Caspase-1 Is Not Required for Type 1 Diabetes in the NOD Mouse

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Interleukin (IL)-1β and IL-18 are two cytokines associated with the immunopathogenesis of diabetes in NOD mice. Both of these cytokines are cleaved by caspase-1 to their biologically active forms. IL-1 is a proinflammatory cytokine linked to β-cell damage, and IL-18 stimulates production of interferon (IFN)γ in synergy with IL-12. To examine the effects produced by caspase-1 deficiency on diabetes development in NOD/Lt mice, a disrupted *Casp1* gene was introduced by a speed congenic technique. *Casp1*^{−/−} bone marrow–derived macrophages stimulated with lipopolysaccharide produced no detectable IL-18, fourfold lower IL-1β, and 20–30% less IL-1α than macrophages from wild-type *Casp1*^{+/+} or *Casp1*^{+/−} controls. Unexpectedly, despite reduced IL-1 and IL-18, there was no change in the rate of diabetes or in total incidence as compared with that in wild-type NOD mice. IL-1 reportedly makes an important pathological contribution in the multidose streptozotocin model of diabetes; however, there was no difference in sensitivity to streptozotocin between NOD mice and NOD.*Casp1*^{−/−} mice at 40 mg/kg body wt or at 25 mg/kg body wt dosage levels. These findings show that caspase-1 processing of IL-1β and IL-18 is not absolutely required for mediation of spontaneous or chemically induced diabetes pathogenesis in the NOD mouse. *Diabetes* 53:99–104, 2004

C aspase 1, also known as IL-1β–converting enzyme, is a cysteine protease involved both in apoptosis and in the proteolytic cleavage of some cytokines into their biologically active forms. The inactive proforms of IL-1β and IL-18, also known as interferon γ (IFNγ)-inducing factor, are cleaved primarily by caspase-1 (1). Caspase-1 is important in the regulation of IFNγ production induced by lipopolysaccharide (LPS)-stimulated secretion of IL-18 (2). Both IL-1 and IL-18 are proinflammatory cytokines associated with Fas-mediated pancreatic β-cell destruction in the spontaneous

development of autoimmune type 1 diabetes in NOD mice (3–5). IL-1β, in combination with tumor necrosis factor (TNF)-α and IFNγ, has also been shown to activate apoptosis in human islet cells (6). Chronic exposure of mouse islet cell monolayers to recombinant IL-1β decreased their insulin biosynthesis and secretion (7). Pretreatment of NOD female mice with soluble IL-1 receptor antagonist prevented cyclophosphamide-induced hyperglycemia, but not insulitis (8). Pretreatment with the soluble IL-1 receptor antagonist also blocked hyperglycemia development in C57BLKS/J males treated with low-dose streptozotocin (STZ) (9). The receptor antagonist further prevented recurrence of disease in spontaneously diabetic NOD females engrafted with syngeneic islets (10). In vitro, IL-1β induces nitric oxide synthase in cultured rodent β-cells; this stimulates production of β-cytotoxic nitric oxide radicals, but IL-1 also elicits stress response proteins, such as heat shock protein 70, that can protect against free radical–mediated stress (11).

Because of its demonstrated β-cytotoxic potential in combination with other cytokines, IL-1 has been assumed to be an important and necessary comediator of insulitic damage in the NOD mouse. However, one of the strain characteristics of NOD mice is a blunted maturation of macrophages (12) and dendritic cells (13,14). The macrophage developmental anomalies include a reduced level of LPS-induced IL-1β secretion (15). Reduced production of this T-cell costimulatory molecule may underlie defects in thymic negative selection and/or maintenance of peripheral tolerance. This defect may be counterbalanced by increased secretion of IL-18, which is capable of contributing to the T-helper 1 polarization that is often associated with rapidly progressive insulitis in the NOD model (16). Given the multiple levels at which these two caspase-1–processed cytokines act, it was difficult to predict how suppression of their processing into their biologically active and secreted forms would affect diabetes pathogenesis in NOD mice.

The *Casp1* gene at the very centromeric end of chromosome 9 (5.2 Mb) has been disrupted by gene targeting. Caspase-1–deficient mice develop normally. Two groups report (17,18) that these mice exhibited impaired secretion of active IL-1β and, unexpectedly, IL-1α as well. As expected, secretion of bioactive IL-18 from LPS-stimulated macrophages is also suppressed (19). Caspase-1–deficient mice are resistant to LPS-induced toxic shock (18) and are less able to successfully overcome *Escherichia coli* infection (20). In general, caspase-1–deficient mice show re-
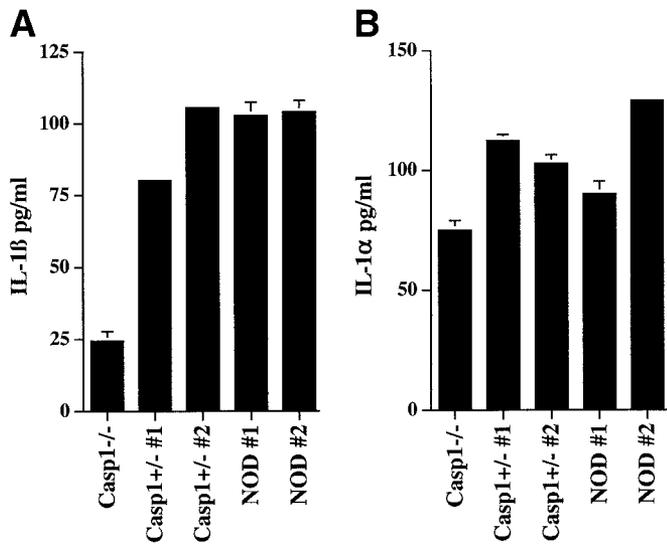
FIG. 1. Caspase-1 deficiency markedly suppresses LPS-stimulated IL-1β secretion from bone marrow–derived macrophages (*A*) and moderately reduces IL-1α secretion (*B*).

duced inflammatory response, as evidenced by resistance to experimentally induced colitis (21). This is presumably a reflection of reduced IL-18 secretion, as neutralization of this cytokine also lessens colitis severity (22).

Our objective in the present study was to transfer the disrupted *Casp1* allele onto the NOD/Lt inbred strain background to assess how essential IL-1 and IL-18 are to the development of spontaneous diabetes in mice of both sexes as well as to experimental induction of diabetes in young pre-diabetic NOD males by treatment with multiple low doses of STZ.

RESEARCH DESIGN AND METHODS

Mice. A stock of mixed C57BL/6;129 mice carrying the *Casp1* targeted gene disruption was imported to The Jackson Laboratory (kindly provided by Dr. Winnie Wong [BASF, Worcester, MA]). The targeted allele was introgressed into the NOD background using a marker-assisted "speed congenic" breeding approach wherein backcross segregants were fixed for homozygosity for NOD alleles at NOD/B6/129 polymorphic markers at known NOD diabetes susceptibility (*Idd*) loci (23). By N6, the disrupted *Casp1* gene was constrained to an interval of <10 cM from the centromere (recombination introduced NOD genome at and below the polymorphic *D9Mit59* marker [22 Mb]), thereby excluding the possibility that the presence of the 129/Sv genome could potentially contribute resistance at the *Idd2* locus on that chromosome or at the gene encoding IL-18. Homozygous NOD.*Casp1*-targeted mutant (−/−) mice were produced by intercross of heterozygotes at the N6 and N10 generations. A permanent line of NOD.*Casp1*$^{−/−}$ congenic mice was established at N10, and standard NOD/Lt mice were used for comparison with the N10 congenic stock. The formal designation of this stock is NOD.129S2(B6) *Casp1*^{tm1Sesh}/Lt (JAX mice stock no. 003847). The NOD/Lt mice used in these experiments have been maintained by sib mating. All mice have acidified water and NIH 31 (6% fat) diet available ad libitum.

Cytokine secretory functions of bone marrow–derived macrophages. Macrophages derived from bone marrow or isolated from thioglycolate-elicited peritoneal exudates were cultured in the presence of LPS, and the levels of cytokines were determined. Briefly, bone marrow harvested from femorae, tibia, and humeri was cultured in RPMI 1640 (Cellgro; Mediatech, Herndon, VA), fortified with 10% fetal bovine serum (Atlanta Biologicals, Norcross, GA) and 10 ng/ml mouse recombinant colony-stimulating factor-1 (R&D Systems, Minneapolis, MN), at 3×10^6 cells/ml for 4 days. For the IL-18 determination, 2.5×10^6 cells/ml in 8 ml were cultured for 5 days in the same conditions except 500 units/ml human recombinant colony-stimulating factor-1 (Cetus, Emeryville, CA) were used in addition to 10 units/ml IFNγ (R&D Systems). Confluent plates were washed to remove nonadherent cells, and 8 ml fresh RPMI 1640 medium containing either LPS (Sigma, St. Louis, MO) at 10 μg/ml or control medium was added. All media contained 100 units/ml

penicillin and 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA) and 50 μmol/l β-mercaptoethanol (Sigma). Plates were cultured at 37°C in a 5% CO_2 humidified atmosphere for 24 h. IL-1α, IL-1β, and IL-18 secretion from bone marrow macrophages was determined by direct enzyme-linked immunosorbent assay (ELISA) of supernatant from stimulated and control cultures after centrifugation to remove particulates and cellular debris. ELISA for each cytokine (all R&D Systems) was performed according to the manufacturer's instructions. The remaining supernatants were assayed using the Beadlyte Mouse Multi-Cytokine Detection System 2 from Upstate Biotechnology (Lake Placid, NY), following the manufacturer's instructions with modification for the use of the high biotin–containing RPMI medium. Briefly, 50 μl of the diluted standards or samples were added to each well in triplicate with the addition of the cytokine capture antibody beads. The samples were gently mixed and incubated overnight to enhance sensitivity, and then the plate was subjected to a vacuum manifold to control for high biotin in the medium. The samples were then gently mixed, 25 μl reporter solution was added, and then the samples were mixed and incubated for 1.5 h in the dark at room temperature. After incubation, 25 μl of diluted streptavidin-phycoerythrin solution was added to each well, and plates were incubated for 30 min. The samples then received the stop solution and were mixed. The plates were then read on the Luminex[100] machine, which was programmed with the proper bead-signature numbers representing the cytokines assayed.

Monitoring for spontaneous and STZ-induced NOD/Lt diabetes frequency. Diabetes development was analyzed at the N6F2 backcross/intercross generation in congenic NOD/Lt mice of both sexes homozygous for the disrupted (−/−) *Casp1* gene. Comparison was made with both *Casp1*-intact (+/+) segregants and with standard NOD/Lt mice. Beginning at 8 weeks of age, mice were tested weekly for spontaneous glycosuria development using Diastix (kindly supplied by Bayer, Elkhart, IN). Mice were considered diabetic after two consecutive weekly values testing >250 mg/dl glucose. Log-rank survival statistics were determined using the JMP 5 statistical package (SAS, Cary, NC). Multiple low-dose STZ (MLDSTZ) (Upjohn, Kalamazoo, MI) or citrate buffer vehicle were administered to young pre-diabetic male NOD and N10 NOD.*Casp1*$^{−/−}$ mice at the specified doses for 5 consecutive days. Plasma glucose concentrations were measured weekly using a glucose analyzer (Beckman Coulter, Fullerton, CA). Comparisons of plasma glucose concentrations were made using a two-tailed *t* test. All mice were killed at the conclusion of the experiment, and the pancreata were examined histologically by aldehyde fuchsin staining for granulated β-cells to determine the level of islet destruction.
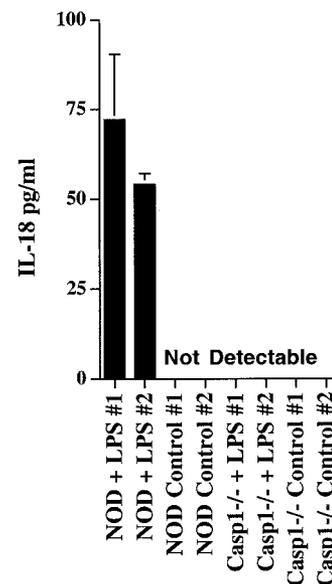

FIG. 2. Caspase-1 deficiency completely suppresses LPS-stimulated IL-18 secretion from cultured bone marrow–derived macrophages. IL-18 ELISA of cell culture supernatants from bone marrow–derived macrophages incubated for 24 h with 10 μg/ml LPS. The data are from two separate cultures of macrophages per genotype assayed in triplicate. The antibody used detects both the active and proform of the cytokine. Bars indicate SE of the assays.
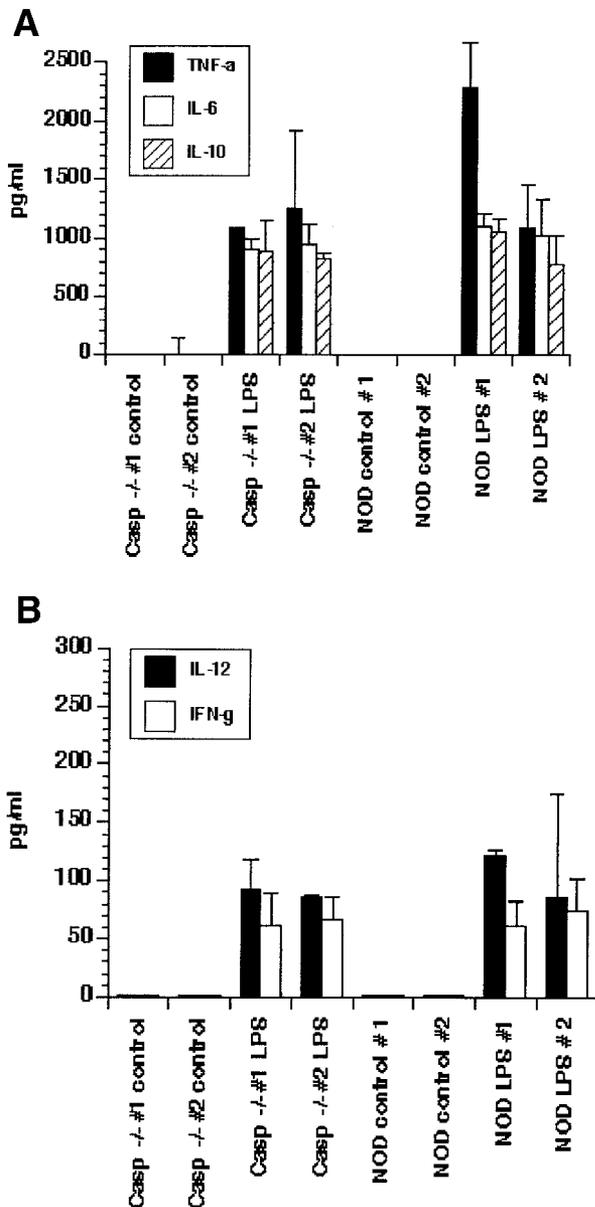
A

B

FIG. 3. *A*: **Caspase-1 deficiency does not affect secretion of TNFα (■), IL-6 (□), or IL-10 (▨) from LPS-stimulated bone marrow–derived macrophages.** *B*: **Additionally, there is no difference in secretion of IL-12 (■) or IFNγ (□). Bars indicate SE of the assays. The supernatants are from the same cultures shown in Fig. 2.**

RESULTS

Marked suppression of LPS-stimulated IL-1β and IL-18 secretion by caspase-1–deficient NOD macrophages and modest reduction of IL-α release. Data for LPS-stimulated IL-1β and IL-1α secretion from cultures of bone marrow–derived macrophages from NOD.*Casp*1$^{-/-}$ versus NOD.*Casp*1$^{+/-}$ and standard NOD wild-type donors are shown in Fig. 1. Consistent with previous studies, macrophages from NOD.*Casp*1$^{-/-}$ mice secreted fourfold less IL-1β than heterozygous littermates or NOD mice. Although IL-1α does not require caspase-1 processing, a modest (~30%) reduction from +/− or wild-type levels was observed in its secretion by the same NOD.*Casp*1$^{-/-}$ macrophages. Also consistent with a known requirement for caspase-1 in processing of pro-IL-18 to its biologically active and secreted form, immunoreactive IL-18 from

LPS-stimulated NOD.*Casp*1$^{-/-}$ macrophages was undetectable by ELISA (Fig. 2). The ELISA would detect both pro-IL-18 as well as processed IL-18 in the medium. However, the lack of caspase-1 did not affect the secretion of TNFα, IFNγ, IL-6, IL-10, or IL-12 by LPS-stimulated bone marrow–derived macrophages (Fig. 3). Similar levels of each cytokine were detectable in LPS-stimulated, but not in unstimulated macrophage supernatants.

Caspase-1 deficiency has no effect on development of spontaneous diabetes. Data in Fig. 4 show that diabetes developed equivalently in N6F1 *Casp1*$^{-/-}$ mice and both control sets (*Casp1*$^{+/+}$ littermate segregants and standard NOD/Lt mice). Diabetes frequencies are high in NOD/Lt mice of both sexes in our research colony at The Jackson Laboratory. Survival analysis showed no significant differences in the frequencies regardless of *Casp1* gene status.

Complete Freund's adjuvant protects caspase-1–deficient NOD mice from spontaneous diabetes development. Complete Freund's adjuvant (CFA) is one of many immunostimulatory protocols capable of preventing or retarding diabetes development in NOD mice (24,25). Indeed, injection of weanling NOD/Lt mice at The Jackson Laboratory with a single 50-μl dose of CFA is routinely used to extend the diabetes-free survival of breeder stock. As noted above, NOD/Lt macrophages secrete reduced levels of IL-1 upon stimulation in vitro compared with control strains. Following CFA administration, increased secretion of proinflammatory cytokines by antigen-presenting cells including macrophages recruited to the popliteal lymph node site of CFA injection would be expected. Hence, we tested whether disruption of normal processing of IL-1β and IL-18 attenuated the protective effect of single-injection CFA administration. As shown in Fig. 5, CFA was as protective in NOD.*Casp1*$^{-/-}$ mice as it was in standard NOD/Lt controls.

Caspase-1 deficiency has no effect on MLDSTZ-induced diabetes. Young pre-diabetic NOD/Lt male mice are sensitive to diabetes induction by MLDSTZ (26). This chemically induced destruction of β-cells is not autoimmune in etiology because NOD.CB17-*Pkrdc*^{scid} mice are also highly sensitive (26). Although lacking functional T- and B-cells, macrophages and neutrophils are highly enriched in NOD.CB17-*Pkrdc*^{scid} mice, and as noted above, IL-1 has been assumed to be an important contributor to pathogenesis in the MLDSTZ model (9). However, as shown in Fig. 6, the expectation that the NOD.*Casp1*$^{-/-}$ congenic males would be resistant to MLDSTZ-induced hyperglycemia was not confirmed. At the higher dose used (40 mg/kg body wt × 5 days), 7 of 7 of the NOD and 4 of 7 of the NOD.*Casp1*$^{-/-}$ male mice had plasma glucose concentrations >250 mg/dl after 1 week from the first injection. Although the mean plasma glucose of the *Casp1*$^{-/-}$ males was significantly lower than that of controls at this 1-week time point, there was no difference between the two MLDSTZ treatment groups at the 2-week time point, when all males except the vehicle controls were severely hyperglycemic. To determine whether the delay in onset of severe hyperglycemia was a statistical fluke, a lower-dose regimen was utilized (25 mg/kg body wt × 5 days). Onset of hyperglycemia was more protracted in mice of both genotypes, but there were no significant differences in degree of hyperglycemia at any time point
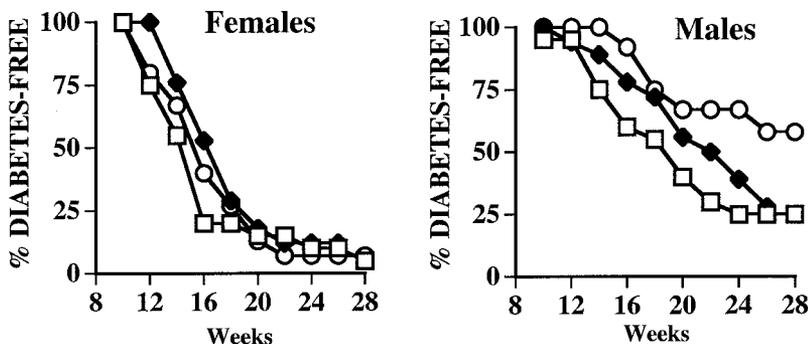
FIG. 4. Incidence of diabetes in $Casp1^{-/-}$ (♦, $n = 17$ females and 18 males), $Casp1^{+/+}$ (○, $n = 15$ females and 12 males), and NOD (□, $n = 20$) mice. Development of hyperglycemia in mice was determined by biweekly testing for glycosuria using Diastix test strips. Mice were considered diabetic after two consecutive readings >250 mg/dl. All three female groups developed diabetes at the same rate. The differences in rate of diabetes development in $Casp1^{-/-}$ and $Casp1^{+/+}$ males as compared with NOD/Lt males were not significant ($P = 0.449$ and 0.180, respectively).

studied. Histological examination of the pancreata of the wild-type and $Casp1^{-/-}$ NOD males killed at the end of the MLDSTZ treatment periods showed no differences in the extent of islet destruction (data not shown).

DISCUSSION

Because IL-1β and IL-18 have heretofore been viewed as central to the pathogenic process in two different mouse models of insulin-dependent diabetes, it is surprising that ablation of the caspase-1 function required for proprotein processing and biologic activation had no major effect in either of the two models (spontaneous T-cell–mediated diabetes in NOD/Lt or toxin-mediated destruction of NOD β-cells). Secretion of bioactive IL-1 did not differ between marrow-derived macrophages from NOD and diabetes-resistant strains in the absence of IFNγ, which is required to achieve full maturation from myeloid precursors in vitro (12). However, NOD macrophages were IL-1 hyposecretors when compared with other strains under conditions of IFNγ-promoted full maturation (12). To explore the possibility that reduced IL-1β secretion by NOD antigen-presenting cells might actually be contributory to defects in thymic negative selection and/or maintenance of peripheral tolerance, and thus diabetes predisposing, we tested whether disruption of the $Casp1$ gene diminished the diabetes-suppressive efficacy of CFA, a potent immunostimulant. The strong diabetes-suppressive effect of CFA treatment was not diminished in NOD.$Casp1^{-/-}$ mice. In aggregate, our findings are consistent with another report (T. Kay, personal communication) that introgression into the NOD genetic background of a disrupted IL-1 receptor ($Il1R1$) gene also failed to affect diabetes frequency. The finding that a "knockout" of a specific gene function fails to alter a phenotype cannot be overly interpreted to mean that the ablated gene product or products are not contributors in an intact mouse. Often, the knockout elicits compensatory upregulation of genes that can potentially substitute for the gene whose function has been depleted (27). In the case of suppressed processing of IL-1 and IL-18, upregulated secretion of TNFα and IL-12 would represent such compensation. However, at least for these two cytokines, as well as for IL-6, such compensatory hypersecretion was not seen in the caspase-1–deficient macrophage cultures. This does not rule out the possibility that increased concentrations of these cytokines or of other cytokines we did not measure may have been elicited in vivo, compensating for the IL-1 and IL-18 deficiencies in the development of autoimmune diabetes. Possibly, the low concentrations of IL-1α detected in

combination with well-preserved TNFα and IFNγ secretion from leukocytes in the insulitic infiltrate are sufficient to allow β-cell destruction in the islets of $Casp1^{-/-}$ mice. The genome of the NOD mouse is associated with multiple immune anomalies that collectively lead to the development of diabetes (28). The removal of a limited number of the active participants in this process may not be sufficient to prevent the disease progress. Despite a wealth of reports (3) indicating that T-helper 1 cytokine gene expression by CD4+ T-cells in the NOD insulitic infiltrate is an important aspect of diabetes pathogenesis, disruption of the IFNγ gene fails to block diabetes and disruption of the IL4 and IL-10 genes individually does not accelerate pathogenesis (25). Moreover, treatment of pre-diabetic NOD
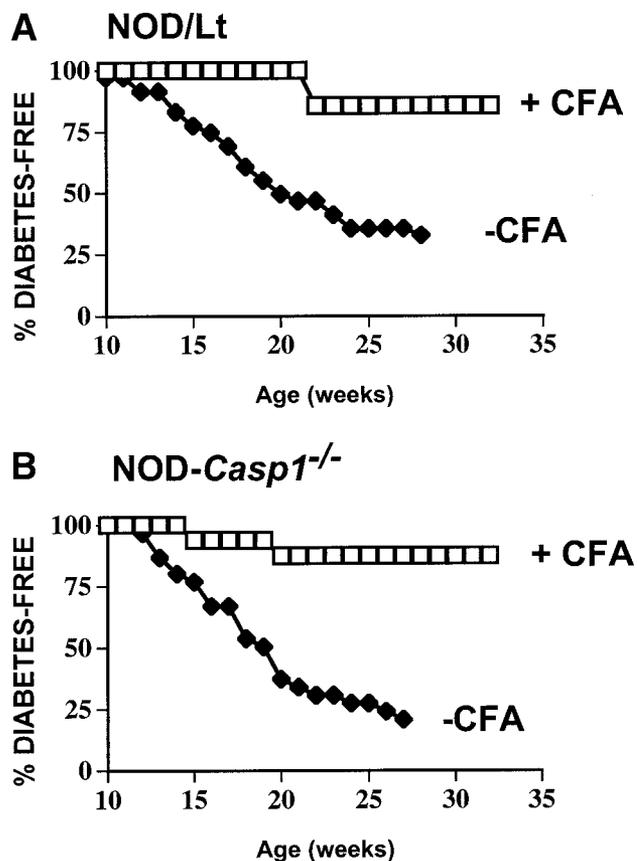

FIG. 5. NOD.$Casp1^{-/-}$ female mice are responsive to the diabetes-suppressive action of a single injection of 50 µl CFA administered to a hind foot at weaning (3–4 weeks). A: □, NOD/Lt, no CFA, $n = 14$; ♦, NOD/Lt, treated with CFA, $n = 35$. B: □, NOD-$Casp1^{-/-}$, no CFA, $n = 16$; ♦, NOD.$Casp1^{-/-}$, treated with CFA, $n = 30$.
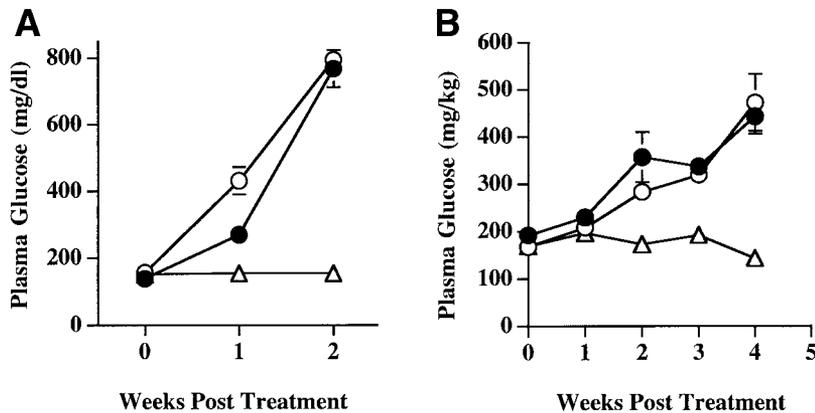
FIG. 6. Multidose STZ induction of hyperglycemia in NOD and NOD.*Casp1*$^{-/-}$ 7.5-week-old male mice. Mice were injected intraperitoneally with STZ at the described dosage or with citrate buffer vehicle for 5 consecutive days and monitored for plasma glucose level weekly. The means ± SE are shown from each group. For the 40-mg/kg experiment (*A*), there were seven mice in each group, and for the 25-mg/kg experiment (*B*), five animals in each group. Only at the 1-week time point in the 40-mg/kg treatment group was there a significant difference ($P = 0.0092$). ○, NOD mice treated with MDSTZ; ●, NOD.*Casp1*$^{-/-}$; △, NOD vehicle controls.

mice with recombinant IL-18 suppressed rather than accelerated diabetogenesis (29). Thus, although it is surprising that elimination of caspase-1 processing of IL-1 and IL-18 failed to retard diabetes etiopathology in the NOD mouse, the finding is consistent with other reports that no single cytokine gene is indispensable for β-cell destruction in this model.

The finding that caspase-1 deficiency did not prevent diabetes induction by the MLDSTZ model in young prediabetic NOD/Lt males was not particularly surprising because the major component of β-cell destruction in this model is not immune mediated, but rather a direct cumulative effect of the toxin on β-cells (30). For example, genetic disruption of *Icam1* prevents diabetes in NOD mice, a recognized model of autoimmune diabetes (31), but the same mutation on a C57BL/6 background failed to block MLDSTZ-induced diabetes (32). However, pathogenic contributions from islet-infiltrating macrophages have been identified in the MLDSTZ model, notably IL-1β–mediated upregulation of nitric oxide. A reduced frequency of hyperglycemia induced by this toxin regimen in mice with a targeted disruption of the inducible nitric oxide synthase gene (*Nos2*) has been reported (33). Similarly, mice with a targeted disruption in the arachidonate 12-lipoxygenase gene acquire resistance to MLDSTZ-induced diabetes, a deviation associated with reduced nitric oxide production by macrophages (34).

In summary, despite literature suggesting an important role for IL-1 and IL-18 in diabetes pathogenesis in the NOD mouse, the present results indicate that both cytokines are dispensable for β-cell destruction in this very complex model.

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