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Islet-Specific Expression of IL-10 Promotes Diabetes in Nonobese Diabetic Mice Independent of Fas, Perforin, TNF Receptor-1, and TNF Receptor-2 Molecules

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Several death-signaling or death-inducing molecules have been implicated in β cell destruction, including Fas, perforin, and TNFR-1. In this study, we examined the role of each death-signaling molecule in the IL-10-accelerated diabetes of nonobese diabetic (NOD) mice. Groups of IL-10-NOD mice, each deficient in either Fas, perforin, or TNFR-1 molecules, readily developed insulitis, and subsequently succumbed to diabetes with an accelerated kinetics and incidence similar to that observed in their wild-type or heterozygous IL-10-NOD littersmates. Similarly, a TNFR-2 deficiency did not block accelerated diabetes in IL-10-NOD mice and spontaneous diabetes in NOD mice. These results demonstrate that pancreatic IL-10 promotes diabetes independent of the Fas, perforin, TNFR-1, and TNFR-2 molecules. Subsequently, when cyclophosphamide, a diabetes-inducing agent, was injected into insulitis-free NOD.lpr/lpr mice, none of these mice developed insulitis or diabetes. Our data suggest that cyclophosphamide-induced but not IL-10-induced diabetes is Fas dependent. Overall, these findings provide evidence that pancreatic expression of IL-10 promotes diabetes independent of the major death pathways and provide impetus for identification of novel death pathways precipitating autoimmune destruction of insulin-producing β cells.

Insulin-dependent diabetes mellitus (IDDM)4 is an autoimmune disease caused by the T cell-mediated destruction of insulin-producing β cells in the pancreatic islets of Langerhans (1, 2). The nonobese diabetic (NOD) mouse spontaneously develops IDDM and has been used as an animal model of human IDDM. In the NOD mouse, insulitis begins at 3–4 wk of age and diabetes by 14 wk of age (3).

Cytokines produced by the mononuclear cell infiltrate (T cells and APC) itself are clearly involved in the propagation of insulitis. Treatment of young NOD mice with anti-IL-10 mAb prevented the development of insulitis (4). Elsewhere, the expression of IL-10 in pancreatic β cells correlated with the insulitis of NOD mice (5). BALB/c mice expressing the IL-10 transgene (tg) in their insulin-producing β cells (IL-10-BALB/c mice) of the pancreas did not develop diabetes, but their offspring (IL-10-NOD mice) from backcrosses (N2-N3) to NOD mice became diabetic at an accelerated rate (6). Similarly, NOD mice expressing the IL-10 transgene in glucagon-producing α cells of the pancreas developed diabetes at an accelerated rate (5). These cumulative findings implied that IL-10 is an immunostimulatory factor in the IDDM of NOD mice. Our recent studies have demonstrated that the promotion of diabetes by IL-10 in NOD mice requires the participation of an autoreactive T cell repertoire (7). Depending upon the circumstances, pancreatic IL-10 promoted autoimmune diabetes via CD4 (8) or CD8 T cells (7).

Several studies have shown that a homozygous deficiency of the Fas, perforin, or TNFR-1 molecules dramatically affects the incidence of spontaneous insulitis and diabetes in NOD mice. Fas-deficient NOD.lpr/lpr mice were free from insulitis and diabetes (9, 10), and a perforin deficiency reduced the incidence of diabetes by a dramatic 80–85%, despite the mild insulitis in pancreata (11) of these mice, as confirmed by others (12). Additionally, TNFR-1-deficient NOD mice exhibited mild insulitis, but were completely resistant to spontaneous diabetes (13). Consequently, each of these pathways participates in either the initiation and/or effector phases of autoimmune diabetes.

In this study, we examined the roles of these death-signaling molecules (Fas, perforin, and TNFR-1), and of TNFR-2 as well, in the IL-10-accelerated autoimmune diabetes of NOD mice. The Fas-Fas ligand (FasL) pathway was also explored in cyclophosphamide (CYP)-induced diabetes of NOD mice. Determining how these molecules function, if at all, in the inception and acceleration of autoimmune diabetes is important to understanding how inflammatory stimuli such as cytokines in the target organ predispose mice to autoimmune diabetes and to devising appropriate therapeutic interventions.

Our results demonstrate that IL-10 promoted diabetes in NOD mice independent of the Fas, perforin, TNFR-1, and TNFR-2 molecules. A homozygous deficiency at perforin gene locus in IL-10-NOD mice slightly delayed the onset, but did not decrease the incidence of their diabetes. Although IL-10-NOD.lpr/lpr mice...
readily developed diabetes, CYP-injected NOD.Ipr/lpr mice did not. This outcome suggests that IL-10-accelerated diabetes is Fas independent, whereas CYP-induced diabetes is Fas dependent. Finally, current findings may provide impetus for the delineation of additional death pathways, under the influence of cytokine-induced inflammation, precipitating autoimmune destruction of insulin-producing β cells.

Materials and Methods

Mice

NOD/shi mice were part of the rodent breeding colony at The Scripps Research Institute (La Jolla, CA). IL-10-BALB/c mice expressing the IL-10 transgene in their islets under control of the human insulin promoter were backcrossed to NOD/shi mice for 10–11 generations to produce IL-10-NOD mice (7). The presence of the transgene was verified by PCR. Mice were housed under specific pathogen-free conditions.

Generation of Fas-deficient NOD.Ipr/lpr mice

Initially, we backcrossed B6.MRL.Ipr/lpr mice to NOD mice for two generations. The heterozygous offspring were intercrossed to get NOD.Ipr/lpr mice. IL-10-NOD mice were then backcrossed to these mice to generate N3 to N4 mice of appropriate combinations.

Subsequently, to generate a later generation of IL-10-NOD.Ipr/lpr, mice, IL-10-NOD mice were backcrossed to NOD.wt/lpr or NOD.lpr/lpr mice of an N9 backcross generation. The Fas mutation (lpr) was verified in tail DNA by using two pairs of primer sets (10). The first pair was composed of NIL-1, 5′-CAG CAG GAA TCC TAT GAG GT-3′ and NIL-2, 5′-CTC GCA ACG TGA AGT CGG-3′, yielding a band of 381 bp for the mutated allele. The second pair was composed of NIL-1, 5′-CAG CAG GAA TCC TAT GAG GT-3′ and NIL-4, 5′-GCA GAG ATG CTA AGC AGC AG-3′, yielding a band of 265 bp for the wild-type allele and a band of 5.7 kb for the mutated allele.

Generation of perforin-deficient NOD mice

Perforin-deficient BALB/c mice were backcrossed onto NOD mice for two to three generations, and the resulting heterozygous mice of generation N2 or N3 were then intercrossed to generate perforin-deficient NOD mice and, subsequently, perforin-deficient IL-10-NOD mice. The perforin genotype was determined with PCR using three primers on DNA prepared from tail biopsies (perforin 12 primer, 5′-TGG CCT AGG GTT CAC ATC CAG-3′; perforin 17 primer, 5′-ATA TTG GCT GCA GGG TCG CTC-3′; perforin 26 primer, 5′-ATG TTG GCT GCA GGG TCG TCT-3′). The PCR yielded a 500-bp fragment for wild-type mice, a 350-bp fragment for KO mice, and 350- and 500-bp fragments for heterozygous mice.

Generation of TNFR-1-deficient NOD mice

TNFR-1-deficient C57BL/6j mice (14) were purchased from The Jackson Laboratory (Bar Harbor, ME) and backcrossed onto NOD mice for three to four generations. NOD.TNFR-1 heterozygous mice of N3 or N4 generations were intercrossed to generate TNFR-1-deficient NOD mice, which were used to introduce the TNFR-1 gene deficiency into IL-10-NOD mice. TNFR-1 genotyping was determined by PCR using three primers on DNA prepared from tail biopsies in one PCR. o1MR448, 5′-TGG CCT AGG GTT CAC ATC CAG-3′ (TNFR-1 wild-type primer); o1MR449, 5′-GCC TGC AGT CCA GCA ACT GG-3′ (TNFR-1 common primer); o1MR450, 5′-ATT CGC CAA TGA TGA CAA GAC GCT GG-3′ (HSV-thymidine kinase primer). The o1MR448 and o1MR449 primer set yielded 470 bp for +/+ mice, The o1MR449 and o1MR450 primer set yielded 300-bp fragment for KO mice and 300-bp fragment for TNFR-1 KO mice.

Generation of TNFR-2-deficient NOD mice

TNFR-2-deficient C57BL/6j mice (15) were purchased from The Jackson Laboratory and were backcrossed onto NOD mice for three to four generations. NOD.TNFR-2 heterozygous mice of N3 or N4 generations were intercrossed to generate TNFR-2-deficient NOD mice, which were used to introduce TNFR-2 gene deficiency into IL-10-NOD mice. The TNFR-2 genotyping was determined by PCR on DNA prepared from tail biopsies using two primer sets in two separate reactions. o1MR338, 5′-CCT CTC ATG CTG TCC CGG AAT-3′ (wild-type primer) (forward); o1MR338, 5′-AGC TCC AGC CAA AGG GCC GG-3′ (wild-type primer) (reverse); o1MR338, 5′-ATT CGC CAA TGA TGA CAA GAC GCT GG-3′ (HSV-thymidine kinase primer) (forward); o1MR331, 5′-ATC CTC GGC GGG CAT GC-3′ (neo primer) (reverse). The o1MR338 and o1MR338 primer set yielded a 200-bp fragment, whereas o1MR340 and o1MR340 yielded a 400-bp fragment.

MHC typing of mice

All the second backcross mice were tested by PCR for NOD, C57BL/6, and BALB/c MHC. The presence of I-Aβ8 was determined on tail DNA by PCR using the following primer set: forward primer, 5′-GAT ACA TCT ACA ACC GGG AGG AG-3′, and reverse primer, 5′-CTG TTC GAC TAC TCG GCG TCT G-3′. PCR amplification yielded a 103-bp product from BALB/c, but not NOD mice. The presence of I-Eα4 was tested in tail DNA by PCR using the following primer set: forward primer, 5′-ATG AGC TTC CAG AAG TCA TGG G-3′, and reverse primer, 5′-GGA GAG ACA GCA GCT CTC AGC-3′. PCR amplification yielded a 277-bp product from BALB/c, but not NOD mice. Mice were also tested for MHC class I molecules at Kβ with anti-Kβ mAb (clone SF1-1.1), Kβ with anti-Kβ mAb (clone AF6-88.5), Dβ1 with anti-Dβ mAb (clone 34-2-12), Dβ with anti-H-2Dβ mAb (clone 28-14-A8), and class II β4-1 molecule with anti-I-Aβ mAb (clone AF6-120.1) by flow cytometry.

Adaptive transfers

Donor splenocytes from the mice indicated were prepared as single cell suspensions in sterile PBS. These cells were injected (at 1 × 107 cells/mouse or 3 × 107/mouse) i.v. into 16-wk-old IL-10-NOD.scid/scid or NOD.scid/scid mice. For adoptive transfer of perforin-deficient splenocytes, we used perforin-deficient (−/−) NOD mice of N10 backcross generation (12).

Assessment of diabetes

Starting at 4–5 wk of age, mice were tested for diabetes by weekly measurements of blood glucose (BG) levels using a one-step Bayer Glucometer Elite (Bayer Corporation, Elkhart, IN). Animals were considered diabetic when BG levels were >300 mg/dl. In most instances, the BG levels exceeded 500 mg/dl. Mice of both sexes were included in all the IL-10-NOD experiments, and female mice were employed for monitoring of diabetes in TNFR-1 KO and TNFR-2 KO mice.

Histological analysis

Lymphocytic infiltration of the islets was evaluated on hematoxylin and eosin (H&E)-stained paraffin sections of the pancreas. For insulin staining, paraffin-embedded sections of the pancreata were stained with an immunoperoxidase method using polyclonal Abs to porcine insulin, followed by a biotinylated secondary Ab and an avidin-biotin complex, as described earlier (16).

Results

Pancreatic IL-10 promotes autoimmune insulitis and diabetes in NOD.Ipr/lpr mice

Autoimmune destruction of insulin-producing β cells involves Fas-FasL interaction, as evident because Fas-deficient NOD.Ipr/lpr mice are free from spontaneous insulitis and diabetes (9, 10). Previous studies from our group demonstrated that IL-10-NOD mice rapidly develop insulitis and diabetes compared with their counterpart NOD mice (7). Because a Fas+ mononuclear cell infiltrate in the pancreatic islets of 5-wk-old diabetic IL-10-NOD mice (not shown) was observed, we tested the requirement for Fas-FasL interaction in the accelerated diabetes of IL-10-NOD mice. After introducing the Fas deficiency into IL-10-NOD mice by breeding them with diabetes-resistant Fas-deficient NOD.Ipr/lpr mice, we monitored their offspring for diabetes at weekly intervals. Surprisingly, IL-10-NOD.Ipr/lpr mice of the N3-N4 backcross generations (n = 8; 88%) (Fig. 1A) and N8-N9 backcross mice (n = 13; 100%) (Fig. 1B) developed the accelerated diabetes. The kinetics and incidence of the disease in these mice duplicated that of the wild-type (Fas/Fas) (N3-N4 backcross n = 5; 100%; or N8-N9 backcross mice n = 12; 100%) as well as of the heterozygous littersmates (Fas/lpr) (N3-N4 backcross n = 9; 89%; or N8-N9 backcross mice n = 12; 83%). Statistical values (p values) for N3-N4 backcross mice were as follows: IL-10-NOD.Ipr/lpr vs IL-10-NOD.Fas/lpr 0.6145; IL-10-NOD.Ipr/lpr vs IL-10-NOD.Fas 0.850. Statistical values for N8-N9 backcross mice were as follows: IL-10-NOD.Ipr/lpr vs IL-10-NOD.Fas/lpr 0.5370; IL-10-NOD.Ipr/lpr vs IL-10-NOD.Fas 0.0751. However, during the same period of time, none of the non-tg littersmates (Fas/Fas or Fas/lpr vs IL-10-NOD.Fas 0.0751).
Fas/lpr or lpr/lpr) became diabetic. The NOD.Fas/Fas and NOD.
Fas/lpr mice developed diabetes after 14 wk of age. In agreement
with the published literature, NOD.lpr/lpr mice did not develop
diabetes over a 24-wk period (not shown). Therefore, the Fas/FasL
interaction was not necessary for IL-10-accelerated diabetes in
NOD mice. Analysis of pancreata from IL-10-NOD.lpr/lpr (−/−) mice, by H&E staining, showed extensive lymphocytic infiltration
of the islets similar to that observed in IL-10-NOD.Fas/lpr (+/−)
littermates used as controls (Fig. 1C). The insulin-positive cells in
these infiltrated islets were completely destroyed (data not shown).

**FIGURE 1.** A and B, Incidence of autoimmune diabetes in IL-10-NOD.lpr/lpr mice and their littermate controls. C, H&E staining of the paraffin-
embedded pancreata from Fas-deficient IL-10-NOD.lpr/lpr (−/−) (N9 backcross) shows severe insulitis compared with that in Fas-sufficient IL-10-
NOD.Fas/Fas (+/+) littermates. Note that pancreata from NOD.lpr/lpr are free from insulitis (×200).
As expected, islets from pancreata of NOD.lpr/lpr mice were free from insulitis. Our results demonstrate that IL-10 promotes autoimmune insulitis and diabetes independent of the Fas/FasL pathway.

**Splenocytes from diabetic IL-10-NOD.lpr/lpr mice transfer disease into NOD.scid/scid mice**

Since, as Fig. 1 shows, IL-10-NOD.lpr/lpr mice readily developed diabetes, but the non-tg NOD.lpr/lpr mice did not, we tested whether splenocytes from diabetic 8-wk-old IL-10-NOD.lpr/lpr mice would transfer disease into NOD-scid/scid mice. As expected (Fig. 2A), splenocytes from littermate non-tg NOD.lpr/lpr mice did not cause diabetes in NOD-scid/scid mice (n = 4). Conversely, splenocytes from diabetic IL-10-NOD.lpr/lpr mice provoked diabetes in recipient NOD-scid/scid mice beginning at 16 wk post-transfer (n = 4) (p = 0.0082). Staining of pancreata by H&E revealed mononuclear cell infiltrates within islets from NOD-scid/scid recipients of splenocytes from diabetic IL-10-NOD.lpr/lpr mice, whereas islets in NOD-scid/scid recipients of splenocytes from NOD.lpr/lpr mice were free from insulitis (Fig. 2B). In a different set of experiments, splenocytes from 5-wk-old diabetic IL-10-NOD mice (Fas/lpr) transferred disease into NOD.scid/scid mice with same kinetics that was observed with 8-wk-old diabetic IL-10-NOD.lpr/lpr mice. Additionally, the kinetics of disease transfer observed with splenocytes from 5-wk-old diabetic IL-10-NOD mice is far different from that observed with splenocytes from 18-wk-old diabetic NOD mice. The results again indicate that the Fas/FasL pathway is not required for autoimmune diabetes in IL-10-NOD mice.

**IL-10 promotes accelerated diabetes in perforin-deficient NOD mice**

Next, we examined the role of perforin in autoimmune destruction of β cells of IL-10-NOD mice by introducing a perforin gene deficiency into IL-10-NOD mice. As Fig. 3A depicts, IL-10-NOD mice of the wild-type (+/+), (n = 8; 88% incidence) or heterozygous (+/−) (n = 15; 87% incidence) or KO (−/−) (n = 9; 89%) for the perforin gene developed the anticipated diabetes. Interestingly, a gene dose effect on the incidence of accelerated diabetes at 5 wk of age was noticed. As compared with 50% incidence of diabetes at 5 wk of age in IL-10-NOD wild-type (+/+), mice, only 7% of heterozygous and 11% of KO mice developed diabetes. However, the cumulative percentage of incidence of diabetes at 12 wk was similar among all the groups (p = 0.8185 for IL-10-NOD.perforin (−/−) vs IL-10-NOD.perforin (+/−); p = 0.3244 for IL-10-NOD.perforin (−/−) vs IL-10-NOD.perforin (+/+)). During the same interval, none of the non-tg perforin-deficient (−/−) NOD mice developed diabetes (n = 10). The pancreatic islets from diabetic IL-10-NOD mice that are heterozygous (+/−) or deficient (−/−) for perforin were completely infiltrated with mononuclear cells (Fig. 3B) and their insulin-producing β cells were destroyed (not shown).

To further confirm that perforin is not required for accelerated diabetes of IL-10-NOD mice, we performed adoptive transfer experiments. To this end, splenocytes from non-diabetic 32-wk-old perforin-deficient (−/−) NOD mice of the N10 backcross generation (12) were injected into tg IL-10-NOD.scid/scid mice (7) or non-tg NOD.scid/scid mice (7). When injected, these splenocytes readily caused diabetes in 13- to 14-wk-old IL-10-NOD.scid/scid mice (n = 4; 100% incidence) at 4 wk post-transfer. However, NOD.scid/scid mice (n = 4; 0% incidence) that received splenocytes from NOD.perforin-deficient (−/−) mice did not develop diabetes (Fig. 3C) (p = 0.0082). Additionally, IL-10-NOD-scid/scid mice not given splenocytes from NOD.perforin-deficient (−/−) mice did not develop diabetes (n = 5) (not shown). These results confirm that perforin is not essential for the accelerated diabetes of IL-10-NOD mice.

Analysis by H&E staining (Fig. 3D) revealed that the pancreatic islets from IL-10-NOD-scid/scid mice that were injected with NOD-perforin deficient (−/−) splenocytes were completely infiltrated with mononuclear cells, causing destruction of most of β cells, leaving few in place. Conversely, pancreatic islets from NOD-scid/scid mice that were injected with NOD.perforin KO mice were free from insulitis, confirming that perforin is not required for accelerated diabetes in IL-10-NOD mice.

**FIGURE 2.** A. Adoptive transfer of splenocytes from female 8-wk-old diabetic IL-10-NOD.lpr/lpr mice causes disease in 8-wk-old female NOD.scid/scid mice, whereas splenocytes from littermate 8-wk-old NOD.lpr/lpr mice do not. Each recipient mouse was injected i.v. with 2 × 10^7 splenocytes in PBS. B. Pancreata from NOD.scid/scid recipients that were injected with splenocytes from diabetic IL-10-NOD.lpr/lpr mice show mononuclear cell infiltration of the islets and the loss of insulin-producing β cells, whereas splenocytes from NOD.lpr/lpr mice did not cause insulitis (×200).

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**FIGURE 3.** A. Incidence of autoimmune diabetes in N2-N3 backcross IL-10-NOD, perforin-deficient (−/−) mice and their littermate controls. B. H&E staining of the paraffin-embedded pancreata from IL-10-NOD, perforin-deficient (−/−) shows severe insulitis compared with that in perforin-sufficient (+/−) IL-10-NOD littermates (magnification, ×400). C. Adoptive transfer of splenocytes from female 32-wk-old nondiabetic perforin-deficient (−/−) NOD mice provokes disease in 8-wk-old female IL-10-NOD, scid/scid, but not NOD, scid/scid mice. Each recipient mouse was injected i.v. of 1 × 10^7 splenocytes in PBS. D. H&E staining of pancreata from IL-10-NOD, scid/scid and NOD, scid/scid recipient mice injected with splenocytes from 32-wk-old nondiabetic perforin-deficient (−/−) NOD mice (×200).
(−/−) splenocytes exhibited only periinsulitis and contained intact insulin-producing β cells. These findings further confirm the data of Fig. 3A that IL-10 promotes diabetes independent of perforin pathway.

TNFR-1-deficient NOD mice are susceptible to IL-10-accelerated diabetes

Since neither Fas nor perforin molecules were essential for the diabetic state of IL-10-NOD mice, we questioned whether the TNFR-1 gene would fill that role. TNFR-1 gene function was disrupted in IL-10-NOD mice, after which their tg progeny and non-tg littermates were monitored for diabetes beginning at 5 wk of age. The results shown in Fig. 4A are from N3-N4 backcross generations. The findings show that IL-10-NOD mice that are wild type (+/+)(n = 9; 89%) or heterozygous (+/−)(n = 12; 83%) for TNFR-1 gene readily developed diabetes. Similarly, IL-10-NOD-TNFR-1-deficient (−/−) mice (n = 10; 90%) developed diabetes with an accelerated kinetics and incidence like that in the tg littermate controls (p = 0.6790 vs IL-10-NOD.TNFR-1 +/+ mice; p = 0.3359 vs IL-10-NOD.TNFR-1 +/− mice). In agreement with previous observations (13), NOD-TNFR-1-deficient mice (−/−) did not develop diabetes (n = 7) over a period of 24 wk. When pancreatic tissues from diabetic IL-10-NOD-TNFR-1 +/− and IL-10-NOD-TNFR-1-deficient (−/−) mice were then stained with H&E, islets from both groups were completely infiltrated with mononuclear cells (Fig. 4B), and their insulin-producing β cells had been destroyed (not shown). Considering that the

![FIGURE 4. A. Incidence of autoimmune diabetes in IL-10-NOD. TNFR-1-deficient (−/−) and sufficient (+/−, +/+ ) mice. Their non-tg TNFR-1-deficient (−/−) littermate controls are also included. The mice used were of the N3-N4 backcross generations. B. H&E staining of paraffin-embedded pancreatic sections from diabetic IL-10-NOD. TNFR-1-deficient (−/−) and sufficient (+/−) mice, respectively (×200).](image)

![FIGURE 5. A. Incidence of autoimmune diabetes in IL-10-NOD mice that are deficient (−/−) and sufficient (+/−) for TNFR-2 molecules. Their non-tg TNFR-1-deficient (−/−) littermate controls are also included. The mice used were of N3-N4 backcross generation. B. H&E staining of paraffin-embedded pancreatic sections from diabetic IL-10-NOD. TNFR-2-deficient (−/−) and sufficient (+/−) mice, respectively (×400).](image)
pancreatic islets from age-matched non-tg TNFR-1-deficient littermates were free from insulitis (data not shown), and their inability to develop diabetes even at 24 wk of age (n = 8), clearly TNFR-1 signaling plays a role in spontaneous autoimmune diabetes of NOD mice, but not in the accelerated diabetes of IL-10-NOD mice.

TNFR-2-deficient NOD mice are susceptible to spontaneous and IL-10-accelerated diabetes

Subsequently, we introduced the disrupted TNFR-2 gene into IL-10-NOD mice. As shown in Fig. 5A, IL-10-NOD mice (generation N4 backcross) that were either heterozygous (+/−) (n = 8; 100%) or deficient (n = 6; 100%) for TNFR-2 gene developed diabetes beginning at 4–5 wk of age. There was no statistical significance between these two groups (p = 0.2059). However, their non-tg KO (n = 5) littermates were diabetes free for that 10-wk period. Furthermore, the pancreatic islets from both groups of mice were extensively infiltrated with autoreactive lymphocytes (Fig. 5B), and their insulin-producing β cells were destroyed (not shown). The islets from pancreata of 5-wk-old littermate NOD-TNFR-2-deficient (+/−) mice exhibited periinsulitis. These mice subsequently progressed to diabetes beginning at 18 wk of age and showed 60% incidence of diabetes by 24 wk of age (n = 5). These findings imply that TNFR-2 signaling is irrelevant for spontaneous and accelerated diabetes of NOD and IL-10-NOD mice, respectively.

CYP fails to provoke autoimmune insulitis and diabetes in the absence of pancreatic inflammation

Finally, to test whether CYP would induce diabetes in a Fas-dependent manner, we injected CYP into Fas-deficient NOD.Fas/lpr mice.
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(-/-) mice and their Fas-sufficient NOD.Fas/lpr (+/-) littermate controls on days 0 and 14. This protocol has been shown earlier to evoke or accelerate diabetes in NOD mice (17, 18). We found that, over a period of 8 wk, NOD.lpr/lpr mice were completely resistant to CYP-induced diabetes. As expected, heterozygous mice (Fas/lpr) (p = 0.004 vs lpr/lpr group) and wild-type (Fas/Fas) mice (p = 0.0019 vs lpr/lpr) rapidly developed diabetes beginning at 2 wk after the first CYP injection (Fig. 6A). Additionally, most of the heterozygous NOD mice (Fas/lpr) (+/-) became diabetic within 2–4 wk of receiving the first inoculation of CYP.

Although approximately 80% of the islets from CYP-injected heterozygous (Fas/lpr) mice (n = 10) showed severe insulitis, none of the islets from CYP-treated NOD.lpr/lpr mice (n = 8) had any sign of lymphocytic infiltration in or near the islets (Fig. 6B). Occasionally, a perivascular infiltrate occupied the pancreatic tissue of CYP-treated NOD.lpr/lpr mice, as pictured in representative H&E-stained sections from NOD.Fas/lpr (+/-) and NOD.lpr/lpr (-/-) (Fig. 6C). Since the pancreatic islets of CYP-treated NOD.lpr/lpr mice were free from lymphocytic infiltration and stained positively for insulin, yet those of CYP-treated heterozygous NOD.Fas/lpr mice were filled with mononuclear cells and stained only weakly for insulin-positive cells, Fas expression is a prerequisite for CYP-induced diabetes in NOD mice.

Discussion

The results from this study demonstrate that the expression of IL-10 in pancreatic islets bypasses the requirement for Fas, perforin, TNFR-1, and TNFR-2 molecules and that IL-10 can otherwise precipitate the diabetic process. In contrast to IL-10-accelerated diabetes in NOD.lpr/lpr mice, CYP fails to provoke diabetes in NOD.lpr/lpr mice. Therefore, our findings demonstrate that IL-10-accelerated diabetes is Fas independent and that IL-10-induced diabetes is Fas dependent. This current study also demonstrates that for the first time, in contrast to the role of TNFR-1 in diabetes of the NOD mouse, deficiency of TNF-2 failed to block spontaneous diabetes of NOD mice.

 Destruction of β cells in the spontaneous diabetes of NOD mice required Fas-FasL interaction. The Fas-FasL pathway appeared to be required for the initiation and/or effector phases of spontaneous autoimmune diabetes in former experiments with NOD mice (9, 10, 19), and with TCR tg NOD mice expressing islet-specific T cells (12, 20). The current study with CYP-induced diabetes of NOD mice further highlights a role for Fas-FasL pathway in destruction of β cells. However, islet transplantation experiments provided differing results. That is, the Fas-FasL pathway did not participate in the effector stages of diabetes, since NOD.lpr/lpr islets transplanted into recently diabetic NOD mice were completely destroyed by an autoimmune attack (21, 22) or following CYP injection (22). Apparently different mechanisms participate in the destruction of ectopically transplanted islet grafts and of β cells in situ. Our current findings demonstrated that expression of the IL-10 transgene in the pancreatic islets promoted accelerated diabetes of NOD mice in situ without a requirement for the Fas signaling. Additionally, we showed that splenocytes from diabetic IL-10-NOD.lpr/lpr mice transferred disease into NOD.scid/scid mice, reinforcing the implication that the Fas-FasL pathway is not required throughout this autoimmune process of IL-10-NOD mice.

If, as seems evident, the Fas-FasL pathway does not participate in this accelerated diabetes, presumably expression of the IL-10 transgene in the islets of NOD mice could awaken other death pathways such as those that use perforin or TNFR-1 or TNFR-2 molecules. However, our findings exclude that possibility. Therefore, the results described in this work contrast with earlier conclusions that perforin is required for spontaneous diabetes (11, 12) and CYP-induced diabetes (11). TNFR-1 molecules were also considered a requirement for the spontaneous and CYP-induced diabetes of NOD mice (13). Since TNFR-2-deficient NOD mice developed spontaneous diabetes, we did not study the effect of TNFR-2 deficiency on CYP-induced diabetes. In addition, our current findings demonstrate that Fas is also required for CYP-induced diabetes of NOD mice. However, the actual cause, for the absence of disease, may have been the lack of intense inflammation in the pancreatic environment that is necessary for efficient APC activation, Ag presentation, and T cell activation, leading to the production of inflammatory mediators.

For example, we have shown that CD40-CD40L pathway is essential for the spontaneous autoimmune insulitis and diabetes of NOD mice, as demonstrated by Ab-blocking studies. This pathway appears to play a role in the initiation but not the effector phase of this disease process (16). The requirement for this pathway in spontaneous diabetes was confirmed by Green and coworkers (23) using CD40L-deficient NOD mice. However, this pathway was found dispensable for the accelerated diabetes of tgl IL-10-NOD (7) and TNF-α-NOD (23) mice. These findings and the results presented in the current study suggest that cytokine-induced inflammation in the pancreatic environment circumvented the requirement for the well-established costimulation pathways, thereby short-circuiting the onset of disease. This hypothesis is further supported by two additional observations: 1) Expression of the IL-10 transgene in diabetes-free BDC2.5 NOD mice leads to the development of diabetes. 2) CYP injection fails to provoke diabetes in insulin-free NOD.lpr/lpr mice. We are of the opinion that the failure to observe diabetes in CYP-treated NOD.lpr/lpr mice is unrelated to the lpr-induced lymphoproliferative effect, because IL-10-NOD.lpr/lpr mice do develop diabetes, and their splenocytes can transfer diabetes into NOD.scid/scid mice with delayed kinetics.

It is well established that IL-10 promotes pathogenic cell-mediated and humoral autoimmunity. In fact, tgl IL-10-C57BL/6 mice expressing IL-10 under control of the salivary amylase promoter developed a Sjögren’s-like syndrome via a Fas-FasL pathway (24). Depending on the circumstances, then, IL-10 could exhibit its autoimmune-stimulatory effect via Fas-dependent and Fas-independent pathways. IL-10 also seems to act as an immunostimulator in humoral autoimmunity through B cells (25). However, our previous findings demonstrate that, in T cell-mediated autoimmunity, IL-10 readily promotes autoimmune diabetes independent of B cells, because B cell-deficient IL-10-NOD mice also developed accelerated diabetes similar to that in their wild-type counterparts (7). Continual administration of IL-10 to NZB/W F1 mice accelerated autoimmunity, whereas treatment with anti-IL-10 Ab delayed its onset (26). Apart from its pathogenic role in autoimmunity, IL-10 further exerted its immunostimulatory capacity with respect to tumor suppression, since IL-10 expressed under a class II promoter of C57BL/6 mice limited the growth of immunogenic tumors (27).

From the foregoing results, we conclude that accelerated diabetes in IL-10-NOD mice does not involve the classical death signaling molecules, Fas, perforin, and TNFR-1. Nor is the TNFR-2 molecule required for diabetes to arise in IL-10-NOD mice. Therefore, pancreatic IL-10 may promote diabetes via unique death pathways(s) involving TRAIL, TWEAK, and LIGHT molecules (28–30), or its expression may promote diabetes via compensatory death pathways. To prevent such disease, these novel death pathways used by autoimmune responses to destroy insulin-producing β cells must be uncovered along with molecules that interfere with their destruction.
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References