Toll-like Receptor 3 and STAT-1 Contribute to Double-stranded RNA+ Interferon-γ-induced Apoptosis in Primary Pancreatic β-Cells*

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Viral infections and local production of cytokines probably contribute to the pathogenesis of Type 1 diabetes. The viral replicative intermediate double-stranded RNA (dsRNA, tested in the form of polyinosinic-polycytidylic acid, PIC), in combination with the cytokine interferon-γ (IFN-γ), triggers β-cell apoptosis. We have previously observed by microarray analysis that PIC induces expression of several mRNAs encoding for genes downstream of Toll-like receptor 3 (TLR3) signaling pathway. In this report, we show that exposure of β-cells to dsRNA in combination with IFN-α, -β, or -γ significantly increases apoptosis. Moreover, dsRNA induces TLR3 mRNA expression and activates NF-κB and the IFN-β promoter in a TRIF-dependent manner. dsRNA also induces an early (1 h) and sustained increase in IFN-β mRNA expression, and blocking IFN-β with a specific antibody partially prevents PIC plus IFN-γ-induced β-cell death. On the other hand, dsRNA plus IFN-γ does not induce apoptosis in INS-1E cells, and expression of TLR3 and type 1 IFNs mRNAs is not detected in these cells. Of note, disruption of the STAT-1 signaling pathway protects β-cells against dsRNA plus IFN-γ-induced β-cell apoptosis. This study suggests that dsRNA plus IFN-γ triggers β-cell apoptosis by two complementary pathways, namely TLR3-TRIF-NF-κB and STAT-1.

Type 1 diabetes mellitus (T1DM) results from a progressive destruction of insulin-producing pancreatic β-cells by a β-cell-specific autoimmune process (1, 2). Although a genetic susceptibility is important for disease development, environmental factors such as viruses may also contribute for the initiation and/or progression of T1DM (3, 4). In line with this hypothesis, cross-sectional and prospective studies indicate that virus infections are involved in the pathogenesis of T1DM (3, 5), and about a dozen viruses have already been reported to be associated with human and animal T1DM (6).

Several mechanisms have been proposed for the deleterious action of viruses on pancreatic β-cells. Thus, viruses may directly infect and destroy pancreatic β-cells or they may trigger (or contribute to) β-cell-specific autoimmunity with or without direct β-cell infection; viruses may also alter some mechanisms of peripheral tolerance (7–9). For example, mouse infection with a high titer of the D variant of the encephalomyocarditis virus causes β-cell destruction and diabetes as a result of viral replication within β-cells, whereas mouse infection with a low titer of encephalomyocarditis D virus leads to diabetes as a chronic process, induced by the destruction of β-cells by macrophage-produced soluble mediators such as IL-1β, tumor necrosis factor-α/β, and NO (10). During viral infection, the pancreatic β-cells themselves initiate antiviral responses but, in this process, may stimulate host immune-mediated and islet-directed damage (11, 12). The molecular mechanisms involved in β-cell damage by viruses, alone or in combination with soluble mediators, remain to be elucidated. During viral infection, the viral replicative intermediate double-stranded RNA (dsRNA) accumulates in the infected cell and stimulates antiviral activities (13, 14). These antiviral responses can be mimicked by the synthetic dsRNA polyinosinic-polycytidylic acid (PIC) (15, 16). In vivo, PIC triggers the development of hyperglycemia in diabetes-resistant BioBreeding rats and accelerates the development of the disease in diabetes-prone BioBreeding rats (17, 18). In vitro, PIC inhibits glucose-stimulated insulin biosynthesis in mouse islets (19) and, when used in combination with IFN-γ, decreases rat islet cell function and viability (11, 20). We have recently characterized by microarray analysis the global pattern of genes induced by PIC, alone or in combination with IFN-γ, in FACS-purified rat β-cells (21). Our data demonstrated that PIC regulates the expression of several genes that participate in the induction of islet inflammation and β-cell death, such as pro-apoptotic transcription factors and diverse chemokines and cytokines (21). We noticed that several of the PIC-affected genes, such as RANTES (regulated on activation normal T cell expressed and secreted), IP-10, MCP-1 and IRF-7, were shown in other cell types to be downstream of the Toll-like receptor 3 (TLR3) signaling pathway (22). Exposure to PIC or to IFN-γ also induced the expression of the transcription factor STAT-1 and of several target genes of STAT-1, such as IRF-1, IRF-7, Mx1, Mx3, and 2',5'-oligoadenylate synthetase (21).

Toll-like receptors (TLRs) belong to a superfamily that includes the interleukin-1 receptors and is essential for the recognition of pathogen-associated molecular patterns. Eleven members of the TLR family have already been identified in mammals; each TLR acts as a primary sensor of conserved microbial components, driving the induction of specific biological responses (22, 23). Activation of TLRs by their specific ligands leads to the production of inflammatory cytokines and triggers expres-
sion of interferon-inducible genes through the JAK-STAT pathway (24, 25). Mammalian TLR3 is specifically involved in the cellular recognition of dsRNA (26). Most TLRs share signaling components with the IL-1 receptor, including IL-1 receptor-associated kinase, the cytoplasmic adaptor molecule MyD88, and TRAF6, but TLR3 signals through a MyD88-independent pathway involving the newly described adaptor molecule TRIF (Toll/interleukin-1 receptor-domain-containing adaptor protein inducing IFN-β (27, 28)). In other cell types, stimulation of TLR3 by dsRNA leads to a TRIF-mediated activation of the transcription factors IRF-3 and NF-κB, resulting in production of pro-inflammatory cytokines and type I interferons (26, 29, 30). Additionally, a dsRNA-induced pathway involving c-Jun NH2-terminal kinase (JNK) activation has been recently described (31). Expression of TLRs 2, 3, and 4 has been recently shown in mouse and human pancreatic islets (32, 33). TLR4, the lipopolysaccharide receptor, seems to be involved in Coxsackievirus B4-induced cytokine production by pancreatic cells (34), whereas TLR3 plays a role in dsRNA-induced diabetes in transgenic mice expressing the B7.1 co-stimulatory molecule under the control of the rat insulin promoter (33).

Against this background, the present study was carried out to clarify the molecular mechanisms involved in dsRNA plus IFN-γ-induced β-cell dysfunction and death and to examine whether these deleterious effects are mediated via TLR3, STAT-1, and JNK stimulation. For this purpose, experiments were conducted in parallel in FACS-purified primary β-cells and in INS-1E cells, an insulin-producing cell line previously shown to be resistant to the deleterious effects of dsRNA plus PIC (12).

EXPERIMENTAL PROCEDURES

Animals—Adults male Wistar rats (Charles River Laboratories, Brussels, Belgium), C57BL/6 mice (Harlan CBP, Zeist, The Netherlands), and STAT-1 knock-out (STAT-1−/−) mice (35) were housed and utilized according to the guidelines of the Belgian Regulations for Animal Care. STAT-1−/− mice were kept under specific pathogen-free conditions. All experiments were conducted with the approval of the Animal Ethics Committees of the Université Libre de Bruxelles and of the Katholieke Universiteit van Leuven.

Islet Cell Isolation and Culture—Pancreatic islets were isolated by collagenase digestion followed by hand picking under a stereomicroscope. They were subsequently dissociated into single cells, and β-cells were purified by autofluorescence-activated cell sorting (FACS, FACStar, BD Biosciences, Sunnyvale, CA) as previously described (36), with the following modifications: (a) pancreatic islets were dissociated into single cells by mechanical dispersion followed by enzymatic dissociation in the presence of dispase (5 mg/ml); (b) after Percoll filtration, dissociated cells were suspended in HEPES-buffered Earle’s medium containing 2.8 mM glucose (5 x 10⁵ cells/ml) and incubated for 15 min at 10 °C; (c) the freshly dissociated cells were then immediately submitted to autofluorescence-activated cell sorting using an argon laser illuminating the cells at 488 nm. Light emission at 560 – 610 nm was taken as sorting criteria; (d) purified β-cells were then cultured in Ham’s F-10 medium (Cambrex Bio Science, Verviers, Belgium) supplemented with 10 mM glucose, 2 mM l-glutamine, 0.5% bovine serum albumin, 50 μM isobutylmethylxanthine, 50 units/ml penicillin, and 50 μg/ml penicillin (37). During the first 18 h of culture, 5% heat-inactivated fetal bovine serum was added to the culture medium.

The insulin-producing INS-1E cells (passages 61–72), a kind gift from Prof. C. Wolheim (Centre Medical Universitaire, Geneva, Switzerland), were cultured in RPMI 1640 (containing Glutamax-1) supplemented with 10 mM HEPES, 10% heat-inactivated fetal bovine serum, 1 mM sodium pyruvate, 50 μM 2-mercaptoethanol, 100 units/ml penicillin, and 100 μg/ml penicillin (38).

For determination of viability (see below), FACS-purified single β-cells (10⁶ cells per well) or INS-1E cells (6 x 10⁵ cells per well) were cultured for 2 or 6 days in Falcon 96-well microtiter plates (BD Biosciences, New Jersey, NJ) containing 200 μl of medium; for β-cell culture, the plates were pre-coated with poly-L-lysine. Culture medium was changed every 2 days, and recombinant rat IFN-γ (100 units/ml, R&D Systems, Oxon, UK) or PIC (100 μg/ml, Sigma) was added. When required, TAT and c-Jun NH2-terminal kinase (JNK) inhibitor (JNKi) peptides, kindly provided by Prof. C. Bonny (Université de Lausanne, Lausanne, Switzerland) were added at a concentration of 1 μM each 1 h before the addition of PIC and IFN-γ. JNKi is a bioactive peptide linked to a TAT-carrier peptide that blocks activation (i.e. phosphorylation of the activation domains) of c-Jun by JNK (39). It has been previously shown that the JNKi, at the concentrations presently utilized, efficiently transfects insulin-secreting cells and protects against IL-1β-induced apoptosis (39); kinase assays indicated that the inhibitor blocks activation of the transcription factor c-Jun by JNK (39). In some experiments, an antibody raised against rat IFN-β (20 μg/ml, R&D Systems) was added 30 min before PIC plus IFN-γ treatment.

For RNA extraction for RT-PCR and real-time RT-PCR analysis (see below), single β-cells (10⁶ cells per 2.5 ml) were re-aggregated for 3 h in a rotary shaking incubator (40), cultured for 14 – 16 h in suspension, and then exposed for 1, 2, 6, or 24 h to IFN-γ (100 units/ml) or PIC (100 μg/ml). In some experiments, recombinant human IL-1β (10 or 50 units/ml, a kind gift of Dr. C. W. Reynolds from NCI, National Institutes of Health, Bethesda, MD) was utilized as positive control. The concentrations of cytokines and PIC, and experimental time points, were selected based on our previous studies in β-cells (11, 12) and aimed to analyze β-cells at time points that precede nonspecific changes in mRNA expression induced by early apoptosis.

Assessment of Cell Viability—The percentage of viable, apoptotic, and necrotic cells was determined after 2 or 6 days of exposure to IFN-γ and/or PIC (11, 12). For this purpose, purified β-cells or INS-1E cells were incubated for 15 min with propidium iodide (10 μg/ml) and Hoechst 33342 (10 μg/ml) (41). This fluorescence assay for single cells is quantitative and has been validated by systematic comparisons with electron microscopy observations (41, 42). The method has been successfully used to evaluate apoptosis/necrosis in rat (11, 12, 41), mouse (43, 44), and human (45) β-cells. Viability was evaluated by at least two independent observers, with one of them unaware of sample identity. The agreement between findings obtained by the two observers was always ≥90%.

RT-PCR and Real-time RT-PCR—Poly(A) plus RNA was isolated from β-cells and INS-1E cells using oligo(dT)25-coupled polystyrene Dynabeads (Dynal, Oslo), and reverse transcribed as previously described (46). For semi-quantitative RT-PCR, the number of cycles was selected to allow linear amplification of the cDNA under study, and the GAPDH housekeeping gene was used as control. The primer sequences used for amplification of rat cDNAs and their respective PCR fragment lengths were as follows: GAPDH (F, 5′-TCCCTCAAGATTTGCGACAAA-3′; R, 5′-AATGTACGTCCTGTAGTCT-3′; 308 bp); TLR3 (F, 5′-TAA-GATGCAAGGTTGACCCA-3′; R, 5′-AGCGGTATATCCTGTT-3′; 426 bp); IFN-α (F, 5′-TTCTTCATCTCTGTGCAAA-3′; R, 5′-AGCGTCCTCCAGACTCTT-3′; 414 bp); and IFN-β (F, 5′-CACTGGTGGAATGGAGAC-3′; R, 5′-AAGACTTCTGCTCGGA-3′; 222 bp). The identity of the PCR products of each gene was confirmed by size after electrophoretic migration on ethidium bromide-stained agarose gels photographed under UV-transillumination.
sequences and their respective PCR fragment lengths were as follows: IFN-α (F, 5'-CTCTACACTCTGGCAGCA-3'; R, 5'-AGCAGATCCAGAAGGCTCA-3', 116 bp); IFN-β (F, 5'-GCGTTGGCATTCAG-3'; R, 5'-AGACAGAGCTTCTGGGA-3', 131 bp); and GAPDH (F, 5'-AGTTCAACGGCGAGTCAAG-3'; R, 5'-TACCTCAGACCCAGCATCACC-3', 118 bp). The method used for quantification is the standard curve approach (42, 43). To obtain the standard curve, the primer sequences and their respective PCR fragment lengths were as follows: IFN-α (F, 5'-GGTGGTTGGTGAGCTACTGGT-3'; R, 5'-CAGAAGTCTGCCCTCTCCAT-3', 525 bp); IFN-β (F, 5'-CTGGCTCTCTCCATCGACTAC-3'; R, 5'-TCCGAGCAGAAGTCTT-3', 453 bp); and GAPDH (see above).

**Promoter Studies**—Purified β-cells or INS-1E cells were transfected using Lipofectamine with a plasmid construct containing the firefly luciferase gene under the control of either multiple copies of the NF-κB consensus sequence (BD Biosciences Clontech), or four copies of the STAT consensus sequence (a plasmid kindly provided by Prof. A. Stephanou, University College, London, UK) or the mouse IFN-β promoter (27). In some experiments cells were co-transfected with one of the plasmids described above and an expression plasmid encoding for a dominant negative form of human TRIF (27) or its empty vector, CMV-Myc (27). Of note, co-transfection with the DNnhTRIF construct reduced the activity of the internal control pRL-CMV, which then represented 72 ± 10% (n = 11) of the activity measured in β-cells transfected with the DNnhTRIF empty vector.

Transfected cells were exposed to IFN-γ (100 units/ml) and/or PIC (100 μg/ml) for 18 h. Luciferase activities were assayed with the Dual-Luciferase Reporter Assay System (Promega, Madison, WI) as previously described (12, 21). Test values were corrected for the luciferase activity value of the internal control plasmid, pRL-CMV.

**Statistical Analysis**—Data are presented as means ± S.E. of at least three independent experiments. Statistical differences between groups were determined either by paired Student’s t test or by ANOVA followed by t test with the Bonferroni correction, as indicated. A p value of <0.05 was considered as statistically significant.

### RESULTS

**Effects of dsRNA and IFN-γ on β-Cell Viability**—FACS-purified primary β-cells exposed to PIC plus IFN-γ for 6 days had a significant increase in the percentage of apoptotic cells when compared against control cells (Fig. 1A). Similar results were obtained after 2 days treatment; thus, there were 17.2 ± 0.9% apoptotic cells following PIC plus IFN-γ treatment, whereas control cells had only 3.4 ± 0.4% apoptosis (n = 4, p < 0.001, ANOVA). Exposure to PIC or IFN-γ alone for 2 or 6 days did not affect β-cell viability indicating the need for a synergistic action of PIC plus IFN-γ to trigger β-cell death (Fig. 1A). There was no significant increase in the percentage of necrotic cells in any of the experimental conditions tested (Fig. 1A).

When INS-1E cells, an insulin-producing cell line, were exposed to PIC plus IFN-γ for 6 days, the percentage of apoptotic cells was similar to that obtained in INS-1E cells treated with IFN-γ alone (Fig. 1B), suggesting that PIC does not potentiate the deleterious effect of IFN-γ on INS-1E cells viability. Of note, when combined to the cytokine IL-1β, IFN-γ significantly increases apoptosis in both INS-1E cells and primary β-cells (data not shown (11, 12)). Altogether, these results indicate that dsRNA, in association with IFN-γ, potentiates apoptosis in primary β-cells but not in the clonal INS-1E cells.

**dsRNA and IFN-γ Induce Toll-like Receptor 3 and Type I Interferons mRNA Expression**—To examine expression of both TLR3 mRNA and of genes downstream of the TLR3 signaling pathway (namely IFN-α and IFN-β), primary β-cells or INS-1E cells were cultured for 2, 6, and 24 h in the presence or absence of PIC and/or IFN-γ. In primary β-cells, exposure to PIC led to an early increase in IFN-β mRNA expression, which was already significant after a 2-h treatment, reached highest levels after 6 h, and then returned to basal levels after 24 h (Fig. 2A). A more prolonged exposure to PIC (6 h) was necessary to induce TLR3 and IFN-α mRNA expression (Fig. 2, B and C). Expression of IFN-α and IFN-β mRNAs was also examined by real-time PCR, and similar expression kinetics were observed. Thus, control expression of IFN-β (i.e. without addition of dsRNA and/or IFN-γ) was 0.03 ± 0.02 ([IFN-β/GAPDH] × 1000; n = 15), whereas in the presence of PIC it increased to 10.8 ± 3.9 after 2 h (n = 5; p < 0.05 versus control), returning to basal level after 6 and 24 h (data not shown). IFN-γ alone did not change IFN-β mRNA expression, but in the presence of PIC, it further increased IFN-β mRNA expression to 20.3 ± 4.7 (n = 4; p < 0.05 versus control), 8.3 ± 2.0 (n = 6; p < 0.05 versus control), and 1.1 ± 0.5 (NS) after, respectively, 2, 6, and 24 h. Control expression of IFN-α was 2.2 ± 0.9 ([IFN-α/GAPDH] × 1000; n = 9), and it increased to 5.7 ± 1.1 (p < 0.05 versus control, n = 5) after a 6-h exposure to PIC. IFN-γ alone did not affect IFN-α mRNA expression. In the presence of PIC, however, IFN-γ up-regulated IFN-α mRNA expression to 3.6 ± 0.7 (n = 4, NS)
and 8.5 ± 1.4 (n = 5, p < 0.05 versus control) after, respectively, 2 and 6 h of exposure. These data suggest that primary β-cells express TLR3 and that expression of this receptor is regulated by dsRNA. Of note, exposure to IFN-γ alone does not affect expression of TLR3 or type I IFNs, but, as indicated by the real-time PCR data, it magnifies the action of dsRNA.

Interestingly, TLR3 mRNA expression was not detected in INS-1E cells in any of our tested conditions (data not shown). Furthermore, PIC alone or in combination with IFN-γ did not affect IFN-β and IFN-β mRNAs expression in INS-1E cells (data not shown).

Inhibition of dsRNA-induced NF-κB Activation by a Dominant Negative Form of TRIF—To investigate the functionality of TLR3 expressed in primary β-cells, we examined whether stimulation of the receptor by its ligand dsRNA induces NF-κB activation. For this purpose, β-cells were transfected with a NF-κB luciferase reporter plasmid and a pRL-CMV plasmid used as internal control; FACS-purified β-cells (A) or INS-1E cells (B) were cultured for 18 h in the absence (control) or presence of PIC (100 μg/ml) or IL-1β (10 units/ml), used as a positive control. When required, β-cells were co-transfected with the NF-κB reporter plasmid, the pRL-CMV plasmid, and either an expression plasmid encoding for the dominant negative form of human TRIF or its empty vector CMV-Myc. Luciferase activities of the NF-κB reporter plasmid were corrected for those obtained with the pRL-CMV plasmid in the same sample. Data are expressed relative to the values obtained in the respective control condition, taken as 1.0. The results represent means ± S.E. of six independent experiments. * p < 0.05, **, p < 0.01 versus control (Student’s paired t test).

shown in Fig. 3A, exposure to PIC induced a >6-fold NF-κB activation in primary β-cells. These results were confirmed by immunocytochemistry for NF-κB (data not shown). PIC-induced NF-κB activation was efficiently prevented by the expression of a dominant negative form of TRIF, indicating that the PIC-induced NF-κB activation is mediated via the TLR3 signaling pathway. Of note, IL-1β-induced NF-κB activation, which is mediated through an MyD88-dependent/TRIF-independent pathway (50), was not prevented in β-cells by the presence of the DNHTRIF (Fig. 3A). On the other hand, PIC did not induce NF-κB activation in INS-1E cells (Fig. 3B). The transcription factor was, however, significantly activated by IL-1β, used as positive control (Fig. 3B).

Inhibition of dsRNA-induced IFN-β Promoter Activation by a Dominant Negative Form of TRIF—We next studied whether the observed dsRNA-induced increase in IFN-β mRNA expression (Fig. 2A) was associated with TRIF activation and increased activity of the IFN-β gene promoter. For this purpose, primary β-cells were transfected with a luciferase reporter construct containing the mouse IFN-β promoter and a pRL-CMV plasmid, used as internal control. To investigate the potential participation of TLR3 in this process, β-cells were co-transfected with a dominant negative form of TRIF. The cells were then cultured for 18 h in control condition or in the presence of PIC. As shown in Fig. 4, exposure to PIC led to a 7-fold increase of IFN-β promoter activity, whereas the presence of single-stranded RNA, used as negative control, did not affect the promoter activity (Fig. 4). Expression of a dominant negative form of the TLR3-specific adaptor molecule efficiently prevented PIC-induced IFN-β promoter activity (Fig. 4).
Toll-like Receptor 3 and β-Cell Death

These data show that TLR3 stimulation by dsRNA induces IFN-β promoter activity in primary β-cells by a TRIF-dependent process.

Effect of IFN-β and IFN-α on β-Cell Viability—Because stimulation of TLR3 by dsRNA up-regulates the expression of mRNAs encoding for type I interferons, we next examined whether type I interferons affect β-cell viability. Primary β-cells or INS-1E cells were cultured for 6 days in the presence of IFN-α or IFN-β, alone or in combination with PIC. As shown in Fig. 5, the presence of IFN-α or IFN-β alone did not affect β-cell viability. In primary β-cells, however, PIC combined with one of the cytokines induced a 3-fold increase in the percentage of apoptotic cells. Of note, the percentage of apoptotic cells induced by PIC plus IFN-α or IFN-β was similar to that observed in β-cells treated with a combination of PIC and IFN-γ (Fig. 1A). None of the tested agents had a significant effect on β-cell necrosis. In INS-1E cells, addition of PIC to IFN-α or IFN-β did not increase cell death (Fig. 5B), which is in good agreement with the absence of potentiating effects of PIC on IFN-γ-induced cell death (Fig. 1).

Inhibition of IFN-β Signaling Partially Protects β-Cells against dsRNA plus IFN-γ-Induced Cell Death—To further examine whether TLR3-TRIF-dependent IFN-β production plays a role in dsRNA plus IFN-γ-induced cell death, we studied the effects of PIC plus IFN-γ on β-cell viability in the absence or presence of an antibody against IFN-β (20 μg/ml). As shown in Fig. 6, addition of the IFN-β antibody partially prevented PIC plus IFN-γ-induced β-cell death (p < 0.05 versus PIC plus IFN-γ). These data suggest that dsRNA-induced IFN-β promoter activation and mRNA up-regulation putatively results in IFN-β production by the β-cell themselves, which will then contribute for the deleterious effects of PIC plus IFN-γ on β-cell viability.

Role for the Transcription Factor STAT-1 in PIC plus IFN-γ-induced β-Cell Death—Because a synergistic action between PIC and interferons is necessary to induce β-cell apoptosis (11, 12), we next investigated whether activation of the transcription factor STAT-1, a key mediator of IFN signaling pathways, was involved in dsRNA plus IFN-γ-induced cell death. Primary rat β-cells were transfected with a luciferase reporter construct containing four STAT-1 binding sites and a pRL-CMV plasmid, used as internal control. When compared with control conditions, exposure to PIC plus IFN-γ for 18 h induced a 2.5 ± 0.3-fold increase in STAT-1 activity (n = 3, p < 0.05). To evaluate whether STAT-1 activation contributes to PIC plus IFN-γ-induced β-cell death, we next cultured FACs-purified β-cells isolated from wild-type mice or STAT-1 knock-out mice (STAT-1−/−) (35) for 6 days either in the presence of PIC plus IFN-γ or under control condition. In FACs-purified β-cells isolated from the wild-type mice, exposure to PIC plus IFN-γ induced a 2-fold increase in β-cell death, mainly through apoptosis (Fig. 7), a situation similar to that observed in primary rat β-cells (Fig. 1). On the other hand, β-cells obtained from STAT-1−/− mice were protected against the combination of PIC plus IFN-γ (Fig. 7). These results indicate that exposure to PIC plus IFN-γ activates STAT-1 and that suppression of the IFN signaling pathway at the STAT-1 level efficiently protects β-cells against the toxic action of PIC plus IFN-γ. This suggests a key role for the transcription factor STAT-1 in PIC plus IFN-γ-induced β-cell death.

JNK Activation Does Not Contribute to PIC plus IFN-γ-induced β-Cell Death—The TLR3-TRIF-dependent pathway activates the transcription factor c-Jun (51), and a novel dsRNA-induced apoptotic pathway involving activation of JNK was recently described (31). We therefore investigated whether inhibition of JNK activation by the use of JNKI, a bioactive cell-permeable peptide inhibitor of JNK (39), prevents dsRNA plus IFN-γ-induced β-cell death. FACs-purified β-cells were thus cultured for 6 days in control condition or exposed to PIC plus IFN-γ in the absence or presence of the transporter peptide TAT (1 μM) or the inhibitory peptide JKNI (1 μM). The percentage of viable cells was not affected by the presence of the TAT or JKNI peptide when compared with control condition (i.e., absence of exogenous peptide) and ranged from 74.5 to 80.5% (n = 4). When β-cells were exposed to dsRNA plus IFN-γ, addition of JKNI did not prevent death (50.5 ± 4.2% of viable cells for PIC plus IFN-γ plus JKNI versus 55.3 ± 3.1% in PIC plus IFN-γ; n = 4); similar results were obtained with PIC plus IFN-γ plus TAT (not shown), suggesting that JNK activation does not play a major role in dsRNA plus IFN-γ-induced β-cell death.

DISCUSSION

Both viral infections and local production of cytokines may contribute to the development of T1DM. We have shown in previous studies that dsRNA cooperates with IFN-γ to induce apoptosis in rat β-cells (11, 12). We next characterized the global profile of dsRNA and/or IFN-γ-modified genes in rat pancreatic β-cells by microarray analysis (21), and noticed that several genes downstream of the TLR3 signaling pathway were up-regulated by dsRNA. To gain further insights in the molecular mechanisms involved in the pro-apoptotic actions of dsRNA plus IFN-γ in β-cells, we presently examined the role of TLR3, the dsRNA cellular receptor, and STAT-1 in such process. Experiments were performed in FACs-purified β-cells and in INS-1E cells, a clonal cell line previously reported to be less susceptible to dsRNA than primary β-cells (12) (a phenomenon confirmed in the present experiments). Our results show that dsRNA, in the presence of IFN-γ, increases cell death in primary β-cells, mainly via apoptosis. On the contrary, INS-1E cells are resistant to dsRNA tested alone or in combination with IFN-γ. Interestingly, primary β-cells, but not INS-1E cells, express TLR3, the dsRNA cellular receptor. TLR3 stimulation in other cell types induces NF-κB and IRF-3 activation, leading to production of pro-inflammatory cytokines and type I interferons (26, 29, 30). TLR3 transduces its signal mainly through TRIF, a newly described adaptor molecule (27, 28). Previous studies demonstrated that activation of the transcription factor NF-κB has mostly pro-apoptotic effects in pancreatic β-cells (52, 53), and we have shown that inhibition of dsRNA plus IFN-γ-induced NF-κB activation by a recombinant adenosine encoding for a nondegradable mutant form of IκBα (AdIkBα(SA)2) prevents β-cell death (12). We therefore investigated whether TLR3 expressed in primary β-cells is involved in NF-κB activation, via a TRIF-dependent

![FIGURE 4. Effect of dsRNA or single-stranded RNA on IFN-β promoter activation in primary β cells. FACS-purified β-cells were co-transfected with a mIFN-β promoter-reporter plasmid, a pRL-CMV plasmid (used as internal control), and, as required, either an expression plasmid encoding for the dominant negative form of human TRIF or its empty vector CMV-Myc; the cells were then treated or not (control (ctrl)) condition for 18 h with PIC (100 μg/ml) or single-stranded RNA (100 μg/ml). Luciferase activities of mIFN-β promoter-reporter plasmid were corrected for those obtained with the pRL-CMV plasmid in the same sample. Data are expressed relative to the value obtained in control condition, taken as 1.0. The results represent means ± S.E. of six independent experiments. *, p < 0.01 versus control; a, p < 0.01 versus PIC (ANOVA).]
versus IFN-γ.

These observations also clarify the puzzling paradox of the lack of dsRNA plus IFN-γ-induced apoptosis in INS-1E cells: these cells neither express TLR3 nor transduce signals via the TRIF-NF-κB pathway, thus deleting one of the main pathways for dsRNA-induced death signaling.

β-Cell exposure to dsRNA also up-regulated IFN-β mRNA and induced IFN-β promoter activation in primary β-cells, whereas single-stranded RNA did not affect IFN-β promoter activity. In line with the observations with the NF-κB reporter (see above), dsRNA-induced IFN-β promoter activation was also TRIF-mediated. The action of dsRNA on gene expression is magnified by IFN-γ, an observation that may explain why the concomitant presence of both agents is necessary to induce β-cell apoptosis. We next investigated whether type 1 interferons, potentially produced by the β-cells themselves or by immune-compotent cells, have a deleterious effect on their viability. Neither IFN-α nor IFN-β affected β-cell viability when tested alone; in the presence of dsRNA, however, both cytokines significantly induced β-cell apoptosis. Moreover, inhibition of IFN-β signaling by a specific antibody partially protected β-cells against PIC plus IFN-γ-induced cell death. Type 1 IFNs have been detected in the pancreas of patients with recent onset of T1DM (56, 57), and transgenic mice expressing IFN-α, -β, or -κ in β-cells develop diabetes (58–60). STAT-1, a transcription factor induced by IFNs, has been implicated in the regulation of pro- and anti-apoptotic genes in other cell types (61), and STAT-1 overexpression enhances apoptotic cell death in cardiac myocytes (62).

We observed up-regulation of STAT-1 mRNA after β-cell exposure to dsRNA or IFN-γ in our previous microarray experiments, and the combination of both agents for 6 h magnified this effect (21). Because our data documented deleterious effects of dsRNA plus IFN-α, -β, or -γ on β-cell viability, we next investigated whether suppression of the interferons signaling pathway at the level of the transcription factor STAT-1 protects β-cells against dsRNA plus IFNs-induced cell death. The results obtained demonstrate that dsRNA plus IFN-γ activates STAT-1 and that purified β-cells isolated from STAT-1-/- mice are totally protected against dsRNA plus IFN-γ-induced apoptosis, emphasizing a key role for STAT-1 in the process. Interestingly, TLR3 stimulation by dsRNA in murine macrophages induces STAT-1 activation as a consequence of TLR3-mediated IFN-β production (30, 63).

TLR signaling and IFN production are usually considered as “cell protections” response against invading pathogens. The data presented here, together with other recent reports (64, 65), suggest that excessive TLR and IFN signaling may also be involved in pro-apoptotic and pro-inflammatory effects, potentially leading to an autoimmune response in genetically predisposed individuals. Of note, expression of human IFN-β in β-cells of NOD mice and non-obese-resistant mice breaks β-cell peripheral tolerance and accelerates progression of insulitis and diabetes (66), and it was recently suggested that the degree of TLR-induced activation of the immune system is related to the development

mechanism. Exposure of primary β-cells to dsRNA led to NF-κB activation, which was prevented by expression of a dominant negative form of TRIF. In INS-1E cells, however, dsRNA did not induce NF-κB activation. These results suggest that the dsRNA plus IFN-γ toxic effects in primary β-cells are mediated, at least in part, through TLR3-mediated NF-κB activation. Studies in other cell types suggest that TRIF acts as a specific pro-apoptotic signal transducer, probably as part of the host innate immune response to bacterial or viral infection (54, 55). These
of virus-induced diabetes (67). Furthermore, TLR3-dependent inflammatory responses in the central nervous system modulate the ability of viruses to invade cells and to induce neuronal injury through inflammation-induced cell death (68).

We outline in Fig. 8 our current hypothesis for the role of viral infection in β-cell death and insulitis. The viral replicative intermediate dsRNA is formed during viral infection and stimulates TLR3 expression and signaling in pancreatic β-cells. TLR3-generated signals activate NF-κB and cooperate with IFN-γ, produced by invading immune cells, to activate STAT-1. Both NF-κB and STAT-1 have a pro-apoptotic role in β-cells (Refs. 12, 52, 53, and present data). TLR3 stimulation also induces IFN-β expression in β-cells via promoter activation (present data). IFN-β was shown in other cell types, to act in an autocrine/paracrine manner through its receptor, leading to STAT-1 activation, chemokine production, and sensitization to apoptosis (24, 30, 69). In pancreatic β-cells, chemokine production via the TRIF-mediated TLR3 pathway may favor recruitment and activation of immune cells leading to exacerbation of insulitis. In line with this possibility, β-cells exposed to dsRNA and/or IFN-γ express several chemokines, including RANTES, IP-10, and MCP-1 (12, 21). Moreover, our present data indicate that autocrine IFN-β production contributes for dsRNA plus IFN-γ-induced β-cell apoptosis.

In conclusion, the present study shows that primary β-cells express TLR3. Activation of TLR3 by dsRNA activates the pro-apoptotic transcription factor NF-κB and induces cytokine production. On the other hand, clonal INS-1E cells do not express TLR3 and are thus resistant to the deleterious action of dsRNA plus IFN-γ. Disruption of the STAT-1 signaling pathway efficiently protects β-cells against the pro-apoptotic effects of dsRNA plus IFN-γ. Taken together, these observations suggest that dsRNA plus IFN-γ induces β-cell apoptosis via the activation of two complementary pathways regulated, respectively, by TLR3-TRIF-NF-κB and STAT-1.

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REFERENCES
1. Tisch, R., and McDevitt, H. (1996) Cell 85, 291–297
2. Lernmark, A., and Falorni, A. (1997) Textbook of Diabetes, 2nd Ed., pp. 15.1–15.23, Blackwell Science, Oxford
3. Hyöty, H., and Taylor, K. W. (2002) Diabetologia 45, 1353–1361
4. Yoon, J. W. (1997) Textbook of Diabetes, 2nd Ed., Blackwell Science, Oxford, pp 14.1–14.14
5. Lonning, P., Korpela, K., Knip, M., Ilonen, J., Simell, O., Korhonen, S., Savolka, K., Muona, P., Simell, T., Koskela, P., and Hyöty, H. (2000) Diabetes 49, 1314–1318
6. Haverkos, H. W., Battula, N., Drotman, D. P., and Rennert, O. M. (2003) Biomed. Pharmacother. 57, 379–385
7. Guidotti, L. G., and Chisari, F. V. (2001) Annu. Rev. Immunol. 19, 65–91
8. Ylipaasto, P., Klingel, K., Lindberg, A. M., Otonkoski, T., Kandolf, R., Hovi, T., and Rovinboren, M. (2004) Diabetes 53, 225–239
9. Jun, H. S., and Yoon, J. W. (2003) Diabetes Metab. Res. Rev. 19, 8–31
10. Jun, H. S., and Yoon, J. W. (2001) Diabetesologia 44, 271–285
11. Liu, D., Darville, M., and Eizirik, D. L. (2001) Endocrinology 142, 2593–2599
12. Liu, D., Cardozo, A. K., Darville, M., and Eizirik, D. L. (2002) Endocrinology 143, 1225–1234
13. Jacobs, B. L., and Langland, J. O. (1996) Virology 219, 339–349
14. Williams, B. R. (1999) Oncogene 18, 6112–6120
15. Sobel, D. O., Ewel, C. H., Zeligs, B., Abbassi, V., Rossio, J., and Bellanti, J. A. (1994) Diabetes 43, 518–522
16. Der, S. D., Yang, Y. L., Weissmann, C., and Williams, B. R. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 3279–3283
17. Ewel, C. H., Sobel, D. O., Zeligs, B. L., and Bellanti, J. A. (1992) Diabetes 41, 1016–1021
18. Sobel, D. O., Newsome, J., Ewel, C. H., Bellanti, J. A., Abbassi, V., Cresswell, K., and Blair, O. (1992) Diabetes 41, 515–520
19. Rhodes, C. L., and Taylor, K. W. (1985) Biochem. J. 228, 87–94
20. Heitmerer, M. R., Scaram, A. L., and Corbett, J. A. (1999) J. Biol. Chem. 274, 12531–12536
21. Rasschaert, J., Liu, D., Kutla, B., Cardozo, A. K., Kruehoffer, M., Orntoft, T., and Eizirik, D. L. (2003) Diabetologia 46, 1641–1657
22. Takeda, K., Kaisho, T., and Akira, S. (2003) Annu. Rev. Immunol. 21, 335–376
23. Beutler, B. (2004) Nature 430, 257–263
24. Doyle, S. E., Vaidya, S. A., O’Connell, R., Dadgostar, H., Dempsey, P., Wu, T., Rao, G., Sun, R., Haberland, M., Modlin, R., and Cheng, G. (2002) Immunity 17, 251–263
25. Akira, S., and Takeda, K. (2004) Nat. Rev. Immunol. 4, 499–511
26. Alexopoulos, L., Holt, A. C., Medzhitov, R., and Flavell, R. A. (2001) Nature 413, 732–738
27. Yamamoto, M., Sato, S., Hemmi, H., Hosshino, K., Kaisho, T., Sanjo, H., Takeuchi, O., Sugiyama, M., Okabe, M., Takeda, K., and Akira, S. (2003) Science 301, 640–643
28. Oshiumi, H., Matsumoto, M., Funami, K., Akazawa, T., and Seya, T. (2003) Nat. Immunol. 4, 161–167
29. Matsumoto, M., Funami, K., Oshiumi, H., and Seya, T. (2004) Microbiol. Immunol. 48, 1225–1234.

FIGURE 8. A proposed model for dsRNA plus IFN-γ-induced β cell death.
