



## IFN- $\gamma$ /TNF- $\alpha$ Synergism as the Final Effector in Autoimmune Diabetes: A Key Role for STAT1/IFN Regulatory Factor-1 Pathway in Pancreatic $\beta$ Cell Death

This information is current as of August 4, 2022.

Kyoung-ho Suk, Sunshin Kim, Yun-Hee Kim, Kyoung-Ah Kim, Inik Chang, Hideo Yagita, Minho Shong and Myung-Shik Lee

*J Immunol* 2001; 166:4481-4489; ;  
doi: 10.4049/jimmunol.166.7.4481  
<http://www.jimmunol.org/content/166/7/4481>

**References** This article **cites 55 articles**, 32 of which you can access for free at:  
<http://www.jimmunol.org/content/166/7/4481.full#ref-list-1>

**Why *The JI*? Submit online.**

- **Rapid Reviews! 30 days\*** from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Fast Publication!** 4 weeks from acceptance to publication

*\*average*

**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>



# IFN- $\gamma$ /TNF- $\alpha$ Synergism as the Final Effector in Autoimmune Diabetes: A Key Role for STAT1/IFN Regulatory Factor-1 Pathway in Pancreatic $\beta$ Cell Death<sup>1</sup>

Kyoungso Suk,<sup>2\*¶</sup> Sunshin Kim,<sup>2\*</sup> Yun-Hee Kim,<sup>\*</sup> Kyoung-Ah Kim,<sup>†</sup> Inik Chang,<sup>\*</sup> Hideo Yagita,<sup>‡</sup> Minho Shong,<sup>§</sup> and Myung-Shik Lee<sup>3\*†</sup>

Fas ligand (FasL), perforin, TNF- $\alpha$ , IL-1, and NO have been considered as effector molecule(s) leading to  $\beta$  cell death in autoimmune diabetes. However, the real culprit(s) in  $\beta$  cell destruction have long been elusive, despite intense investigation. We and others have demonstrated that FasL is not a major effector molecule in autoimmune diabetes, and previous inability to transfer diabetes to Fas-deficient nonobese diabetic (NOD)-*lpr* mice was due to constitutive FasL expression on lymphocytes from these mice. Here, we identified IFN- $\gamma$ /TNF- $\alpha$  synergism as the final effector molecules in autoimmune diabetes of NOD mice. A combination of IFN- $\gamma$  and TNF- $\alpha$ , but neither cytokine alone, induced classical caspase-dependent apoptosis in insulinoma and pancreatic islet cells. IFN- $\gamma$  treatment conferred susceptibility to TNF- $\alpha$ -induced apoptosis on otherwise resistant insulinoma cells by STAT1 activation followed by IFN regulatory factor (IRF)-1 induction. IRF-1 played a central role in IFN- $\gamma$ /TNF- $\alpha$ -induced cytotoxicity because inhibition of IRF-1 induction by antisense oligonucleotides blocked IFN- $\gamma$ /TNF- $\alpha$ -induced cytotoxicity, and transfection of IRF-1 rendered insulinoma cells susceptible to TNF- $\alpha$ -induced cytotoxicity. STAT1 and IRF-1 were expressed in pancreatic islets of diabetic NOD mice and colocalized with apoptotic cells. Moreover, anti-TNF- $\alpha$  Ab inhibited the development of diabetes after adoptive transfer. Taken together, our results indicate that IFN- $\gamma$ /TNF- $\alpha$  synergism is responsible for autoimmune diabetes in vivo as well as  $\beta$  cell apoptosis in vitro and suggest a novel signal transduction in IFN- $\gamma$ /TNF- $\alpha$  synergism that may have relevance in other autoimmune diseases and synergistic anti-tumor effects of the two cytokines. *The Journal of Immunology*, 2001, 166: 4481–4489.

Understanding of the final effector phase of autoimmune diabetes is becoming an area of intense research, due to recent realization of the importance of apoptosis in a variety of physiological or pathological conditions. Autoreactive T lymphocytes are the most important effector cells in autoimmune diabetes (1–3), and they ultimately induce apoptosis of  $\beta$  islet cells (4, 5). Previous adoptive experiments indicated that CD4<sup>+</sup> T lymphocytes are the final effector cells in  $\beta$  cell destruction, whereas CD8<sup>+</sup> T lymphocytes play a role in the initiation period of islet destruction (2, 6). The role of macrophages as effector cells has also been suggested. Besides their role as APCs, they may also be a source of oxygen radicals or other soluble cytotoxic mediators (7).

Although effector cells and their roles are rather clearly defined, it is far from clear which molecule(s) are the real effector(s) in autoimmune diabetes. Perforin, Fas ligand (FasL),<sup>4</sup> TNF- $\alpha$ , IL-1, and NO have been implicated in  $\beta$  cell destruction. Although the role of perforin in autoimmune diabetes has been reported, perforin alone accounts for only part of  $\beta$  cell death by CD8<sup>+</sup> T lymphocytes (8). We and others recently reported that FasL is not a major effector molecule in autoimmune diabetes (5, 9, 10), and previous inability to transfer diabetes to Fas-deficient nonobese diabetic (NOD)-*lpr* mice (11) was due to constitutive FasL expression on lymphocytes from these mice (12). The role of TNF- $\alpha$  as an effector has been extremely ambiguous. Its diabetogenic role was suggested in some studies (13–18), whereas opposite effects were reported in other studies (19, 20). Neonatal islet-specific expression of TNF- $\alpha$  promoted diabetes by enhancing the presentation of islet Ags (16, 17). The period between 21 and 25 days following the initiation of TNF- $\alpha$  expression was characterized as the time point when the decision to progress from nondestructive to destructive insulinitis is made, underscoring the temporal importance of TNF- $\alpha$  expression in the development of diabetes (18). Also, treatment of newborn NOD mice with TNF- $\alpha$  led to an earlier onset of diabetes, and the administration of an anti-TNF- $\alpha$  Ab before 3 wk of age resulted in the complete prevention of diabetes (13). In contrast, other transgenic mouse studies failed to demonstrate the role of TNF- $\alpha$  as an effector molecule (21, 22). In these reports, transgenic expression of TNF- $\alpha$  in pancreatic islets led to insulinitis, but not diabetes, or even prevented diabetes (21, 22). Thus, it appears that TNF- $\alpha$  either inhibits or promotes diabetes

\*Clinical Research Center, Samsung Biomedical Research Institute, and <sup>†</sup>Department of Medicine, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul, Korea; <sup>‡</sup>Department of Immunology, Juntendo University School of Medicine, Tokyo, Japan; <sup>§</sup>Department of Medicine, Chungnam University, Taejeon, Korea; and <sup>¶</sup>Graduate School of East-West Medical Science, Kyunghee University, Seoul, Korea

Received for publication September 5, 2000. Accepted for publication January 30, 2001.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

<sup>1</sup> This work was supported by National Research Laboratory grants from the Korea Institute of Science & Technology Evaluation and Planning (2000-N-NL-01-C-232), and Science Research Center grants from the Korea Science & Engineering Foundation. M.-S.L. is an awardee of the Juvenile Diabetes Foundation International Research Grant (1-1999-760). K.S. was supported by Brain Korea 21 project, Ministry of Education, Korea.

<sup>2</sup> K.S. and S.K. contributed equally to this work.

<sup>3</sup> Address correspondence and reprint requests to Dr. Myung-Shik Lee, Department of Medicine, Samsung Medical Center, Sungkyunkwan University School of Medicine, 50 Ilwon-dong, Kangnam-ku, Seoul, 135-710, Korea. E-mail address: mslee@smc.samsung.co.kr

<sup>4</sup> Abbreviations used in this paper: FasL, Fas ligand; NOD, nonobese diabetic; IRF, IFN regulatory factor; NMMA, N-monomethyl-L-arginine; iNOS, inducible NO synthase.

depending on the condition under which it acts. The effect of IL-1 or NO on islet cell death was studied mostly *in vitro* (23, 24), and their *in vivo* effects were not demonstrated (14, 19). IFN- $\gamma$  has been regarded as a sensitizing or immunomodulatory cytokine rather than an effector molecule (25).

In our effort to identify the final effector molecule(s) in autoimmune diabetes, we have found that IFN- $\gamma$  and TNF- $\alpha$  synergistically induce  $\beta$  cell apoptosis, and we demonstrated a critical role for STAT1/IFN regulatory factor (IRF)-1 in the signal transduction of the cytokine synergism, which was also found to be pertinent to the *in vivo*  $\beta$  cell death and diabetes in NOD mice.

## Materials and Methods

### Cell line and reagents

MIN6N8 cells, SV40 T-transformed insulinoma cells derived from NOD mice (kindly provided by J.-i. Miyagaki, Osaka University, Osaka, Japan) (26), were grown in DMEM containing 15% FBS, 2 mM glutamine, and penicillin-streptomycin (Life Technologies, Gaithersburg, MD). Recombinant rat IFN- $\gamma$  was generously provided by P. H. van der Meide (Utrecht University, Utrecht, The Netherlands). Recombinant mouse TNF- $\alpha$  and recombinant human IL-1 $\beta$  were purchased from R&D Systems (Minneapolis, MN). Recombinant human IL-1 $\beta$  has been shown to be active in rodent systems (27). Agonistic anti-TNFR1 Ab and antagonistic anti-TNFR2 Ab were obtained from R&D Systems. Caspase inhibitors (z-VAD-fmk, Ac-YVAD-cmk, Ac-YVAD-fmk, Ac-YVAD-CHO, Ac-DEVD-fmk, and Ac-IETD-fmk) were purchased from Enzyme Systems Products (Livermore, CA). *N*-monomethyl-L-arginine (NMMA) was obtained from Calbiochem (San Diego, CA). All other chemicals were obtained from Sigma (St. Louis, MO) unless stated otherwise.

### Isolation of mouse pancreatic islets

Islets were isolated from overnight-fasted ICR or NOD mice using the collagenase digestion technique. In brief, after 2.5 ml of collagenase P (0.8 mg/ml) was injected into the bile duct of an anesthetized mouse, the swollen pancreas was gently pulled out and other attached tissues were removed. The pancreas was then incubated with collagenase at 37°C for 15 min with gentle shaking. After incubation, cold HBSS was added to stop the digestion. The tissue was then passed through a 400- $\mu$ m screen and centrifuged on 25, 23, 21.5, and 11.5% Ficoll gradients. Islets were collected from the interface. Collected islets were washed with M199 medium, and individual islets were hand-picked using micropipettes. They were treated with trypsin-EDTA for 5 min to yield single-islet cells.

### Assessment of cytotoxicity by MTT assays

Cells ( $3 \times 10^4$ /well for MIN6N8 cells and  $2 \times 10^4$ /well for mouse single-islet cells) were seeded in 96-well microtiter plates and treated with various combinations of cytokines for the indicated time periods. The optimal concentrations of cytokines for the cytotoxic action were 1000 U/ml for IFN- $\gamma$ , 10 ng/ml for TNF- $\alpha$ , and 17.5 ng/ml for IL-1 $\beta$ . In some experiments, cells were pretreated with caspase inhibitors or NMMA for 1 h before cytokine treatment. After cytokine treatment, the medium was removed and MTT (0.5 mg/ml) was added and followed by incubation at 37°C for 2 h in a CO<sub>2</sub> incubator. After a brief centrifugation, supernatants were carefully removed and DMSO was added. After insoluble crystals were completely dissolved, absorbance at 540 nm was measured using a Thermomax microplate reader (Molecular Devices, Menlo Park, CA).

### Morphological analysis of apoptotic cells

Morphological changes in the nuclear chromatin of cells undergoing apoptosis were detected by staining with 2.5  $\mu$ g/ml DNA-binding bis-benzimide Hoechst 33258 fluorochrome (Calbiochem), followed by an examination on a fluorescence microscope. In some experiments, islet cells were stained with anti-insulin Ab (Dako, Carpinteria, CA) and then with FITC-conjugated anti-guinea pig IgG Ab (Zymed, San Francisco, CA), followed by Hoechst 33258 staining. For transmission electron microscopy, cells were fixed in 4% glutaraldehyde, 1% paraformaldehyde, and 0.2 M phosphate (pH 7.2) at 4°C for 2 h. After two washes in 0.2 M phosphate, the cell pellets were postfixated with 2% OsO<sub>4</sub> in the same buffer for 30 min. The pellets were dehydrated in ethanol and then in 100% propylene oxide, followed by embedding at 37°C overnight and 60°C for another 3 days. Ultrafine sections were cut and examined on an electron microscope (Hitachi H7100, 75 kV; Hitachi, Tokyo, Japan).

### Agarose gel electrophoresis of DNA

For the isolation of genomic DNA, MIN6N8 cells were lysed by incubation in the extraction buffer overnight at 55°C (10 mM Tris-HCl (pH 8.0), 0.1 M EDTA, 0.5% SDS, and 100 mM NaCl), followed by phenol/chloroform extraction and ethanol precipitation. The final pellet was dissolved in distilled water containing 0.1 mg/ml RNase A. Isolated genomic DNA was electrophoresed on 1.5% agarose gel and stained with ethidium bromide to detect internucleosomal cleavage.

### DNA ploidy analysis

Cells were suspended in PBS-5 mM EDTA and fixed by adding 100% ethanol dropwise. RNase A (40  $\mu$ g/ml) was added to the resuspended cells, and incubation was conducted at room temperature for 30 min. Propidium iodide (50  $\mu$ g/ml) was then added for flow cytometric analyses (FACS-Vantage; Becton Dickinson, Mountain View, CA).

### RT-PCR and RNase protection assays

Total RNA was extracted from MIN6N8 cells by a sequential addition of 4 M guanidinium thiocyanate, 2 M sodium acetate, and acid phenol/chloroform. Reverse transcription was conducted using Superscript (Life Technologies) and oligo(dT) primer. PCR amplification using primer sets specific for each caspase was conducted at 60°C annealing temperature for 30 cycles. Nucleotide sequences of the primers were based on published cDNA sequences (caspase-1 forward, AAG ATG GCA CAT TTC CAG GAC; caspase-1 reverse, GGG CAC TTC AAA GTG TTC ATC; caspase-2 forward, ATG CTA ACT GTC CAA GTC TA; caspase-2 reverse, GTC TCA TCT TCA TCA ACT CC; caspase-3 forward, CTA AGC CAT GGT GAT GAA GGG; caspase-3 reverse, CTG CAA AGG GAC TGG ATG AAC; caspase-7 forward, GAA GTA ACC GTC CAC AAT GAC; caspase-7 reverse, TGC CAT GCT CAT TCA GGA TGG; caspase-8 forward, GCT CTT CTA CCT CTT GAT AAG; caspase-8 reverse, GAC CCT GTA GGG AGA AAT CTG; caspase-9 forward, AGC TCT TCT TCA TCC AGG; caspase-9 reverse, CCC CCA GCC TCA TGA AGT T; caspase-11 forward, CTT CAC AGT GCG AAA GAA CT; caspase-11 reverse, GGT CCA CAC TGA AGA ATG TCT GGA GAA GCA TTT CA). Nucleotide sequences of forward and reverse primers for mouse  $\beta$ -actin were ATC CTG AAA GAC CTC TAT GC and AAC GCA GCT CAG TAA CAG TC, respectively. Isolated total RNA was also subjected to RNase protection assay, which was conducted using the HybSpeed RPA kit (Ambion, Austin, TX) and RiboQuant Multi-Probe Template set (BD Pharmingen, San Diego, CA) according to the manufacturer's protocols, except that hybridization of the probe with sample RNA was done for 2 h.

### Western blot analyses

Cells were lysed in triple-detergent lysis buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.02% sodium azide, 0.1% SDS, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 1 mM PMSF). Protein concentration in cell lysates was determined using the Bio-Rad protein assay kit (Bio-Rad, Richmond, CA). An equal amount of protein for each sample was separated by 10 or 12% SDS-PAGE and transferred to Hybond ECL nitrocellulose membrane (Amersham Pharmacia Biotech, Piscataway, NJ). After blocking with 5% skim milk, the membranes were sequentially incubated with one of the primary Abs (rabbit anti-mouse IRF-1, obtained from Santa Cruz Biotechnology (Santa Cruz, CA); rabbit anti-STAT1 and anti-phospho-STAT1, obtained from New England Biolabs (Beverly, MA); and rabbit anti-mouse caspase-1, which was generously provided by P. Vandennebe, (University of Ghent, Ghent, Belgium) and then HRP-conjugated secondary Abs (anti-rabbit IgG; Amersham Pharmacia Biotech), followed by ECL detection (Amersham Pharmacia Biotech).

### Assessment of caspase activity

Caspase-3 or -8-like activity was measured using a commercial caspase assay kit (BD Pharmingen) according to the supplier's instruction. In brief, caspase-3 or -8 fluorogenic substrates (Ac-DEVD-AMC or Ac-IETD-AMC) were incubated with cytokine-treated cell lysate for 1 h at 37°C. AMC liberated from Ac-DEVD-AMC or Ac-IETD-AMC was measured using a fluorometric plate reader with an excitation wavelength of 380 nm and an emission wavelength of 420–460 nm.

### Transient transfection of MIN6N8 cells

MIN6N8 cells in 6-well plates were cotransfected with 1  $\mu$ g human STAT1 cDNA, dominant negative STAT1 cDNA (provided by T. Hirano, Osaka University) or mouse *IRF-1* cDNA (provided by T. Taniguchi, University of Tokyo, Tokyo, Japan) together with 0.2  $\mu$ g *lacZ* (pCHI10; Amersham Pharmacia Biotech) using lipofectamine reagent (Life Technologies), according to the supplier's instruction. At 48 h after transfection,

cells were treated with cytokines. After another 72 h, cells were washed with PBS and fixed with 0.5% glutaraldehyde for 10 min at room temperature and stained with 5-bromo-4-chloro-3-indolyl  $\beta$ -D-galactoside (1 mg/ml) in 4 mM potassium ferricyanide, 4 mM potassium ferrocyanide, and 2 mM magnesium chloride at 37°C for blue cell counting. Viable blue cells in 10 random high power ( $\times 200$ ) fields were counted for each sample, excluding dark blue apoptotic cells. For the transfection of IRF-1 antisense oligonucleotides, antisense and sense oligonucleotides with phosphothioated-terminal 3 bases were designed. A pair of sense and antisense oligonucleotides (S and AS1) were based on the previously published work (S, GCA TCT CGG GCA TCT TTC; AS1, GAA AGA TGC CCG AGA TGC) (28). An additional antisense oligonucleotide (AS2) was designed to encompass the translational start codon of IRF-1 (AS2, AGT GAT TGG CAT GGT GGC; bold-typed sequences indicate antisense start codon). The cells were similarly transfected with either sense or antisense oligonucleotides in the presence of lipofectAMINE reagent. At 24 h after the transfection, the cells were exposed to the cytokines for 48 h, then viability was assessed by MTT assays. For IRF-1 immunoblotting, transfected cells were treated with IFN- $\gamma$  for 2 h before the cells were lysed for SDS-PAGE.

#### Immunohistochemistry and TUNEL staining

Formalin-fixed sections of the mouse islet tissues were deparaffinized and briefly microwaved in 0.01 M sodium citrate buffer (pH 6.0). They were then incubated with the appropriate dilution of anti-STAT1 (New England Biolabs), anti-phospho-STAT1 (New England Biolabs), or anti-IRF-1 Ab (Santa Cruz Biotechnology) after goat serum blocking. Incubation with biotinylated anti-rabbit IgG Ab and then with avidin-biotin-peroxidase complex (Vector Laboratories, Burlingame, CA) followed. Diaminobenzidine was used as a color substrate. In some experiments, islet sections were double-stained for IRF-1 expression and apoptosis. For the double staining, tissue sections were first stained using TUNEL reagents (Roche Biomedical Laboratories, Burlington, NC) and diaminobenzidine. Immunostaining using primary Ab, biotinylated anti-rabbit IgG Ab, streptavidin-alkaline phosphatase, and then chromogenic substrate solutions containing Naphtol AS-MX salt (Sigma), Fast Blue (Sigma), and levamisole in TBS buffer followed.

#### Adoptive transfer of diabetes

The spleens from diabetic NOD mice were harvested, and  $2 \times 10^7$  splenocytes were infused into the tail vein of each 9-wk-old NOD mouse. Recipient mice were irradiated (775 rad) 6 h before the transfer of splenocytes. The incidence of diabetes was  $>80\%$  at 4 wk after the adoptive transfer in our previous experiments (5). From 2 wk after adoptive transfer, the mice were bled retro-orbitally every week, and nonfasting blood glucose levels were determined by the glucose oxidase method. Mice were considered diabetic if their blood glucose level was  $>300$  mg/dl. To determine the severity of insulinitis,  $>30$  pancreatic islets from more than three parallel sections of different cut levels were analyzed per mouse unless the mice were devoid of pancreatic islets due to diabetes. The degree of insulinitis was classified into four categories: 0, no insulinitis; 1, peri-insulinitis with minimal lymphocytic infiltration in islets; 2, invasive insulinitis with islet destruction  $<50\%$ ; and 3, invasive insulinitis with islet destruction  $>50\%$ . Animal care was in accordance with institutional guidelines.

#### Anti-TNF- $\alpha$ Ab treatment

A hybridoma producing anti-TNF- $\alpha$  Ab, MP6-XT22.11, was injected i.p. in nude mice pretreated with pristane. Ab was purified using a protein G-agarose (Amersham Pharmacia Biotech) column. Bound IgG was eluted with 50 mM glycine-HCl (pH 2.5). Collected fractions were dialyzed against PBS and then sterilized by filtration. Ab (0.5 mg) was injected i.p. three times a week into NOD mice before and after adoptive transfer. Control NOD mice were treated with PBS or control IgG in the same manner.

#### Statistical analysis

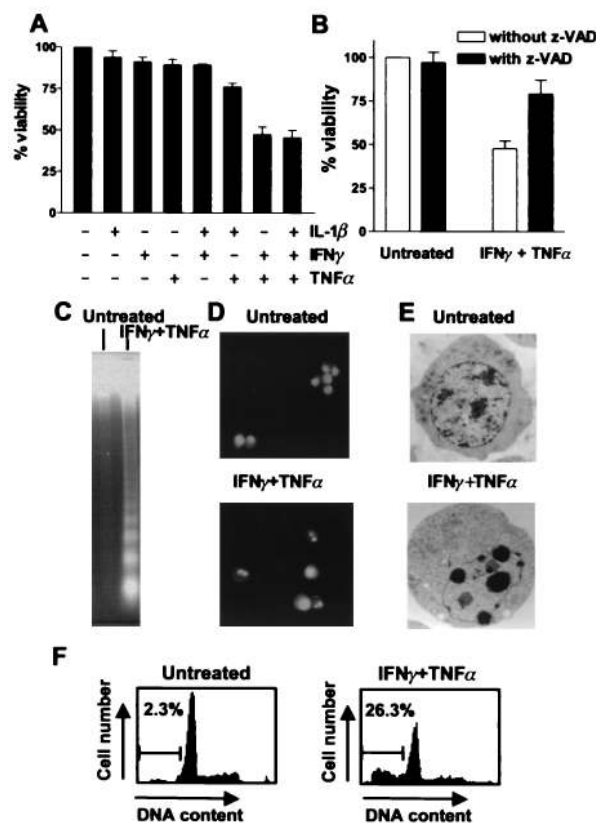
The incidence of diabetes with or without anti-TNF- $\alpha$  Ab treatment was plotted according to the Kaplan-Meier method. The incidences between the two groups were compared using the logrank test. Other statistical analyses were performed using the Student's *t* test.

## Results

#### Synergistic induction of $\beta$ cell death by IFN- $\gamma$ and TNF- $\alpha$

TNF- $\alpha$ , IFN- $\gamma$ , and IL-1 $\beta$  are the major proinflammatory cytokines that have been implicated in the islet cell destruction in vitro. To determine how these proinflammatory cytokines participate in

the  $\beta$  cell destruction in vitro, we first tested the cytotoxic effects of various combinations of these cytokines using MIN6N8 insulinoma cells. The combination of IFN- $\gamma$  and TNF- $\alpha$  significantly reduced the viability of MIN6N8 cells as judged by the morphological changes on light microscopy (data not shown) and MTT assays (Fig. 1A). The addition of IL-1 $\beta$  to IFN- $\gamma$ /TNF- $\alpha$  combination or IL-1 $\beta$  alone had only a marginal effect. The effect of IFN- $\gamma$  and TNF- $\alpha$  was synergistic, because neither of the single cytokines alone had a significant cytotoxic effect on insulinoma cells. NO production measured by Griess reaction was negligible from the insulinoma cells treated with an IFN- $\gamma$ /TNF- $\alpha$  combination (data not shown). NMMA, an inhibitor of inducible NO synthase (iNOS), did not affect IFN- $\gamma$ /TNF- $\alpha$ -induced insulinoma cell death, indicating that NO had no significant effects on the insulinoma/islet cell viability in this condition (data not shown). TNF- $\alpha$  signaling in IFN- $\gamma$ /TNF- $\alpha$  synergism was mediated through p55 TNFR (TNFR1) because agonistic Ab against TNFR1 together with IFN- $\gamma$  induced apoptosis of  $\beta$  cells, and antagonistic Ab against p75 TNFR (TNFR2) did not interfere with  $\beta$  cell apoptosis by IFN- $\gamma$ /TNF- $\alpha$  synergism (data not shown).



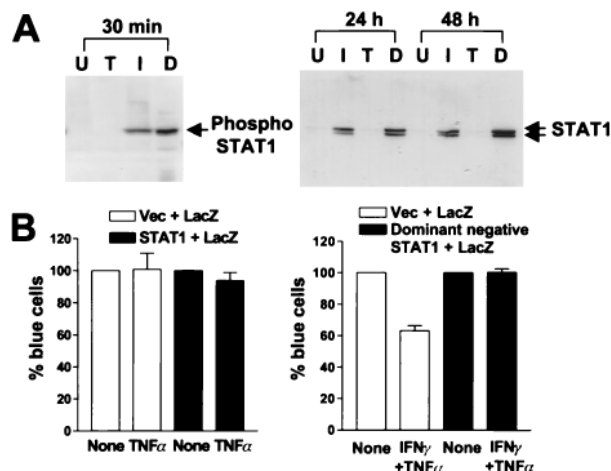
**FIGURE 1.** Caspase-dependent apoptosis of insulinoma cells by IFN- $\gamma$ /TNF- $\alpha$  synergism. **A**, MIN6N8 cells were treated for 2 days with recombinant cytokines alone or in combinations as indicated. A combination of IFN- $\gamma$  (1000 U/ml) and TNF- $\alpha$  (10 ng/ml), but neither cytokine alone, induced MIN6N8 cell death as measured by MTT assays. IL-1 $\beta$  (17.5 ng/ml) had negligible effects. The OD value at 450 nm of the untreated cells was set to 100%. Results are means  $\pm$  SE from three independent experiments. **B**, z-VAD-fmk (50  $\mu$ M), a broad-spectrum caspase inhibitor, inhibited insulinoma cell death by IFN- $\gamma$ /TNF- $\alpha$  synergism. DNA fragmentation (**C**) and nuclear condensation demonstrated by Hoechst staining (**D**) or electron microscopy (**E**) revealed that insulinoma cell death by IFN- $\gamma$ /TNF- $\alpha$  was a classical apoptosis. **F**, SubG1 peak was detected by DNA ploidy assays after treatment with IFN- $\gamma$ /TNF- $\alpha$ .

### IFN- $\gamma$ /TNF- $\alpha$ -induced $\beta$ cell death is a typical apoptosis

Next, we studied whether cell death by IFN- $\gamma$ /TNF- $\alpha$  synergism was the classical apoptosis. The cytotoxic effects of the cytokines on MIN6N8 cells were effectively blocked by z-VAD-fmk, indicating that the cytokine combination induced cytotoxicity in a caspase-dependent manner (Fig. 1B). The electrophoresis of genomic DNA, nuclear staining with Hoechst 33258, electron microscopy, and DNA ploidy analysis all indicated that the two cytokines synergistically induced apoptosis of MIN6N8 cells (Fig. 1, C–F). DNA ploidy assays indicated that the effect of the IFN- $\gamma$ /TNF- $\alpha$  combination was not due to the growth arrest, as was shown by the absence of a decrease in the S phase cell percentage (Fig. 1F).

### IFN- $\gamma$ induces phosphorylation of STAT1 and the expression of IRF-1: a key role for STAT1/IRF-1 in IFN- $\gamma$ /TNF- $\alpha$ synergism

To study the mechanism of IFN- $\gamma$ /TNF- $\alpha$  synergism on  $\beta$  cell apoptosis, MIN6N8 cells were sequentially treated with the two cytokines. Upon pretreatment with IFN- $\gamma$ , TNF- $\alpha$  alone was sufficient to induce cytotoxic effects comparable to those by the combination of cytokines (Table I). However, pretreatment with TNF- $\alpha$  followed by IFN- $\gamma$  treatment did not induce significant insulinoma cell death, suggesting that IFN- $\gamma$  pretreatment sensitizes MIN6N8 cells to TNF- $\alpha$ -mediated cytotoxicity by inducing IFN- $\gamma$ -responsive genes (Table I). We studied the possible involvement of STAT protein, an important element of IFN- $\gamma$  signal transduction, in the induction of susceptibility to TNF- $\alpha$ . Phosphorylated STAT1 was detected as early as 30 min after IFN- $\gamma$  treatment (STAT1 activation) and rapidly disappeared (Fig. 2A, left). Interestingly, the level of unphosphorylated form of STAT1 was also increased 24–48 h after IFN- $\gamma$  treatment (STAT1 induction; Fig. 2A, right). TNF- $\alpha$  treatment alone did not activate or induce STAT1 protein. We first thought STAT1 induction might be related to the sensitization of islet cells to TNF- $\alpha$ -mediated apoptosis because it has been reported that transfection of wild-type or phosphorylation-defective STAT1 rendered STAT1-deficient U3A cells susceptible to TNF- $\alpha$ -mediated apoptosis (29). However, transfection of *STAT1* failed to induce susceptibility to TNF- $\alpha$ -mediated apoptosis in insulinoma cells (Fig. 2B, left), reflecting the differences in signal transduction between the two cell types. In contrast, IFN- $\gamma$ /TNF- $\alpha$ -mediated insulinoma cell death was inhibited by transfection with dominant-negative phosphorylation-defective *STAT1* (Fig. 2B, right), suggesting that STAT1 activation, rather than STAT1 induction, plays a critical role in inducing susceptibility to TNF- $\alpha$ -mediated apoptosis. We further traced the signal mediators downstream of STAT1 activation. Among the genes acting downstream of STAT1 activation, we



**FIGURE 2.** Induction of STAT1 by IFN- $\gamma$  and a role for phosphorylated STAT1 in IFN- $\gamma$ /TNF- $\alpha$  synergism. **A**, After MIN6N8 cells were left untreated (U) or treated with TNF- $\alpha$  (T; 10 ng/ml), IFN- $\gamma$  (I; 1000 U/ml), or IFN- $\gamma$  plus TNF- $\alpha$  (D) for the indicated time, cell lysates were prepared for the detection of phosphorylated STAT1 and unphosphorylated STAT1 by Western blot analysis. Two isoforms of unphosphorylated STAT1 (STAT1 $\alpha$  and STAT1 $\beta$ ) were detected ( $\leftarrow$ ). Phosphorylated STAT1 and unphosphorylated STAT1 were detected 30 min and 24–48 h, respectively, after treatment with IFN- $\gamma$  or IFN- $\gamma$ /TNF- $\alpha$ , but not with TNF- $\alpha$  alone. **B**, To evaluate the role for STAT1 in the cytokine synergism, MIN6N8 cells were transiently cotransfected with *STAT1* plus *LacZ* gene (left) or dominant negative *STAT1* plus *LacZ* (right). At 48 h after transfection, the cells were treated for 72 h with either TNF- $\alpha$  (10 ng/ml) alone or IFN- $\gamma$  (1000 U/ml) plus TNF- $\alpha$  (10 ng/ml). Transfection with *STAT1* did not induce susceptibility to TNF- $\alpha$  in insulinoma cells (left), whereas transfection with dominant-negative *STAT1* abolished sensitivity to IFN- $\gamma$ /TNF- $\alpha$  combination (right). “Vec” indicates an empty vector control. The number of blue cells in untreated wells was set to 100%.

found that IRF-1 was induced at 24–48 h after IFN- $\gamma$  treatment but not by TNF- $\alpha$  (Fig. 3A). The inhibition of IFN- $\gamma$ -mediated IRF-1 induction by antisense oligonucleotides abolished the cytotoxic effects of IFN- $\gamma$ /TNF- $\alpha$ , strongly suggesting that IRF-1 is the key player in IFN- $\gamma$ /TNF- $\alpha$  synergism (Fig. 3B). Decreased expression of IRF-1 protein in antisense oligonucleotide-transfected cells was confirmed by Western blot analyses (Fig. 3C). Furthermore, *IRF-1* transfection alone rendered insulinoma cells susceptible to TNF- $\alpha$  (Fig. 3D), suggesting that IRF-1 was both necessary and sufficient for the induction of TNF- $\alpha$  sensitivity in insulinoma cells.

### Induction of caspase-1 and -11 by IFN- $\gamma$

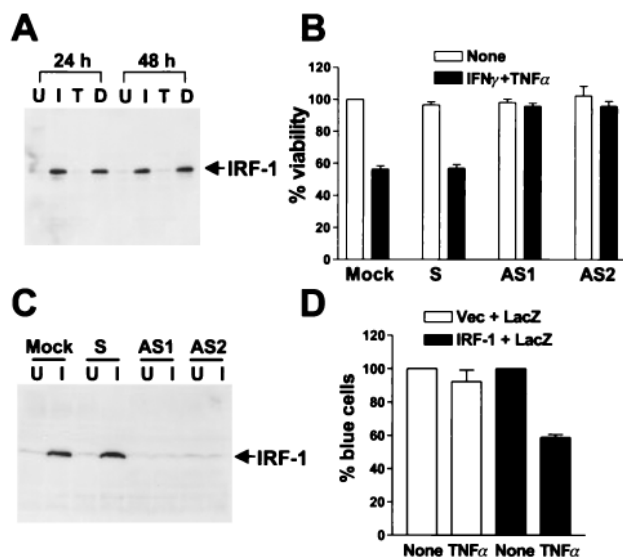
Because those papers linking IRF-1 to apoptosis demonstrated the induction of caspases by IRF-1 (30, 31), we next studied the possibility of caspase induction as a downstream event following IRF-1 induction. When the expression of various caspases was assessed by RT-PCR, the expression of caspase-1 and -11 was induced by IFN- $\gamma$ , whereas constitutive expression of other caspases (-2, -3, -7, -8, and -9) was not affected by the cytokine treatment (Fig. 4A). TNF- $\alpha$  alone appeared to have induced the expression of caspase-11, but not caspase-1. Western blot analysis also showed that caspase-1 was induced at the protein level by IFN- $\gamma$  but not by TNF- $\alpha$  (Fig. 4C). Although caspase-1 has been regarded as a proinflammatory caspase, its involvement in apoptosis has also been described in a number of instances, particularly in relation to IRF-1 (30, 32). The involvement of caspase-1 in TNF- $\alpha$ -induced apoptosis also has been reported (33). Caspase-11 is essential for the activation of caspase-1 by physically interacting

**Table I.** Cytotoxic effects of sequential treatment of cytokines

Treatment <sup>a</sup> (48 h)	% Viability <sup>b</sup>
None	100
IFN- $\gamma$ + TNF- $\alpha$	48.2 $\pm$ 3.4
IFN- $\gamma$	96.2 $\pm$ 2.1
IFN- $\gamma$ and then TNF- $\alpha$	65.8 $\pm$ 3.9
TNF- $\alpha$	94.5 $\pm$ 2.7
TNF- $\alpha$ and then IFN- $\gamma$	90.3 $\pm$ 3.8

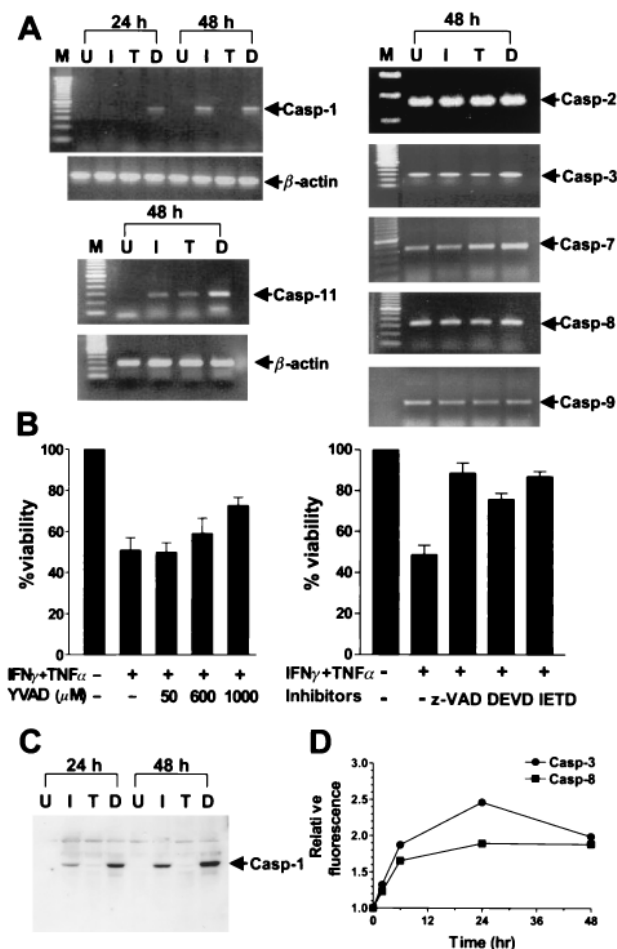
<sup>a</sup> MIN6N8 cells were treated with cytokines as indicated, either simultaneously or sequentially. Treatment with IFN- $\gamma$  (1000 U/ml) for 48 h followed by TNF- $\alpha$  (10 ng/ml) treatment for 48 h induced a significant cytotoxicity. However, the sequential treatment with the two cytokines in a reverse order did not significantly affect the MIN6N8 cell viability, indicating the priming role of IFN- $\gamma$  in TNF- $\alpha$ -induced MIN6N8 cell death.

<sup>b</sup> Cell viability was assessed by MTT assays. The viability of untreated cells was set to 100%. Results are means  $\pm$  SE ( $n = 3$ ).



**FIGURE 3.** Induction of IRF-1 expression by IFN- $\gamma$  and a critical role for IRF-1 in IFN- $\gamma$ /TNF- $\alpha$  synergism. *A*, IRF-1 was detected by Western blot analysis at 24–48 h after treatment of MIN6N8 cells with IFN- $\gamma$  or IFN- $\gamma$ /TNF- $\alpha$ , but not with TNF- $\alpha$  alone (U, untreated; I, 1000 U/ml of IFN- $\gamma$ ; T, 10 ng/ml of TNF- $\alpha$ ; D, 1000 U/ml of IFN- $\gamma$  plus 10 ng/ml of TNF- $\alpha$ ). IRF-1 was also detected as early as 2 h after IFN- $\gamma$  treatment (data not shown). *B*, Transfection of IRF-1 antisense oligonucleotides inhibited IFN- $\gamma$ /TNF- $\alpha$ -induced cytotoxicity as determined by MTT assays. Two antisense oligonucleotides (AS1 and AS2) corresponding to different regions of *IRF-1* mRNA completely inhibited cytotoxic synergism between IFN- $\gamma$  and TNF- $\alpha$  in MIN6N8 cells, whereas a sense oligonucleotide (S) or mock transfection (Mock) was without effect. The OD value at 450 nm of the untreated cells was set to 100%. *C*, IRF-1 was not induced by IFN- $\gamma$  in antisense oligonucleotide-transfected cells as determined by Western blot analyses (U, uninduced; I, induced by IFN- $\gamma$ , 1000 U/ml). *D*, Transfection with *IRF-1* induced susceptibility to TNF- $\alpha$  alone, suggesting the key role of IRF-1 in the IFN- $\gamma$ /TNF- $\alpha$  synergism. Transfection and blue cell counting were done in the same manner as in Fig. 2*B*. The results are means  $\pm$  SE from three independent experiments.

with procaspase-1 (34). Induction of caspase-11 by IFN- $\gamma$  alone or IFN- $\gamma$ /TNF- $\alpha$ , as observed in this study, could be essential for apoptosis mediated by caspase-1, which itself was induced by IFN- $\gamma$ . Thus, IFN- $\gamma$  may induce susceptibility to TNF- $\alpha$  by inducing both caspase-1 and caspase-11 that form a complex. To confirm caspase-1 involvement in the cytokine synergism, we studied the effect of Ac-YVAD-cmk, an inhibitor of caspase-1. Only high concentrations of Ac-YVAD-cmk (600–1000  $\mu$ M) were able to block the cytokine-induced cytotoxicity in MIN6N8 cells (Fig. 4*B*, left). Similar results were obtained using another caspase-1 inhibitor, Ac-YVAD-CHO (data not shown). Because Ac-YVAD of this high concentration may nonspecifically inhibit other caspases, we could not at present definitively determine whether caspase-1 is involved in  $\beta$  cell apoptosis by IFN- $\gamma$ /TNF- $\alpha$  synergism. In contrast to Ac-YVAD, Ac-IETD-fmk and Ac-DEVD-fmk at 50  $\mu$ M significantly inhibited cytotoxic activity of IFN- $\gamma$ /TNF- $\alpha$  (Fig. 4*B*, right), suggesting the involvement of other caspases such as caspase-8 and -3 in the cytokine-induced apoptosis. Caspase assays using fluorogenic substrates also indicated that caspase-3 and caspase-8 were activated after IFN- $\gamma$ /TNF- $\alpha$  treatment of MIN6N8 cells (Fig. 4*D*), further supporting the involvement of these caspases in the apoptosis of insulinoma cells. Besides caspases, IFN- $\gamma$  is known to modulate the expression of other apoptosis-related genes including TNFRI (35). However, the expression of TNFRI, which mediated apoptotic signaling of TNF- $\alpha$  in



**FIGURE 4.** Involvement of caspases in IFN- $\gamma$ /TNF- $\alpha$ -induced cytotoxicity. *A*, After cytokine treatment of MIN6N8 cells (U, untreated; I, 1000 U/ml of IFN- $\gamma$ ; T, 10 ng/ml of TNF- $\alpha$ ; D, 1000 U/ml of IFN- $\gamma$  plus 10 ng/ml of TNF- $\alpha$ ), total RNA was isolated and subjected to RT-PCR with the primers specific for caspase-1, -2, -3, -7, -8, -9, or -11. The PCR products were separated on a 1.5% agarose gel and visualized by ethidium bromide staining. A DNA size marker (M) is shown on the left. IFN- $\gamma$  induced caspase-1 and -11 but did not significantly affect the constitutive expression of caspase-2, -3, -7, -8, or -9. TNF- $\alpha$  induced caspase-11 but not caspase-1 expression. *B*, MIN6N8 cells were pretreated with the indicated concentrations of Ac-YVAD-cmk (left) or 50  $\mu$ M of z-VAD-fmk, Ac-DEVD-fmk, or Ac-IETD-fmk (right) for 1 h before treatment with IFN- $\gamma$  (1000 U/ml) plus TNF- $\alpha$  (10 ng/ml). After 48 h, cytotoxicity was evaluated by MTT assays. z-VAD-fmk (as shown in Fig. 1*B*), Ac-DEVD-fmk, and Ac-IETD-fmk at 50  $\mu$ M significantly inhibited the cytokine-induced cytotoxicity, whereas Ac-YVAD-cmk at the same concentration did not. Much higher concentrations of Ac-YVAD were required for inhibitory effects. The OD value of the untreated cells at 450 nm was set to 100%. The results are means  $\pm$  SE from four independent experiments. *C*, The induction of caspase-1 by IFN- $\gamma$  was confirmed by Western blot analysis. IFN- $\gamma$ , but not TNF- $\alpha$ , induced the caspase-1 expression after 24–48 h treatment. *D*, IFN- $\gamma$ /TNF- $\alpha$  treatment induced the cleavage of Ac-DEVD-AMC and Ac-IETD-AMC to a lesser extent, indicating the appearance of caspase-3 and -8-like activities, respectively.

MIN6N8 insulinoma cells, was not significantly affected by IFN- $\gamma$  as demonstrated by RNase protection assays (data not shown). Similarly, the expression of *Bcl-2* and other important apoptosis-related genes such as *TRADD*, *RIP*, *FADD*, *FAF*, and *Fas* was not significantly influenced by IFN- $\gamma$  treatment of insulinoma cells as demonstrated by Western blot analyses or RNase protection assays (data not shown).

### STAT1 activation and IRF-1 induction by IFN- $\gamma$ in mouse primary islets

We next studied whether this scheme of cytotoxic synergism between IFN- $\gamma$  and TNF- $\alpha$  found in insulinoma cells could be applicable to primary islet cells. A similar synergistic cytotoxic effect of IFN- $\gamma$  and TNF- $\alpha$  was observed in single-islet cells isolated from ICR mice (Fig. 5A) as well as humans (data not shown). The addition of IL-1 $\beta$  again had only negligible effects on the islet cell viability. Moreover, the cytokine combination also induced classical apoptosis on pancreatic islet cells. Typical apoptotic morphology, such as nuclear condensation and fragmentation, was observed in the cytokine-treated mouse islet cells as indicated by Hoechst 33258 staining (Fig. 5B). STAT1 phosphorylation and IRF-1 induction by IFN- $\gamma$  were also observed in murine single-islet cells (Fig. 5C), indicating that a similar cytokine synergism is operative in MIN6N8 cells and primary islet cells. To confirm the death of islet  $\beta$  cells by IFN- $\gamma$ /TNF- $\alpha$ , immunostaining of insulin was conducted simultaneously with Hoechst 33258 nuclear staining. About 90% of islet cells were insulin-positive, indicating that the majority of islet cells were  $\beta$  cells. The percentage of apoptotic  $\beta$  cells with nuclear condensation increased from 16.6% (incubation for 3 days without cytokines) to 62.7% upon IFN- $\gamma$ /TNF- $\alpha$  treatment for 3 days, indicating that insulin-positive  $\beta$  cells were killed by IFN- $\gamma$ /TNF- $\alpha$  (>200 insulin-positive  $\beta$  cells were counted). We next asked whether a similar cytokine-induced death of islet cells could be observed in studies using islet cells isolated from insulinitis-free NOD mice at 7 wk of age. IFN- $\gamma$ /TNF- $\alpha$  treatment led to 49.3% viability compared with untreated control (viability set to 100%), indicating that IFN- $\gamma$ /TNF- $\alpha$  combination induced the death of islets from NOD mice as well.

### The expression of IRF-1 is up-regulated and colocalized with apoptotic cells in islets of diabetic mice

Having shown that IFN- $\gamma$ -mediated up-regulation of IRF-1 plays a central role in cytokine-induced islet destruction in vitro, we speculated that a similar cytokine synergism between IFN- $\gamma$  and TNF- $\alpha$  may be responsible for  $\beta$  cell death in natural diabetes of NOD mice. IFN- $\gamma$  secreted by islet-infiltrating T lymphocytes may activate or induce STAT1 and/or IRF-1 in islet cells, conferring susceptibility to apoptosis by TNF- $\alpha$  derived mainly from macrophages. To test our hypothesis, we evaluated by immunohistochemical staining the expression of STAT1 and IRF-1 in pancreatic islets of NOD mice. In pancreatic islets of nondiabetic ICR mice or young (5- to 8-wk-old) female NOD mice, minimal or no

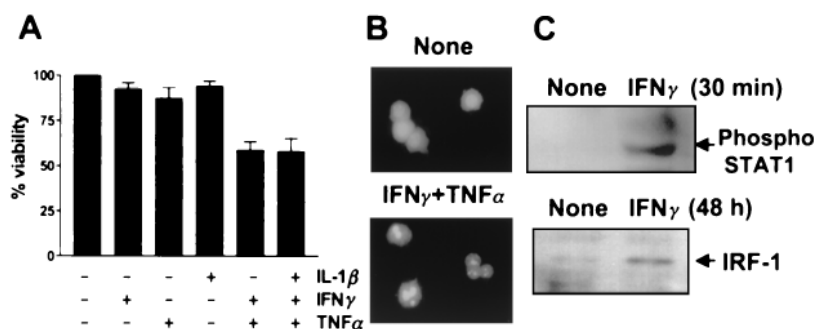
expression of STAT1/IRF-1 was observed (Fig. 6, C and F). However, in adoptive transferred-NOD mice (Fig. 6, A and D) or 20-wk-old female prediabetic NOD mice with (peri)insulinitis (Fig. 6, B and E), a strong expression of STAT1 and IRF-1 was observed in several islet cells (Fig. 6, A, B, D, and E). Moreover, some IRF-1-positive cells were colocalized with apoptotic nuclei in pancreatic islets of diabetic NOD mice (Fig. 6G), further supporting the role of IRF-1 in the cytokine-induced apoptosis of pancreatic  $\beta$  cells. The expression of phosphorylated STAT1 was not detected in pancreatic islets of NOD mice regardless of age, probably because its expression in vivo was transient as was in vitro (data not shown).

### Anti-TNF- $\alpha$ Ab blocks diabetes development

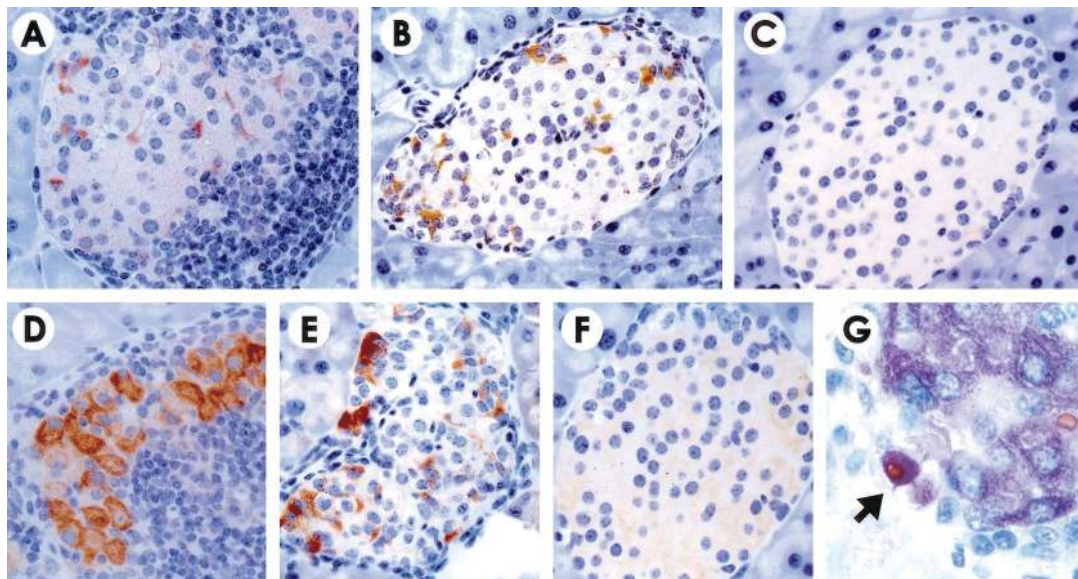
Because the expression of STAT1 and IRF-1 in pancreatic islets of NOD mice suggested the possibility that IFN- $\gamma$  induces susceptibility to TNF- $\alpha$  on islet cells through IRF-1 induction, we investigated whether the blockade of TNF- $\alpha$  could abrogate diabetes development after adoptive transfer of lymphocytes from diabetic NOD mice. As we hypothesized, administration of anti-TNF- $\alpha$  Ab during adoptive transfer blocked the development of diabetes (Fig. 7) ( $p < 0.05$ ), supporting the role of TNF- $\alpha$  as the final effector molecule in the  $\beta$  cell death and diabetes. The administration of anti-TNF- $\alpha$  Ab also significantly decreased the insulinitis score from  $2.13 \pm 0.45$  ( $n = 6$ ) to  $1.44 \pm 0.22$  ( $n = 7$ ) ( $p < 0.01$ ).

### Discussion

Our initial test of cytokines for cytotoxicity toward insulinoma cells led us to conclude that synergism between IFN- $\gamma$  and TNF- $\alpha$  is responsible for  $\beta$  cell apoptosis in vitro. In previous papers that reported the significant effects of IL-1 $\beta$  on the viability of islet cells, the effects of IL-1 $\beta$  varied widely among different species from which islets were isolated. Although IL-1 $\beta$  alone appears to be a major effector in the destruction of rat islets, the combinations of proinflammatory cytokines such as TNF- $\alpha$  or IFN- $\gamma$  were necessary for the induction of mouse or human islet cell death (24). In vitro effects of NO have also been reported, mostly using islet cells from rats (23, 36). NO production by cytokine-stimulated islet cells seems to be negligible in species other than rats. Thus, the effects of IL-1 $\beta$  and NO observed in the studies using rat islets cannot be generalized to other species. A previous paper described the cytotoxicity of pancreatic islets by IFN- $\gamma$  and TNF- $\alpha$  combination; however, the nature of cell death was not investigated (37). Also, although a few reports suggested the involvement of DNA



**FIGURE 5.** IFN- $\gamma$ /TNF- $\alpha$  synergism in the apoptosis of mouse primary islet cells and induction of STAT1 and IRF-1 by IFN- $\gamma$ . **A**, Isolated single-islet cells were treated for 3 days with a recombinant cytokine alone or in combinations as indicated. After the cytokine treatment, viability of the cells was quantified by MTT assays. IFN- $\gamma$ /TNF- $\alpha$  combination, but neither cytokine alone, induced primary murine islet cell death. IL-1 $\beta$  had negligible effects. The OD value of the untreated cells at 450 nm was set to 100%. The results are means  $\pm$  SE of the four independent experiments. **B**, Nuclear condensation and fragmentation was detected by staining with a DNA-binding fluorochrome, Hoechst 33258. **C**, Single-islet cells were either untreated or treated with 1000 U/ml of IFN- $\gamma$  for 30 min or 48 h as indicated, then cell lysates were subjected to Western blot analyses. IFN- $\gamma$  induced the expression of phosphorylated STAT1 30 min after treatment and that of IRF-1 48 h after treatment.



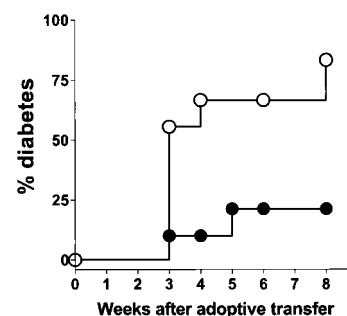
**FIGURE 6.** Up-regulated expression of STAT and IRF-1 and colocalization of IRF-1 expression with apoptotic nuclei in islets of diabetic NOD mice. As revealed by immunohistochemical staining, the expression of STAT1 (A–C) and IRF-1 (D–F) was significantly higher in the pancreatic islets of adoptive transferred-NOD mice (A and D) or 20-wk-old female NOD mice with (peri)insulinitis (B and E) than that in ICR mice (C and F) or 5- to 8-wk-old NOD mice without (peri)insulinitis (data not shown). Some TUNEL-positive apoptotic islet cells in diabetic NOD mice were expressing IRF-1 (G,  $\times 400$ ). An arrow indicates an apoptotic islet cell expressing IRF-1.

strand break or DNA fragmentation in cytokine-induced  $\beta$  cell death (38, 39), detailed morphological changes associated with apoptosis or caspase involvement were not demonstrated. Moreover, in other *in vitro* studies in which apoptosis of  $\beta$  cells have been reported, apoptosis was induced by nonphysiological agents, such as thapsigargin or serum deprivation, that were irrelevant to natural autoimmune diabetes (40, 41). Thus, we have established a physiological *in vitro*  $\beta$  cell apoptosis model, which could be used not only for further investigation on the mechanism of  $\beta$  cell destruction, but also for the screening of anti-apoptotic agents that could be used for the treatment of autoimmune diabetes.

We also demonstrated that IFN- $\gamma$  induced IRF-1 expression following STAT1 activation and that the transfection of IRF-1 could be substituted for IFN- $\gamma$  treatment to induce apoptosis of  $\beta$  cells. These results suggest a collaborative signal transduction mechanism for the cytokine synergism in the destruction of pancreatic  $\beta$  cells. IFN- $\gamma$  induces susceptibility to TNF- $\alpha$ -mediated apoptosis by sequentially activating or inducing STAT1 and IRF-1. Previous studies have indicated the role of IRF-1 in apoptosis induced by DNA damage or IFN- $\gamma$  (30, 31, 42), which supported the proapoptotic action of IRF-1. However, the role of IRF-1 in conferring susceptibility to TNF- $\alpha$ -induced apoptosis has not been reported. Caspase induction has been suggested as a possible downstream event following IRF-1 induction in IFN- $\gamma$ -induced apoptosis (31). Our results showing inhibitory effect of Ac-YVAD only at concentrations above 500  $\mu$ M suggests that caspase-1, although induced by IFN- $\gamma$ , might not be directly involved in IFN- $\gamma$ /TNF- $\alpha$ -induced  $\beta$  cell apoptosis. Caspase-11 has been reported to be crucial for the activation of caspase-1 (34); however, recent papers demonstrated that caspase-11 also activated caspase-3 as well (43). Furthermore, caspase-11-deficient mice are partly resistant to the induction of experimental allergic encephalomyelitis (44) and to the development of stroke after middle cerebral artery occlusion (43). Thus, IFN- $\gamma$ -induced caspase-11, as noted in our experiment, may play a role in the activation of caspase-3 during apoptosis of  $\beta$  cells. Besides caspases, IRF-1 has also been shown to induce iNOS (45) that catalyzes the production of NO. However, our re-

sults argued against the possible involvement of NO in IRF-1-mediated apoptosis. A significant amount of NO was not generated by MIN6N8 cells treated with IFN- $\gamma$ /TNF- $\alpha$ , and iNOS inhibitor did not affect the cytokine-induced cytotoxicity (data not shown).

In addition to our *in vitro* findings, we also present evidence that IFN- $\gamma$ /TNF- $\alpha$  synergism involving STAT1/IRF-1 pathways plays a critical role as the final effector in the development of diabetes *in vivo*: 1) IFN- $\gamma$  activated and induced STAT1/IRF-1 in primary islet cells; 2) STAT1/IRF-1 was expressed in pancreatic islets of diabetic mice and colocalized with apoptotic cells; and 3) anti-TNF- $\alpha$  Ab inhibited diabetes development. The role of TNF- $\alpha$  in NOD mice has been extensively studied. However, the *in vivo* role of the cytokine in diabetes development has not yet been fully elucidated. Its diabetogenic role was suggested in some studies (13–18), whereas opposite effects were reported in other studies (19, 20). Our IFN- $\gamma$ /TNF- $\alpha$  synergism model nicely explains why transgenic expression of TNF- $\alpha$  alone could not induce diabetes in some of the previous studies in which pancreatic expression of



**FIGURE 7.** Blockade of autoimmune diabetes by anti-TNF- $\alpha$  Ab in NOD mice. Anti-TNF- $\alpha$  Ab (0.5 mg) or the same volume of control IgG was administered i.p. during and after adoptive transfer three times a week. Compared with control IgG ( $\circ$ ,  $n = 9$ ), anti-TNF- $\alpha$  Ab administration ( $\bullet$ ,  $n = 10$ ) significantly decreased the incidence of diabetes after adoptive transfer ( $p < 0.05$ ).

TNF- $\alpha$  alone did not accelerate diabetes development (21). It is also possible that the effects of systemic TNF- $\alpha$  administration in previous studies were mostly due to its immunomodulatory effects rather than to the direct effects on target (islet) cell viability (13, 19). There is another previous report that demonstrated the inability of anti-TNF- $\alpha$  Ab treatment to inhibit diabetes in NOD mice (46). However, in that report, the dose and duration of Ab administration might not have been sufficient. In the current study, we used anti-TNF- $\alpha$  Ab whose neutralizing activity was confirmed, and the dose was sufficient to block TNF- $\alpha$  action in vivo (47). We have also shown that the administration of anti-TNF- $\alpha$  Ab significantly decreased the insulinitis score in adoptive transfer experiments. Inhibition of  $\beta$  cell apoptosis by anti-TNF- $\alpha$  Ab could decrease the release of islet Ags from dead islet cells and further insulinitis. In addition to its direct effect on islet cell death, anti-TNF- $\alpha$  Ab treatment may affect Ag presentation or chemotaxis potentially mediated by TNF- $\alpha$ . The inhibition of diabetes by anti-TNF- $\alpha$  Ab was not complete in our current study. This result might be explained by the residual TNF- $\alpha$  activity, lymphotoxin, or perforin. Targeted disruption of perforin significantly decreased diabetes incidence in NOD mice (8); however, such results do not necessarily mean that perforin is the major effector in  $\beta$  cell death. Perforin is predominantly used by CD8 $^{+}$  T lymphocytes that initially infiltrate pancreatic islets, and immunostaining disclosed that perforin was expressed on CD8 $^{+}$  but not on CD4 $^{+}$  T lymphocytes in pancreatic islets of NOD mice (48). Thus, the findings observed in perforin-deficient NOD mice seem to be due to abrogation of the initial islet cell injury by CD8 $^{+}$  T lymphocytes that is critical for the sensitization of the majority of islet-specific CD4 $^{+}$  T lymphocytes. Pakala et al. (14) established a valuable model to test the effect of single-gene disruption on the  $\beta$  cell apoptosis and diabetes. In their model, diabetogenic BDC2.5 T cells were transferred into streptozotocin-treated NOD.*scid/scid* mice grafted with islets deficient in the single gene of interest. They demonstrated that p55 TNFRI is essential for  $\beta$  cell apoptosis in vivo, consistent with our result showing significant inhibition of diabetes transfer by anti-TNF- $\alpha$  Ab. However, IFN- $\gamma$ R was not essential for the development of diabetes in their model, in contrast to our results. IFN $\alpha$  that can induce STAT1 activation and IRF-1 induction might compensate for the absence of IFN- $\gamma$  action on those islets. It is also possible that apoptosis by BDC2.5 T cells does not represent naturally occurring  $\beta$  cell apoptosis in toto by diabetogenic lymphocytes.

According to our model, blockade of IFN- $\gamma$  is also expected to inhibit  $\beta$  cell death and diabetes, although IFN- $\gamma$  itself does not exert apoptosis on islet cells. Ablation of the IFN- $\gamma$  gene in NOD mice did not completely prevent diabetes; however, the development of diabetes was delayed (49). Anti-IFN- $\gamma$  Ab (50) or targeted disruption of IFN- $\gamma$ R (51) abrogated diabetes in NOD mice. On the contrary, it has been reported that  $\beta$  cells are not the direct targets of IFN- $\gamma$  in autoimmune diabetes (52). Thus, the role of IFN- $\gamma$  in diabetes development is still controversial. Our preliminary experiments showed that anti-IFN- $\gamma$  Ab partially inhibited adoptive transfer of diabetes (S. Kim, K. Suk and M.-S. Lee, unpublished data). Incomplete abrogation of diabetes in IFN- $\gamma$ -deficient NOD mice or IFN- $\gamma$  Ab-treated mice could be due to the overlapping effects of other cytokines, such as IFN $\alpha$ , which might compensate for the absence of IFN- $\gamma$ . IRF-1, a key mediator of islet cell apoptosis, can be also induced by type I IFN (53). Importantly, IRF-1-deficient NOD mice did not have insulinitis or diabetes (54), consistent with the proposed key role of IRF-1 in islet cell death. In our model, IFN- $\gamma$  plays a priming role for the cytotoxic action of TNF- $\alpha$  in pancreatic islet cells, and this priming role of IFN- $\gamma$  is mediated by IRF-1 induction. Although previous

reports showed that IFN- $\gamma$  alone could induce apoptosis on various types of cells, such as primary hepatocytes (31) or HeLa, (55) through IRF-1 induction, the cytokine seems to require another cytotoxic signaling event to kill islet  $\beta$  cells.

Based on the results presented here, we propose that CD4 $^{+}$  T lymphocytes (as a major source of IFN- $\gamma$ ) act in collaboration with macrophages (as a major source of TNF- $\alpha$ ) to induce  $\beta$  cell death through delayed-type hypersensitivity-like reaction. This type of cooperative immune response between innate and adaptive immune responses may be also responsible for organ-specific autoimmune diseases other than autoimmune diabetes. Moreover, IFN- $\gamma$ /TNF- $\alpha$  synergism has been reported in numerous tumor cell death models (56). The signal transduction of IFN- $\gamma$ /TNF- $\alpha$  synergism we postulated may be relevant to the pathophysiology of other autoimmune diseases and cytokine-induced tumor cell apoptosis.

## References

- Wicker, L. A., B. J. Miller, and Y. Mullen. 1986. Transfer of autoimmune diabetes mellitus with splenocytes from nonobese diabetic (NOD) mice. *Diabetes* 35:855.
- Wang, Y., O. Pontesilli, R. D. Gill, F. G. La Rosa, and K. J. Lafferty. 1991. The role of CD4 $^{+}$  and CD8 $^{+}$  T cells in the destruction of islet grafts by spontaneously diabetic mice. *Proc. Natl. Acad. Sci. USA* 88:527.
- Katz, J. D., C. Benoist, and D. Mathis. 1995. T helper cell subset in insulin-dependent diabetes. *Science* 268:1185.
- Kurrer, M. O., S. V. Pakala, H. L. Hanson, and J. D. Katz. 1997.  $\beta$  cell apoptosis in T cell-mediated autoimmune diabetes. *Proc. Natl. Acad. Sci. USA* 94:213.
- Kim, Y.-H., S. Kim, K.-A. Kim, H. Yagita, N. Kayagaki, K.-W. Kim, and M.-S. Lee. 1999. Apoptosis of pancreatic  $\beta$ -cells detected in accelerated diabetes of NOD mice: no role of Fas-Fas ligand interaction in autoimmune diabetes. *Eur. J. Immunol.* 29:455.
- Christianson, S. W., L. D. Shultz, and E. H. Leiter. 1993. Adoptive transfer of diabetes in immunodeficient NOD-*scid/scid* mice. *Diabetes* 42:44.
- Appels, B., V. Brunkart, G. Kantwerk-Funke, J. Funda, V. Kolb-Bachofen, and H. Kolb. 1989. Spontaneous cytotoxicity of macrophages against pancreatic islet cells. *J. Immunol.* 142:3803.
- Kagi, D., B. Odermatt, P. Seiler, R. M. Zinkernagel, T. W. Mak, and H. Hengartner. 1997. Reduced incidence and delayed onset of diabetes in perforin-deficient nonobese diabetic mice. *J. Exp. Med.* 186:989.
- Allison, J., and A. Strasser. 1998. Mechanisms of  $\beta$  cell death in diabetes: a minor role for CD95. *Proc. Natl. Acad. Sci. USA* 95:13818.
- Thomas, H. E., R. Darwiche, J. A. Corbett, and T. W. Kay. 1999. Evidence that  $\beta$  cell death in the nonobese diabetic mouse is Fas independent. *J. Immunol.* 163:1562.
- Chervonsky, A. V., Y. Wang, F. S. Wong, I. Visintin, R. A. Flavell, C. A. Janeway, and L. A. Matis. 1997. The role of Fas in autoimmune diabetes. *Cell* 89:17.
- Kim, S., K.-A. Kim, D.-Y. Hwang, T. H. Lee, N. Kayagaki, H. Yagita, and M.-S. Lee. 2000. Inhibition of autoimmune diabetes by Fas ligand: the paradox is solved. *J. Immunol.* 164:2931.
- Yang, X.-D., R. Tisch, S. M. Singer, Z. A. Cao, R. S. Liblau, R. D. Schreiber, and H. O. McDevitt. 1994. Effect of tumor necrosis factor  $\alpha$  on insulin-dependent diabetes mellitus in NOD mice. I. The early development of autoimmunity and the diabetogenic process. *J. Exp. Med.* 180:995.
- Pakala, S. V., M. Chivetta, C. B. Kelly, and J. D. Katz. 1999. In autoimmune diabetes the transition from benign to pernicious insulinitis requires an islet cell response to tumor necrosis factor  $\alpha$ . *J. Exp. Med.* 189:1053.
- Stephens, L. A., H. E. Thomas, L. Ming, M. Grell, R. Darwiche, L. Volodin, and T. W. Kay. 1999. Tumor necrosis factor- $\alpha$ -activated cell death pathways in NIT-1 insulinoma cells and primary pancreatic beta cells. *Endocrinology* 140:3219.
- Green, E. A., E. E. Eynon, and R. A. Flavell. 1998. Local expression of TNF- $\alpha$  in neonatal NOD mice promotes diabetes by enhancing presentation of islet antigens. *Immunity* 9:733.
- Green, E. A., F. S. Wong, K. Eshima, C. Mora, and R. A. Flavell. 2000. Neonatal tumor necrosis factor  $\alpha$  promotes diabetes in nonobese diabetic mice by CD154-independent antigen presentation to CD8 $^{+}$  T cells. *J. Exp. Med.* 191:225.
- Green, E. A., and R. A. Flavell. 2000. The temporal importance of TNF- $\alpha$  expression in the development of diabetes. *Immunity* 12:459.
- Jacob, C. O., S. Aiso, S. A. Michie, H. O. McDevitt, and H. Acha-Orbea. 1990. Prevention of diabetes in nonobese diabetic mice by tumor necrosis factor (TNF): similarities between TNF- $\alpha$  and interleukin 1. *Proc. Natl. Acad. Sci. USA* 87:968.
- Satoh, J., H. Seino, T. Abo, S. Tanaka, S. Shitani, S. Ohta, K. Tamura, T. Sawai, T. Nobunaga, T. Oteki, K. Kumagai, and T. Toyota. 1989. Recombinant human tumor necrosis factor  $\alpha$  suppresses autoimmune diabetes in nonobese diabetic mice. *J. Clin. Invest.* 84:1345.
- Picarella, D. E., A. Kratz, C. Li, N. H. Ruddle, and R. A. Flavell. 1993. Transgenic tumor necrosis factor (TNF)- $\alpha$  production in pancreatic islets leads to insulinitis, not diabetes. *J. Immunol.* 150:4136.
- Grewal, I. S., K. D. Grewal, F. S. Wong, D. E. Picarella, C. A. Janeway, and R. A. Flavell. 1996. Local expression of transgene encoded TNF  $\alpha$  in islets

- prevents autoimmune diabetes in nonobese diabetic (NOD) mice by preventing the development of autoreactive islet-specific T cells. *J. Exp. Med.* 184:1963.
23. Kaneto, H., J. Fujii, H. G. Seo, K. Suzuki, T. Matsuoka, M. Nakamura, H. Tatsumi, Y. Yamasaki, T. Kamada, and N. Taniguchi. 1995. Apoptotic cell death triggered by nitric oxide in pancreatic  $\beta$ -cells. *Diabetes* 44:733.
  24. Mandrup-Poulsen, T. 1996. The role of interleukin-1 in the pathogenesis of IDDM. *Diabetologia* 39:1005.
  25. Lee, M.-S., M. von Herrath, H. Reiser, M. B. A. Oldstone, and N. Sarvetnick. 1995. Sensitization to self (virus) antigen by in situ expression of murine interferon- $\gamma$ . *J. Clin. Invest.* 95:486.
  26. Yagi, N., K. Yokono, K. Amano, M. Nagata, K. Tsukamoto, Y. Hasegawa, R. Yoneda, N. Okamoto, H. Moriyama, M. Miki, et al. 1995. Expression of intercellular adhesion molecule 1 on pancreatic  $\beta$ -cells accelerates  $\beta$ -cell destruction by cytotoxic T-cells in murine autoimmune diabetes. *Diabetes* 44:744.
  27. Rabinovitch, A., W. L. Suarez-Pinzon, Y. Shi, A. R. Morgan, and R. C. Bleackley. 1994. DNA fragmentation is an early event in cytokine-induced islet  $\beta$ -cell destruction. *Diabetologia* 37:733.
  28. Horiuchi, M., T. Yamada, W. Hayashida, and V. J. Dzau. 1997. Interferon regulatory factor-1 up-regulates angiotensin II type 2 receptor and induces apoptosis. *J. Biol. Chem.* 272:11952.
  29. Kumar, A., M. Commane, T. W. Flickinger, C. M. Horvath, and G. R. Stark. 1997. Defective TNF- $\alpha$ -induced apoptosis in STAT1-null cells due to low constitutive levels of caspases. *Science* 278:1630.
  30. Tamura, T., M. Ishihara, M. S. Lamphier, N. Tanaka, I. Oishi, S. Aizawa, T. Maatsuyama, T. W. Mak, S. Take, and T. Taniguchi. 1995. An IRF-1-dependent pathway of DNA damage-induced apoptosis in mitogen-activated T lymphocytes. *Nature* 376:596.
  31. Kano, A., T. Haruyama, T. Akaike, and Y. Watanabe. 1999. IRF-1 is an essential mediator in IFN- $\gamma$ -induced cell cycle arrest and apoptosis in primary cultured hepatocytes. *Biochem. Biophys. Res. Commun.* 257:672.
  32. Enari, M., R. V. Talanian, W. W. Wong, and S. Nagata. 1996. Sequential activation of ICE-like and CPP32-like proteases during Fas-mediated apoptosis. *Nature* 380:723.
  33. Miura, M., R. M. Friedlander, and J. Yuan. 1995. Tumor necrosis factor-induced apoptosis is mediated by a CrmA-sensitive cell death pathway. *Proc. Natl. Acad. Sci. USA* 92:8318.
  34. Wang, S., M. Miura, Y.-K. Jung, H. Zhu, and J. Yuan. 1998. Murine caspase-11, an ICE-interacting protease, is essential for the activation of ICE. *Cell* 92:501.
  35. Aggarwal, B. B., T. E. Eessalu, and P. E. Hass. 1985. Characterization of receptors for human tumour necrosis factor and their regulation by  $\gamma$ -interferon. *Nature* 318:665.
  36. Corbett, J. A., and M. L. McDaniel. 1992. Does nitric oxide mediate autoimmune destruction of  $\beta$ -cells? *Diabetes* 41:897.
  37. Campbell, I. L., A. Iscaro, and L. C. Harrison. 1988. IFN- $\gamma$  and tumor necrosis factor- $\alpha$ : cytotoxicity to murine islets of Langerhans. *J. Immunol.* 141:2325.
  38. Delaney, C. A., D. Pavlovic, A. Hoorens, D. G. Pipeleers, and D. L. Eizirik. 1997. Cytokines induce deoxyribonucleic acid strand breaks and apoptosis in human pancreatic islet cells. *Endocrinology* 138:2610.
  39. Iwahashi, H., T. Hanafusa, Y. Eguchi, H. Nakajima, J. Miyagawa, N. Itoh, K. Tomita, M. Namba, M. Kuwajima, T. Noguchi, Y. Tsujimoto, and Y. Matsuzawa. 1996. Cytokine-induced apoptotic cell death in a mouse pancreatic  $\beta$ -cell line: inhibition by Bcl-2. *Diabetologia* 39:530.
  40. Zhou, Y., D. Teng, F. Dralyuk, D. Ostrega, M. W. Roe, L. Philipson, and K. S. Polonsky. 1998. Apoptosis in insulin-secreting cells. *J. Clin. Invest.* 101:1623.
  41. Mizuno, N., H. Yoshitomi, H. Ishida, H. Kuromi, J. Kawaki, Y. Seino, and S. Seino. 1998. Altered bcl-2 and bax expression and intracellular  $\text{Ca}^{2+}$  signaling in apoptosis of pancreatic cells and the impairment of glucose-induced insulin secretion. *Endocrinology* 139:1429.
  42. Tanaka, N., M. Ishihara, M. Kitagawa, H. Harada, T. Kimura, T. Matsuyama, M. S. Lamphier, S. Aizawa, T. W. Mak, and T. Taniguchi. 1994. Cellular commitment to oncogene-induced transformation or apoptosis is dependent on the transcriptional factor IRF-1. *Cell* 77:829.
  43. Kang, S.-J., S. Wang, H. Hara, E. P. Peterson, S. Namura, S. Amin-Hanjani, Z. Huang, A. Srinivasan, K. J. Tomaselli, N. A. Thornberry, M. A. Moskowitz, and J. Yuan. 2000. Dual role of caspase-11 in mediating activation of caspase-1 and caspase-3 under pathological conditions. *J. Cell Biol.* 149:613.
  44. Hisahara, S., J. Yuan, T. Momoi, H. Okano, and M. Miura. 2001. Caspase-11 mediates oligodendrocyte cell death and pathogenesis of autoimmune-mediated demyelination. *J. Exp. Med.* 193:111.
  45. Kamijo, R., H. Harada, T. Matsuyama, M. Bosland, J. Gerecitan, D. Shapiro, J. Le, S. I. Koh, T. Kimura, S. J. Green, et al. 1994. Requirement for transcriptional factor IRF-1 in NO synthase induction in macrophages. *Science* 263:1612.
  46. Jacob, C. O., S. Aiso, R. D. Schreiber, and H. O. McDevitt. 1992. Monoclonal anti-tumor necrosis factor Ab renders non-obese diabetic mice hypersensitive to irradiation and enhances insulinitis development. *Int. Immunol.* 4:611.
  47. Hattori, K., T. Hirano, H. Miyajima, N. Yamakawa, M. Tateno, K. Oshimi, N. Kayagaki, H. Yagita, and K. Okumura. 1998. Differential effects of anti-Fas ligand and anti-TNF $\alpha$  Abs on acute graft-versus-host disease pathologies. *Blood* 91:4051.
  48. Young, L. H. Y., L. B. Peterson, L. S. Wicker, P. M. Persechini, and J. D.-E. Young. 1989. In vivo expression of perforin by CD8 $^{+}$  lymphocytes in autoimmune disease. *J. Immunol.* 143:3994.
  49. Hultgren, B., X. Huang, N. Dybdal, and T. A. Stewart. 1996. Genetic absence of  $\gamma$ -interferon delays but does not prevent diabetes in NOD mice. *Diabetes* 45:812.
  50. Campbell, I., T. W. Kay, L. Oxbrow, and L. C. Harrison. 1991. Essential role for interferon- $\gamma$  and interleukin-6 in autoimmune insulin-dependent diabetes in NOD/Wehi mice. *J. Clin. Invest.* 87:739.
  51. Wang, B., I. Andre, A. Gonzalez, J. D. Katz, M. Aguet, C. Benoist, D. Mathis. 1997. Interferon- $\gamma$  impacts at multiple points during the progression of autoimmune diabetes. *Proc. Natl. Acad. Sci. USA* 94:13844.
  52. Thomas, H. E., J. L. Parker, R. D. Schreiber, and T. W. Kay. 1998. IFN- $\gamma$  action on pancreatic  $\beta$  cells causes class I MHC upregulation but not diabetes. *J. Clin. Invest.* 102:1249.
  53. Lehtonen, A., S. Matikainen, and I. Julkunen. 1997. Interferons up-regulate STAT1, STAT2, and IRF family transcription factor gene expression in human peripheral blood mononuclear cells and macrophages. *J. Immunol.* 159:794.
  54. Nakazawa, T., J. Satoh, K. Takahashi, Y. Sakata, T. Masuda, T. Taniguchi, I. Kato, H. Okamoto, and T. Toyota. 1998. IRF-1-deficient nonobese diabetic mice do not develop autoimmune diabetes. Presented at the 3rd Immunology of Diabetes Society Conference.
  55. Chin, Y. E., M. Kitagawa, K. Kuida, R. A. Flavell, X. Y. Fu. 1997. Activation of the STAT signaling pathway can cause expression of caspase 1 and apoptosis. *Mol. Cell. Biol.* 17:5328.
  56. Fransen, L., J. Heyden, R. Ruyschaert, and W. Fiers. 1986. Recombinant tumor necrosis factor: its effect and its synergism with interferon- $\gamma$  on a variety of normal and transformed human cell lines. *Br. J. Cancer Clin. Oncol.* 22:419.