

Proapoptotic BH3-Only Protein Bid Is Essential For Death Receptor–Induced Apoptosis of Pancreatic β -Cells

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OBJECTIVE—Apoptosis of pancreatic β -cells is critical in both diabetes development and failure of islet transplantation. The role in these processes of pro- and antiapoptotic Bcl-2 family proteins, which regulate apoptosis by controlling mitochondrial integrity, remains poorly understood. We investigated the role of the BH3-only protein Bid and the multi-BH domain proapoptotic Bax and Bak, as well as prosurvival Bcl-2, in β -cell apoptosis.

RESEARCH DESIGN AND METHODS—We isolated islets from mice lacking Bid, Bax, or Bak and those overexpressing Bcl-2 and exposed them to Fas ligand, tumor necrosis factor (TNF)- α , and proinflammatory cytokines or cytotoxic stimuli that activate the mitochondrial apoptotic pathway (staurosporine, etoposide, γ -radiation, tunicamycin, and thapsigargin). Nuclear fragmentation was measured by flow cytometry.

RESULTS—Development and function of islets were not affected by loss of Bid, and Bid-deficient islets were as susceptible as wild-type islets to cytotoxic stimuli that cause apoptosis via the mitochondrial pathway. In contrast, Bid-deficient islets and those overexpressing antiapoptotic Bcl-2 were protected from Fas ligand–induced apoptosis. Bid-deficient islets were also resistant to apoptosis induced by TNF- α plus cycloheximide and were partially resistant to proinflammatory cytokine-induced death. Loss of the multi-BH domain proapoptotic Bax or Bak protected islets partially from death receptor–induced apoptosis.

CONCLUSIONS—These results demonstrate that Bid is essential for death receptor–induced apoptosis of islets, similar to its demonstrated role in hepatocytes. This indicates that blocking Bid activity may be useful for protection of islets from immune-mediated attack and possibly also in other pathological states in which β -cells are destroyed. *Diabetes* 57:1284–1292, 2008

Death of islet β -cells lies at the core of all forms of diabetes. In type 1 diabetes, β -cell apoptosis may be required in the initiation phase of the disease for the release of self-antigens and during the effector phase, when infiltrating leukocytes

cause progressive β -cell destruction (1). Several mechanisms have been implicated in the destruction of β -cells, including cytotoxic granule exocytosis (perforin/granzymes), Fas/Fas ligand (FasL), pro-inflammatory cytokines and cellular stress (2). In type 2 diabetes, decline in β -cell numbers and function in the face of insulin resistance is required for disease development (3,4). Additionally β -cell loss occurs during islet isolation, during engraftment following transplantation and in some forms of secondary diabetes such as chronic pancreatitis (5). In none of these diseases are the mechanisms of β -cell death fully understood.

In mammalian cells, two distinct apoptotic pathways have been defined, the extrinsic and mitochondrial pathways (6). The pathways ultimately converge by activating common downstream effector cysteine proteases (caspases), that cleave a large number of intra-cellular proteins thereby causing cell death. The extrinsic pathway is initiated by activation of ‘death receptors’, members of the tumor necrosis factor receptor (TNF-R) family that have an intra-cellular death domain (e.g., Fas, TNF-R1). Upon ligation, these receptors recruit a so-called DISC (death inducing signaling complex) in which the initiator caspase, caspase-8, is activated by its adaptor Fas-associated death domain (FADD) (7). Both FADD and caspase-8 are essential for “death receptor”-induced apoptosis. The mitochondrial (also called ‘intrinsic’ or ‘Bcl-2-regulated’) pathway is induced by developmental cues, growth factor deprivation, anoxia and a large number of cytotoxic drugs. This pathway does not require FADD and caspase-8, but instead is characterized by mitochondrial release of apoptogenic molecules, such as cytochrome c, and Apaf-1-mediated activation of the ‘initiator caspase’, caspase-9.

The Bcl-2 family of proteins are critical regulators of the mitochondrial apoptotic pathway. Bcl-2 proteins share one or more Bcl-2 homology (BH) domains and are divided into three groups according to their structure and function. The anti-apoptotic members, including Bcl-2, Bcl-x_L, Bcl-w, Mcl-1 and A1, prevent mitochondrial outer membrane permeabilization (MOMP) and consequent release of cytochrome c and are critical for cell survival. The pro-apoptotic members (Bax, Bak and Bok) and the BH3-only proteins (Bid, Bad, Bik, Bim, Bmf, Hrk, Noxa and Puma) promote MOMP and are essential for apoptosis (8). The interactions and balance between members of the different subgroups determine cell fate. Genetic and biochemical studies have shown that BH3-only proteins are activated in a death stimulus- and cell type-specific manner (9). BH3-only proteins bind with high affinity and specificity to anti-apoptotic Bcl-2 family members (10), thereby liberating Bax/Bak to elicit MOMP and activation of the caspase cascade.

The pro-apoptotic BH3-only protein Bid is of particular

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CHX, cycloheximide; CTL, cytotoxic T-lymphocyte; dnFADD, dominant-negative Fas-associated death domain; ER, endoplasmic reticulum; FADD, Fas-associated death domain; FasL, Fas ligand; FLIP, FADD-like IL-1 β -converting enzyme inhibitory protein; IFN- γ , γ -interferon; IL, interleukin; NMMA, N^G-monomethyl-L-arginine; TNF, tumor necrosis factor; zVAD.fmk, z-Val-Ala-Asp-fluoromethylketone.

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relevance for immune-mediated β -cell death because it is activated by caspase-8 following activation of the Fas death receptor (11–13) and granzyme B (14,15). Although BH3-only proteins are generally associated with the mitochondrial apoptotic pathway, experiments with gene-targeted mice have shown that Bid is essential for Fas-mediated apoptosis in hepatocytes but not other cell types such as thymocytes or mature T cells (16,17). Cells in which Fas/FasL-induced apoptosis occurs independent of Bid have been termed type I cells, whereas those that require Bid have been termed type II cells (18). The reasons for the differences in Fas-induced apoptosis between these two cell types are presently unknown, but they may have consequences for the design of therapies to prevent apoptosis.

We have previously shown that the pro-apoptotic BH3-only protein Bid is required for β cell apoptosis induced by recombinant perforin and granzyme B (15). Here we describe experiments with genetically modified mice to investigate the mechanisms by which other death stimuli relevant to diabetes, including FasL and TNF α cause apoptosis in β -cells. Our data demonstrate that Bid is essential for death receptor- and inflammatory cytokine-induced apoptosis in β -cells, indicating that they are type II cells like hepatocytes.

RESEARCH DESIGN AND METHODS

Mice. All mice used were bred and maintained under specific pathogen-free conditions at the St. Vincent's Institute animal facility (Fitzroy, Victoria, Australia). C57BL/6 and NOD/Lt mice were obtained from the Walter and Eliza Hall Institute animal breeding facility (MuriGen, Victoria, Australia). Bid-deficient (*bid*^{-/-}) mice were generated on an inbred C57BL/6 background using C57BL/6-derived embryonic stem cells (17). Homozygous H-2^{bmi1} RIP-Bcl-2 and NOD.RIP-Bcl-2 transgenic mice (backcrossed ≥ 10 generations), which express human Bcl-2 in β -cells under control of the rat insulin promoter, have previously been described (19,20). The generation of *bax*^{-/-} (21), *bak*^{-/-} (22) and *bak*^{-/-}*bax*^{+/-} (23) mice has previously been described, and those used here had been backcrossed for >10 generations onto a C57BL/6 background. Perforin-deficient OT-I T-cell receptor transgenic mice have been described (24). All animal experiments were approved by the institutional animal ethics committee.

Reagents. Recombinant murine γ -interferon (IFN- γ) and tumor necrosis factor (TNF)- α were obtained from Genentech (San Francisco, CA) and used at 1–100 units/ml and 1,000–2,400 units/ml, respectively. Human recombinant interleukin-1 α from Dr. C. Reynolds (National Cancer Institute, Bethesda, MD) was used at 100 units/ml. IL-1 β from R&D systems (Minneapolis, MN) was used at 10–150 units/ml. MegaFasL (APO-010), provided by Dr. M. Dupuis (Apoxis, Lausanne, Switzerland), was used at 100 nmol/l. N^G-monomethyl-L-arginine (NMMA) (Sigma-Aldrich, St. Louis, MO) and z-Val-Ala-Asp-fluoromethylketone (zVAD.fmk) (Enzyme Systems Products, Livermore, CA) were used at 2 mmol/l and 100 μ mol/l, respectively. Cycloheximide (CHX) (Sigma-Aldrich) was used at 5 μ g/ml. Staurosporine (Sigma-Aldrich), etoposide (Pfizer, Kalamazoo, MI), tunicamycin (Sigma-Aldrich), and thapsigargin (Calbiochem, San Diego, CA) were used at the concentrations indicated.

Preparation of islets. Islets were isolated from mice using collagenase P (Roche, Basel, Switzerland) and histopaque-1077 density gradients (Sigma-Aldrich) as previously described (24). Islets were handpicked and cultured overnight at 37°C and 5% CO₂ in CMRL medium-1066 (Gibco products; Invitrogen, Grand Island, NY) supplemented with 100 units/ml penicillin, 100 μ g/ml streptomycin, 2 mmol/l glutamine, and 10% fetal calf serum (KS; JRH Biosciences) (referred to below as complete CMRL).

Islet size distribution and determination of islet yields. Isolated islets from age-matched male and female mice were cultured overnight and then washed in complete CMRL to remove cellular debris. Islet yields were determined by counting the total number of islets isolated per mouse. For quantification of size, isolated islets were blind scored under 40 \times magnification with a graduated eyepiece to determine islet diameter. A reference grid was placed under each dish to ensure that each islet was analyzed only once.

Intracellular insulin staining. The expression of insulin in islet β -cells was determined as previously described (20). Freshly isolated islets were trypsinized, fixed, and permeabilized. Cells were then stained with either guinea pig anti-insulin antibody (diluted 1:1,000; DakoCytomation, Carpinte-

ria, CA) or isotype-matched control guinea pig IgG (diluted 1:2,000; Sigma-Aldrich) followed by biotinylated donkey anti-guinea pig IgG antibodies (diluted 1:400; Jackson ImmunoResearch Laboratories) plus streptavidin allophycocyanin (diluted 1:600; BD Pharmingen, San Diego, CA).

Intra-peritoneal glucose tolerance tests. Mice were injected intraperitoneally with 2 mg D-glucose/g body wt. Blood samples were obtained from tail-tip bleeds and blood glucose concentrations determined using Advantage II Glucose Strips with Advantage glucometer (Roche).

Cell death assays. The day after isolation, 50–100 uniformly sized islets (excluding very large or necrotic islets) per sample were handpicked into 3.5-cm Petri dishes containing 1.1 ml complete CMRL. Islets were cultured with the appropriate stimuli to induce cell death. For assays with FasL, 50 islets ($1.8 \times 10^4 \pm 0.3 \times 10^4$ cells) ranging in size from 50 to 200 μ m were used. For γ -radiation, islets were irradiated at the indicated doses and then washed and cultured. At the end of the culture period, nonattached cells and islets were transferred into polypropylene tubes and washed in PBS. Islets were then dispersed (0.1 mg/ml bovine trypsin [Calbiochem] and 2 mmol/l EDTA in PBS) for 5 min at 37°C. Islets were mechanically dispersed using a pipette, washed in PBS, and recovered in complete CMRL medium for 1 h at 37°C in 5% CO₂. Cells were then washed in PBS and resuspended in 250 μ l hypotonic buffer containing 50 mg/ml propidium iodide (Sigma-Aldrich), 0.1% sodium citrate, and 0.1% TritonX-100, which stains nuclear DNA. Cells were then analyzed on a FACSCalibur (Becton Dickinson, Franklin Lakes, NJ) using the FL3 channel. Apoptotic cells were identified by their subdiploid DNA content as previously described (25). DNA fragmentation of untreated cells was always <10%.

Cr release assays. Activated perforin-deficient OT-I cytotoxic T-lymphocytes (CTLs) were generated in vitro as previously described (24). Wild type and Bid-deficient islets were pulsed with specific ovalbumin (OVA_{257–264}) or nonspecific herpes simple virus (HSV_{498–505}) peptides and used in ⁵¹Cr release assays as previously described (24).

Cytochrome c release assay. Islets were cultured with IFN- γ , IL-1 β , and FasL in complete CMRL. After 3 days, islets were dispersed into single cells using trypsin, and cytochrome c release was measured as previously described (26). This technique relies on the principle that cytochrome c diffuses through cells with mitochondrial outer membrane damage. After plasma membrane permeabilization, diffused cytochrome c leaks out of the cell, resulting in low fluorescence when cells are stained with anti-cytochrome c antibody. Cells with intact mitochondria retain cytochrome c and have high fluorescence after staining (26). Cells were permeabilized and fixed and then stained overnight at 4°C with mouse anti-cytochrome c antibody (Clone 6H2B4, BD Pharmingen) followed by phycoerythrin-conjugated sheep anti-mouse IgG (Silenus, Hawthorn, Australia) in blocking buffer for 1 h at room temperature. Cells were analyzed by flow cytometry on a FACSCalibur.

Fas staining. Islets were cultured with IL-1 β and IFN- γ in complete CMRL for 2 days. Islets dispersed with trypsin were allowed to recover in complete CMRL for 1 h before staining with hamster anti-mouse Fas antibodies (Jo2; BD Pharmingen) followed by biotinylated anti-hamster Ig (BD Pharmingen) and phycoerythrin-conjugated streptavidin (Caltag, Burlingame, CA). Dead cells and leukocytes were excluded by staining with propidium iodide (3.3 μ g/ml) and anti-CD45-PerCP-Cy5.5 (30-F11; BD Pharmingen) respectively. Cells were analyzed on a FACSCalibur.

Statistical analysis. Analyses of data were performed using GraphPad Prism (GraphPad Software, San Diego, CA). Data are represented as means \pm SE. Data were analyzed by one-way ANOVA with Bonferroni's posttest for comparison of multiple columns. ⁵¹Cr release assays were analyzed with paired *t* tests.

RESULTS

Pancreatic islets from Bid-deficient mice develop and function normally. Islet development and function were studied in Bid-deficient mice. We observed no significant difference in the islet yields from Bid-deficient and wild-type mice (Fig. 1A), and islets from both sets of animals displayed a similar size distribution (Fig. 1B). Moreover, intracellular insulin expression, measured by flow cytometry, was similar in freshly isolated islets from Bid-deficient and wild-type mice (Fig. 1C). There was also no significant difference in intraperitoneal glucose tolerance tests between Bid-deficient and wild-type mice (Fig. 1D). These results demonstrate that Bid is not required for normal development and function of islets or apoptosis

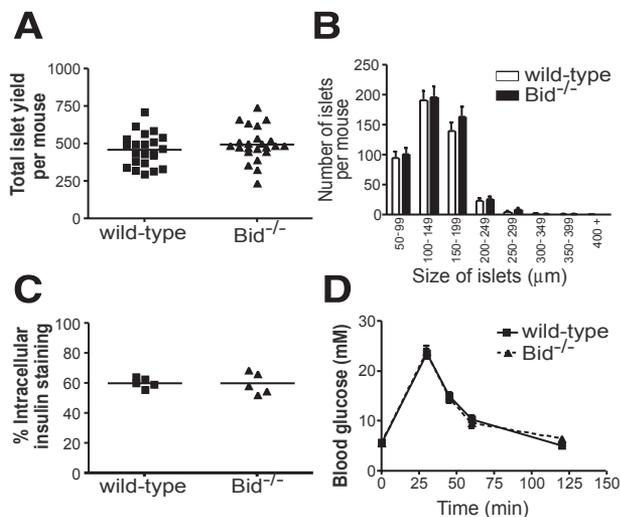


FIG. 1. Loss of Bid does not affect the development or function of islets. **A:** Islet yields from individual mice were determined. Data were pooled from three independent experiments; wild-type, $n = 22$; $bid^{-/-}$, $n = 23$. **B:** Isolated islets from individual mice were scored blind for size; wild-type, $n = 8$; $bid^{-/-}$, $n = 8$. **C:** Percentages of cells positive for insulin after intracellular staining on freshly isolated trypsinized islets. Data represent staining from individual mice; wild-type, $n = 5$; $bid^{-/-}$, $n = 5$. **D:** Blood glucose levels after intraperitoneal injection of 2 mg D-glucose/g body wt. Data are representative of three independent experiments; wild-type, $n = 8$; $bid^{-/-}$, $n = 8$. No significant differences detected.

due to physical stress that occurs during the isolation process.

Bid-deficient islets are normally sensitive to a broad range of cytotoxic stimuli that activate the mitochondrial apoptotic pathway. Bid has been reported to be essential for apoptosis induced by a broad range of cytotoxic stimuli (27) and direct DNA damage (28,29) or endoplasmic reticulum (ER) stress (30). In addition, Bid can be activated by effector caspases and has therefore been proposed to function as a general amplifier of apoptosis signaling, but whether this is essential for cell killing has not been determined. Islets from Bid-deficient and wild-type mice were exposed to stimuli that activate the mitochondrial apoptotic pathway and cell death measured by intracellular DNA content analysis (apoptotic cells exhibit a sub-G1 DNA content). Bid-deficient islets underwent apoptosis to the same extent as wild-type islets in response to treatment with staurosporine, a broad-spectrum kinase inhibitor (Fig. 2A). Bid-deficient islets were also normally sensitive to DNA damage induced with the topoisomerase inhibitor etoposide (Fig. 2B) or γ -radiation (Fig. 2C), consistent with previous analysis of fibroblasts and hemopoietic cells (17,31). Islets are thought to be particularly susceptible to ER stress because of their high rate of protein synthesis and secretion (32). We found that Bid-deficient and wild-type islets were equally susceptible to treatment with the ER stressors tunicamycin (Fig. 2D) or thapsigargin (Fig. 2E). While as many as 40% of the cells in islets represent non- β cells, apoptosis observed in these assays always exceeded 40%, suggesting that β -cells at least in part contributed to the cell death. These results demonstrate that Bid is not required for mitochondrial apoptosis signaling by a broad range of cytotoxic stimuli. **Loss of Bid prevents FasL-induced apoptosis of islets in vitro.** Overexpression of the caspase-8 inhibitor CrmA (33) or dominant-negative FADD (dnFADD) (20) both inhibit FasL-induced apoptosis of β -cells. However, the

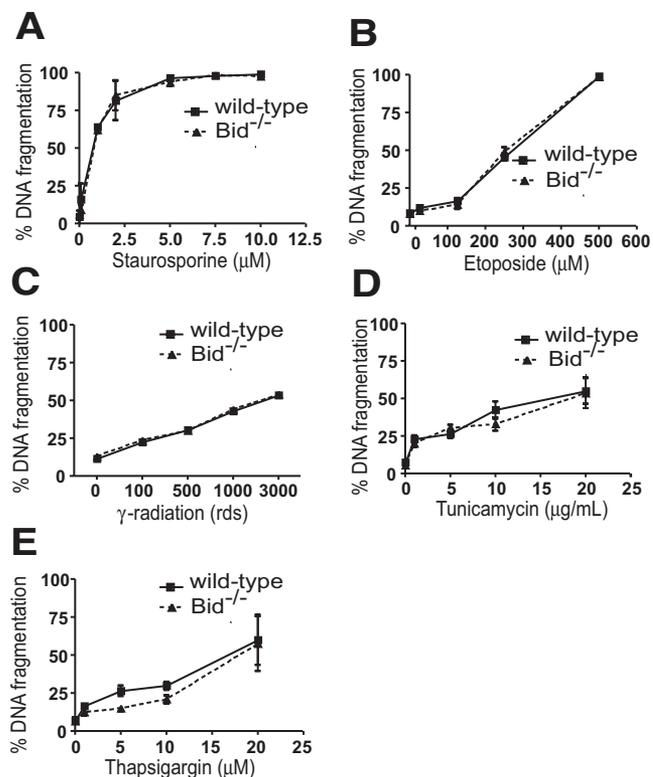


FIG. 2. β -cells from $bid^{-/-}$ mice are normally sensitive to a broad range of stimuli that activate the mitochondrial apoptotic pathway. Approximately 100 islets isolated from individual mice were cultured in increasing concentrations of etoposide of staurosporine for 36 h (A), increasing concentrations of etoposide for 3 days (B), complete CMRL for 7 days after γ -radiation (C), increasing concentrations of tunicamycin for 3 days (D), and increasing concentrations of thapsigargin for 3 days (E). Percentages of apoptotic cells (staining with a sub-G1 DNA content) determined by flow cytometry. Data represent means \pm SE of 3–6 mice of each genotype. No significant differences detected.

connection between the death receptor pathway and the mitochondrial pathway, and Bid in particular, in FasL-induced β -cell killing, has not yet been explored. Islets were cultured with IFN- γ plus IL-1 β to induce Fas expression (which is not constitutively expressed on islet cells [34]) and were cotreated with FasL. Because high concentrations of IFN- γ plus IL-1 β are toxic to islets even without adding FasL, the concentrations of these cytokines were optimized to obtain efficient Fas upregulation with minimal cytotoxicity (1 unit/ml IFN- γ and 150 units/ml IL-1 β) (data not shown). After a 4-day incubation with IFN- γ , IL-1 β , and FasL, apoptotic cells were identified by flow cytometry (Fig. 3A). Significant killing was seen in wild-type islets ($65.3 \pm 6.6\%$) and, as expected, this was inhibited by the broad spectrum caspase inhibitor zVAD.fmk (34). In contrast, Bid-deficient islets were highly resistant to FasL, exhibiting only $12.6 \pm 1.5\%$ ($bid^{-/-}$ vs. wild type; $P < 0.001$) (Fig. 3B). As previously reported (17), Bid-deficient thymocytes (type I cells) were normally sensitive to FasL (Fig. 3C).

We also examined whether Bid was essential for FasL-induced cytochrome *c* release from mitochondria in islet cells. Upon treatment with IFN- γ , IL-1 β , and FasL, wild-type islets released cytochrome *c* ($46.6 \pm 4.5\%$ cytochrome *c* low), whereas Bid-deficient islets showed only little release of cytochrome *c* above background ($25.5 \pm 5.9\%$) (Fig. 4A and B). These data demonstrate that Bid is essential for FasL-induced mitochondrial cytochrome *c*

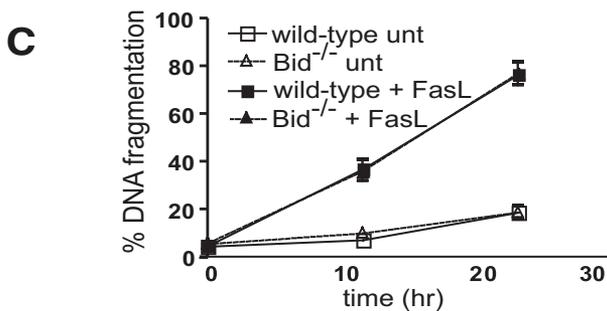
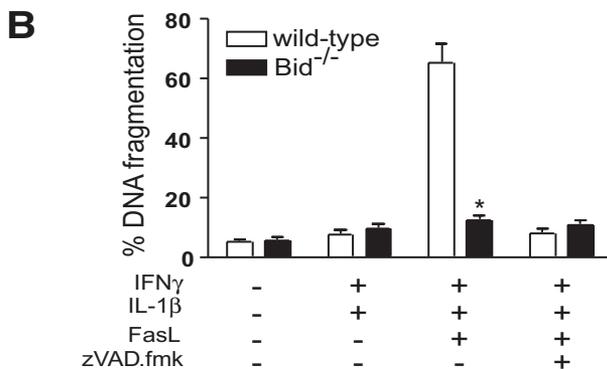
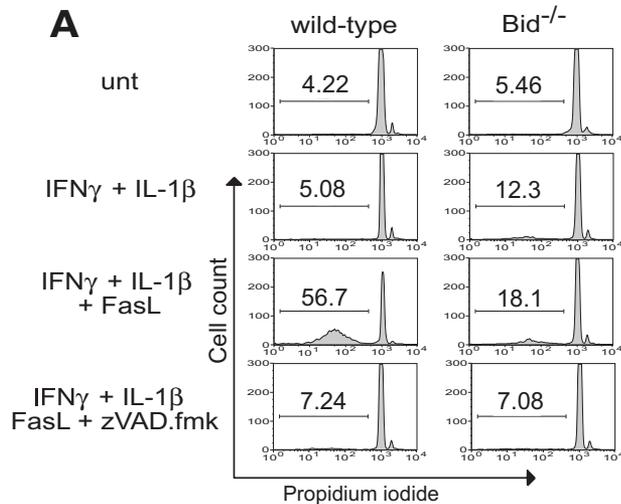


FIG. 3. Loss of Bid protects islets from FasL-induced apoptosis in vitro. Islets from bid^{-/-} or wild-type mice were cultured for 4 days with IFN- γ , IL-1 β , and FasL with or without zVAD.fmk. The percentages of apoptotic cells were determined as described in Fig. 2. **A:** The histograms from a representative experiment are shown. Cells with a sub-G1 DNA content are shown in the gated area. **B:** Data represent means \pm SE of three individual experiments ($n = 6$ mice of each genotype). Statistical significance: * $P < 0.001$ compared with wild-type islets treated with IFN- γ , IL-1 β , and FasL (one-way ANOVA). **C:** Thymocytes from bid^{-/-} or wild-type mice were treated with FasL and percentages of apoptotic cells determined as described in Fig. 2. Data represent means \pm SE from 4–5 mice of each genotype.

release and apoptosis of islet cells, indicating that they display a type II phenotype.

Bcl-2 overexpression protects β -cells from FasL-induced apoptosis. Because Bid needs to activate Bax/Bak to trigger apoptosis and because Bax and Bak are controlled by the antiapoptotic members of the Bcl-2 family (35), we tested whether Bcl-2 overexpression can inhibit FasL-induced killing of islets. Islets from RIP-Bcl-2 transgenic or wild-type mice were cultured in the presence

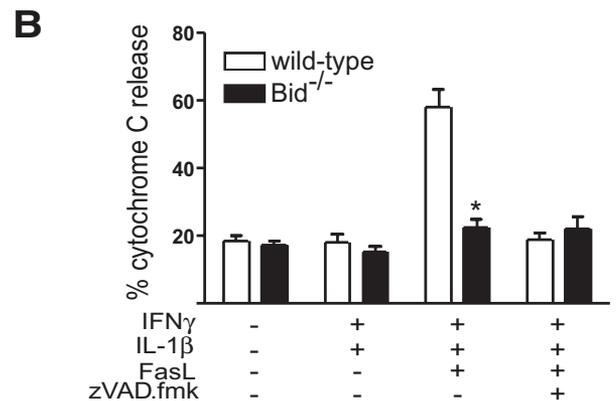
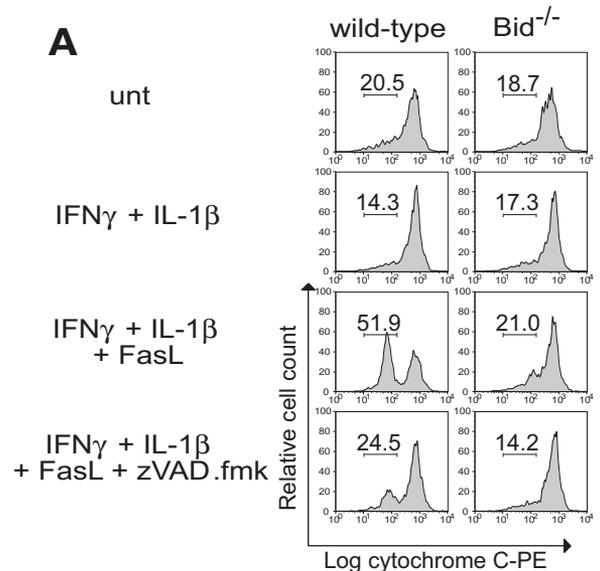


FIG. 4. Bid is required for FasL-induced cytochrome c release from islet cells. Islets from bid^{-/-} or wild-type mice were cultured for 3 days with IFN- γ , IL-1 β , and FasL with or without zVAD.fmk. Islets were assayed for cytochrome c release by flow cytometry. **A:** The histograms from one representative experiment are shown. Percentages of cells that have released cytochrome c are shown in the gated area. **B:** Means \pm SE from three individual mice per genotype are shown. Statistical significance: * $P < 0.001$ compared with wild-type islets treated with IFN- γ , IL-1 β , and FasL (one-way ANOVA).

of IL-1 β , IFN- γ , and FasL and cell death examined after 4 days. Bcl-2-overexpressing islets were as resistant to FasL as those from bid^{-/-} mice, and this was seen on two separate genetic backgrounds: bm1 (21.4 \pm 1.8% RIP.Bcl-2 vs. 69.8 \pm 9.3% wild type) and NOD (25.5 \pm 3.0% NOD.RIP.Bcl-2 vs. 71.5 \pm 7.9% wild type) (Fig. 5A and B).

Fas upregulation on Bid-deficient and Bcl-2-overexpressing islets treated with cytokines was similar to that observed in wild-type islets (Fig. 5C and D), excluding the possibility that they are abnormally resistant to FasL as a result of a defect in Fas expression. These results show that Bcl-2 overexpression can protect islet β -cells from FasL-induced apoptosis.

Loss of Bid protects islets from FasL-dependent CTL-induced cytotoxicity. The protection of Bid-deficient islets from FasL-induced killing prompted us to test whether they were protected from CTL that kill in a FasL-dependent manner. To do this, we used in vitro-activated, perforin-deficient, ovalbumin-specific OT-I CTL as effector cells and peptide-pulsed islets from wild-type or Bid-deficient mice as targets. We have previously shown

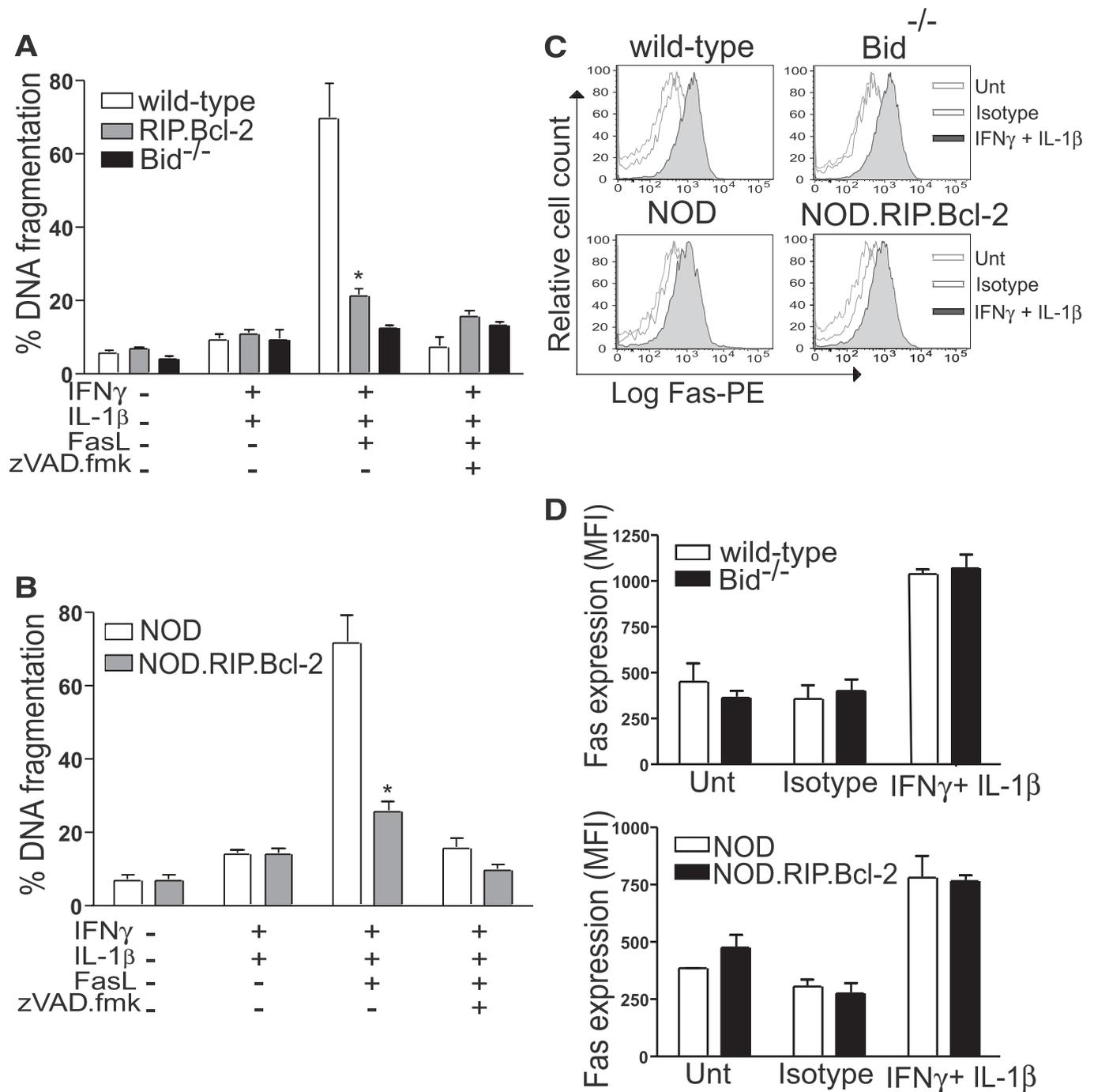


FIG. 5. Overexpression of Bcl-2 protects β -cells from FasL-induced apoptosis. Islets from homozygous RIP.Bcl-2, bid^{-/-}, or wild-type mice (A) and 6- to 8-week-old NOD mice or homozygous NOD.Bcl-2 mice (B) were cultured for 4 days with IFN- γ , IL-1 β , and FasL. Percentages of dead cells were determined as described for Fig. 2. The results represent the means \pm SE from 3–4 mice of each genotype. Statistical significance: * $P < 0.001$ compared with wild-type islets treated with IFN- γ , IL-1 β , and FasL (one-way ANOVA). C: Islets were either left untreated or treated with cytokines for 2 days before Fas expression on β -cells was examined by flow cytometry. The histograms from one representative experiment are shown. D: Mean fluorescence intensity of Fas staining on β -cells from two experiments is shown.

that perforin-deficient OT-I cells kill peptide-pulsed islets in a FasL-dependent manner (24). OVA_{257–264}-pulsed Bid-deficient islets were significantly protected from killing at both 10:1 and 20:1 effector:target ratios (supplementary Fig. 1, available in an online appendix at <http://dx.doi.org/10.2337/db07-1692>).

Loss of Bid or overexpression of Bcl-2 protects islets from TNF- α -induced cell death in the presence of cycloheximide. To assess whether Bid and linkage to the mitochondrial apoptotic pathway are also required for

apoptosis triggered by other death receptors, we treated islets from Bid-deficient, Bcl-2-overexpressing and wild-type mice with TNF- α in the presence of CHX. In most primary cells, including β -cells, TNF- α does not cause cell death on its own because its receptors, TNF-R1 and TNF-R2, activate the Rel/nuclear factor- κ B pathway that promotes expression of inhibitors of apoptosis such as FADD-like IL-1 β -converting enzyme inhibitory protein (FLIP), which blocks caspase-8. However, when new protein synthesis is blocked by CHX or when the Rel/

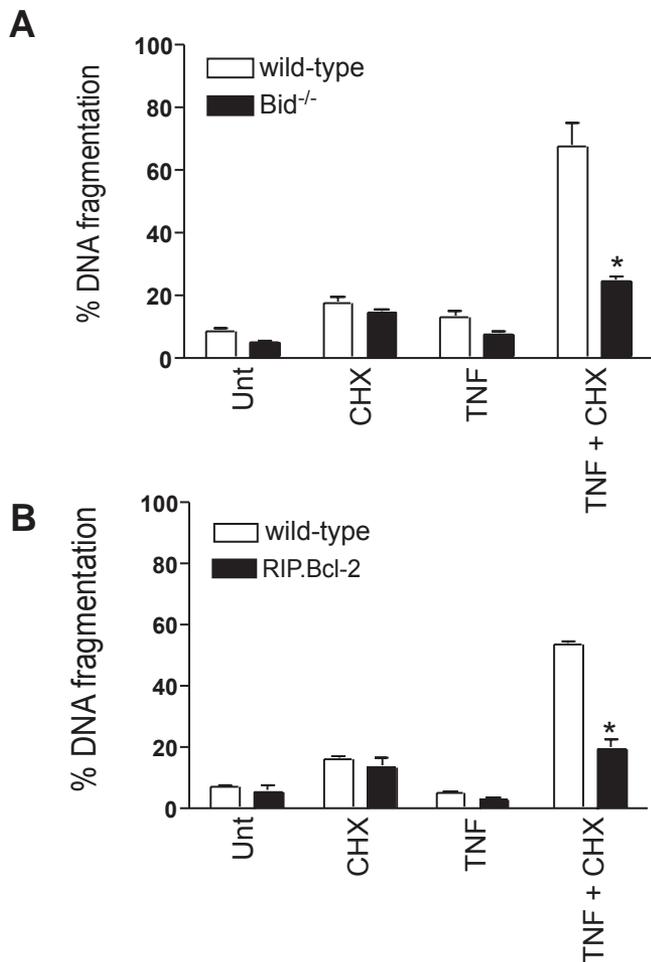


FIG. 6. Bid-deficient and Bcl-2-overexpressing islets are protected from TNF α -induced apoptosis when protein synthesis is inhibited. Islets from wild-type (A), bid^{-/-}, or RIP.Bcl-2 (B) transgenic mice were treated with TNF- α plus CHX and after 48 h. Percentages of dead cells were determined as described for Fig. 2. Data represent means \pm SE from analysis of 3–9 mice of each genotype. Statistical significance: * $P < 0.001$ compared with wild-type islets treated with TNF plus CHX (one-way ANOVA).

NF κ B pathway is incapacitated (e.g., by deficiency of a critical component such as RelA), TNF- α elicits cell death by recruitment of TRADD (TNF receptor-associated DD protein) and FADD to TNF-R1, resulting in the activation of caspase-8 (36). Figure 6A and B shows that, in comparison to wild-type islets, those lacking Bid or overexpressing Bcl-2 were resistant to treatment with TNF- α plus CHX ($24.4 \pm 2.0\%$ bid^{-/-}, $67.4 \pm 7.5\%$ wild type [Fig. 6A]; $19.4 \pm 3.1\%$ RIP.Bcl-2, $53.6 \pm 1.1\%$ wild type [Fig. 6B]). These results demonstrate that Bid is essential not only for FasL but also for TNF- α -induced apoptosis of islet cells.

Loss of Bid partially protects islets from apoptosis induced by inflammatory cytokines. Islets treated with the combination of IFN- γ , IL-1 α/β , and TNF- α (in the absence of CHX) undergo cell death in culture. The main mechanism for this cell death is believed to be dependent on generation of nitric oxide (NO) (2). We treated islets from wild-type and Bid-deficient mice for 4 days with IL-1 β , IFN- γ , and TNF- α and measured cell death by flow cytometry. Combined treatment with IFN- γ , IL-1 β , and TNF- α (Fig. 7A) or IFN- γ plus TNF- α (Fig. 7B) killed 59.6 ± 1.9 or $23.6 \pm 2.6\%$, respectively, of the wild-type islet cells. Bid-deficient islets showed significantly less killing: $44.8 \pm$

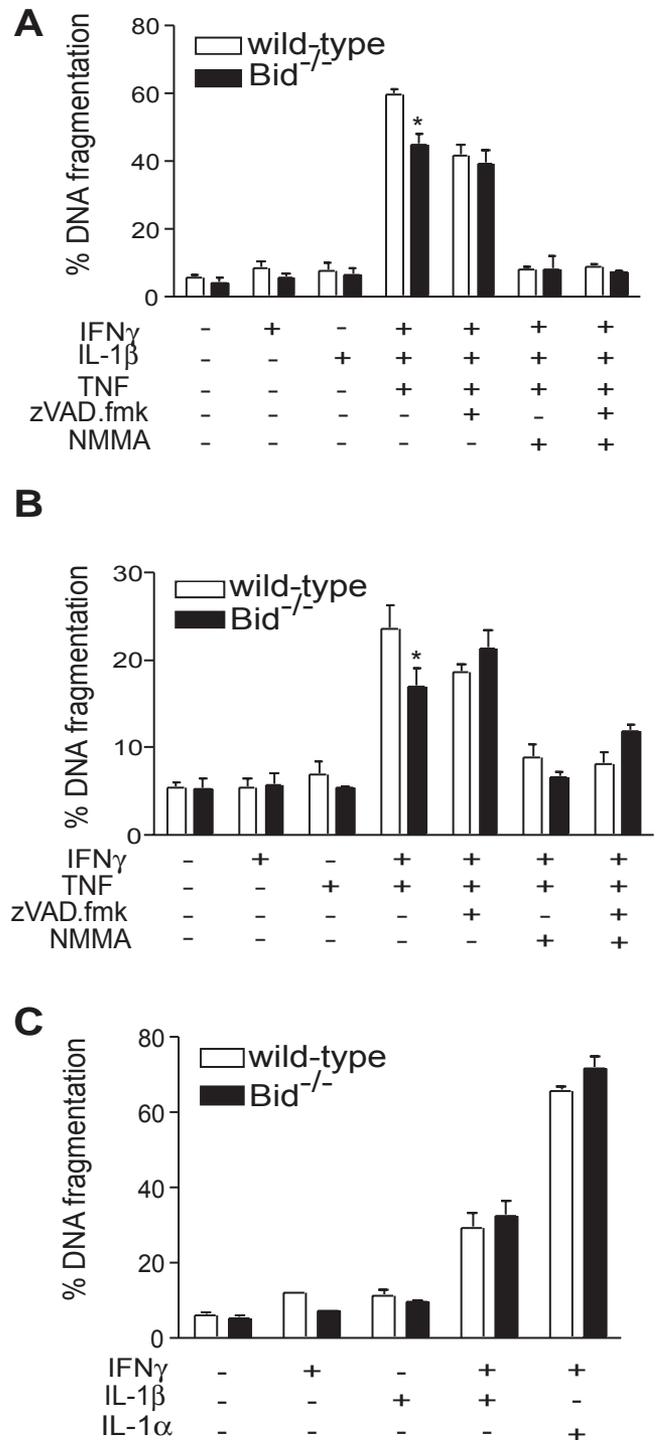


FIG. 7. Loss of Bid partially protects islets from apoptosis induced by treatment with IFN- γ , IL-1 β , and TNF- α . Islets from bid^{-/-} or wild-type mice were cultured with the following: A) IFN- γ , IL-1 β , and TNF- α for 4 days. Statistical significance: * $P < 0.001$ compared with wild-type treated with all three cytokines (one-way ANOVA). B) IFN- γ plus TNF- α for 6 days. Statistical significance: * $P < 0.05$ compared with wild-type mice treated with both cytokines (one-way ANOVA). C) IL-1 α or IL-1 β in combination with IFN- γ for 4 days. The percentage of dead cells was determined as described for Fig. 2. NMMA was used to block NO production, and zVAD.fmk was used to inhibit caspases. Results represent means \pm SE of at least three independent experiments with 3–6 mice per genotype.

3.1% for treatment with IFN- γ , IL-1 β plus TNF- α (Fig. 7A), and $17 \pm 2.2\%$ for stimulation with IFN- γ plus TNF- α (Fig. 7B). Bid-deficient islets were, however, normally sensitive

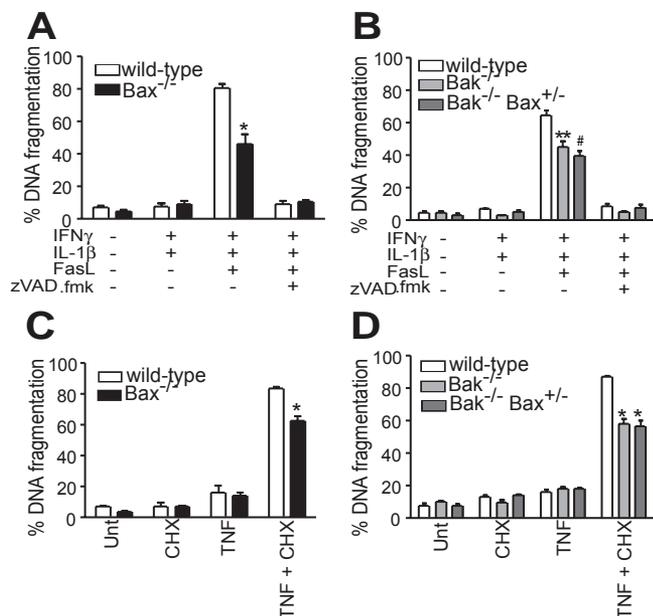


FIG. 8. Loss of Bax or Bak partially protects islets from death receptor-induced apoptosis. Islets from wild-type, bid^{-/-}, bak^{-/-}, or bak^{-/-}bax^{+/-} mice were treated with IFN- γ , IL-1 β , and FasL (A and B) or with TNF- α plus CHX (C and D). The percentages of dead cells was determined as described for Fig. 2. Results represent the means \pm SE of three mice of each genotype. Statistical significance: * P < 0.001 compared with treated wild-type islets; ** P < 0.05, IFN- γ , IL-1 β , FasL, and Bak^{-/-} compared with wild type; # P < 0.01 IFN- γ , IL-1 β , FasL, and Bak^{-/-}Bax^{+/-} compared with wild type (ANOVA).

to treatment with IFN- γ plus IL-1 α or IL-1 β (Fig. 7C). Similar to loss of Bid, addition of the caspase inhibitor zVAD.fmk afforded partial protection from apoptosis induced by treatment with IFN- γ , IL-1 β , and TNF α ($41.5 \pm 3.2\%$) (Fig. 7A) or IFN- γ plus TNF α ($18.7 \pm 0.9\%$) (Fig. 7B) but had no impact on killing by IFN- γ plus IL-1 α or IL-1 β (data not shown). Addition of the inducible NO synthase inhibitor NMMA almost completely prevented cell death induced by any of these three treatments ($7.9 \pm 1.1\%$ IFN- γ , IL-1 β , and TNF α ; $9.0 \pm 1.4\%$ IFN- γ plus TNF α). These data indicate that TNF- α in the cytokine cocktail triggers an apoptotic pathway that requires caspase activity and Bid, which can operate in parallel to the cytokine (IFN- γ plus IL-1 α or IL-1 β)-induced cell-killing pathway, which is mediated by NO. IL-1 α and IL-1 β , on the other hand, only induce NO-dependent cell death, which can be blocked by the inducible NO synthase inhibitor NMMA and appears to be independent of zVAD.fmk-sensitive caspases and Bid.

Single deficiency of Bax or Bak reduces death receptor-induced apoptosis. The multi-BH domain proapoptotic Bcl-2 family members Bax and Bak are released from antiapoptotic Bcl-2 family members when BH3-only proteins such as Bid are activated and have essential, albeit largely overlapping roles in developmentally programmed and cytotoxic stress-induced apoptosis (10,37). Islets deficient in either Bax or Bak displayed reduced apoptosis after treatment with cytokines plus FasL (Fig. 8A and B) ($45.9 \pm 6.0\%$ bax^{-/-} and $80.7 \pm 2.2\%$ wild type [Fig. 8A]; $45.3 \pm 3.5\%$ bak^{-/-} and $64.4 \pm 3.3\%$ wild type [Fig. 8B]) or TNF- α plus CHX (Fig. 8C and D) ($62.6 \pm 3.0\%$ bax^{-/-} and $83.6 \pm 1.1\%$ wild type [Fig. 8C]; $57.9 \pm 3.2\%$ bak^{-/-} and $87.1 \pm 0.4\%$ wild type [Fig. 8D]). Bak-deficient islets with only a single allele of Bax showed no additional protection over Bak deficiency alone. This indicates that at least one

allele of either Bax or Bak is required for death receptor killing in islets and that without one of these proteins apoptosis is reduced.

DISCUSSION

β -cell destruction underlies or at least contributes to development of type 1 as well as type 2 diabetes (2). Multiple cell death pathways, including perforin/granzyme, Fas/FasL, TNF- α , and other cytokines, as well as excessive ER stress (2), have been implicated in β -cell killing, but the mechanisms are still unclear. We have found that the proapoptotic BH3-only protein Bid is critical for FasL- and TNF- α -induced apoptosis of islet cells and also plays a minor role in their killing by inflammatory cytokines (IFN- γ , IL-1 β , and TNF- α). This shows that, like in hepatocytes (16,17), death receptor-induced apoptosis requires amplification of the caspase cascade through Bid-mediated activation of the mitochondrial pathway for efficient killing of β -cells. In other cell types, such as thymocytes or mature T-cells, ligation of death receptors leads to efficient cell killing solely through activation of caspase-8 and the downstream effector caspases (-3, -6, and -7) without the need for Bid- and mitochondrial pathway-mediated amplification of the caspase cascade. Therefore, islets are one of only a handful of primary cell types in which Fas-induced apoptosis is dependent on Bid (16,17,38). No pathology in the pancreas was seen when mice were injected with agonistic anti-Fas antibodies. This may be due to the fact that β -cell killing takes longer than the killing of hepatocytes, which leads to death of the animals. Alternatively, and probably more likely, β -cells are not killed because, in contrast to hepatocytes, they do not constitutively express Fas in vivo.

Like Bid deficiency, overexpression of Bcl-2 also protected islet β -cells from death receptor-induced killing, and loss of either Bak or Bax could also partially inhibit this process. This further confirms that the connection between the death receptor and the mitochondrial pathway is essential for FasL-induced apoptosis in β -cells. Curiously, although Bcl-2 overexpression can protect β -cells from FasL, it is unable to do so in hepatocytes (39,40), which also require Bid for Fas-mediated apoptosis (16,17). There are two possible explanations. Since activated Bid binds only relatively weakly to Bcl-2, the very high levels of transgene expression achieved in β -cells may be sufficient for protection, whereas the levels achieved in hepatocytes might be inadequate. Alternatively, since Bcl-2 was shown to be able to control only Bax but not Bak (23), it is possible that FasL-induced apoptosis is mostly dependent on Bak in hepatocytes, while either Bax or Bak can mediate this death in β -cells.

Loss of Bid did not protect β -cells against a range of apoptotic stimuli that can be countered by Bcl-2 overexpression (19), such as ER stress or DNA damage. It appears likely that these death stimuli activate other BH3-only proteins, such as Bim in the case of ER stress (41) or Puma in the case of DNA damage (42). Since β -cell killing induced by IFN- γ plus IL-1 β , which requires NO production, cannot be inhibited by either loss of Bid, caspase blockade, or Bcl-2 overexpression (20), it appears likely that this death occurs through a process independent of this pathway.

Apoptotic β -cell death is central to the pathogenesis of type 1 diabetes and islet graft rejection. β -cell killing in type 1 diabetes is likely to involve at least the perforin/

granzyme and death receptor pathways. The importance of the perforin pathway is probably best documented by the finding that loss of perforin greatly reduces the incidence of diabetes in NOD mice (43). Experiments with transgenic NOD mice expressing dnFADD in their β -cells indicated that death receptor signaling plays a partial role in diabetes development (20). Antigen-specific autoreactive cytotoxic T-cells kill β -cells in vitro using the Fas/FasL pathway in the absence of perforin (24,44), indicating that this mechanism may still contribute to diabetes development. Therefore, simultaneous inhibition of multiple cell killing pathways will be needed to prevent β -cell destruction and diabetes development. For this reason, genetic strategies to inhibit intracellular pathways of β -cell apoptosis have been generally less successful than hoped in blocking diabetes or allograft destruction. Inhibition of extrinsic (i.e., dnFADD, cFLIP, or A20) or intrinsic (i.e., Bcl-2 or Bcl-X_L) signals of apoptosis has only limited impact on islet cell apoptosis and diabetes development. Overexpression of XIAP, which prevents activation of effector caspases late in apoptosis, has shown promising results (45,46). However, caspase inhibition is generally not sufficient for survival following mitochondrial outer membrane permeabilization (47,48). Collectively, the observations that loss of Bid protects β -cells against FasL, TNF- α , inflammatory cytokines (albeit only partially), and (at least in vitro) perforin plus granzyme B indicate that pharmacological inhibition of Bid may be able to prevent or limit apoptosis induced by multiple pathways without interfering with normal physiological β -cell function. Inhibitors targeted at Bid have been recently described (49), making this a feasible approach for preserving β -cell survival and prevention of diabetes.

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