in our synchimeric model system (Fig. 4), we next tested directly our hypothesis that restoration of a normal IL-2 gene by the Idd3 locus in NOD mice would restore the ability of costimulation blockade to delete alloreactive CD8 T-cells. To directly identify alloreactive CD8 T-cell function in NOD.B6 Idd3 mice, we used an in vivo cytotoxicity assay (23). In NK cell–depleted mice, all in vivo cytotoxic activity is due to alloreactive CD8 T-cells (34).

We first confirmed (24) that the anti-CD122 antibody would delete all NK cell cytotoxic activity in NOD mice (Fig. 6A). As expected, NK-depleted C57BL/6 mice treated with costimulation blockade exhibited low in vivo cytotoxicity, whereas alloreactive CD8 T-cell activity in NOD mice was high (Fig. 6B). In NK-depleted NOD.B6 Idd3 mice, in vivo cytotoxicity was significantly lower than that observed in NOD mice and was comparable to that observed in C57BL/6 mice (Fig. 6B). In all three strains, priming NK cell–depleted mice with a DST alone induced strong in vivo alloreactive CD8 T-cell cytotoxicity (Fig. 6B).

**DISCUSSION**

We have confirmed in this article that islet allograft survival in NOD.B6 Idd3 mice treated with costimulation blockade is prolonged compared with NOD mice (1). We now document that some but not all Idd resistance loci synergize with Idd3 to enhance islet allograft survival. We further show that a major tolerance defect in NOD mice is the resistance of alloreactive CD8 T-cells to deletion by costimulation blockade, a defect that can be reversed by addition of the Idd3 gene or by administration of exogenous IL-2.

Disease-resistant alleles at the Idd10 Idd18 region provide moderate protection against diabetes (45–55%) (27,28,35), and together, the Idd3 Idd10 and Idd18 protective alleles confer almost complete resistance (8,27,29). We hypothesized that NOD.B6 Idd10 Idd18 mice and NOD.B6 Idd3 Idd10 Idd18 mice would demonstrate a stepwise improvement in costimulation blockade–induced islet allograft survival. Surprisingly, islet allograft survival in NOD.B6 Idd10 Idd18 mice was not increased over that achieved in NOD mice and did not increase over that achieved with Idd3 alone.

Genes located within the Idd10 and Idd18 intervals include Ptpn22, which is orthologous to PTPN22, a human
gene that is associated with the development of diabetes and other autoimmune diseases (28,36). The disease-associated allele of human PTPN22 is a gain-of-function variant that in vitro suppresses TCR signaling in response to TCR/CD28 ligation to a greater extent than the more common allele (37). The functional outcome of TCR signaling in the PTPN22 gain-of-function variant reduces expression of IL-2. Studies that altered the allelic status of the Ptpn22 region in NOD congenic mice have demonstrated that the B6-derived interval confers susceptibility to type 1-like autoimmune diabetes (L.S.W., L.B.P., unpublished data), therefore providing a potential explanation for the inability of the B6-derived Idd10 Idd18 region to increase islet allograft survival.

Given the surprising results seen with the NOD.B6 Idd3 Idd10 Idd18 islet allograft studies, we next determined whether a synergistic effect could be found between Idd3 and Idd5. NOD.B10 Idd5 mice have a lower frequency of diabetes than NOD mice, and when combined with resistance alleles at Idd3, the frequency of spontaneous diabetes is <2% (30,32). Importantly, islet allograft survival in NOD.B6 Idd3 Idd5 mice was increased to levels achieved in C57BL/6 mice.

The Idd5 congenic strain used in our study contains at least three diabetes resistance genes, termed Idd5.1, Idd5.2, and Idd5.3 (30). The Idd5.1 gene is most likely a variant of Cita4, with the diabetes-prone NOD allele producing less of the ligand-independent CTLA-4 (lCTLA-4) molecule than the resistant B10 allele (38). CTLA-4 is critical for the induction of tolerance using costimulation blockade (39). Slc11a1 (formerly known as Nramp1) is likely to be the causal Idd5.2 gene (40). Interestingly, the B10 diabetes-resistant Nramp1 allele encodes a nonfunctional protein (41). However, Idd5.2/Nramp1 is not required for the decreased diabetes frequency in NOD.B6 Idd3 B10 Idd5 mice, although the B10-derived Idd5.2 region is required to maintain the reduced insulitis present in NOD.B6 Idd3 B10 Idd5 mice (31). In addition, the non–NOD-derived Idd5.1/Cita4 and Idd3 resistance alleles did not increase protection from diabetes compared with Idd3 alone (31). These results are consistent with our islet allograft survival data. The synergy observed between Idd5 and Idd3 that results in nearly complete protection from diabetes and insulitis and increases islet allograft survival to a C57BL/6-like frequency is dependent on the B10-derived Idd5.2 region, either alone or in combination with the other Idd5 loci. To extend this observation, additional congenic strain combinations would have to be developed and tested: Idd3/Idd5.3, Idd3/Idd5.2, and Idd3/Idd5.2/Idd5.3.

Idd3, which is partially protective of diabetes, significantly improves islet allograft survival in the NOD mouse, with the strongest effect seen in the NOD.B6 Idd3 B10 Idd5 congenic strain. The Idd3 effect likely results from differential expression of IL-2 that modulates CD4+ CD25+ Treg cell function in NOD mice (9). IL-2 is also required for the development of self-tolerance and for costimulation blockade–induced allograft tolerance (10,15).

Based on the NOD.B6 Idd3 congenic data, we hypothesized that the inability to induce tolerance in NOD mice is due to a failure to efficiently delete host alloreactive CD8+ T-cells and that injection of exogenous IL-2 would correct this defect. As expected, (KB5 CBA × C57BL/6)F1 synchimeric mice treated with costimulation blockade showed a marked depletion of alloreactive CD8+ T-cells compared with (KB5 CBA × NOD)F1 synchimeric mice.
tute, the National Institute of Allergy and Infectious Diseases, and the JDRF. No other potential conflicts of interest relevant to this article were reported.

We thank Linda Paquin, Cindy Bell, Linda Leethy, Dan Rainbow, and Jean Leif for technical assistance.

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