Transgenic Expression of Dominant-Negative Fas-Associated Death Domain Protein in β Cells Protects against Fas Ligand-Induced Apoptosis and Reduces Spontaneous Diabetes in Nonobese Diabetic Mice

Janette Allison, Helen E. Thomas, Tara Catterall, Thomas W. H. Kay and Andreas Strasser

*J Immunol* 2005; 175:293-301; doi: 10.4049/jimmunol.175.1.293
http://www.jimmunol.org/content/175/1/293

**References**
This article cites 48 articles, 20 of which you can access for free at:
http://www.jimmunol.org/content/175/1/293.full#ref-list-1

**Subscription**
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Transgenic Expression of Dominant-Negative Fas-Associated Death Domain Protein in β Cells Protects against Fas Ligand-Induced Apoptosis and Reduces Spontaneous Diabetes in Nonobese Diabetic Mice

Janette Allison, Helen E. Thomas, Tara Catterall, Thomas W. H. Kay, and Andreas Strasser

In type 1 diabetes, many effector mechanisms damage the β cell, a key one being perforin/granzyme B production by CD8⁺ T cells. The death receptor pathway has also been implicated in β cell death, and we have therefore generated NOD mice that express a dominant-negative form of the Fas-associated death domain protein (FADD) adaptor to block death receptor signaling in β cells. Islets developed normally in these animals, indicating that FADD is not necessary for β cell development as it is for vasculogenesis. β cells from the transgenic mice were resistant to killing via the Fas pathway in vitro. In vivo, a reduced incidence of diabetes was found in mice with higher levels of dominant-negative FADD expression. This molecule also blocked signals from the IL-1R in culture, protecting isolated islets from the toxic effects of cytokines and also marginally reducing the levels of Fas up-regulation. These data support a role for death receptors in β cell destruction in NOD mice, but blocking the perforin/granzyme pathway would also be necessary for dominant-negative FADD to have a beneficial clinical effect. The Journal of Immunology, 2005, 175: 293–301.

In humans, type 1 diabetes (T1D) is the result of progressive, immune-mediated destruction of β cells. The NOD mouse provides a useful model for studying T1D in humans, because initiation and progression of diabetes can be dissected using transgenic and knockout technology as well as experimental manipulation of the immune system, the environment, and the genetic background.

CD8⁺ T cells are essential for diabetes initiation in NOD mice, and animals globally lacking class I MHC or given lytic anti-CD8 Ab (before 3 wk of age) do not develop β cell infiltrates (1, 2). CD8⁺ CTLs are also important in the effector phase of the disease, because NOD mice lacking perforin have islet infiltrates, but a greatly reduced incidence of diabetes (3). Likewise, when β cells alone were engineered to lack class I expression by cre-lox-mediated deletion (4) or overexpression of the adenoviral E19 protein (which impairs transport of class I MHC chains to the surface) (5), infiltration eventually occurred, but the diabetes incidence was reduced.

Several mechanisms may account for β cell damage in the absence of direct recognition by CTL. Fas ligand (FasL), TNF-α, certain other cytokines (IFNs and IL-1), and conditions of stress (e.g., anoxia, NO, and free radicals) may all contribute (6, 7). β cells have undetectable levels of Fas, but it can be up-regulated in inflammatory lesions (8) or in vitro by IFNγ and TNF-α, or IL-1 (9). The data on the contribution of Fas/FasL signaling to β cell death during the initiation or effector phase have been controversial. Mice lacking Fas expression (NODlpr mice) do not develop islet infiltrates and are protected from diabetes, but instead succumb to other autoimmune and lymphoproliferative diseases (10, 11). However, given the distorted lymphocyte repertoire that develops in these mice due to lymphadenopathy (11), this finding tells us little about the contribution of Fas to diabetes initiation or progression.

Several mechanisms may account for β cell damage in the absence of direct recognition by CTL. Fas ligand (FasL), TNF-α, certain other cytokines (IFNs and IL-1), and conditions of stress (e.g., anoxia, NO, and free radicals) may all contribute (6, 7). β cells have undetectable levels of Fas, but it can be up-regulated in inflammatory lesions (8) or in vitro by IFNγ and TNF-α, or IL-1 (9). The data on the contribution of Fas/FasL signaling to β cell death during the initiation or effector phase have been controversial. Mice lacking Fas expression (NODlpr mice) do not develop islet infiltrates and are protected from diabetes, but instead succumb to other autoimmune and lymphoproliferative diseases (10, 11). However, given the distorted lymphocyte repertoire that develops in these mice due to lymphadenopathy (11), this finding tells us little about the contribution of Fas to diabetes initiation or progression. Transfer of NOD, Fas-expressing, effector cells into NODlpr adult mice can give an invalid readout, because we showed that donor cells are deleted before they have time to reach the islets (12), although this problem can be partly circumvented by using very young (7-day-old) NODlpr recipients (13). We found that Fas on β cells played a minor role in β cell death during the effector phase of diabetes, and this has been confirmed by others (13, 14). In fact, the lack of infiltration into NODlpr islets may have more to do with the reduced proliferative ability of T cells, rather than a role for Fas on the islets themselves (15). Recently, better tools have been made to test the significance of Fas on β cells without the complications brought about by the lpr mutation. Transgenic mice expressing a dominant-negative form of Fas (FasΔ) in β cells showed a delayed and reduced incidence of diabetes in the spontaneous NOD model (16). Preventing caspase 8 activation in β cells by transgenic expression of the serpin, crmA, protected mice from transferred diabetes, although not spontaneous diabetes (17). In contrast, β cell-specific deletion of Fas using the cre-lox system had no impact on diabetes initiation or progression in a C57BL/6 TCR transgenic model of T1D (18).

TNFR1 signaling can contribute directly or indirectly to β cell death; for example, by causing β cells to produce chemokines that
recruit pathogenic lymphocytes (19, 20) or by inducing tissue resident macrophages to secrete IL-1 (21, 22) or other mediators. Much has been published about the toxic effects of IL-1 on β cells (7, 23). The IL-1R signals through the MyD88 adaptor to activate NF-κB, leading to up-regulation of inducible NO synthase (iNOS) and NO production. However, although IL-1R deficiency affords some protection against spontaneous diabetes in NOD mice (22), iNOS deficiency had little effect (24). In vivo, therefore, IL-1R signals may have more relevance to Fas up-regulation marking β cells for Fas-dependent destruction (25) rather than for NO-induced death.

We are interested in studying the contribution of death receptors such as Fas to diabetes initiation and progression. Fas, TNFR1, TRAIL receptors (TRAILR), DR3, and DR6 all carry a death domain (DD) in their cytoplasmic tail, and they therefore belong to a subgroup of the TNFR family called death receptors (26). Apoptosis signaling from death receptors requires recruitment of the DD in the adaptor molecule, Fas-associated DD protein (FADD), either directly (to Fas and TRAILR) or indirectly via the DD-containing adaptor TNFR-associated DD protein (TRADD; to TNFR1, DR3, and DR6). The FADD adaptor contains an N-terminal death effector domain (DED) that, in turn, recruits procaspase 8 (and in humans also caspase 10), leading to caspase activation. Activated caspase 8 can then process downstream effector caspases, such as caspase 3, leading to dismantling of the cell. In vivo, Fas or TRAILR engagement generally results in cell death through caspase 8 activation unless the pathway is blocked by the caspase 8 inhibitor, c-FLIP (27). TNFR1, DR3, or DR6 can induce cell death through caspase 8, but these receptors normally signal through TRADD, TNFR-associated factor 2 (TRAF2), and TRAF5 to three other pathways (NF-κB, JNK, and p38/MAPK), which control the induction of inflammatory proteins (such as TNF, IL-8, FasL, and IL-1) as well as antiapoptotic proteins (such as c-FLIP, cellular inhibitor of apoptosis 1/2, A20, Bcl-xL, and A1).

The death receptor-FADD-caspase 8 pathway can be blocked experimentally by the expression of a dominant-negative form of the adaptor FADD (dnFADD) that lacks the DED (28, 29). The dnFADD competes with wild-type FADD for recruitment to the DD of Fas, TRAILR, or TRADD, preventing caspase 8 recruitment and activation. There is evidence from studies with cultured cells that FADD may also transduce death signals from the IL-1R via MyD88 to caspase 8 (30) and that dnFADD can block this pathway. In addition, by associating with the DD of MyD88, the DD of dnFADD or wild-type FADD may block signals through the IL-1R-MyD88-IL-1R-associated kinase pathway to NF-κB and JNK (31). Furthermore, in lymphocytes, dnFADD prevented caspase 8-mediated apoptosis induced by Fas ligation, but, unexpectedly, it also inhibited T cell proliferation (28, 32). This is not a nonspecific effect of dnFADD, because T cells lacking FADD (32) or caspase 8 (33) were also found to have defects in mitogen-induced proliferation. Therefore, FADD and caspase 8 have functions in addition to death receptor-induced apoptosis signaling, and this is also demonstrated by the early embryonic lethality of mice lacking FADD or caspase 8 (32, 34), which appears to be due to a defect in vasculogenesis (35).

In this study we have expressed a dnFADD transgene in islet β cells of NOD mice to test the consequence of blocking death receptor signaling for β cell death and autoimmunity. Islets developed normally in these animals, but diabetes incidence was reduced, demonstrating a role for FADD-transduced signals in diabetes. In addition, culture experiments showed that the overexpressed dnFADD molecule blocked signals from the IL-1R.

Materials and Methods

Mice

Animals were bred under specific pathogen-free conditions at the University of Melbourne. Experiments using animals were performed according to the guidelines of University of Melbourne’s animal experimentation ethics committee. Transgenic mice expressing human dnFADD were generated by microinjecting fertilized eggs of NOD/LtJ mice with a construct containing the rat insulin promoter (RIP); position −695 to +8 (36), linked to the BglII-XbaI fragment of the human dnFADD construct, PEF-FLAG-FADD-DN79 (28), and the blunt-ended Small-EcoRI 3’ sequences containing the polyadenylation signals of the human growth hormone gene (37). The construct (RIP-dnFADD) was built in pGL2OH, and the vector sequences were removed before microinjection. Six transgenic lines were obtained, one of which expressed dnFADD in the islet β cells. The transgene copy number for this line was approximately two or three, as determined by Southern analysis. Offspring were screened for the transgene by PCR analysis of tail-tip DNA using oligonucleotides specific for sequences within the RIP (forward primer, 5’-ccca tca ata gga cca aca gaa aac t-3’), and the dnFADD (reverse primer, 5’-gaa cct ctt gta cca ggt cag cca c-3’). RIP-dnFADD mice were made homozygous for the transgene (Homo RIP-dnFADD mice) by brother/sister mating and then breeding to test the offspring for homozygosity. RIP-Bcl-2-transgenic mice (NOD.Bgl/Tnfafs69/J) were made by backcrossing the lpr mutation from the C3H/HeJ strain to NOD/LtJ for 10 generations and then intercrossing (11). NOD mice deficient in Fas and TNFR1 (NODlpr mice) were made by intercrossing NODlpr mice with backcross 10 NOD mice containing the targeted mutation in the TNFR1 gene (B6.129-Tnfrsf1a11, 39). RIP-dnFADD/Bcl-2-double-transgenic mice were made by crossing NOD RIP-Bcl-2 hemizygous mice to NOD RIP-dnFADD hemizygous mice. Offspring were screened by PCR analysis of tail-tip DNA using oligonucleotides specific to sequences in the RIP (forward primer, see above) and human Bcl-2 (reverse primer, 5’-ttc act atc act ccc tgg tct ctc-3’).

Reagents

Recombinant murine IFN-γ and TNF-α were obtained from Genentech. Human rIL-1β was obtained from Dr. C. Reynolds (National Cancer Institute, Bethesda, MD) as was human rIL-1α (used in Fig. 5C only). Mega Fasl, was a gift from Dr. J. Tschopp (Institute for Biochemistry, Lausanne, Switzerland). Abs used for intracellular immunofluorescent staining and FACS analysis were mouse anti-FLAG mAb (M2, Sigma-Aldrich; diluted 1/1000) and mouse anti-human Bcl-2 mAb supernatant (Bcl-2–100 hybridoma; diluted 1/3); both were detected with sheep anti-mouse IgG-FTC (Silenus; Melbourne Australia; diluted 1/500). Guinea pig anti-insulin Ab (DakoCytomation; diluted 1/1000) was detected with biotinylated goat anti-guinea pig IgG (Jackson Immunoresearch Laboratories; diluted 1/500) plus streptavidin-alkaline phosphocyanin (BD Pharmingen; diluted 1/600). Guinea pig IgG (Sigma-Aldrich; diluted 1/1000) was used as a negative control for insulin staining. For surface staining, hamster anti-Fas mAb (JO2; diluted 1/100) was detected with biotinylated mouse anti-hamster IgM mAb mixture (diluted 1/200) plus streptavidin-alkaline phosphocyanin (diluted 1/400; all from BD Pharmingen). The IL-1R antagonist was purchased from Amgen, and N-α-mono-methyl-l-arginine (NMMA) was obtained from Sigma-Aldrich.

Preparation of islets

The preparation of islets was previously described (40, 41). Briefly, the mouse bile duct was cannulated and injected with unsupplemented CMRL medium 1066 (Invitrogen Life Technologies) containing 0.4 mg/ml collagenase P (Roche) to digest the pancreas. A maximum of one or two pancreata were digested together. After digestion at 37°C, islets were separated from exocrine tissue by density gradient centrifugation through Histopaque 1077 (Sigma-Aldrich) and then hand picked and cultured overnight in 9-cm untreated petri dishes (~700 islets/dish) containing 9.5 ml of CMRL medium 1066 supplemented with antibiotics, 0.2 mM l-glutamine, and 10% FCS (CSL) at 37°C in 5% CO2. The next day, islets were repicked and placed in fresh, filtered, supplemented CMRL medium. Islets from about three to five pancreata were pooled per strain to generate enough islets for the assays.

Cell death assays

The day after isolation, 100 islets were picked into 3.5-cm, untreated petri dishes containing 1 ml of supplemented CMRL medium. A uniform size range of islets (excluding very large or necrotic islets) was selected by eye. Downloaded from http://www.jimmunol.org/ by guest on August 13, 2017

294 DOMINANT-NEGATIVE FADD PROTECTS AGAINST β CELL DEATH
For the FasL killing assay, IL-1β (1 U/ml) and IFN-γ (100 U/ml) were added to up-regulate Fas. Mega FasL (0.1 μg/ml) was added at the same time to induce β cell death over a 4-day culture period (9). For the cytokine killing assays, IL-1β (10 U/ml) and IFN-γ (100 U/ml) was added for 2–3 days or TNF-α (1000 U/ml) and IFN-γ (100 U/ml) was added for 4 days. IL-1R antagonist (5 μg/ml) was used to block IL-1R signaling, and NMMA (20 mM) was used to block NO production. At the end of the incubation period, the culture medium containing nonattached cells and the islets were transferred to a polypyrrole tube. Islets were washed twice with PBS, dispersed in 2% bovine trypsin (Calbiochem) containing 0.1% chick serum, washed in PBS, and allowed to recover in supplemented CMRL medium for 30 min at 37°C in 5% CO₂. After centrifugation and washing with PBS, the cells were resuspended in 250 μl of hypotonic buffer containing 50 μg/ml propidium iodide (Sigma-Aldrich), 0.1% sodium citrate, and 0.1% Triton X-100 as previously described (42). The percentages of cells with fragmented nuclei were determined by flow cytometry on a FACSort analyzer using the fluorescence (FL1)3 channel. Fragmented nuclei have an apparent DNA content of <2 C.

**Immunofluorescent staining and flow cytometric analysis**

The expression of insulin, human Bcl-2, and the FLAG tag epitope in the dnFADD protein was revealed in islet β cells using an intracellular staining protocol (43). Single-cell suspensions of islet cells were fixed in 2% paraformaldehyde/PBS at room temperature. After washing twice in PBS, cells were resuspended in saponin buffer, which was comprised of PBS, 5% FCS, and 0.3% saponin (Calbiochem), for 15 min. Cells were then stained with the appropriate primary Ab diluted in 150 μl of saponin buffer, vortexed for 20 s, and incubated on ice for 30 min. After washing twice with saponin buffer, cells were stained for 30 min on ice with the appropriate secondary reagents diluted in saponin buffer. Flow cytometry was performed using a FACSort (BD Biosciences) upgraded with a red laser. A forward scatter vs side scatter gate was set to exclude debris and small cells (lymphocytes). An FL1 or FL4 baseline was set on unstained forward scatter/side scatter-gated islet cells. Gated events (5,000–10,000) were collected. For analysis of surface Fas expression, single-cell suspensions of islet cells were allowed to recover for 1 h in supplemented CMRL at 37°C in 5% CO₂. Cells were then stained with IOX2, mAb, fol- lowed by biotinylated mouse anti-hamster Ig mixture and streptavidin-al lophycocyanin. A forward scatter vs FL1 gate was set to include cells with higher FL1 fluorescence (β cells) and to exclude debris and small cells (lymphocytes) (9).

**Western analysis**

Western blotting was performed as described by Newton et al. (28). Isolated islets from NOD and Het RIP-dnFADD mice were trypsinized and solubilized in lysis buffer (0.25 M Tris-HCl (pH 6.8), 10% SDS, 20% glycerol, 5% 2-ME, 0.02% bromphenol blue, and 0.5 mg/ml Pefabloc (Roche)). After SDS-PAGE electrophoresis and transfer to nitrocellulose membranes, the dnFADD was detected with 3 μg/ml anti-FLAG M2 mAb, followed by rabbit anti-mouse IgG (Fc-specific) Abs and 125I-labeled protein A.

**Monitoring of diabetes**

Urinary glucose levels were monitored daily or weekly depending on the nature of the experiment, with two consecutive readings of >111 mM glucose taken to indicate the onset of diabetes. Blood glucose readings were taken at this time, and the mice were killed.

**Diabetes transfer**

Spleen cells (2 × 10⁶) were isolated from diabetic NOD female donors, washed in PBS, and injected i.v. into irradiated (850 rad), <10-wk-old male NOD control, Het RIP-dnFADD, or Hom RIP-dnFADD mice. Mice were kept on antibiotic water and analyzed for diabetes onset 10 days after irradiation.

**Statistical analysis**

Analyses of data were performed using the program PRISM (GraphPad). Data are presented as the mean and SE. Data were analyzed using one-way ANOVA with Bonferroni’s post test for comparison of multiple columns. Survival curves were analyzed using the log-rank test.

**Results**

**Transgenic mice expressing dnFADD in islet β cells**

A dnFADD containing an intact DD, but lacking the DED, was linked to the RIP. This mutant dnFADD is known to compete out the binding of endogenous FADD to the DD of Fas and to other death receptors, thereby preventing recruitment of caspase 8 to the DED. We were able to detect wild-type FADD expression in islet β cells of normal mice by immunohistochemistry (data not shown), indicating that this adaptor may play a role in these cells. Of six primary transgenic lines derived on a NOD genetic background, one line expressed the dnFADD protein in β cells (Het RIP-dnFADD mice). This line was made homozygous for the transgene (Hom RIP-dnFADD mice). Western blotting of extracts from isolated islets of transgenic mice with an Ab to the FLAG epitope revealed a band of the appropriate size (~16 kDa) for the dnFADD molecule (Fig. 1A). Protein levels of dnFADD in β cells were also determined by intracellular immunofluorescent staining with the anti-FLAG mAb and flow cytometry. Although the bulk of the islet is made up of the large β cells, numerically these make up only ~55–60% of all cells in an islet, other cell types being the α, δ, and pancreatic polypeptide cells; endothelial cells; resident macrophages; fibroblasts; and neuronal cells. Although 53% of the islet cells from Het RIP-dnFADD mice expressed insulin after overnight culture (Fig. 1B), only ~39% of the cells expressed dnFADD rather than the expected 50–60% (mean, 36 ± 6.7% from 16 experiments). This may have been because transgenic dnFADD was toxic or detrimental to β cells or because the dnFADD was expressed at a broad range in β cells. Analysis of freshly isolated islets from Het RIP-dnFADD and Hom RIP-dnFADD mice was therefore performed (Fig. 1C). Hom RIP- dnFADD mice expressed a higher level of dnFADD (mean fluorescence intensity, 436 compared with 278) and a higher proportion of β cells (62%) stained for FLAG. This suggested that dnFADD was expressed in all β cells, but levels in some β cells of Het RIP-dnFADD mice were too low to be resolved from the negative peak. To check that dnFADD was not toxic to β cells, the RIP-dnFADD mice were mated to RIP-Bcl-2 mice in which all the β cells expressed human Bcl-2 at uniform levels (Bcl-2 transgene-expressing cells, 50 ± 6.6% from 11 experiments). Approximately 50% of islet cells from double-transgenic animals expressed human Bcl-2 as usual, but only ~35% expressed dnFADD (Fig. 2). This indicates that β cells from Het RIP-dnFADD mice expressed dnFADD at varying levels, but that dnFADD was not toxic to the β cells.

Islet yields from Het RIP-dnFADD mice were similar to those of control NOD mice. Fig. 1D shows the yield of islets from individual NOD and Het RIP-dnFADD mice isolated simultaneously and then plotted against each other. Similar yields were found for Hom RIP-dnFADD islets. However, Het (and Hom) RIP-dnFADD islets appeared to be more degranulated after overnight culture, as indicated by the reduced side light scatter profile from FACs analysis (Fig. 1B, top panels).

**dnFADD protects islet β cells against FasL-induced cell death in culture**

To determine whether the transgenic dnFADD was functional, whole islets isolated from Het RIP-dnFADD and control NOD mice were cultured with cytokines (IL-1β and IFN-γ) to induce the expression of Fas and with FasL to induce cell death. It has been shown previously that at concentrations of 1 U/ml IL-1β and 100 U/ml IFN-γ, Fas is efficiently up-regulated, but islets are not damaged by the cytokines (9). After 4 days of combined treatment with IL-1β, IFN-γ, and FasL, islets and media were recovered and analyzed for nuclei with an apparent DNA content of less than 2C (<2C) as previously described (42). The results of six experiments, presented in Fig. 3A, show that dnFADD-expressing islet cells were resistant to FasL-induced cell death in culture (by ANOVA for NOD vs Het RIP-dnFADD, p < 0.001 on day 4 and
p < 0.01 on day 5). Fas up-regulation on islets by the cytokines was confirmed by flow cytometric analysis of an aliquot of islet cells from the assay (Fig. 4A). Fas-deficient, NOD/lpr islets were used as a control for the death assay and flow cytometry. The dnFADD-expressing islets had more FasL-induced death than the NOD/lpr islets (Fig. 3A), which would be consistent with deaths of those cells that did not express the dnFADD transgene.

Levels of Fas on Het RIP-dnFADD islets were marginally lower than those on NOD islets (mean, 22 ± 8% lower levels from seven experiments; Fig. 4B). Hom RIP-dnFADD islets had a similar reduction in Fas levels (not shown). In principle, this slightly lower level of Fas could contribute to the protection of Het RIP-dnFADD islets from killing by FasL. To test this possibility, FasL killing of NOD/lpr (w/o) islets was performed. Islets from NOD/lpr (w/o) mice that carry one normal and one mutant Fas allele, expressed Fas at ~50% the level seen on NOD islets after treatment with cytokines (mean, 53 ± 3% lower levels from three experiments; Fig. 4, C and D). This reduced level prevented killing by FasL in culture (Fig. 3B; by ANOVA for NOD vs Het RIP-dnFADD, p < 0.01; for NOD vs NOD/lpr (w/o), p < 0.001). In principle, therefore, the marginally lower levels of Fas on Het RIP-dnFADD islets could also have provided protection against FasL killing. Thus, dnFADD could work to protect islets from Fas-induced death by blocking caspase 8 recruitment and by blocking the up-regulation of Fas by cytokines. This block of Fas up-regulation might be explained by the findings presented below.

DnFADD protects islet β cells against cytokine-induced cell death in culture

Cultured islet β cells undergo cell death in response to treatment with high levels of IL-1β and IFN-γ (7). The mechanism for this involves NO production by the β cells themselves. Whole islets from mice expressing dnFADD in β cells or from control animals were cultured with or without IL-1β and IFN-γ for 2–3 days and then analyzed for nuclei with an apparent DNA content of <2C (Fig. 5A). After this treatment, NOD islets contained ~45% dead

![FIGURE 1.](http://www.jimmunol.org/)

Expression of dnFADD in islet β cells. The dnFADD protein was identified by the presence of the FLAG tag epitope using an anti-FLAG Ab. A, Western blot analysis of whole islet cell extracts. Lane 1, Marker; lanes 2 and 3, NOD islets (10⁴ and 10⁵ cells); lanes 4 and 5, Het RIP-dnFADD islets (10⁴ and 10⁵ cells). B, Intracellular staining of total islet cells to detect insulin expression (guinea pig anti-insulin Ab) and dnFADD expression (mouse anti-FLAG mAb, M2). The percentages of cells stained are given. The stippled line shows staining with guinea pig IgG as a negative control. A forward/side scatter gate was set around the islet cell population to exclude lymphocytes, dead cells, debris, and large clumps. Profiles represent staining on overnight-cultured islets from three to five pooled male mice, 6–8 wk of age. C, Intracellular FLAG staining of total islet cells from Het RIP-dnFADD and Hom RIP-dnFADD mice. The mean fluorescence intensity (MFI) is given. Profiles represent staining on freshly isolated islets from three to five pooled male mice, 6–8 wk of age. D, Islet yields. Islets were isolated at the same time from Het RIP-dnFADD transgenic mice and NOD littermate controls. Each dot represents the islet yield per mouse for one islet isolation experiment. The graph includes data from male and female mice between 4 and 10 wk of age.
The dnFADD protein has a broad range of expression in β cells. Intracellular staining of total islet cells from the indicated mice was performed using anti-FLAG mAb (M2) to detect dnFADD and mouse anti-human Bcl-2 mAb (Bcl-2–100) to detect transgenic Bcl-2. A forward/side scatter gate was set around the islet cell population to exclude lymphocytes, dead cells, debris, and large clumps. Profiles represent staining on overnight cultured islets from three to five pooled male mice, 6–8 wk of age.

cells, whereas islets that expressed dnFADD had significantly fewer dead cells (~25%; by ANOVA for NOD vs dnFADD, p < 0.001). The experiments were repeated to include islets from RIP-Bcl-2, Het RIP-dnFADD/Bcl-2, and Hom RIP-dnFADD mice. Others have shown that Bcl-2 overexpression in β cells can protect against cytokine-mediated death (44), but we did not find that Bcl-2 provided protection (Fig. 5B; by ANOVA for NOD vs RIP-Bcl-2, p > 0.05), nor did we find protection when C57BL/6 β cells expressing Bcl-2 were used (45). In contrast, dnFADD on its own or in combination with Bcl-2 overexpression inhibited cytokine-induced cell death in all experiments (Fig. 5B; by ANOVA for NOD vs Het RIP-dnFADD and NOD vs Hom RIP-dnFADD, p < 0.001). In four experiments, Het RIP-dnFADD/Bcl-2 double-transgenic islets were studied, but death of these cells was not statistically different between the double-transgenic group and the Het RIP-dnFADD group (Fig. 5B; p > 0.05).

We next tested whether dnFADD could protect against β cell killing by TNF-α and IFN-γ. This mechanism is believed to be mediated by NO production by β cells, but in this case, TNF-α impacts on resident macrophages, causing them to make IL-1, which then engages the IL-1R on β cells (21, 22). This killing can be blocked by using the IL-1R antagonist (IL-1Ra) or NMMA (to block synthesis of NO). In this 4-day assay, dnFADD protected NOD β cells against killing by TNF-α and IFN-γ with an efficiency comparable to that achieved with the IL-Ra blocker (Fig. 5C; by ANOVA for NOD with TNF-α plus IFN-γ vs RIP-dnFADD with TNF-α plus IFN-γ, p < 0.01). Addition of IL-1Ra afforded additional protection to the RIP-dnFADD islets (by ANOVA for RIP-dnFADD without IL-1Ra vs RIP-dnFADD with IL-1Ra, p < 0.05). NODlpr mice (which lack both Fas and TNFRI) on all cell types) were killed by IL-1α and IFN-γ, but not at all by TNF-α and IFN-γ. It is possible, therefore, that in RIP-dnFADD islets some of the cell death seen after treatment with TNF-α and IFN-γ was due to nonislet cells, and this was blocked by the inhibitors, IL-1Ra and NMMA. Alternatively, dnFADD was not expressed at sufficient levels in all β cells to provide complete protection.

Apparently, the overexpressed dnFADD molecule could block signals through the IL-1R, resulting in protection of β cells from cytokine-induced death. A block of IL-1R signaling should also impact on Fas up-regulation and may explain the marginally reduced levels of Fas on Het (and Hom) RIP-dnFADD β cells (Fig. 4, A and B).

Incidence of spontaneous diabetes in NOD RIP-dnFADD mice

Female Het RIP-dnFADD transgenic mice and their littermate controls were followed for diabetes onset over 250 days. Het RIP-dnFADD mice had a diabetes incidence similar to that of control animals, but the rate was slightly lower, although not statistically different between the two groups (Fig. 6A). Pancreata from seven diabetic Het RIP-dnFADD and seven littermate control mice had similar histology scores (not shown). A separate analysis was performed for control NOD, Hom RIP-dnFADD, and Het RIP-dnFADD mice (the latter being generated from matings of Hom RIP-dnFADD males to NOD females). Again, compared with NOD mice, the Het RIP-dnFADD mice had a slightly reduced...
incidence of diabetes, and the incidence was reduced even more in Hom RIP-dnFADD animals (Fig. 6B; by ANOVA for NOD vs Hom RIP-dnFADD, $p < 0.01$). Additional diabetes incidence studies were performed for offspring from matings of Het RIP-dnFADD mice with Het RIP-Bcl-2 mice. These matings gave rise to nontransgenic, RIP-dnFADD only, RIP-Bcl-2 only, and RIP-dnFADD/Bcl-2 double-transgenic mice (Fig. 6C). The numbers of animals in this experiment were relatively small, but Bcl-2 did not seem to synergize with dnFADD to protect mice from diabetes.

Diabetes was also transferred to male NOD, Het RIP-dnFADD, and Hom RIP-dnFADD recipients (Fig. 6D). A slightly reduced incidence was seen in the transgenic mice, but this was not statistically significant.

**Discussion**

Islets overexpressing dnFADD appeared to develop normally, indicating that FADD is not essential for β cell development as it is for vasculogenesis (35) or T cell proliferation (28, 32). The dnFADD molecule protected β cells against FasL-induced killing in culture after up-regulation of Fas with the cytokines IFN-γ and IL-1β. However, it was possible that dnFADD did this by blocking both caspase 8 activation and Fas up-regulation by IL-1.

In vivo, Het RIP-dnFADD NOD mice were marginally protected against spontaneous diabetes, but greater protection was found when the mice were made homozygous for the transgene. These results are similar to those reported by Savinov et al. (16), who found that higher levels of dominant-negative Fas (Fas<sup>dn</sup>)
were needed to provide protection against spontaneous diabetes in NOD mice. The incidence and kinetics of disease in dnFADD and Fascg transgenic mice were remarkably similar, implicating Fas as the main death receptor blocked by dnFADD. We have yet to test whether blocking of other death receptors (TRAILR, TNFR1, DR3, or DR6) with dnFADD has any role in cell death and diabetes, but the lack of additional protection over that observed with blockade of Fas signaling alone (16) suggests that this is unlikely. As yet, TRAILR, DR3, and DR6 expression by cells is not proven. We have been unable to detect TRAILR (DR5) on islet cells cultured with TNF-α and IFN-γ using flow cytometric analysis, when Fas up-regulation was easily detected (our unpublished observations), and have yet to test for DR3 or DR6 expression. TNFR1 is expressed on cells, but is not thought to signal through the caspase 8 pathway (46). Rather, TNFR1 signaling in cells may serve to induce chemokine production and lymphocyte recruitment through TRADD-TRAF-mediated activation of NF-κB transcription factors (19).

As yet, we cannot say whether dnFADD protects NOD mice from diabetes by delaying disease initiation or by inhibiting cell killing at the effector phase. Het and Hom RIP-dnFADD mice injected with diabeticogenic spleen cells became diabetic at a similar rate as NOD control animals, showing that dnFADD has little protective effect against high frequency, potent, diabeticogenic T cells. Because such T cells use the perforin/granzyme system as well as FasL for target cell killing, this result is not surprising. A time course of infiltration into islets of NOD and Het RIP-dnFADD mice showed similar kinetics by histology (our unpublished observations). A clearer role for dnFADD might become more apparent in Het RIP-dnFADD mice that also lack perforin. Finally, we also tested whether Bcl-2 overexpression would synergize with dnFADD to protect NOD mice from spontaneous diabetes, but this did not seem to be the case, demonstrating that the Bcl-2-regulated apoptosis pathway (47) does not play a major role in cell destruction in NOD mice (38).

It is well established that killing of mouse β cells in culture with IL-1β and IFN-γ or TNF-α and IFN-γ is mediated by IL-1R signaling and NO toxicity (7, 21, 22). We did not find protection against this form of killing when Bcl-2 was expressed in β cells, in agreement with the report by Barbu et al. (48), although others
have found protection (44). This may relate to slight differences in the assay procedure or the level of Bcl-2 expression achieved in the different systems. Isolated RIP-Bcl-2 islets were more healthy than control islets, indicating that Bcl-2 could protect against the stress caused by collagenase digestion and tissue culture. We did, however, find that dnFADD could protect β cells against killing with IL-1β and IFN-γ or TNF-α and IFN-γ. The dnFADD molecule appears to block IL-IR signals, and this might explain the marginally lower levels of Fas on Het (and Hom) RIP-dnFADD islets, because IL-IR signals are necessary for Fas up-regulation in β cells. One possible mechanism to explain this is that the DD of dnFADD interacts with the DD of the MyD88 adaptor that associates with the IL-IR. This association may prevent recruitment of IL-1R.

300 DOMINANT-NEGATIVE FADD PROTECTS AGAINST β CELL DEATH

References

4. Hamilton-Williams, E. E., S. E. Palmer, B. Charlton, and R. M. Slattery. 2003. β cell overexpression of a wild-type FADD could suppress activation of NF-κB by LPS and IL-1β in cultured human cell lines and that FADD-deficient, mouse embryonic fibroblasts were more sensitive to NF-κB activation. We speculate that in our culture experiments, the DD of dnFADD could block signals from MyD88 to NF-κB, thus preventing iNOS production in β cells and, hence, death by cytokines. Because the in vitro killing of NOD islet cells by cytokines is essentially all NO mediated (i.e., it can be almost completely blocked by the NMMA inhibitor), we do not think that dnFADD is blocking caspase 8 recruitment to the IL-IR as suggested by Aliprantis et al. (30). Whether blocking IL-IR signals by dnFADD has any physiological relevance in vivo is not proven. It has been suggested that iNOS deficiency in NOD mice had little effect on diabetes incidence (24), so iNOS killing of islets in vitro may not have a counterpart mechanism in vivo. In fact, IL-IR signals may have more relevance to Fas up-regulation marking β cells for Fas-dependent destruction (25) than for NO-induced death. Whether dnFADD interacts with the β cell IL-IR pathway in vivo has yet to be formally shown. We are currently testing whether dnFADD reduces the in vivo up-regulation of Fas on islets from mice expressing the 8.3 TCR.

In conclusion, our results show that dnFADD has a protective effect in the NOD mouse model, implicating Fas signaling as a contributing, albeit minor, mechanism of β cell death. In addition, in vitro data suggest that dnFADD may interact with the IL-7 pathway as well. We are currently creating NOD RIP-dnFADD mice that lack perforin to test whether blocking FADD function in β cells can synergize with loss of perforin in reducing the manifestations of diabetes. The dnFADD molecule may be a useful inhibitor of Fas-mediated β cell death and possibly IL-1-mediated killing, two pathways known to cause β cell damage.

Acknowledgments

We thank Hansa Puthalakath (Walter and Eliza Hall Institute, Melbourne, Australia) for performing the Western blot. We much appreciate the excellent technical help of Dina Stockwell and Stacey Fynch. We thank Jurg Tschopp for purified FasL, and Mark Smyth for anti-DR5 Ab, Rima Serreze, D. V., H. D. Chapman, D. S. Varnum, I. Gerling, E. H. Leiter, and L. D. Shultz. 1997. Initiation of autoimmune diabetes in NOD/Lt mice is MHC class I-dependent. J. Immunol. 158: 3978–3986.
4. Hamilton-Williams, E. E., S. E. Palmer, B. Charlton, and R. M. Slattery. 2003. β cell overexpression of a wild-type FADD could suppress activation of NF-κB by LPS and IL-1β in cultured human cell lines and that FADD-deficient, mouse embryonic fibroblasts were more sensitive to NF-κB activation. We speculate that in our culture experiments, the DD of dnFADD could block signals from MyD88 to NF-κB, thus preventing iNOS production in β cells and, hence, death by cytokines. Because the in vitro killing of NOD islet cells by cytokines is essentially all NO mediated (i.e., it can be almost completely blocked by the NMMA inhibitor), we do not think that dnFADD is blocking caspase 8 recruitment to the IL-IR as suggested by Aliprantis et al. (30). Whether blocking IL-IR signals by dnFADD has any physiological relevance in vivo is not proven. It has been suggested that iNOS deficiency in NOD mice had little effect on diabetes incidence (24), so iNOS killing of islets in vitro may not have a counterpart mechanism in vivo. In fact, IL-IR signals may have more relevance to Fas up-regulation marking β cells for Fas-dependent destruction (25) than for NO-induced death. Whether dnFADD interacts with the β cell IL-IR pathway in vivo has yet to be formally shown. We are currently testing whether dnFADD reduces the in vivo up-regulation of Fas on islets from mice expressing the 8.3 TCR.

In conclusion, our results show that dnFADD has a protective effect in the NOD mouse model, implicating Fas signaling as a contributing, albeit minor, mechanism of β cell death. In addition, in vitro data suggest that dnFADD may interact with the IL-7 pathway as well. We are currently creating NOD RIP-dnFADD mice that lack perforin to test whether blocking FADD function in β cells can synergize with loss of perforin in reducing the manifestations of diabetes. The dnFADD molecule may be a useful inhibitor of Fas-mediated β cell death and possibly IL-1-mediated killing, two pathways known to cause β cell damage.

Acknowledgments

We thank Hansa Puthalakath (Walter and Eliza Hall Institute, Melbourne, Australia) for performing the Western blot. We much appreciate the excellent technical help of Dina Stockwell and Stacey Fynch. We thank Jurg Tschopp for purified FasL, and Mark Smyth for anti-DR5 Ab, Rima Darwiche for help with the Fas staining, and John Scanlon for some of the transfer data and histology.

Disclosures

The authors have no financial conflict of interest.


