Type 1 diabetes (T1D) is a chronic autoimmune disease targeting pancreatic beta cells. Genome-wide association studies and gene expression analysis identified interferon (IFN)-driven gene networks as crucial pathways in the pathogenesis of T1D. IFNs are linked to the response to viral infections and might contribute to the initiation of the autoimmune process in T1D. We presently analyzed the role of ubiquitin-specific peptidase 18 (USP18), an interferon-stimulated gene 15-specific protease, on IFN-induced pancreatic beta cell inflammation and apoptosis. Our findings indicate that USP18 inhibition induces inflammation by increasing the STAT signaling and exacerbates IFN-induced beta cell apoptosis by the mitochondrial pathway of cell death. USP18 regulates activation of three BH3-only proteins, namely, DP5, Bim and PUMA in pancreatic beta cells, suggesting a direct link between regulators of the type I IFN signaling pathway and members of the BCL-2 family. USP18 depletion increases the expression of the T1D candidate gene MDA5, leading to an upregulation of double-stranded RNA-induced chemokine production. These data suggest a cross talk between the type I IFN signaling pathway and a candidate gene for T1D to increase pro-inflammatory responses in beta cells. The present study shows that USP18 is a key regulator of IFN signaling in beta cells and underlines the importance of this pathway in beta cell inflammation and death.

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level; (2) how can this regulation modulate beta cell death and the generation of signals that contribute to trigger and amplify the local inflammation (insulitis); and (3) how can this regulation cross talk with relevant candidate genes for T1D.

Ubiquitin-specific peptidase 18 (USP18; UBP43) is a major IFN-stimulated gene 15 (ISG15)-specific protease and its activity is crucial for proper regulation of ISG15-conjugated proteins. ISG15 is an IFN-stimulated ubiquitin-like protein (Ubl) that conjugates to a number of cellular substrates via an ubiquitination-like process named 'ISGylation'. Both USP18 expression and conjugation of ISG15 are strongly induced by viral infections and type I IFNs, suggesting that protein modifications by USP18-regulated ISG15 have a role in responses to viruses and type I IFN signaling.

We presently analyzed the role of USP18 in type I IFN-induced beta cell inflammation and apoptosis. Our findings indicate that USP18 inhibition increases IFN-induced beta cell apoptosis via the mitochondrial pathway of cell death and increases inflammation by exacerbating the STAT signaling pathway and the expression of the T1D candidate gene MDA5 (melanoma differentiation-associated protein 5).

Results

Cytokines upregulate USP18 expression in INS-1E cells and primary rat beta cells. Untreated INS-1E cells showed low USP18 mRNA expression, but IFNα upregulated USP18 expression from 2 to 24 h (Figure 1a). The basal expression of USP18 in primary beta cells was higher than in INS-1E cells, and IFNα treatment increased its expression by nearly 30-fold after 48 h (Figure 1b). We also observed an upregulation in USP18 expression by IFNγ alone or in combination with interleukin (IL)-1β or tumor necrosis factor-α in INS-1E cells (Supplementary Figures S1A–C). During viral infections, beta cells release type I IFNs that act via IFNAR. We have previously shown that polynosinic:polycytidylic acid (PIC; a synthetic viral double-stranded RNA (dsRNA) analog)-induced beta cell death is partially mediated by type I IFNs released by the beta cells.

These data clarify the role of USP18 as a master regulator of type I IFN signaling in beta cells and underlines the importance of this pathway in beta cell inflammation and death in T1D.
themselves. Interestingly, a time course of PIC transfection in INS-1E cells indicated that USP18 mRNA expression was already induced after 2 h, increasing progressively up to 24 h (Supplementary Figure S1D).

Two different small interfering RNAs (siRNAs) targeting USP18 (siUSP18#1 and siUSP18#2) efficiently decreased USP18 mRNA expression in untreated condition and after treatment with IFN_α at different time points (Figure 1c). In primary rat beta cells, siUSP18#1 inhibited USP18 mRNA expression by 80% and 60%, respectively, in the untreated condition and after 48 h of IFN_α treatment (Figure 1d).

In human-dispersed islets, USP18 protein expression was undetectable by Western blot under basal condition (e.g., not treated with IFN_α). A 48 h treatment with IFN_α, however, upregulated its expression and this was efficiently inhibited (>70%) by a specific siRNA against human USP18 (Figure 1e).

USP18 inhibition activates IFN_α-induced STAT signaling pathway and increases pro-inflammatory chemokine production in pancreatic beta cells. We next examined the effect of USP18 inhibition on the kinetics of IFN_α-induced STAT signaling activation. IFN_α-induced STAT1 and STAT2 phosphorylation was markedly enhanced in INS-1E cells exposed to siUSP18 after 2–4 h of IFN_α treatment, reaching a peak after 8 h that was maintained until 24 h (Figure 2a). USP18 inhibition increased IFN_α-induced total STAT1 and STAT2 protein expression after 8 h, and this effect was prolonged up to 24 h after IFN_α treatment (Figure 2a). STAT1 mRNA expression was also upregulated after treatment with IFN_α in USP18-inhibited INS1-E cells (Figure 2b) and primary rat beta cells (Figure 2c).

Figures 2a, b, c, d: INS-1E cells were transfected with siCTRL (C) or two different siRNAs against USP18 (U1 and U2). After 2 days of recovery, they were left untreated or treated with IFN_α (1000 U/ml) for 0.5, 2, 4, 8, 16 or 24 h. Phospho-STAT1, total STAT1, phospho-STAT2, total STAT2 and α-tubulin (used as loading control) were evaluated by western blot. The results are representative of three independent experiments. (b,c) INS-1E cells (b) or primary rat beta cells (c) were transfected with siCTRL or siUSP18 and, after 48 h of recovery, they were left untreated or treated with IFN_α for 24 h (b) or 48 h (c). STAT1 expression was assayed by RT-PCR and normalized for the housekeeping gene GAPDH. The results are means ± S.E.M. of 3–4 independent experiments. *P<0.05 and ****P<0.001 versus untreated (i.e., not treated with cytokines) transfected with the same siRNA; **P<0.01 and $$$P<0.001 versus siCTRL treated with IFN_α; ANOVA.

Figures 2d, e: INS-1E cells were transfected with siCTRL or siUSP18 and co-transfected with an ISRE (d) or GAS reporter construct (e) plus a pRL-CMV plasmid (used as internal control). After 12 h of recovery, they were left untreated or treated with IFN_α for 16 h and luciferase activities were measured. Results are means ± S.E.M. of three independent experiments. **P<0.01 and $$$P<0.001 versus untreated (i.e., not treated with cytokines) transfected with the same siRNA; **P<0.01 versus siCTRL treated with IFN_α; ANOVA.
(Figure 2d). However, IFNα-induced GAS (IFNγ-activated site) reporter activity was not affected by USP18 knockdown (Figure 2e).

To evaluate the role of USP18 in IFNα-induced chemokine production, we analyzed the mRNA expression of CXCL10 (C-X-C motif chemokine 10), CCL5 (chemokine (C-C motif) ligand 5) and IL-15 in USP18-silenced INS-1E cells (Figure 3a), primary rat beta cells (Figure 3b) and human-dispersed islet cells (Figure 3c). Treatment with IFNα for 24 h or 48 h did not affect chemokine mRNA expression in siCTRL-transfected INS-1E cells, but inhibition of USP18 exacerbated IFNα-induced expression of CXCL10, CCL5 and IL-15 by 280-, 35- and 9-fold, respectively (Figure 3a). These results were confirmed in primary rat beta cells (Figure 3b) in which USP18 inhibition upregulated CXCL10, CCL5 and IL-15 mRNA expression. IFNα-induced CXCL10, CCL5 and IL-15 expression was also upregulated by 18-, 21- and 2.5-fold, respectively, in USP18-silenced dispersed human pancreatic islets (Figure 3c).

The upregulation of CXCL10 mRNA expression in USP18-inhibited INS-1E, primary rat beta and human-dispersed islet cells was confirmed at the protein level, as USP18-silenced cells secreted higher amounts of CXCL10 compared with control cells after IFNα treatment (Figures 3d–f). These data indicate an inhibitory role of USP18 in the expression of IFNα-induced pro-inflammatory chemokines in pancreatic beta cells.

The increase in chemokine expression in USP18-silenced cells is mediated by the transcription factors STAT1 and STAT2. To evaluate the implication of STAT1 and STAT2 in IFNα-induced chemokine expression in

![Figure 3](link)
USP18-inhibited cells, we performed a double-knockdown approach to silence USP18 in combination with STAT1 or STAT2. As shown in Figure 4a, the individual siRNAs targeting STAT1 or STAT2 efficiently inhibited their target protein alone or in combination with siUSP18. The siRNA targeting USP18 inhibited its mRNA expression by 50–78%, alone or when combined with STAT1 (Figure 4b) or STAT2 (Figure 4c). As shown above (Figure 3), inhibition of USP18 exacerbated IFN-α-induced chemokine expression but double knockdown of USP18 and STAT1 (Figure 4d) or STAT2 (Figure 4e) reverted this effect for all the chemokines tested.

Knockdown of USP18 also exacerbated mRNA expression of the transcription factor IRF7 (interferon regulatory factor 7) in USP18-inhibited INS-1E cells (Supplementary Figure S2A). The increase in IRF7 expression in USP18-silenced cells was reverted after double inhibition of USP18 and STAT1 (Supplementary Figure S2B) or STAT2 (Supplementary Figure S2C), suggesting that IRF7 is downstream of the STAT signaling pathway. IRF7 is a major regulator of chemokine production in beta cells. To assess whether IRF7 mediates chemokine production in USP18-silenced cells, we silenced USP18 and IRF7 (using a previously validated siRNA against IRF7). Knockdown of USP18 and IRF7 (between 30 and 61%) was confirmed by RT-PCR (Supplementary Figures S2D and E). USP18 inhibition increased IFN-α-induced mRNA expression of CXCL10, CCL5 and IL-15; however, double knockdown of USP18 and IRF7 failed to revert this exacerbation in chemokine expression (Supplementary Figure S2F). These results suggest that IRF7 is not a key mediator in IFN-α-induced chemokine upregulation in USP18-inhibited cells.

Inhibition of USP18 in INS-1E cells exacerbates IFN-α-induced mRNA expression of the T1D candidate gene MDA5. MDA5 (IFIH1) is a T1D candidate gene, and we have previously shown that it has a crucial role in dsRNA-induced chemokine production in pancreatic beta cells. IFN-α did not induce MDA5 expression in siCTRL-transfected cells, but MDA5 mRNA was upregulated by 24-fold in USP18-inhibited INS-1E cells after IFN-α treatment (Figure 5a). To test the functional significance of MDA5 mRNA upregulation in USP18-inhibited cells, we analyzed CXCL10 and CCL5 mRNA expression under IFN-α and PIC (synthetic dsRNA) treatment. Inhibition of USP18 increased IFN-α-induced CCL5 and CXCL10 expression by 120- and 280-fold, respectively, and this effect was further exacerbated (by 12- and 2.6-fold) after 10 h of PIC transfection (Figures 5b and c).

Figure 4: The transcription factors STAT1 and STAT2 mediate IFN-α-induced chemokine production in USP18-silenced INS-1E cells. (a-c) INS-1E cells were transfected with siCTRL (C), siUSP18 (U), siSTAT1 (S1) or siSTAT2 (S2) or double transfected with siUSP18 + siSTAT1 (U + S1) or siUSP18 + siSTAT2 (U + S2) and, after 48 h of recovery, they were left untreated or treated with IFN-α for 24 h. (a) Protein expression of STAT1, STAT2 and α-tubulin (as loading control) was determined by western blot analysis to confirm the inhibition efficiency of siRNAs targeting STAT1 and STAT2, alone or in combination with siUSP18. The results are representative of 3 independent experiments. (b, c) Inhibition efficiency of siUSP18 alone or in combination with siSTAT1 (b) or siSTAT2 (c) was determined by USP18 mRNA expression analysis by RT-PCR. Expression results were normalized for the housekeeping gene GAPDH. The results are means ± S.E.M. of three independent experiments; ***P < 0.001 versus untreated (i.e., not treated with cytokines) transfected with the same siRNA; *P < 0.05, **P < 0.01 and ***P < 0.001 versus siCTRL; ANOVA. (d-e) INS-1E cells were transfected with siCTRL, siUSP18, siSTAT1 or siSTAT2 or double transfected with siUSP18 + siSTAT1 (d) or siUSP18 + siSTAT2 (e). After 48 h of recovery, they were left untreated or treated with IFN-α for 24 h and CXCL10, CCL5 and IL-15 expression assayed by RT-PCR and normalized for the housekeeping gene GAPDH. The results are means ± S.E.M. of three independent experiments; #P < 0.05, ##P < 0.01 and ###P < 0.001 versus untreated (i.e., not treated with cytokines) transfected with the same siRNA; *P < 0.01 and **P < 0.001 versus siCTRL treated with IFN-α; °P < 0.05 °°P < 0.01 and °°°P < 0.001 versus siUSP18 treated with IFN-α; ANOVA.
Inhibition of USP18 by specific siRNAs exacerbates IFN- and PIC-induced beta cell apoptosis. USP18 inhibition by two independent siRNAs increased beta cell apoptosis following exposure to IFN$_\alpha$ or IFN$_\gamma$ (Figure 6a), whereas siCTRL has no such effect. USP18 inhibition also increased apoptotic cell death by nearly 4-fold in primary rat beta cells after 48 h of treatment with IFN$_\alpha$ (Figure 6b). Inhibition of USP18 in INS-1E cells also increased cleaved caspases-9 and -3 after 24 h of IFN$_\alpha$ treatment (Figure 6c), suggesting apoptosis via the mitochondria-driven intrinsic pathway of cell death.

Transfection with PIC increased beta cell death, and this effect was also exacerbated after inhibition of USP18 by two different siRNAs (Figure 6d).

IFN$_\alpha$-induced beta cell apoptosis in USP18-silenced cells is mediated via three members of the BH3-only protein family. Death protein 5 (DP5), p53 upregulated modulator of apoptosis (PUMA) and BCL2-like protein 11 (Bim) are members of the BCL-2 (B-cell lymphoma 2) family and have been shown to contribute to cytokine-induced beta cell death. USP18 inhibition in INS-1E cells induced Bim expression in untreated and IFN$_\alpha$-treated conditions (Figure 7a), whereas DP5 (Figure 7b) and PUMA (Figure 7c) mRNA expression was significantly upregulated when compared to untreated or IFN$_\alpha$-treated cells. Post-transcriptional phosphorylation of Bim at a serine residue increases its pro-apoptotic activity in beta cells, but USP18 inhibition in INS-1E cells did not increase Bim phosphorylation at serine 65 (data not shown).

To clarify whether Bim, DP5 and PUMA contribute to IFN$_\alpha$-induced beta cell death in USP18-silenced cells, we silenced USP18 and each of the three BH3-only proteins in a double-knockdown approach. All siRNAs used have been previously validated and they presently inhibited all target mRNAs (Supplementary Figure S3). Inhibition of USP18 significantly exacerbated beta cell apoptosis after treatment with IFN$_\alpha$ (Figure 7d), confirming the findings from Figure 6a. This effect was counteracted by DP5, PUMA and Bim knockdown, which protected INS-1E cells against IFN$_\alpha$-induced apoptosis in USP18-silenced cells by 47%, 58% and 78%, respectively (Figure 7d).

Discussion

The current study shows that USP18 is a key regulator of type I IFN signaling pathway in pancreatic beta cells and its inhibition contributes to IFN-induced beta cell apoptosis and inflammation. Thus, we show that inhibition of USP18 exacerbates pro-inflammatory chemokine production via induction of the STAT signaling pathway and increases IFN-induced beta cell apoptosis by activation of the mitochondrial-driven pathway of cell death. Moreover, we demonstrate a direct cross talk between a candidate gene for T1D (MDA5) and the USP18-regulated type I IFN signaling pathway that amplifies the viral dsRNA-induced chemokine production.
USP18 is a 43-kDa protein highly upregulated by type I IFNs, viral infections and bacterial lipopolysaccharide. We presently show that IFNα and dsRNA induce USP18 expression in human islets, primary rat beta cells and INS-1E cells, supporting its role in IFN-related signaling pathways and antiviral responses in pancreatic beta cells.

Low and constitutive expression of type I IFNs have been detected in many cell types, and their pleiotropic activities are highly regulated in time and space by several mechanisms that coexist to attenuate IFN-initiated STAT signaling. Upon binding of type I IFNs to their cell surface receptor (IFNAR), the tyrosine kinases Tyk2 and JAK1 are autophosphorylated and activated, leading to phosphorylation of the transcription factors STAT1 and STAT2. In USP18-silenced beta cells (present data), activation of IFNα-induced STAT signaling pathway is exacerbated and leads to an increase in ISRE reporter activity. These results are in line with previous studies in USP18 knockout mice in which IFNα induced long-lasting activation of JAK-STAT signaling and increased IFN-inducible gene expression.

Increased STAT signaling pathway in USP18-inhibited beta cells leads to an upregulation of several pro-inflammatory chemokines, including CCL5, CXCL10 and IL-15. This is in line with previous findings that bone marrow-derived macrophages of USP18 knockout mice have increased chemokine and chemokine receptor expression after exposure to IFNα. Of note, CXCL10 is the most expressed chemokine in USP18-silenced rodent and human beta cells (present data). CXCL10 is a key chemokine expressed in vivo in islets T1D patients, and human insulinitis is characterized by expression of CXCL10 in endocrine cells, while the infiltrating lymphocytes express the corresponding chemokine receptor CXCR3.

Chemokine expression in USP18-inhibited cells is regulated by STAT1 and STAT2 (present data). STAT1 has an important role in cytokine-induced inflammation in beta cells by regulating the expression of several pro-inflammatory chemokines, such as CXCL10, CXCL9, CXCL1 and CCL20. STAT1 was originally described as a transcription factor essential for type I IFN signaling in other cell types, but recent findings in STAT1 knockout mice indicate the presence of STAT2-mediated antiviral mechanisms. We presently observed that STAT2 has a key role in the regulation of chemokine expression in USP18-silenced beta cells, and its inhibition prevents USP18-induced chemokine expression. Unphosphorylated STAT1 and STAT2 are also upregulated by USP18 knockdown, suggesting their possible implication in IFNα-induced chemokine expression. Unphosphorylated STATs may prolong the expression of several IFN-induced genes, but whether unphosphorylated STATs have a role in increased chemokine expression in USP18-inhibited pancreatic beta cells needs further investigation.

In recent years, > 50 candidate genes have been identified for human T1D, many of them are related to type I IFN signaling and antiviral responses. The expression of the T1D candidate gene MDA5 in USP18-silenced cells is highly increased after IFNα treatment (present data) and this upregulation amplifies dsRNA-induced chemokine production, suggesting a cross-talk between the type I IFN signaling pathway and a candidate gene for T1D that increase pro-inflammatory responses in beta cells. Thus, combination

Figure 6  Inhibition of USP18 exacerbates IFNα-, IFNγ- and PIC-induced cell apoptosis in INS-1E cells and primary rat beta cells. (a, b) INS-1E cells (a) or primary rat beta cells (b) were transfected with siCTRL or siUSP18. After 48 h of recovery, INS-1E cells were left untreated or treated with IFNα (1000 U/ml) or IFNγ (100 U/ml) for 24 h (a) and primary rat beta cells with IFNα for 48 h (b). Apoptosis was measured using HO/PI staining. Results are means ± S.E.M. of 3–5 independent experiments. P < 0.01 and P < 0.001 versus untreated (i.e., not treated with cytokines) transfected with the same siRNA; ***P < 0.001 versus siCTRL treated with the same cytokine; ANOVA. (c) INS-1E cells were transfected with siCTRL (C) or siUSP18 (U) and exposed to IFNα or IFNγ for 24 h. Expression of cleaved caspases-9 and -3 and β-tubulin (used as loading control) were measured by western blot. Results are representative of three independent experiments. (d) INS-1E cells were transfected as in (a). After 48 h of recovery, they were left untransfected or transfected with PIC for 24 h. Apoptosis was measured using HO/PI staining. Results are means ± S.E.M. of three independent experiments; ***P < 0.001 versus untreated (i.e., not transfected with PIC); ***P < 0.001 versus siCTRL transfected with PIC; ANOVA.
of a viral infection with upregulation of the type I IFN signaling pathway might lead to an excessive immune response against beta cells in genetically susceptible individuals (e.g., individuals with risk alleles in MDA5), increasing the risk of autoimmunity and T1D.

Exacerbation of STAT signaling pathway and chemokine production in USP18-silenced beta cells is accompanied by exacerbation of cell death. In fact, USP18 knockdown contributes to IFN- and PIC-induced caspases-9 and -3 activation and beta cell apoptosis. Activation of caspase-9 suggests triggering of the mitochondrial pathway of cell death, characterized by the loss of mitochondrial membrane integrity, mitochondrial cytochrome c release to the cytosol and activation of caspases-9 and -3. Mitochondrial integrity is controlled by interactions between pro- and antiapoptotic members of the Bcl-2 protein family and several of these proteins are crucial regulators of beta cell apoptosis induced by pro-inflammatory cytokines or lipotoxicity. In USP18-silenced cells, mRNA expression of the BH3-only proteins DP5 and PUMA is highly enhanced after IFN- treatment, whereas Bim expression is upregulated in USP18-depleted cells in both untreated and IFN- treated cells. It is noteworthy that upregulation of Bim mRNA expression secondary to IFN- inhibition is not sufficient to trigger beta cell apoptosis in the absence of IFN-2. This suggests that increased basal expression of Bim by USP18 knockdown together with signals provided by the other BH3-only proteins, for example, PUMA and DP5, are required to overcome anti-apoptotic signals provided by BCL-2, MCL-1 and BCL-XL and thus trigger beta cell death in USP18-silenced cells. In line with this hypothesis, double knockdown of USP18 and DP5, PUMA or Bim protects against USP18 knockdown-induced beta cell death. This suggests the implication of the intrinsic mitochondrial pathway of cell death in this process and emphasizes the need for multiple pro-apoptotic signals to initiate beta cell apoptosis. DP5 and PUMA expression is regulated by the transcription factors STAT1 and STAT2 in USP18-silenced cells. In fact, double inhibition of USP18 and STAT1 or STAT2 reverted the supportive effect of USP18 knockdown in IFN-z-induced beta cell apoptosis. Both DP5
and PUMA have been previously shown to be implicated in cytokine- and ER stress-induced beta cell death, and their transcriptional activation has been shown to be regulated by NF-κB and STAT1, respectively. This is the first study, however, reporting the implication of both STAT1 and STAT2 in IFNα-induced DP5 and PUMA transcriptional regulation.

The current study shows that USP18 has a crucial role in type I IFN signaling in pancreatic beta cells via regulation of the STAT signaling pathway (Figure 8). Under physiological conditions, USP18 inhibits STAT signaling, decreasing IFNα-induced chemokine production and activation of several members of the BH3-only protein family. There is also a cross talk between a candidate gene for T1D (MDA5) and the USP18-regulated type I IFN signaling pathway that amplifies the viral dsRNA-induced chemokine production. These data suggest that a perturbation of the type I IFN signaling in beta cells may induce an excessive inflammatory process and increased activation of proapoptotic signaling pathways, resulting in increased beta cell inflammation and apoptosis following, for instance, a local viral infection. These results further support the implication of type I IFN-driven pathways and antiviral responses in the pathogenesis of T1D and point out the regulators of these pathways as potential targets for pharmacological interventions in T1D.

Materials and Methods

Culture of FACS-purified rat beta cells, human islets and INS-1E cells. Male Wistar rats (Charles River Laboratories, Brussels, Belgium) were housed and used according to the Belgian Regulations for Animal Care and with permission from the local Ethical Committee. Rat islets were isolated by collagenase digestion and handpicked under a stereomicroscope. For beta cell isolation, islets were dispersed and beta cells were purified by FACS (FACSaria, BD Bioscience, San Jose, CA, USA) as described. The beta cell preparations used in this study contained 90 ± 3% beta cells (n = 6). Purified beta cells were cultured for 48 h in Ham’s F-10 medium containing 10 mM glucose, 2 mM glutamax, 50 μM isobutyl-L-methylxanthine, 5% fetal bovine serum, 0.5% charcoal-absorbed bovine serum albumin (bovine serum albumin fraction V, Boehringer, IN, USA), 50 μM penicillin and 50 μg/ml streptomycin. For cytokine treatment, cells were cultured in the same medium but without serum. For siRNA and PIC transfection, bovine serum albumin-free and antibiotic-free medium was used.

Human islets were isolated from four non-diabetic organ donors (age: 61 ± 4 years; body mass index: 28 ± 3 kg/m²) in Pisa, Italy, after approval of the local Ethics Committee. Islets were isolated by enzymatic digestion and density-gradient purification. After overnight recovery in Ham’s F-10 containing 6.1 mM glucose, 10% fetal bovine serum, 2 mM Glutamax, 50 μM 3-isobutyl-1-methylxanthine, 1% bovine serum albumin, 50 μM penicillin and 50 μg/ml streptomycin, islets were dispersed for treatment and viability assays as previously described. The percentage of beta cells, examined in the four dispersed islet preparations by staining with anti-insulin antibody (1:1000, Sigma, Bornem, Belgium) and donkey anti-mouse IgG rhodamine (1:200, Lucron Bioproducts, De Pinte, Belgium), was 48 ± 7%. In all experiments, islets from each individual donor were counted as n = 1. The results shown are thus mean ± SEM of 3–4 independent donors.

The insulin-producing INS-1E cell line (a kind gift from Dr. C Wollheim, Center Medical Universitaire, Geneva, Switzerland) was cultured in RPMI 1640 Glutamax-I, 5% fetal bovine serum, 10 mM HEPES, 1 mM Na-pyruvate and 50 μg/ml 2-mercaptoethanol.

RNA interference. The siRNA used in the present study are listed in Supplementary Table 1. The optimal concentrations (30 nM), and conditions for siRNA transfection were established based on previous dose–response studies. The cells were transfected using Lipofectamine RNAiMAX lipid reagent (Invitrogen, Paisley, UK) as described. After transfection, cells were cultured for a 48 h recovery period and subsequently exposed to cytokines (see below).

Cytokine exposure and dsRNA transfection. The following cytokines were used based on dose–response experiments previously performed by our group (11,16, unpublished data): recombinant IFNα (specific activity: 1 × 10^6 U/mg, PBL Biomedical Laboratories, Piscataway, NJ, USA) at 1000 U/ml, recombinant human IFNβ at 2000 U/ml (specific activity: 1.8 × 10^6 U/mg, PeproTech, Rocky Hill, USA), recombinant IFNγ at 1000 U/ml (specific activity: 8 × 10^5 U/mg, PBL Biomedical Laboratories), recombinant IFNγ at 100 U/ml (specific activity: 2 × 10^5 U/mg, R&D Systems, Abingdon, UK), recombinant human IL-1β (specific activity: 1.8 × 10^6 U/mg; a gift from C.W. Reynolds, National Cancer Institute, Bethesda, MD) at 10 U/ml and recombinant murine tumor necrosis factor-α (specific activity: 2 × 10^5 U/mg; Innogenetics, Gent, Belgium) at 1000 U/ml.

The synthetic dsRNA analog PIC (Invivogen, San Diego, CA, USA) was used at the final concentration of 1 μg/ml. PIC transfection was performed in the same conditions as described for siRNA but using 0.15 μl of Lipofectamine 2000.

Assessment of cell viability. The percentage of living, apoptotic and necrotic cells was determined after 15 min incubation with the DNA-binding dyes propidium iodide (PI, 5 μg/ml, Sigma, Bornem, Belgium) and Hoechst 33342 (HO, 5 μg/ml, Sigma) as described. Apoptosis was confirmed by western blot analysis of cleaved caspases 9 and 3 (see below).

mRNA extraction and RT-PCR. mRNA extraction and reverse transcription were performed as previously described and the real-time PCR amplification was done using SYBR Green and compared with a standard curve. Expression values were corrected for the housekeeping genes glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or β-actin; GAPDH expression in rat beta cells and β-actin in human samples are not affected by cytokine treatment. The primers used for mRNA expression analysis are listed in Supplementary Table 2.

Evaluation of chemokine accumulation in the medium by ELISA. INS-1E, rat primary beta and human-dispersed islet cells were transfected with siCTRL or siUSP18 and subsequently left untreated or treated with IFNα as described above. Supernatants from INS-1E and primary rat beta cells were collected after 24 h or 48 h of treatment for determination of CXCL10 using the commercially available ELISA kit for rat CXCL10 (Abnova, Taipei City, Taiwan). Supernatants from human-dispersed islet cells were collected after 48 h of treatment with IFNα and determination of CXCL10 was performed using an ELISA kit for human CXCL10 (R&D Systems, Abingdon, UK).
Western blot analysis. Cells were washed with cold PBS and lysed in Laemmi buffer. Immunoblot analysis was performed with the antibodies listed in Supplementary Table 3 and immunoreactive bands were revealed using the SuperSignal West Fermo chemiluminescent substrate (Thermo Scientific, Chicago, IL, USA), detected using a Bio-Rad Molecular Imager ChemiDoc XR+ with ImageLab software (Bio-Rad, Nazareth-Eke, Belgium).

Luciferase reporter assays. INS-1E cells (10^5) were plated in 24-well plates and transfected with siRNAs as described above. After 12 h of recovery, cells were co-transfected with pRL-CMV encoding Renilla luciferase (Promega, Madison, WI, USA) and either a firefly luciferase promoter-reporter construct containing three GAS consensus sequences (GAS reporter—LR0075, Panomics, Fremont, CA, USA) or four ISRE consensus sequences (ISRE reporter—LR0040). After 24 h of recovery and 16 h of treatment with IFNz, luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega) and corrected for the luciferase activity of the internal control plasmid, pRL-CMV.44

Statistical analysis. Data are presented as mean ± SEM. Comparisons were performed by two-tailed paired Student’s t-test, ratio test or ANOVA followed by Student’s Htest with Bonferroni correction as indicated. A P-value < 0.05 was considered statistically significant.

Conflict of Interest The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Cell Death and Disease website (http://www.nature.com/cddis)