IL-1 Receptor Deficiency Slows Progression to Diabetes in the NOD Mouse

Helen E. Thomas,[1] Windy Irawaty,[1] Rima Darwiche,[1] Thomas C. Brodnicki,[2] Pere Santamaria,[3] Janette Allison,[4] and Thomas W.H. Kay[1]

Proinflammatory cytokines are believed to be important in pancreatic β-cell destruction in the development of type 1 diabetes. They act by upregulation of genes including Fas and inducible nitric oxide synthase (iNOS), which have both been shown to lead to β-cell death in vitro. We used mice deficient in the interleukin (IL)-1 receptor (IL-1R) to assess the contribution of IL-1 to different models of diabetes. IL-1R–deficient islets were protected from the damaging effects of tumor necrosis factor (TNF) and interferon (IFN)-γ in vitro, and β-cell expression of iNOS was reduced, suggesting that IL-1 mediates the induction of iNOS by TNF and IFN-γ. IL-1 action was not required for induction of class I major histocompatibility complex or Fas by TNF and IFN-γ. IL-1R–deficient nonobese diabetic (NOD) mice developed diabetes significantly slower than wild-type mice. IL-1R deficiency did not affect diabetes in 8.3 TCR transgenic NOD mice but prolonged the time to diabetes in BDC2.5 TCR transgenic NOD mice. We conclude that IL-1R deficiency slows progression to diabetes in NOD mice but on its own does not prevent diabetes. *Diabetes* 53:113–121, 2004

Interleukin (IL)-1 is produced by activated macrophages and is involved in acute-phase inflammatory responses (1,2). Functional IL-1 receptors (IL-1Rs) are present on pancreatic β-cells (3,4). In vitro, IL-1 is toxic to pancreatic β-cells, and it has been suggested that it may be involved in the pathogenesis of type 1 diabetes. IL-1, together with interferon (IFN)-γ, has been shown to inhibit the function of rodent and human pancreatic islets in vitro (5) as well as induce DNA damage and β-cell death (6,7). IL-1 and IFN-γ induce expression of inducible nitric oxide synthase (iNOS), which leads to production of the toxic molecule nitric oxide (NO). NO is believed to be largely responsible for islet cell damage in response to IL-1 and IFN-γ (8,9). Evidence suggests that IL-1 and IFN-γ are directly toxic to β-cells and that NO production by the β-cell itself induces damage. It is likely that NO produced by other cells within the islet, such as resident leukocytes, is at an insufficient local concentration to induce β-cell damage (10).

In addition to the effects of exogenous IL-1 on β-cells, there is also evidence that IL-1 is produced endogenously within islets by resident leukocytes (11). Inflammatory cytokines, such as tumor necrosis factor (TNF) and IFN-γ, and lipopolysaccharide (LPS) induce NO production within islets, but this is blocked by inhibition of IL-1 action with the IL-1R antagonist (IL-1Ra), suggesting that the cytotoxic action of these molecules depends on stimulation of IL-1 production within the islet (12,13).

Inflammatory cytokines including IL-1, TNF, and IFN-γ are all found within islets during the pathogenesis of diabetes (14–16). Because of the effects of these cytokines on islets in vitro, it is likely that they are playing some role in diabetes development in vivo. IFN-γ has been shown to be present in islets from pre-diabetic NOD mice at sufficient concentrations to upregulate class I major histocompatibility complex (MHC) gene expression on β-cells in vivo (17). Additionally, islet cell iNOS expression has been detected (16), presumably upregulated by the cytokines present in the inflammatory lesion in islets. Inhibition of IL-1 in vivo by injection of anti–IL-1β antisera had a modest effect on reducing the incidence of cyclophosphamide-induced diabetes (18), as did treatment with soluble IL-1R (19).

A further way that IL-1 and other cytokines may lead to β-cell destruction in vivo is via induction of the cell death receptor Fas. Whereas Fas does not contribute to IL-1–induced β-cell damage in vitro, it is a potential mechanism of β-cell death in vivo if cells expressing the Fas ligand are nearby. IL-1 upregulates Fas on β-cells, and this is enhanced by IFN-γ (20,21). TNF and IFN-γ also induce Fas expression and whether IL-1 is required for this, as it is for β-cell iNOS expression, is unknown.

We obtained IL-1R–deficient mice to examine whether IL-1 action plays a role in diabetes development in the NOD mouse and other models of diabetes. IL-1R–deficient mice develop normally; however, they are highly susceptible to *Listeria* infection and have reduced acute-phase and delayed-type hypersensitivity responses (1,2). The IL-1R is composed of two chains: IL-1RI and IL-1R AcP. Both are required for all signaling in response to IL-1 (22). IL-1R– and IL-1R AcP–deficient mice were used to analyze the effect of intraislet IL-1 on TNF- and IFN-γ–induced

113

gene regulation. We also backcrossed both type I IL-1R– and IL-1R AcP–deficient mice on to the NOD genetic background to examine the incidence of diabetes in different models.

RESEARCH DESIGN AND METHODS

Mice and reagents. IL-1R–deficient and IL-1R AcP–deficient mice were obtained from Dr. M. Labow (Roche). Mice were backcrossed from a mixed 129/C57Bl/6 background onto the NOD genetic background for 10 generations and were then intercrossed to produce knockout and wild-type backcrossed mice. In addition to screening for the knockout locus, DNA samples from the established knockout strain were genotyped across the genome (~15 cM genome-wide screen) using polymorphic markers that also flanked previously described *Idd* loci (23). The genome-wide screen was performed using fluorescently labeled primers at the Australian Genome Research Facility (Melbourne, Australia). PCR products were detected on a Perkin Elmer 377 and analyzed using Genescan and Genotyper software (Applied Biosystems) as described (24).

NOD8.3 mice expressing the TCRαβ rearrangements of the H-2K^d–restricted β-cell–reactive CD8$^+$ T-cell clone NY8.3 (25) were provided by Pere Santamaria (University of Calgary). NOD BDC2.5 TCR (26) transgenic mice carrying a TCR transgene derived from an I-A^{g7} β-cell–reactive CD4$^+$ T-cell clone were provided by D. Mathis and C. Benoist (Harvard University). NOD/Lt and 129/Sv mice were bred in specific pathogen-free conditions and housed in the animal facility at the Walter and Eliza Hall Institute. All animal studies were carried out in accordance with accepted standards of humane animal care and were approved by the institutional animal ethics committee.

Recombinant murine IFN-γ (used at 100 units/ml) and recombinant murine TNF-α (used at 1,000 units/ml) were obtained from Genentech (South San Francisco, CA). Recombinant human IL-1 (used at 10 units/ml) was obtained from Dr. C. Reynolds (National Cancer Institute), and IL-1Ra (5 μg/ml) was obtained from Amgen. N^G-monomethyl-L-arginine (2 mmol/l) was purchased from Sigma (St. Louis, MO).

Islet isolation. Islets of Langerhans were isolated from mice as previously described (10,27). Purified islets were washed, hand picked, and cultured at 37°C in 5% CO_2 in CMRL medium-1066 (Life Technologies, Gaithersburg, MD) containing antibiotics, glutamine, and 10% FCS.

Cell death assay. Quantitation of apoptosis was determined according to the method of Nicoletti et al. (28). Islets were cultured for 4 days with cytokines, followed by analysis for DNA fragmentation on the flow cytometer (Becton Dickinson, Mountain View, CA) as previously described (21). Data from multiple experiments were pooled, as indicated in the figure legends.

Flow cytometry. Islet cells were analyzed for expression of Fas by flow cytometry as previously described (21). Islets were dispersed into single cells using trypsin (0.2% trypsin [Calbiochem, La Jolla, CA], 10 mmol/l EDTA in Hank's balanced salt solution) and stained using standard procedures. Antisera used were anti-Fas (Jo2; Pharmingen, San Diego, CA) followed by biotinylated anti-hamster Ig (Pharmingen) and phycoerythrin-conjugated streptavidin (Caltag, Burlingame, CA). Islet cells were also analyzed for expression of class I MHC expression using biotinylated anti-mouse H-2D^b (clone 28-14-8; Pharmingen) and H-2K^d (clone SF1-1.1; Pharmingen) followed by phycoerythrin-conjugated streptavidin. Cells were analyzed on a FACScan (Becton Dickinson, Franklin Lakes, NJ). β-Cells were identified based on their high autofluorescence (21). Infiltrating inflammatory cells were excluded from analysis by gating out cells stained with anti-CD45 (anti-leukocyte common antigen) conjugated to Percp-Cy5.5 (3F11; Pharmingen).

Western blotting. Islets were incubated for 48 h with cytokines, transferred to microcentrifuge tubes, and washed three times in PBS; they were then resuspended in 25 μl lysis buffer (10 mmol/l HEPES, pH 7.4, 2 mmol/l EDTA, 0.1% saponin, 5 mmol/l dithiothreitol, 1 mmol/l phenylmethylsulfonyl fluoride, and 20 μg/ml leupeptin). Tubes were then subjected to three cycles of freezing and thawing, followed by centrifugation at 13,000 rpm for 15 min at 4°C. Lysed islets were kept at −70°C until required. Samples were boiled for 5 min in 2× sample buffer (125 mmol/l Tris, pH 6.8, 4% SDS, 2% 2-mercaptoethanol, 0.1% bromophenol blue, and 20% glycerol). Samples were separated by SDS-PAGE and transferred to nitrocellulose using standard procedures. Western blotting was performed with anti-NOS2 Ab (Santa Cruz Biotechnology, Santa Cruz, CA) followed by horseradish peroxidase (HRP)-conjugated anti-rabbit Ig (Silenus Laboratories, Hawthorn, Australia) and detection with Lumi-Light Western blotting substrate (Roche Diagnostics, Mannheim, Germany).

Immunostaining. Islets were cultured for 48 h with cytokines in complete CMRL medium-1066. For cytospins, islets were dispersed into single cells with trypsin. Cell suspensions (4 × 10^4 cells per slide) were centrifuged onto glass slides. Slides were fixed for 30 min in 4% paraformaldehyde, air dried, and stored at −20°C until staining. Two-color immunofluorescence was performed

using standard procedures. Blocking was with 10% FCS and 2% skim milk in PBS; antisera were incubated for 30 min with 5-min washes in PBS. Antisera used were anti-NOS2 Ab (Santa Cruz Biotechnology) with anti-rabbit Ig–Texas red (Molecular Probes, Eugene, OR), anti-insulin Ab (Dako, Carpenteria, CA) with anti–guinea pig Ig–fluorescein isothiocyanate (Becton Dickinson, Franklin Lakes, NJ), and anti-CD45 Ab (clone 30-F-11; Pharmingen) with anti-rabbit Ig–fluorescein isothiocyanate (Silenus). Staining was visualized using confocal microscopy.

Spontaneous diabetes and insulitis. Backcrossed female wild-type and IL-1R–deficient mice were kept for 350 days to assess the spontaneous incidence of diabetes. Mice were visually monitored regularly, and blood glucose analyses were performed on any mice that appeared diabetic. Mice with a blood glucose >12 mmol/l were considered diabetic. For histological analysis, female wild-type and IL-1R–deficient mice were killed at 120 days of age, the pancreas was removed and placed in Bouin's fixative, and 5-μm paraffin-embedded sections were cut at 40-μm intervals. Hemotoxylin and eosin–stained sections were scored for insulitis using a 0–4 scoring system as previously described (29). The scores are expressed as percentage infiltration. Three mice were analyzed per group, with ~100 islets scored in each group. For induction of diabetes with cyclophosphamide, 12- to 15-week-old male mice were given a single injection of 300 mg/kg cyclophosphamide i.p. and monitored for onset of diabetes.

Adoptive transfer of diabetes. Splenocytes from newly diabetic (blood glucose >12 mmol/l) NOD donors were purified free of erythrocytes. Cells were injected intravenously into the tail vein of irradiated (900R) recipient wild-type or IL-1R–deficient mice at 2 × 10^7 cells/mouse. After 14 days, mice were monitored for diabetes by urine and blood glucose measurements. BDC2.5*scid* mice develop diabetes at 3–4 weeks of age (30). Newly diabetic mice were used as donors for adoptive transfer experiments as described above. Recipient mice were given 1 × 10^6 donor cells.

Fetal pancreas grafts. Fetal pancreata from wild-type or IL-1R–deficient mice were harvested on embryonic days 15–18 and cultured for 10 days. Pancreata were then grafted under the kidney capsule of NOD*scid* mice, with one wild-type and one IL-1R–deficient graft at each pole of one recipient kidney (31). Mice recovered for 3 weeks to allow the grafts to become established before adoptive transfer of 2 × 10^7 splenocytes from diabetic NOD mice (n = 13). To assess the effects of IL-1Ra, two recipients were given a daily injection of 8 mg/kg IL-1Ra i.p., and two mice received placebo (Amgen). Grafts were harvested from the IL-1Ra and placebo groups on day 28 posttransfer, before the onset of diabetes. The remaining mice were left until diabetes developed, at which time the graft was harvested. Grafts were placed in Bouin's fixative and embedded in paraffin. The 5-μm sections were cut, and serial sections were stained with hemotoxylin and eosin and with anti-insulin followed by anti–guinea pig HRP and anti-glucagon followed by anti-rabbit HRP (all antisera from Dako). Grafts were histologically scored using the following scale: 0 = no infiltrate; 1 = peri-islet infiltrate; 2 = intraislet infiltrate <50% destruction; 3 = intraislet infiltrate >50% destruction; and 4 = total β-cell loss, glucagon staining without insulin.

Enzyme-linked immunosorbent assay. To measure IL-1β secretion, islets isolated from wild-type and IL-1R–deficient mice were cultured at 200 islets per 250 μl complete CMRL with cytokines. Supernatants were harvested after 4 days and assayed by enzyme-linked immunosorbent assay according to the manufacturer's instructions (R&D Systems, Minneapolis, MN).

Statistical analysis. Analyses of data were performed using the program GraphPad Prism (GraphPad Software, San Diego, CA). Data are represented as means and SE from three or more independent experiments. Data were analyzed using one-way ANOVA with Bonferroni's posttest for comparison of multiple columns. Survival curves were analyzed using the log-rank test.

RESULTS

The contribution of intraislet IL-1 production to islet cell death induced by TNF plus IFN-γ. In islets, TNF plus IFN-γ plus LPS induces iNOS expression, NO production, and inhibition of glucose-stimulated insulin secretion (12). It is believed that intraislet IL-1 produced by resident leukocytes, detectable by immunostaining, directly contributes to this toxicity of TNF plus IFN-γ plus LPS to islets. By inhibiting IL-1 action within islets, it is possible to assess the contribution of intraislet IL-1 production to islet cell death and iNOS expression induced by TNF plus IFN-γ in mouse islets.

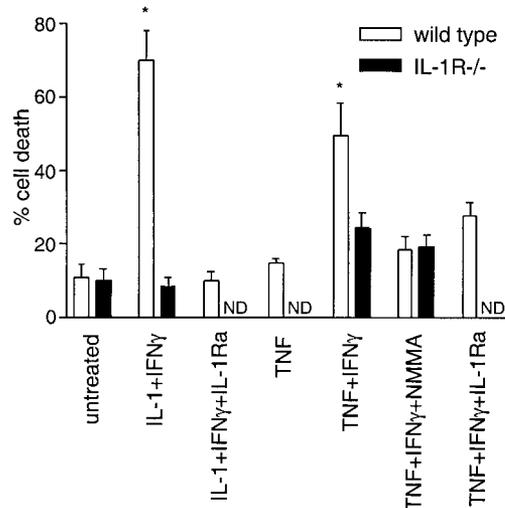IL-1 action was inhibited in two ways. First, we coincubated islets with IL-1Ra, and second, we isolated islets

FIG. 1. Cytokine-dependent killing of islet cells in vitro depends on IL-1. Wild-type or IL-1R–deficient islets were cultured in vitro for 4 days with cytokines with or without IL-1Ra. After cytokine treatment, islets were dispersed into single cells and percent nuclear fragmentation was analyzed by flow cytometry as a measure of cell death. Means ± SE of data from four to five separate experiments are shown. *P < 0.001 by ANOVA, cytokine-treated groups vs. untreated. ND, not done; NMMA, N^G-monomethyl-L-arginine.

from mice deficient in IL-1R. Islets were incubated in culture with cytokines, and nuclear fragmentation was analyzed after 4 days by flow cytometry. Whereas TNF alone had no effect on islet viability, the combination of TNF plus IFN-γ induced nuclear fragmentation of islet cells (Fig. 1). Inhibition of IL-1 action in islets with either IL-1Ra or IL-1R deficiency attenuated cell death of islets treated with TNF plus IFN-γ (Fig. 1). As expected, IL-1 plus IFN-γ did not induce death of islets from IL-1R–deficient mice or of wild-type islets cocultured with IL-1Ra. IL-1 or IFN-γ alone were unable to kill mouse islets (10).

We also measured IL-1β production after culture of islets in vitro with cytokines. Because IL-1 is produced by resident leukocytes in islets and the number of these cells per islet is very low, IL-1β was not detected in the wild-type islets (untreated, 9.1 pg/ml IL-1β; TNF, 7.4 pg/ml IL-1β; TNF plus IFN-γ, 10.6 pg/ml IL-1β). However, we were able to detect very low levels of IL-1β secreted by IL-1R–deficient islets (untreated, 9.3 pg/ml IL-1β; TNF, 18.4 pg/ml IL-1β; TNF plus IFN-γ, 23.8 pg/ml IL-1β). The inability to detect these low amounts of IL-1 in wild-type islets is most likely due to the receptor binding IL-1, thus preventing its detection by enzyme-linked immunosorbent assay. The low but detectable levels of IL-1β secreted by IL-1R–deficient islets indicates that the reduced cell death in these islets treated with TNF plus IFN-γ is due to a lack of IL-1 action and not a lack of IL-1 production.

These data confirm that TNF plus IFN-γ induces intra-islet IL-1 production in mouse islets, and this IL-1 contributes to cell death induced by TNF plus IFN-γ. It remains possible that TNF plus IFN-γ act directly on islet cells to induce iNOS expression and NO production independent of IL-1 production. To address this, we looked at TNF plus IFN-γ–induced gene expression in IL-1R–deficient islets.

TNF plus IFN-γ–induced gene expression in islet cells. TNF plus IFN-γ is known to induce expression of

iNOS in islet cells, and NO production is believed to be at least one mechanism by which these cytokines damage islets in vitro. To investigate the islet cell types upon which TNF plus IFN-γ act, we used iNOS expression as a marker for cytokine action. Western blotting and immunofluorescence were performed to examine iNOS expression in cytokine-treated islets (Fig. 2). In wild-type islets, combinations of IL-1 plus IFN-γ and TNF plus IFN-γ induced iNOS expression (Fig. 2A). Inhibition of IL-1, with either IL-1Ra or IL-1R deficiency, reduced but did not prevent iNOS expression. Two-color immunofluorescence of cyto-spins from TNF plus IFN-γ–treated islets revealed iNOS expression in both β-cells and non–β-cells from wild-type islets. IL-1R–deficient islets, on the other hand, had very few (if any) iNOS-expressing β-cells, whereas the number of iNOS-positive non–β-cells was the same as in wild-type islets (Fig. 2B). These data indicate that whereas non–β-cell expression of iNOS is induced directly by TNF plus IFN-γ, the same cytokine combination requires intraislet IL-1 production to induce iNOS expression in β-cells. Expression of iNOS in β-cells correlated with cytokine-induced cell death of islets, confirming that this is a major mechanism for cytokine-induced islet cell death.

Fas (CD95), induced on β-cells by TNF plus IFN-γ, was also studied in IL-1R–deficient islets. In contrast to iNOS expression, Fas was induced by TNF plus IFN-γ in both wild-type and IL-1R–deficient β-cells (Fig. 3A). Fas expression induced by TNF plus IFN-γ in IL-1R–deficient islets was functional because these islet cells were killed by the addition of soluble FasL (not shown). Similarly, H-2K^d expression was increased by TNF equally in IL-1R–deficient or wild-type β-cells (Fig. 3B). Therefore, local IL-1 production is not required to stimulate Fas expression and function in β-cells, nor is it required for class I MHC upregulation.

Spontaneous diabetes is delayed in IL-1R–deficient NOD mice. We examined the effects of IL-1R deficiency on spontaneous diabetes in the NOD mouse. IL-1R–deficient mice were backcrossed onto the NOD genetic background for 10 generations and then intercrossed to produce either IL-1R–deficient or backcrossed wild-type mice. Apart from the knockout locus, a genome-wide scan confirmed that all genetic markers, including those flanking previously described Idd loci, were homozygous for the NOD allele. Because this genotyping was performed after intercrossing at the tenth backcross generation, there is a statistical chance that small genomic intervals may yet retain donor alleles, but based on the genome-wide scan, it is unlikely that these intervals encompass loci previously linked to diabetes onset. In addition, the 129-derived interval encompassing the IL-1R knockout has been localized, at present, between D1Mit167 and D1Mit322 (not included). This interval is ~6.9 cM proximal to the previously described boundary for Idd5.1 and, to date, has not been implicated in disease susceptibility or resistance (32,33).

Female mice (IL-1R deficient and backcrossed wild type) were kept to 350 days of age to determine the incidence of spontaneous diabetes (Fig. 4A). Although we detected a slight but significant delay in diabetes incidence in the absence of IL-1 receptors, the overall incidence of diabetes remained substantial at 75%. There was no difference in diabetes between homozygous wild-type and het-
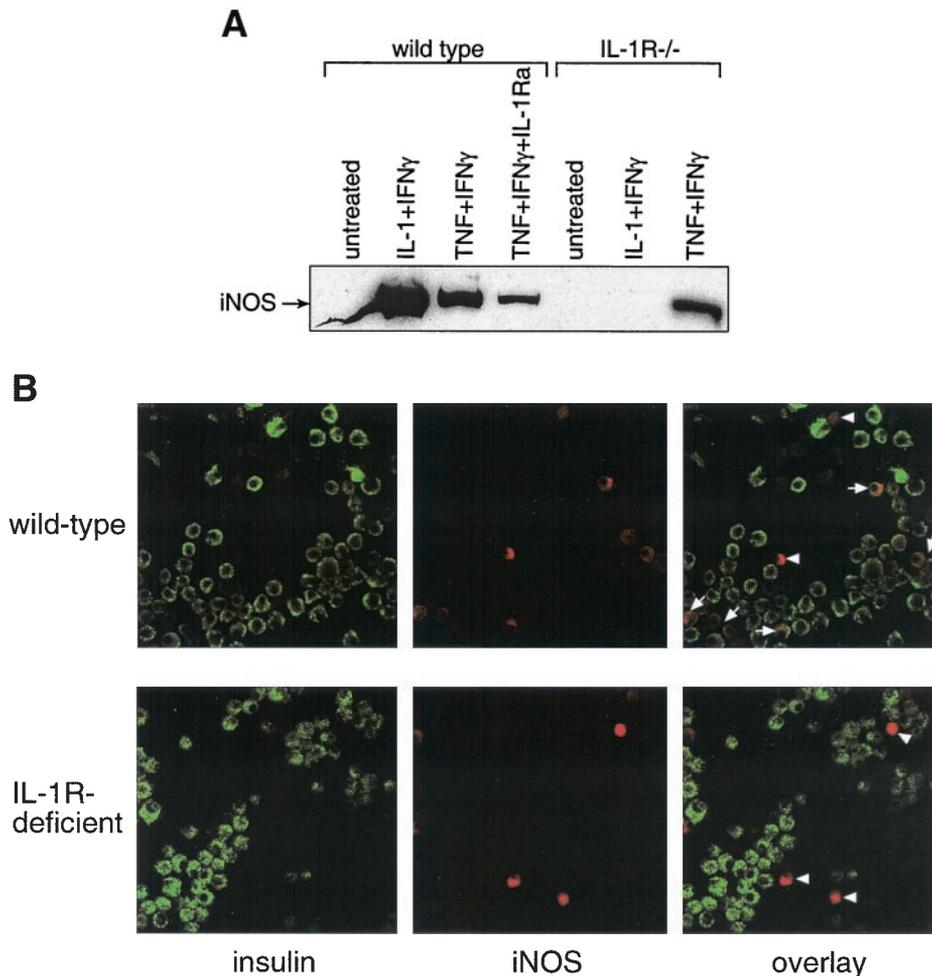
FIG. 2. Cytokine-induced iNOS expression in β-cells is reduced by IL-1Ra or in IL-1R–deficient islets. Islets from wild-type or IL-1R–deficient mice cultured for 48 h with cytokines were analyzed for iNOS expression. *A*: Western blot shows iNOS protein expression in extracts from cytokine-treated islets. iNOS expression after TNF and IFN-γ is reduced by IL-1Ra and IL-1R deficiency but not to basal levels. Gel is representative of three independent experiments. *B*: Two-color immunofluorescence of dispersed islet cells for iNOS expression. Cytospins were stained for insulin (green) and iNOS (red). Overlayed confocal images show cells that coexpress insulin and iNOS. Small arrows indicate insulin-positive β-cells that express iNOS. Arrowheads indicate iNOS-positive but insulin-negative cells. Residual iNOS expression after TNF and IFN-γ in IL-1R–deficient islets was in non–β-cells. Magnification 400×. The images are representative of three independent experiments.

erozygous mice (not shown). Wild-type mice had an insulitis score of 46.5 ± 17% infiltration, whereas IL-1R–deficient mice had a score of 41.8 ± 7% infiltration. We also examined the incidence of diabetes in IL-1R–deficient mice after adoptive transfer of diabetogenic NOD splenocytes. Mice receiving 2×10^7 splenocytes from newly diabetic NOD donors developed diabetes by 50 days posttransfer, even in the absence of IL-1R (Fig. 4*B*). We recently demonstrated that Fas is expressed on β-cells after adoptive transfer, although not during spontaneous diabetes (34). Fas expression after adoptive transfer was not affected by IL-1R deficiency (Fig. 4*C*).

Previous data suggest that cyclophosphamide-induced diabetes is reduced in NOD mice treated with either soluble IL-1R (19) or anti–IL-1β (18). We therefore tested the susceptibility of IL-1R–deficient NOD mice to diabetes induced by cyclophosphamide (Fig. 4*D*). Whereas IL-1R–deficient NOD mice developed cyclophosphamide-induced diabetes at a slightly reduced rate (66%) compared with wild-type mice (78%), the difference was not statistically significant ($P > 0.1$, log-rank test).

IL-1R deficiency does not protect syngeneic grafts from destruction. It has been reported that systemic administration of IL-1Ra prevents recurrence of disease after syngeneic transplantation of islets into diabetic NOD mice. This protection, however, only occurred for as long as IL-1Ra was administered (35). Using IL-1R–deficient islets, we are able to determine whether the protection afforded by IL-1Ra in this previous experiment is due to direct effects on β-cells or systemic effects on other cells such as macrophages, dendritic cells, or other immune cells. We therefore performed fetal pancreas grafts with wild-type and IL-1R–deficient donors into NOD*scid* recipients. Diabetes was then induced by adoptive transfer of splenocytes from newly diabetic NOD mice. Some mice were also treated daily with IL-1Ra or placebo.

Deficiency of IL-1R did not protect fetal pancreas grafts from autoimmune attack. Grafts were histologically scored as described in RESEARCH DESIGN AND METHODS, with a score of 0 being no infiltration and 4 being total β-cell destruction. Wild-type grafts had a score of 2.93 ± 0.1 compared with IL-1R–deficient grafts (2.77 ± 0.7). Both wild-type and IL-1R–deficient grafts had an infiltrate of mononuclear cells and destruction of insulin-producing β-cells (Fig. 5). This is consistent with results of adoptive transfer of diabetic cells into IL-1R–deficient NOD mice.
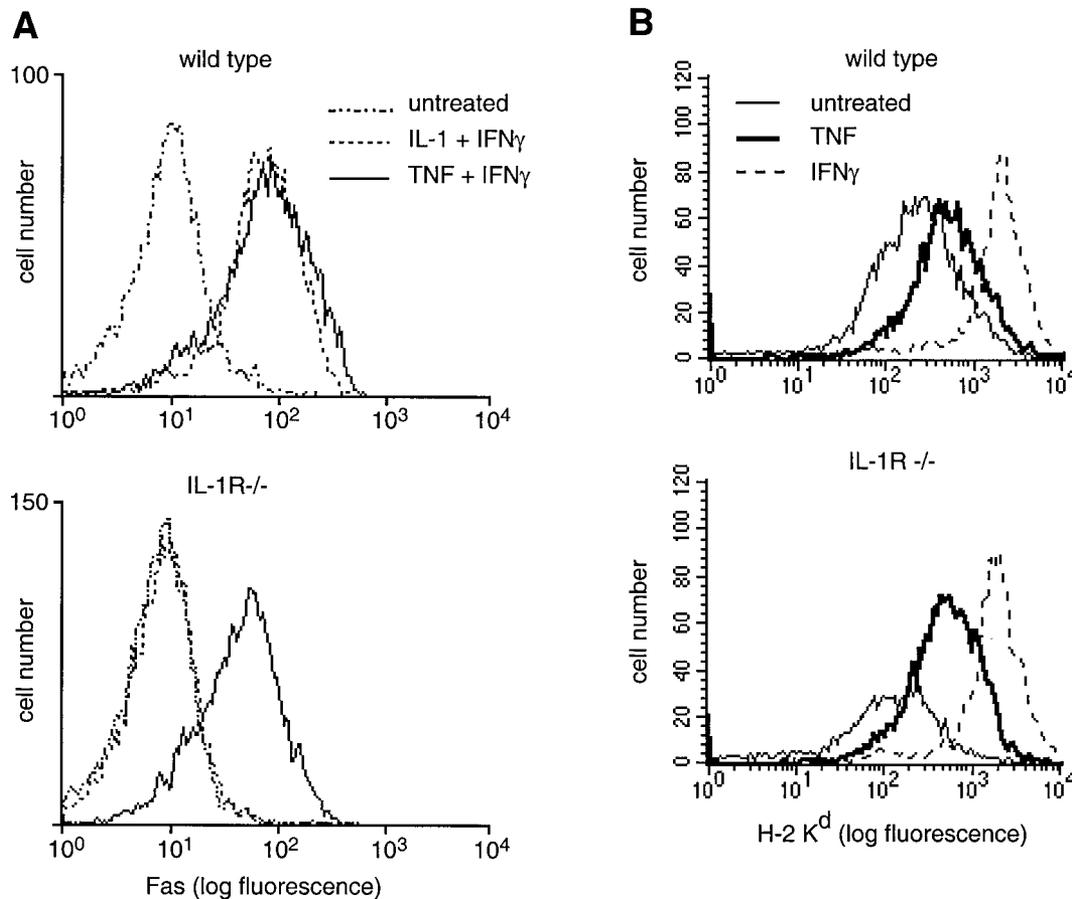
FIG. 3. TNF and IFN-γ induce Fas and class I MHC expression on β-cells from IL-1R–deficient mice. Flow cytometry profiles of Fas (*A*) and class I MHC (H-2K^d) (*B*) expression on β-cells. Islets were treated 24 h (for Fas) or 48 h (for class I MHC) in vitro with cytokines then dispersed into single cells. High autofluorescence cells (predominantly β-cells) were electronically gated and analyzed for Fas expression. Top panels show cells from wild-type islets, and bottom panels shows cells from IL-1R–deficient islets. Figure is representative of at least three independent experiments.

Mice treated with IL-1Ra had well-preserved grafts compared with placebo-treated mice, confirming that this treatment does help to prolong graft survival. However, there was no difference between wild-type (graft score 1.33 ± 0.36) and IL-1R–deficient grafts (graft score 1.12 ± 0.6). These results suggest that deficiency of IL-1R on β-cells alone does not prevent autoimmune destruction and that the effects of IL-1 in development of diabetes in this setting are likely to be systemic rather than direct effects on β-cells.

Adoptive transfer of diabetes by BDC2.5 cells is delayed in IL-1R–deficient NOD mice. BDC2.5 transgenic mice have diabetogenic CD4$^+$ T-cells that rapidly kill β-cells by an as yet unknown mechanism that involves activation of the cells through the type I TNF receptor (36). After treatment of BDC2.5 NOD mice with cyclophosphamide, cytokines such as IL-1, TNF, and IFN-γ are present in the insulitis lesions and treatment of these mice with neutralizing antisera to IL-1 or TNF reduces the incidence of cyclophosphamide-induced diabetes, suggesting that inflammatory cytokines are involved in diabetes in this model (37). On a NOD*scid* genetic background, BDC2.5 transgenic mice develop diabetes at 3–4 weeks of age, and splenocytes from these diabetic mice can transfer diabetes very rapidly (30). We used this CD8$^+$ T-cell–independent model to determine the effects of IL-1R deficiency on CD4$^+$ T-cell–mediated diabetes. Adoptive

transfer of BDC2.5*scid* splenocytes into irradiated wild-type mice led to diabetes in all mice by 11 days posttransfer (Fig. 6). There was a significant delay in diabetes after transfer into IL-1R–deficient mice (all mice were diabetic by 20 days posttransfer). This result suggests that inflammatory cytokines such as IL-1 do play a role in β-cell destruction by these CD4$^+$ T-cells; however, other mechanisms can compensate for the lack of IL-1 action, although this takes a longer time.

Diabetes in NOD8.3 TCR transgenic mice. NOD8.3 TCR transgenic mice express a T-cell receptor from a CD8$^+$ clone that was derived from infiltrating T-cells of an acutely diabetic mouse. The NOD8.3 TCR transgenic mice develop diabetes rapidly, with a mean age of diabetes of ~40 days. Perhaps, surprisingly, progression to diabetes occurs normally on a perforin-deficient background, and the T-cells from these mice appear to kill β-cells in a Fas-dependent manner (38). To test if IL-1 is required for this, for example, to upregulate Fas expression on β-cells, we produced NOD8.3 mice deficient in the IL-1R. These mice and their IL-1R wild-type littermates were studied for development of diabetes. No difference in frequency or age of onset of diabetes was observed (Fig. 7*A*). Furthermore, when islets from these mice were isolated before onset of diabetes, there was no evidence that Fas expression on β-cells was affected by IL-1R deficiency (Fig. 7*B*).
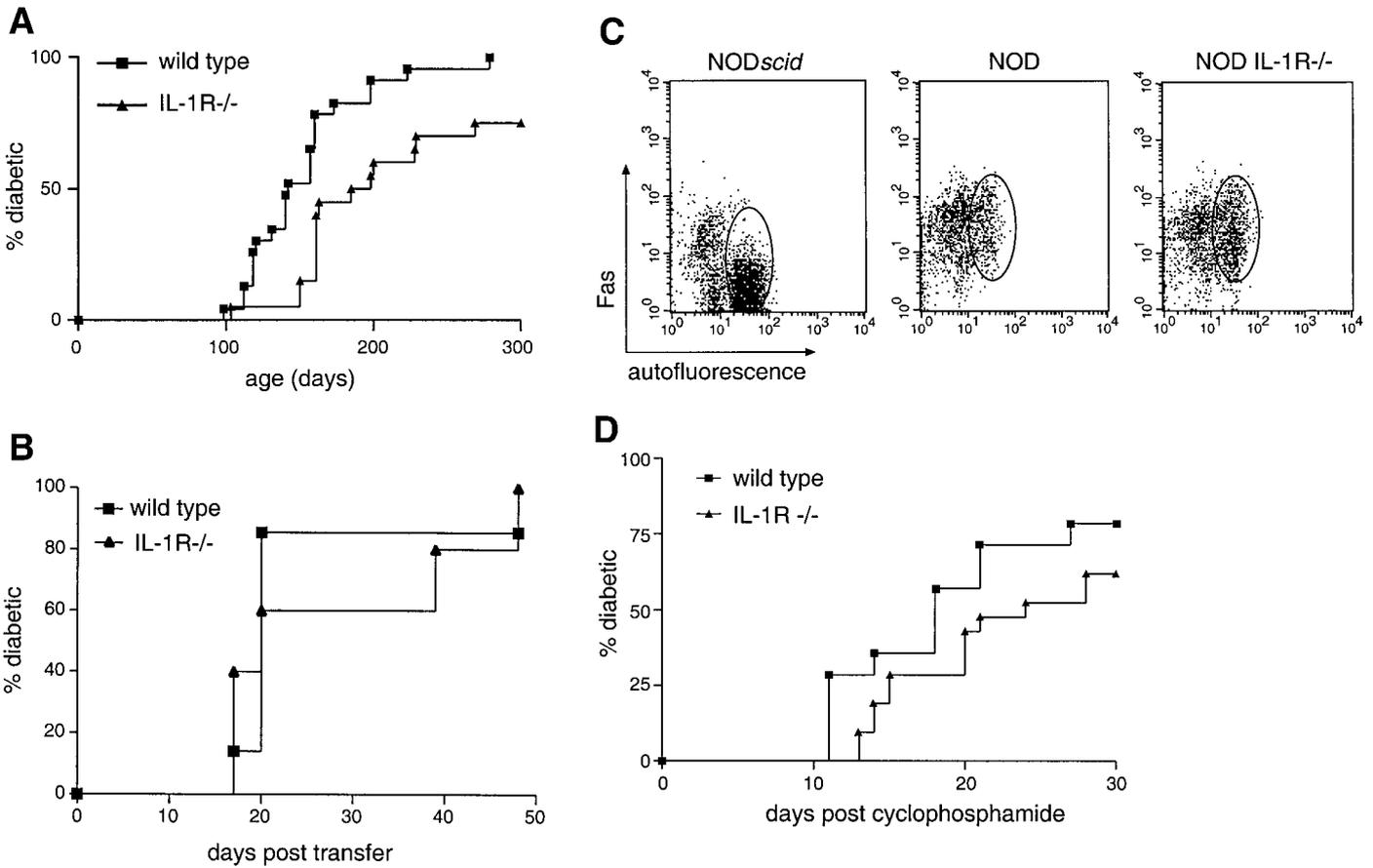
FIG. 4. Diabetes frequency in IL-1R–deficient NOD mice. *A*: Diabetes frequency was studied in cohorts of 23 female wild-type (■) and 20 IL-1R–deficient (▲) mice. Diabetes was diagnosed by measuring blood glucose in mice with clinical features of diabetes. The groups are statistically significantly different from each other ($P < 0.05$, log-rank test). *B*: Diabetes after adoptive transfer of 2×10^7 splenic T-cells from diabetic wild-type NOD mice into irradiated wild-type or IL-1R–deficient recipients (five mice per group). No difference was observed (NS). *C*: Fas expression on islet cells isolated from wild-type or IL-1R–deficient NOD recipients of splenic T-cells from diabetic wild-type NOD mice. Cells with high autofluorescence (predominantly β-cells) are circled. Islets were isolated 25 days after adoptive transfer and dispersed before staining. Leukocytes were excluded from analysis by staining with anti-CD45. The figure is representative of at least four individual mice. *D*: Diabetes frequency was studied after administration of cyclophosphamide. Cohorts of 14 wild-type and 21 IL-1R–deficient male mice were given a single dose of 300 mg/kg cyclophosphamide at 12–15 weeks of age. Mice were then monitored for diabetes. The groups are not statistically different ($P > 0.1$, log-rank test).

DISCUSSION

The main result from these studies is that IL-1 is not essential for progression to diabetes in several variations of the NOD mouse model of type 1 diabetes. Spontaneous diabetes occurred in ~75% of IL-1R–deficient NOD mice, which indicates a minor impact on diabetes frequency. This result is consistent with the lack of impact on diabetes frequency observed in caspase-1–deficient NOD mice (38a). We observed a delay in onset of diabetes in NOD mice and in NOD mice given diabetogenic BDC2.5 T-cells. This is the first time that the in vivo role of IL-1 in an animal model of diabetes has been directly tested with the use of genetic deficiency of IL-1 or IL-1 action.
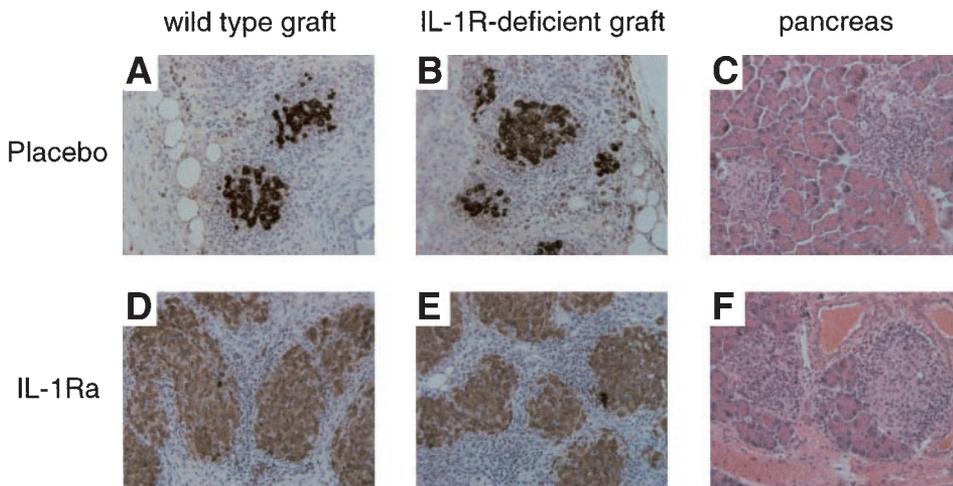
IL-1 kills mouse β-cells in vitro primarily by iNOS

FIG. 5. IL-1R deficiency does not protect syngeneic grafts from destruction. Fetal pancreas grafts were harvested from NOD*scid* mice after adoptive transfer of splenocytes from diabetic NOD mice. Recipient mice were nondiabetic treated with placebo (*A* and *B*) or nondiabetic treated with IL-1Ra (*D* and *E*). Serial sections were stained with insulin and glucagon to visualize islet structures. Representative wild-type and IL-1R–deficient grafts of the two groups stained with insulin are shown. *C* and *F*: Pancreas sections from each group stained with hemotoxylin and eosin are also shown to demonstrate that insulitis caused by the adoptive transfer was unaffected by the treatment.
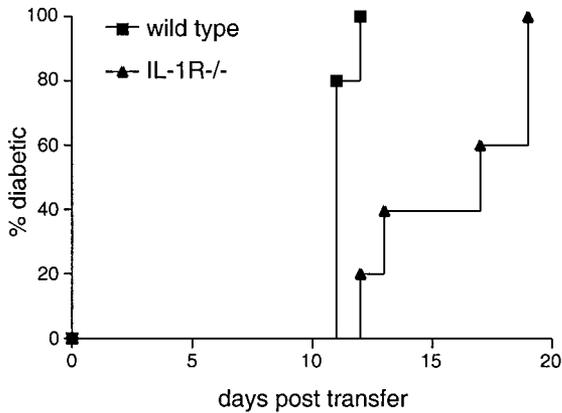
FIG. 6. Diabetes is delayed after adoptive transfer of BDC2.5 T-cells into IL-1R–deficient NOD mice. BDC2.5 cells (2×10^6) obtained from diabetic BDC2.5*scid* mice were transferred into irradiated wild-type or IL-1R–deficient NOD recipients, and the mice were followed for diabetes. Five mice per group were used ($P < 0.05$, log-rank test).

upregulation and production of NO, although some residual cell death is seen in IL-1–treated iNOS-deficient islets (39). This is also the main way that TNF kills primary mouse β-cells in combination with IFN-γ (40,41). TNF-stimulated β-cell iNOS expression is at least partly due to intraislet IL-1 production (11). In our studies, as expected, lack of IL-1 signaling eliminated β-cell iNOS induction due to TNF, IL-1, and IFN-γ. Immunoreactive iNOS was still produced in IL-1R–deficient islet non–β-cells by TNF plus IFN-γ, indicating that regulation of iNOS differs between β-cells in which it is IL-1 dependent and other cell types in which it is not. The amount of iNOS induced in non–β-cells is insufficient to damage β-cells in vitro (10). Consequently, IL-1R–deficient islets are protected from TNF and IFN-γ as well as from IL-1 and IFN-γ in vitro. IL-1R–deficient islets were not protected in vivo, indicating that cytokine-induced iNOS induction is not the dominant pathway to β-cell death in vivo. The lack of substantial impact on diabetes frequency is consistent with the reported lack of impact of iNOS deficiency on diabetes in NOD mice.

Redundancy with TNF that has very similar effects to IL-1 may be an explanation for the unchanged frequency of diabetes. Both activate the nuclear factor–κB and mitogen-activated protein kinase pathways and induce an overlapping set of genes involved in inflammation (42). If TNF effects on β-cells are mediated by intraislet production of IL-1, then TNF effects should be blocked in IL-1R–deficient β-cells. Our data using NOD IL-1R–deficient islets or wild-type islets in the presence of IL-1Ra showed that this is true for iNOS expression but not for Fas or class I MHC upregulation by TNF. These do not require local IL-1 expression, indicating substantial variation in how genes are regulated by TNF plus IFN-γ. Therefore, if both IL-1 and TNF are being produced in the islets of NOD mice, perhaps removing IL-1 would have effects limited to genes regulated like iNOS that require IL-1. Other IL-1–inducible genes regulated like class I MHC, which can be upregulated by TNF alone or Fas and can be upregulated by TNF and IFN-γ independently of IL-1, would still be expressed on β-cells of IL-1R–deficient NOD mice as long as TNF is present.

Blockade of IL-1 would also not be effective if IL-1 is not

significantly expressed in NOD islets. There are reports that IL-1 has been detected in NOD islets (43,44), but this may be at too low levels or, alternatively, inhibitors of IL-1 such as IL-1Ra may be concomitantly expressed (43). Fas expression on β-cells is one way of tracking local expression of IL-1 and TNF. Our data suggest that Fas cannot be detected on β-cells during spontaneous diabetes in NOD mice (21,34). This suggests that there is not sufficient local IL-1 (or TNF) action to induce Fas by itself or with IFN-γ. In contrast, in accelerated models of diabetes, β-cell expression of Fas can be detected, indicating that sufficient IL-1 or TNF may be present. Fas expression in these settings is not affected by IL-1 signaling deficiency and may be due to TNF, although other inducers of Fas on β-cells have also been studied in vitro (45).

Another possible reason why IL-1R deficiency did not protect more fully from diabetes is that it may not block the effects of perforin and granzymes. These factors are secreted by the cytotoxic granule of the $CD8^+$ T-cell and are important mediators of β-cell destruction in NOD diabetes (46). If an intervention to block β-cell death does not block perforin-dependent killing either by blocking the interaction between β-cells and CTL (e.g., by downregulation of class I MHC) or by blocking the function of perforin/granzymes, then it may not significantly reduce diabetes in NOD mice. There is no direct way that IL-1
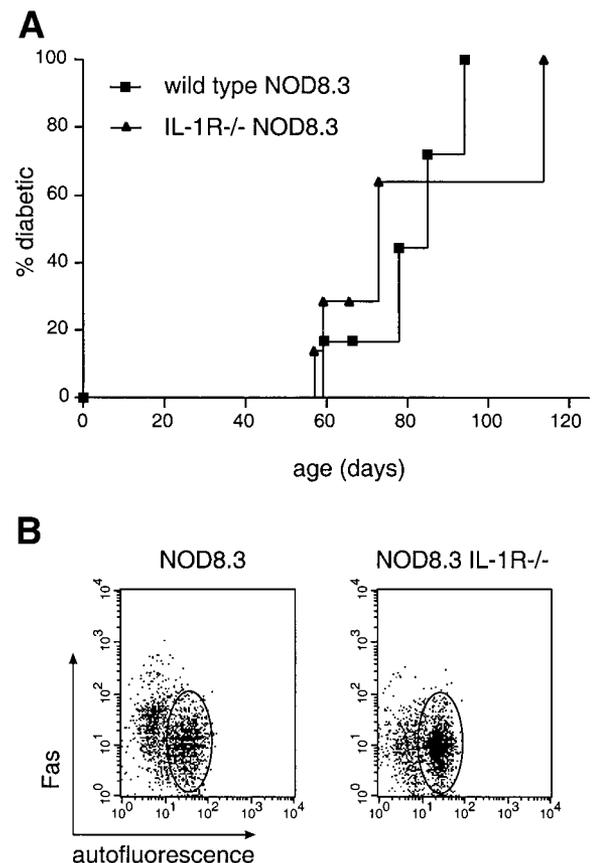

FIG. 7. Diabetes in NOD8.3 mice is not affected by IL-1R deficiency. *A*: Frequency of diabetes was studied in 5 wild-type and 10 IL-1R–deficient NOD8.3 mice. No difference in diabetes frequency or onset was seen ($P > 0.05$). *B*: Fas expression on islet cells from NOD8.3 mice or IL-1R–deficient NOD8.3 mice. Islets were isolated at 35 days of age. Cells with high autofluorescence (predominantly β-cells) are circled. Representative data from four individual mice are shown.

receptor deficiency would block perforin/granzyme function in β-cells. It is likely, however, that multiple pathways (not only perforin) can lead to β-cell death. In perforin-deficient NOD mice, there is residual delayed diabetes (~17% of mice become hyperglycemic) (46). We hypothesized that in settings where β-cell destruction is thought to be perforin independent, particularly those mediated by cytokines, an effect of IL-1R deficiency might be more apparent. Therefore, the IL-1R–deficient mice were bred with NOD TCR transgenic mice BDC2.5, a CD4$^+$ TCR transgenic and 8.3, a CD8$^+$ TCR transgenic in which β-cell killing is believed to be perforin independent (38). In both cases, the frequency of diabetes was unaffected by IL-1R deficiency, although an increase in the time to diabetes was observed in BDC2.5 mice.

We performed fetal pancreas graft experiments to determine whether the small effects of IL-1R deficiency were due to action of IL-1 on β-cells or on effector cells of the immune system. Systemic treatment with IL-1Ra improved both wild-type and IL-1R–deficient graft survival. This treatment is equivalent to the total absence of IL-1 responses in the IL-1R–deficient mice and had similar effects to spontaneous diabetes (i.e., a slight reduction in diabetes incidence). However, IL-1R–deficient grafts survived as poorly as wild-type grafts in placebo-treated mice. This treatment is equivalent to the adoptive transfer of wild-type splenocytes into IL-1R–deficient mice (Fig. 4B), where no change in diabetes incidence was observed. In this experiment, the transferred cells are responsive to IL-1, whereas the recipient mice remain IL-1R deficient. These data suggest that IL-1 action on the effector cells may be more important than that on the β-cells. The exact effect that IL-1 has on these cells remains to be elucidated.

That IL-1 has relatively small effects on diabetes in NOD mice does not rule out a more important role in other rodent models of autoimmune diabetes or in human type 1 diabetes. There are subtle differences between mouse, rat, and human islet responses to cytokines, which may make IL-1 action more important in human diabetes. These differences include regulation of the iNOS gene and its less important role in cytokine-induced death of human β-cells (47,48). Also, Fas appears to be expressed in islets from biopsies of diabetic patients (49), whereas we are unable to detect Fas expression on β-cells of spontaneously diabetic NOD mice (34).

The reduction observed in diabetes frequency in the NOD mouse may reflect more the difficulty of achieving large reductions in diabetes by blockade of a single pathway than the lack of involvement of IL-1. Therefore, it remains to be tested whether blockade of other cytokine pathways together with IL-1R deficiency will have a greater impact on diabetes. These results, however, must make it less of a priority to test neutralization of IL-1 or protection from its effects as monotherapy in human diabetes than if the current experiments had completely blocked diabetes in NOD mice. It also focuses attention on the effector mechanisms more likely to be dominant, including perforin, Fas, and possibly TNF, although TNF has been claimed to affect T-cell activation more than it does β-cell death (36,50). IL-1 has been a major focus of efforts to understand and prevent β-cell death over many years, and we believe our data clarify its importance in the NOD mouse.

REFERENCES

1. Labow M, Shuster D, Zetterstrom M, Nunes P, Terry R, Cullinan EB, Bartfai T, Solorzano C, Moldawer LL, Chizzonite R, McIntyre KW: Absence of IL-1 signaling and reduced inflammatory response in IL-1 type I receptor-deficient mice. *J Immunol* 159:2452–2461, 1997
2. Glaccum MB, Stocking KL, Charrier K, Smith JL, Willis CR, Maliszewski C, Livingston DJ, Peschon JJ, Morrissey PJ: Phenotypic and functional characterization of mice that lack the type I receptor for IL-1. *J Immunol* 159:3364–3371, 1997
3. Deyerle KL, Sims JE, Dower SK, Bothwell MA: Pattern of IL-1 receptor gene expression suggests role in noninflammatory processes. *J Immunol* 149:1657–1665, 1992
4. Scarim AL, Arnush M, Hill JR, Marshall CA, Baldwin A, McDaniel ML, Corbett JA: Evidence for the presence of type I IL-1 receptors on beta-cells of islets of Langerhans. *Biochim Biophys Acta* 1361:313–320, 1997
5. Mandrup-Poulsen T, Bendtzen K, Nerup J, Dinarello CA, Svenson M, Nielsen JH: Affinity-purified human interleukin I is cytotoxic to isolated islets of Langerhans. *Diabetologia* 29:63–67, 1986
6. Delaney CA, Green MH, Lowe JE, Green IC: Endogenous nitric oxide induced by interleukin-1 beta in rat islets of Langerhans and HIT-T15 cells causes significant DNA damage as measured by the 'comet' assay. *FEBS Lett* 333:291–295, 1993
7. Bolaffi JL, Rodd GG, Wang J, Grodsky GM: Interrelationship of changes in islet nicotine adeninedinucleotide, insulin secretion, and cell viability induced by interleukin-1 beta. *Endocrinology* 134:537–542, 1994
8. Corbett JA, Sweetland MA, Wang JL, Lancaster JR Jr, McDaniel ML: Nitric oxide mediates cytokine-induced inhibition of insulin secretion by human islets of Langerhans. *Proc Natl Acad Sci U S A* 90:1731–1735, 1993
9. Eizirik DL, Tracey DE, Bendtzen K, Sandler S: An interleukin-1 receptor antagonist protein protects insulin-producing beta cells against suppressive effects of interleukin-1 beta. *Diabetologia* 34:445–448, 1991
10. Thomas HE, Darwiche R, Corbett JA, Kay TW: Interleukin-1 plus γ-interferon-induced pancreatic β-cell dysfunction is mediated by β-cell nitric oxide production. *Diabetes* 51:311–316, 2002
11. Corbett JA, McDaniel ML: Intraislet release of interleukin 1 inhibits beta cell function by inducing beta cell expression of inducible nitric oxide synthase. *J Exp Med* 181:559–568, 1995
12. Arnush M, Heitmeier MR, Scarim AL, Marino MH, Manning PT, Corbett JA: IL-1 produced and released endogenously within human islets inhibits beta cell function. *J Clin Invest* 102:516–526, 1998
13. Arnush M, Scarim AL, Heitmeier MR, Kelly CB, Corbett JA: Potential role of resident islet macrophage activation in the initiation of autoimmune diabetes. *J Immunol* 160:2684–2691, 1998
14. Fox CJ, Danska JS: IL-4 expression at the onset of islet inflammation predicts nondestructive insulitis in nonobese diabetic mice. *J Immunol* 158:2414–2424, 1997
15. Held W, MacDonald HR, Weissman IL, Hess MW, Mueller C: Genes encoding tumor necrosis factor alpha and granzyme A are expressed during development of autoimmune diabetes. *Proc Natl Acad Sci U S A* 87:2239–2243, 1990
16. Rabinovitch A, Suarez-Pinzon WL, Sorensen O, Bleackley RC: Inducible nitric oxide synthase (iNOS) in pancreatic islets of nonobese diabetic mice: identification of iNOS-expressing cells and relationships to cytokines expressed in the islets. *Endocrinology* 137:2093–2099, 1996
17. Thomas HE, Parker JL, Schreiber RD, Kay TW: IFN-gamma action on

pancreatic beta cells causes class I MHC upregulation but not diabetes. *J Clin Invest* 102:1249–1257, 1998

18. Cailleau C, Diu-Hercend A, Ruuth E, Westwood R, Carnaud C: Treatment with neutralizing antibodies specific for IL-1β prevents cyclophosphamide-induced diabetes in nonobese diabetic mice. *Diabetes* 46:937–940, 1997

19. Nicoletti F, Di Marco R, Barcellini W, Magro G, Schorlemmer HU, Kurrle R, Lunetta M, Grasso S, Zaccone P, Meroni P: Protection from experimental autoimmune diabetes in the non-obese diabetic mouse with soluble interleukin-1 receptor. *Eur J Immunol* 24:1843–1847, 1994

20. Stassi G, Todaro M, Richiusa P, Giordano M, Mattina A, Sbriglia MS, Lo Monte A, Buscemi G, Galluzzo A, Giordano C: Expression of apoptosis-inducing CD95 (Fas/Apo-1) on human beta-cells sorted by flow-cytometry and cultured in vitro. *Transplant Proc* 27:3271–3275, 1995

21. Thomas HE, Darwiche R, Corbett JA, Kay TW: Evidence that beta cell death in the nonobese diabetic mouse is Fas independent. *J Immunol* 163:1562–1569, 1999

22. Cullinan EB, Kwee L, Nunes P, Shuster DJ, Ju G, McIntyre KW, Chizzonite RA, Labow MA: IL-1 receptor accessory protein is an essential component of the IL-1 receptor. *J Immunol* 161:5614–5620, 1998

23. Ewen KR, Bahlo M, Treloar SA, Levinson DF, Mowry B, Barlow JW, Foote SJ: Identification and analysis of error types in high-throughput genotyping. *Am J Hum Genet* 67:727–736, 2000

24. Serreze DV, Leiter EH: Genes and cellular requirements for autoimmune diabetes susceptibility in nonobese diabetic mice. *Curr Dir Autoimmun* 4:31–67, 2001

25. Verdaguer J, Schmidt D, Amrani A, Anderson B, Averill N, Santamaria P: Spontaneous autoimmune diabetes in monoclonal T cell nonobese diabetic mice. *J Exp Med* 186:1663–1676, 1997

26. Katz JD, Wang B, Haskins K, Benoist C, Mathis D: Following a diabetogenic T cell from genesis through pathogenesis. *Cell* 74:1089–1100, 1993

27. Liu M, Shapiro ME: A new method for isolation of murine islets with markedly improved yields. *Transpl Proc* 27:3208–3210, 1995

28. Nicoletti I, Migliorati G, Pagliacci MC, Grignani F, Riccardi C: A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. *J Immunol Methods* 139:271–279, 1991

29. Charlton B, Mandel TE: Progression from insulitis to β-cell destruction in NOD mouse requires L3T4+ T-lymphocytes. *Diabetes* 37:1108–1112, 1988

30. Kurrer MO, Pakala SV, Hanson HL, Katz JD: Beta cell apoptosis in T cell-mediated autoimmune diabetes. *Proc Natl Acad Sci U S A* 94:213–218, 1997

31. Allison J, Strasser A: Mechanisms of beta cell death in diabetes: a minor role for CD95. *Proc Natl Acad Sci U S A* 23:13818–13822, 1998

32. Hill NJ, Lyons PA, Armitage N, Todd JA, Wicker LS, Peterson LB: NOD Idd5 locus controls insulitis and diabetes and overlaps the orthologous CTLA4/IDDM12 and NRAMP1 loci in humans. *Diabetes* 49:1744–1747, 2000

33. Lamhamedi-Cherradi SE, Boulard O, Gonzalez C, Kassis N, Damotte D, Eloy L, Fluteau G, Levi-Strauss M, Garchon H: Further mapping of the Idd5.1 locus for autoimmune diabetes in NOD mice. *Diabetes* 50:2874–2878, 2001

34. Darwiche R, Chong MW, Santamaria P, Thomas HE, Kay TW: Fas is detectable on beta cells in accelerated, but not spontaneous, diabetes in nonobese diabetic mice. *J Immunol* 170:6292–6297, 2003

35. Sandberg JO, Eizirik DL, Sandler S: IL-1 receptor antagonist inhibits recurrence of disease after syngeneic pancreatic islet transplantation to spontaneously diabetic non-obese diabetic (NOD) mice. *Clin Exp Immunol* 108:314–317, 1997

36. Pakala SV, Chivetta M, Kelly CB, Katz JD: In autoimmune diabetes the transition from benign to pernicious insulitis requires an islet cell response to tumor necrosis factor alpha. *J Exp Med* 189:1053–1062, 1999

37. Andre-Schmutz I, Hindelang C, Benoist C, Mathis D: Cellular and molecular changes accompanying the progression from insulitis to diabetes. *Eur J Immunol* 29:245–255, 1999

38. Amrani A, Verdaguer J, Anderson B, Utsugi T, Bou S, Santamaria P: Perforin-independent beta-cell destruction by diabetogenic CD8(+) T lymphocytes in transgenic nonobese diabetic mice. *J Clin Invest* 103:1201–1209, 1999

38a. Schott WH, Haskell BD, Tse HM, Milton MJ, Piganelli JD, Choisy-Rossi CM, Riefsnyder PC, Chervonsky AV, Leiter EH: Caspase-1 is not required for type 1 diabetes in the NOD mouse. *Diabetes* 53:99–104, 2003

39. Liu D, Pavlovic D, Chen MC, Flodstrom M, Sandler S, Eizirik DL: Cytokines induce apoptosis in beta-cells isolated from mice lacking the inducible isoform of nitric oxide synthase (iNOS-/-). *Diabetes* 49:1116–1122, 2000

40. Stephens LA, Thomas HE, Ming L, Grell M, Darwiche R, Volodin L, Kay TW: Tumor necrosis factor-alpha-activated cell death pathways in NIT-1 insulinoma cells and primary pancreatic beta cells. *Endocrinology* 140:3219–3227, 1999

41. Irawaty W, Kay TWH, Thomas HE: Transmembrane TNF and IFNgamma induce caspase-independent death of primary mouse pancreatic beta cells. *Autoimmunity* 35:369–375, 2002

42. Eizirik DL, Mandrup-Poulsen T: A choice of death: the signal-transduction of immune-mediated beta-cell apoptosis. *Diabetologia* 44:2115–2133, 2001

43. Welsh M, Welsh N, Bendtzen K, Mares J, Strandell E, Oberg C, Sandler S: Comparison of mRNA contents of interleukin-1 beta and nitric oxide synthase in pancreatic islets isolated from female and male nonobese diabetic mice. *Diabetologia* 38:153–160, 1995

44. Reddy S, Young M, Ginn S: Immunoexpression of interleukin-1beta in pancreatic islets of NOD mice during cyclophosphamide-accelerated diabetes: co-localization in macrophages and endocrine cells and its attenuation with oral nicotinamide. *Histochem J* 33:317–327, 2001

45. Liu D, Darville M, Eizirik DL: Double-stranded ribonucleic acid (RNA) induces beta-cell Fas messenger RNA expression and increases cytokine-induced beta-cell apoptosis. *Endocrinology* 142:2593–2599, 2001

46. Kagi D, Odermatt B, Seiler P, Zinkernagel RM, Mak TW, Hengartner H: Reduced incidence and delayed onset of diabetes in perforin-deficient nonobese diabetic mice. *J Exp Med* 186:989–997, 1997

47. Eizirik DL, Sandler S, Welsh N, Cetkovic-Cvrlje M, Nieman A, Geller DA, Pipeleers DG, Bendtzen K, Hellerstrom C: Cytokines suppress human islet function irrespective of their effects on nitric oxide generation. *J Clin Invest* 93:1968–1974, 1994

48. Rabinovitch A, Suarez-Pinzon WL, Strynadka K, Schulz R, Lakey JR, Warnock GL, Rajotte RV: Human pancreatic islet beta-cell destruction by cytokines is independent of nitric oxide production. *J Clin Endocrinol Metab* 79:1058–1062, 1994

49. Moriwaki M, Itoh N, Miyagawa J, Yamamoto K, Imagawa A, Yamagata K, Iwahashi H, Nakajima H, Namba M, Nagata S, Hanafusa T, Matsuzawa Y: Fas and Fas ligand expression in inflamed islets in pancreas sections of patients with recent-onset type I diabetes mellitus. *Diabetologia* 42:1332–1340, 1999

50. Green EA, Eynon EE, Flavell RA: Local expression of TNFalpha in neonatal NOD mice promotes diabetes by enhancing presentation of islet antigens. *Immunity* 9:733–743, 1998