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MDA5 and PTPN2, two candidate genes for type 1 diabetes, modify pancreatic β-cell responses to the viral by-product double-stranded RNA

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β-Cell destruction in type 1 diabetes (T1D) is at least in part consequence of a 'dialog' between β-cells and immune system. This dialog may be affected by the individual's genetic background. We presently evaluated whether modulation of MDA5 and PTPN2, two candidate genes for T1D, affects β-cell responses to double-stranded RNA (dsRNA), a by-product of viral replication. These genes were selected following comparison between known candidate genes for T1D and genes expressed in pancreatic β-cells, as identified in previous array analysis. INS-1E cells and primary fluorescence-activated cell sorting-purified rat β-cells were transfected with small interference RNAs (siRNAs) targeting MDA5 or PTPN2 and subsequently exposed to intracellular synthetic dsRNA (polyinosinic–polycitidilic acid—PIC). Real-time RT–PCR, western blot and viability assays were performed to characterize gene/protein expression and viability. PIC increased MDA5 and PTPN2 mRNA expression, which was inhibited by the specific siRNAs. PIC triggered apoptosis in INS-1E and primary β-cells and this was augmented by PTPN2 knockdown (KD), although inhibition of MDA5 did not modify PIC-induced apoptosis. In contrast, MDA5 silencing decreased PIC-induced cytokine and chemokine expression, although inhibition of PTPN2 induced minor or no changes in these inflammatory mediators. These findings indicate that changes in MDA5 and PTPN2 expression modify β-cell responses to dsRNA. MDA5 regulates inflammatory signals, whereas PTPN2 may function as a defence mechanism against pro-apoptotic signals generated by dsRNA. These two candidate genes for T1D may thus modulate β-cell apoptosis and/or local release of inflammatory mediators in the course of a viral infection by acting, at least in part, at the pancreatic β-cell level.

INTRODUCTION

Type 1 diabetes (T1D) is a chronic autoimmune disease with a strong inflammatory component. Islet inflammation (insulitis) probably takes place in the context of a 'dialog' between invading immune cells and the target β-cells. This dialog is partially mediated by cytokines and chemokines released by both β-cells and immune cells and by immunogenic signals delivered by dying β-cells. This leads to induction and amplification or, in some cases, resolution of insulitis (1). The evolution of islet inflammation, and its potential progression to clinical diabetes, probably depends on the interplay between the patient's genetic background and environmental triggers, such as viral infections and/or dietetic components (1–4).

Identification of genetic-based pathways for complex diseases, such as T1D, provides the initial framework for investigations of environmental influences on a given genetic background (5). The relevance of this approach has already been shown in rheumatoid arthritis (6), and was recently confirmed in the context of T1D by a study showing interaction between polymorphisms in the candidate gene PTPN22 and the early introduction of cow's milk in the emergence of islet autoantibodies and diabetes in a Finnish population (7). These population studies, however, cannot clarify the molecular mechanisms involved in the interactions between the

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genetic background and environmental factors. To address this issue in the context of candidate genes that may affect pancreatic β-cell survival and insulitis development in T1D, we used a three-pronged strategy.

(1) Compare the list of known candidate genes for T1D (8–12) with genes expressed in pancreatic β-cells and modified by inflammatory cytokines and/or double-stranded (ds) RNA/virus as determined by our previous microarray analysis (13–18). We observed that at least 30% of the candidate genes for T1D are expressed in β-cells (data not shown), confirming that these cells may have an active role in the emergence of insulitis (1). Two of the identified candidate genes were of particular interest, namely MDA5 (melanoma differentiation-associated gene 5; also known as *IFIH1*) and PTPN2 (protein tyrosine phosphatase N2; also known as TC-PTP or PTP-S2). MDA5 is a cytoplasmic receptor for viral nucleic acids involved in the innate immune response to viruses (19). Rare polymorphisms of MDA5, leading to inhibited function, decrease the risk of T1D by nearly 50% (20). Since MDA5 is a member of the retinoic acid-inducible gene I (RIG-I)-like receptor (RLR) family, we investigated in parallel the role of the other helicase, RIG-I, previously shown to be involved in the recognition of cytoplasmic 5′-triphosphate single-stranded RNA and double-stranded RNA (dsRNA) (21), and of some of the key signalling patterns involved in dsRNA-induced β-cell apoptosis. The phosphatase PTPN2 functions as a negative regulator of several signalling pathways, including Janus kinases (JAKs), signal transducer and activator of transcription (STATs), p42/44 MAPK (ERK), epidermal growth factor receptor and insulin receptor β, and modulates β-cell apoptosis induced by interferon (IFN)-γ (18);
(2) Design specific small interference RNAs (siRNAs) targeting these genes to evaluate their function in β-cells, with special focus on the induction of apoptosis and production of inflammatory cytokines and chemokines;
(3) Expose β-cells transfected with siRNAs targeting the candidate genes to intracellular double-stranded (ds) RNA. dsRNA is a by-product generated during replication and transcription of both RNA and DNA viruses and an efficient inducer of apoptosis, type I interferons and other cytokines/chemokines important for the host immune response to viral infection (22,23). We thus aimed to model *in vitro* and under well-controlled conditions the putative genetic/environmental interactions that may take place in early T1D.

The data obtained suggest that MDA5 and PTPN2 are induced by dsRNA in pancreatic β-cells. Of interest, blocking MDA5 expression prevents dsRNA-induced expression of cytokines and chemokines, important mediators of insulitis. PTPN2 seems to play a different role in this process, since PTPN2 silencing sensitized β-cells to dsRNA-induced apoptosis but had limited effects on the expression of inflammatory mediators. These observations indicate that two candidate genes for T1D may act, at least in part, at the β-cell level, modulating apoptosis and the generation of inflammatory signals in the course of a viral infection.

RESULTS

siRNAs against MDA5 and RIG-I prevent polyinosinic–polycitidilic acid-induced activation of interferon β/NF-κB and chemokines, but not apoptosis in insulin-producing cells

We used the synthetic dsRNA polyinosinic–polycitidilic acid (PIC) with different lengths (21) to selectively evaluate the role of MDA5 and RIG-I (Supplementary Material, Fig. S1A). The largest PIC (>2000 bp; PIC2) preferentially induced MDA5, whereas the PIC with <2000 bp (PIC1) induced mostly RIG-I (data not shown). Both forms of PIC induced a significant increase in apoptosis in INS-1E cells detected 12 h after exposure [Supplementary Material, Figs S1B and S2C; there was no increase in apoptosis at 6 h (data not shown)]. Transfection with respective PICs increased the expression of MDA5 (Fig. 1A and Supplementary Material, Fig. S2A) and RIG-I (Fig. 1B) by 23- and 40-fold after 24 h. To evaluate the functional role of MDA5 and RIG-I, INS-1E cells were pre-treated either with an inactive siRNA (siControl) or with siRNAs targeting MDA5 (siMDA5) or RIG-I (siRIG-I). The siControl has been validated by previous studies from our group (18,24), and a recent array analysis indicated that it does not modify the expression of MDA5, RIG-I or other genes related to β-cell function and survival (unpublished data). PIC-induced MDA5 or RIG-I expression was prevented by >80% by the specific siRNAs against MDA5 or RIG-I, but not by the siControl (Fig. 1A and B). To further confirm the efficiency of these KDs, we performed reporter assays using an IFN-β promoter reporter or a reporter containing multiple NF-κB-binding sites. Both reporters were activated by PIC, and MDA5 KD partially inhibited their activation (Fig. 1C and D). Similar results were observed with siRNA-mediated inhibition of RIG-I, with the difference that siRIG-I did not prevent NF-κB induction (Supplementary Material, Fig. S3A and B). We additionally studied the effect of knocking down these helicases in PIC-induced apoptosis. Both PICs induced apoptosis, but neither MDA5 nor RIG-I silencing prevented cell death 24 h after PIC transfection (Fig. 1E and F). To exclude that this absence of protection was due to redundancy between the two helicases (i.e. MDA5 would compensate for the absence of RIG-I in the recognition of dsRNA and vice versa), we performed double-KD of MDA5 and RIG-I but this also failed to prevent PIC-induced apoptosis (data not shown). This suggests that both helicases are not crucial regulators of intracellular PIC-induced β-cell death.

One of the mechanism by which viruses may contribute to T1D is by promoting the expression of pro-inflammatory mediators (1,25). Against this background, we next examined the effect of MDA5 and RIG-I KD on PIC-induced expression of cytokines and chemokines in INS-1E cells. There was a marked induction of the expression of mRNAs encoding for the cytokines IFN-β and interleukin (IL) 15 and the chemokines CCL2, CCL5 and CXCL10 after 24 h of PIC exposure compared with untreated cells (Fig. 2A and B). MDA5 KD significantly reduced PIC-induced expression of all cytokines and chemokines studied, whereas RIG-I KD decreased PIC-induced expression of IFN-β, CCL2 and CXCL10, but not IL15 or CCL5 (Fig. 2B). A similar pattern was observed

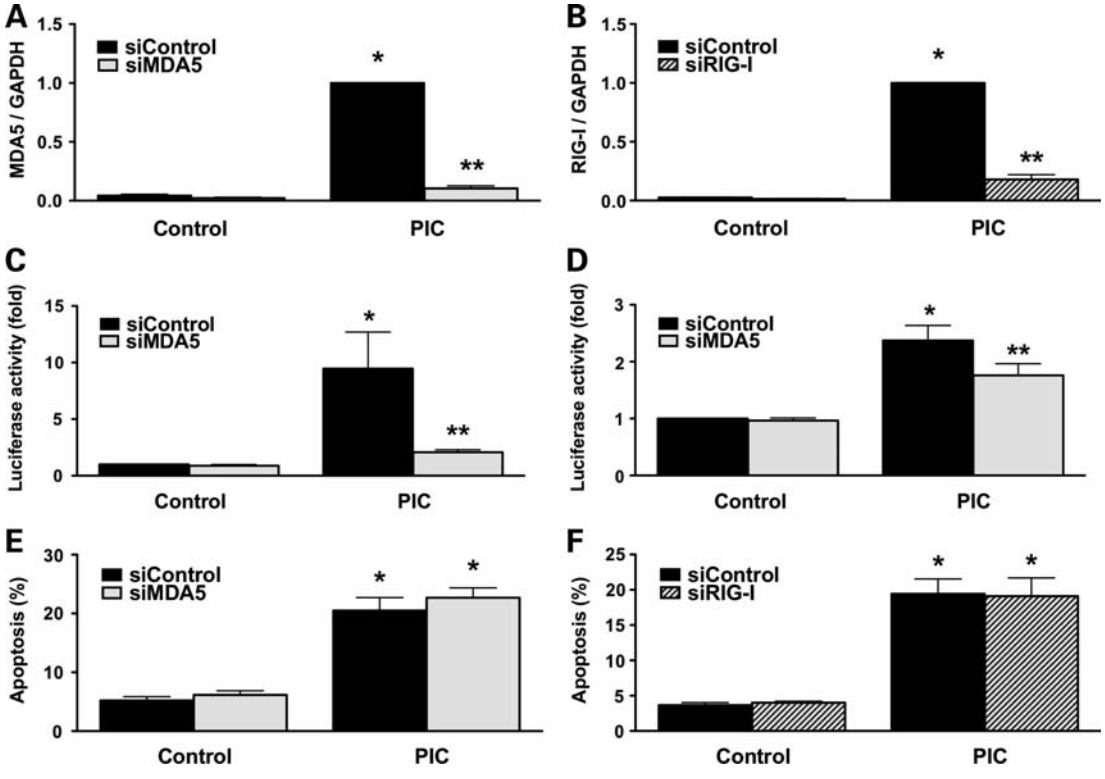

Figure 1. siMDA5 and siRIG-I prevent PIC-induced activation of interferon-β promoter and NF-κB reporter, but do not modify INS-1E cell death. INS-1E cells were transfected with either an siControl (black bars), or siMDA5 (grey bars) or siRIG-I (striped bars). After 24 h of recovery, cells were left untreated or transfected with PIC1 (<2000 bp, used for siRIG-I experiments) or PIC2 (>2000 bp, siMDA5 experiments) as described in Materials and Methods. (**A** and **B**) MDA5 and RIG-I mRNA expression was assayed by real-time RT–PCR and corrected for the housekeeping gene GAPDH. Results are mean ± SEM of three to four independent experiments; $^{*}P < 0.01$ versus siControl; $^{**}P < 0.01$ versus siControl+PIC; ANOVA. (**C** and **D**) Twenty-four hours after transfection with siMDA5, cells were transfected with IFN-β (C) or NF-κB (D) reporters plus a pRL-CMV plasmid (used as internal control); cells were then exposed to internal PIC for 24 h and luciferase activity was assayed. The values were corrected for the activity of the internal control, pRL-CMV, and are presented as fold induction in relation to siControl. Results are mean ± SEM of 5–11 independent experiments; $^{*}P < 0.05$ versus siControl, $^{**}P < 0.05$ versus siControl+PIC, paired *t*-test. (**E** and **F**) INS-1E cells were transfected with siMDA5 or siRIG-I and then PIC as described earlier. Apoptosis was evaluated using HO/PI staining. Results are mean ± SEM of five independent experiments; $^{*}P < 0.01$ versus siControl; ANOVA.

at the protein level for CCL5 (Fig. 3). These observations were confirmed by the use of a second siRNA against MDA5 (Supplementary Material, Fig. S4).

Primary β-cells, different from INS-1E cells, express an additional receptor for dsRNA, namely the toll-like receptor 3 (TLR3) (26), potentially creating a more complex response to dsRNA. Similar to INS-1E cells, PIC induced a marked increase of MDA5 and RIG-I expression in primary β-cells. siMDA5 and siRIG-I prevented PIC-induced MDA5 and RIG-I mRNA expression by >80% (Fig. 4A and B). In line with the observations made in INS-1E cells, KD of both helicases did not modify β-cells apoptosis after PIC exposure (Fig. 4C and D). Expression of mRNAs encoding for IFN-β, IL15, CCL2, CCL5 and CXCL10 was also induced in β-cells, and this was partially prevented by KD of MDA5, except for IL15 (Fig. 4E). On the other hand, RIG-I KD failed to prevent PIC-induced expression of the cytokines and chemokines studied (Fig. 4F). These findings were confirmed at the protein level for the chemokine CCL5 (Supplementary Material, Fig. S5A and B), and as whole they suggest that MDA5 plays a more important role than RIG-I for the recognition of intracellular dsRNA in β-cells.

Mechanisms contributing for PIC-induced apoptosis in insulin-producing cells

Since KD of the helicases MDA5 and RIG-I failed to prevent β-cell apoptosis induced by intracellular dsRNA (Figs 1 and 4), we next examined the putative role of the dsRNA-dependent protein kinase (PKR) in this process. PKR was previously shown to contribute for external dsRNA+-IFN-γ-induced apoptosis in mouse islet cells (27). PIC induced a 20-fold increase in PKR expression, which was prevented by >80% using a specific siRNA against PKR (Supplementary Material, Fig. S6A). PKR KD, however, did not prevent PIC-induced apoptosis (Supplementary Material, Fig. S6B) or the induction of cytokines or chemokines (Supplementary Material, Fig. S6C) in primary β-cells. Unexpectedly, PKR KD augmented PIC-induced CCL2 expression (Supplementary Material, Fig. S5C).

These, and our previous observations, suggest that the pattern recognition receptors TLR3 (28), MDA5, RIG-I and PKR (present data) are not the main mediators of internal dsRNA-induced β-cell apoptosis. Of note, we have previously shown that TLR-3 plays a key role for β-cell apoptosis triggered by external PIC+IFN-γ (26). To further clarify

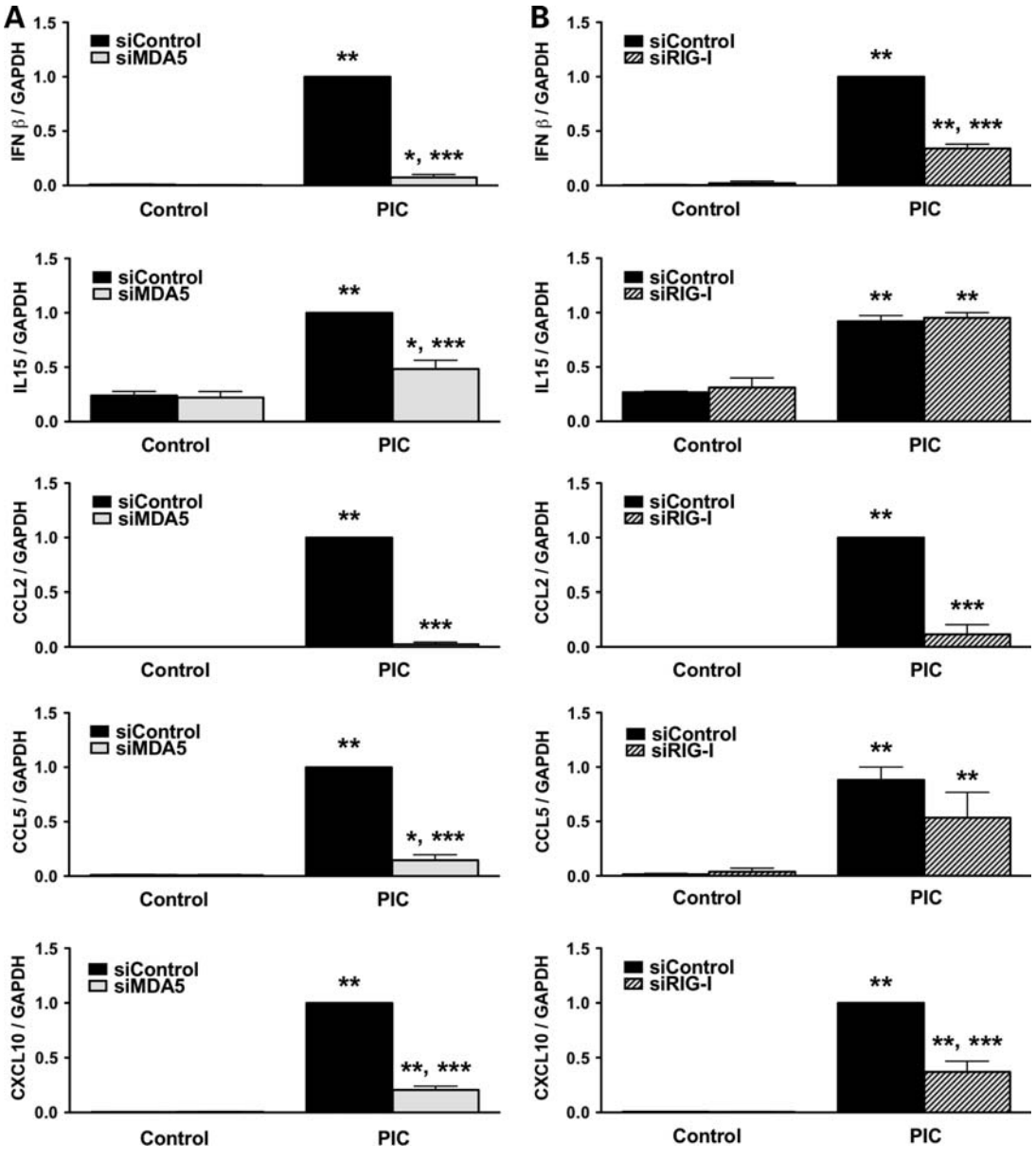

Figure 2. MDA5 or RIG-I KD partially prevents PIC-induced upregulation of mRNA expression of cytokines and chemokines in INS-1E cells. INS-1E cells were transfected with siControl (black bars), siMDA5 (grey bars) or siRIG-I (striped bars), and after recovery left untreated or exposed to different internal PICs (PIC1 for siRIG-I experiments and PIC2 for siMDA5 experiments) for 24 h. Poly(A)+ mRNA was extracted, assayed by real-time RT–PCR for rat IFN-β, IL15, CCL2, CCL5 and CXCL10 mRNAs and corrected for the housekeeping gene GAPDH. Results are mean ± SEM of three to four independent experiments; $^{*}P < 0.05$ versus siControl, $^{**}P < 0.01$ versus siControl, $^{***}P < 0.01$ versus siControl+PIC, ANOVA.

the mechanisms involved in internal PIC-induced β-cell apoptosis, we next examined the downstream apoptosis regulators Jun N-terminal kinase (JNK)/JunB (29). PIC transfection induced concomitant JNK phosphorylation and decreased JunB protein expression (Fig. 5A and B). To investigate whether the decline in JunB expression was secondary to JNK activation, as previously shown in other experimental models (30), we used the JNK inhibitor SP600125. SP600125 decreased PIC-induced JNK phosphorylation and prevented PIC-induced decline in JunB protein expression (Fig. 5C). We have previously observed a similar phenomenon in β-cells treated with cytokines (29), suggesting that JunB degradation is indeed secondary to JNK activation in β-cells.

JNK activation is one of the mechanisms by which dsRNA induces apoptosis in other cell types (31). In line with these observations, two different JNK inhibitors, namely the chemical inhibitor SP600125 and the D-TAT JNKi peptide (32), induced a partial protection against PIC-induced apoptosis in INS-1E cells (Fig. 5D and E). JunB reduces cytokine-mediated endoplasmic reticulum (ER) stress and prevents apoptosis in pancreatic β-cells (29); since one of the effects of JNK activation is to reduce JunB protein levels (Fig. 5B and C), we next examined whether decreased JunB expression indeed contributes for PIC-induced apoptosis. For this purpose, JunB was knocked down by the use of a specific and previously validated siRNA (29). KD of JunB (Fig. 6A and B)

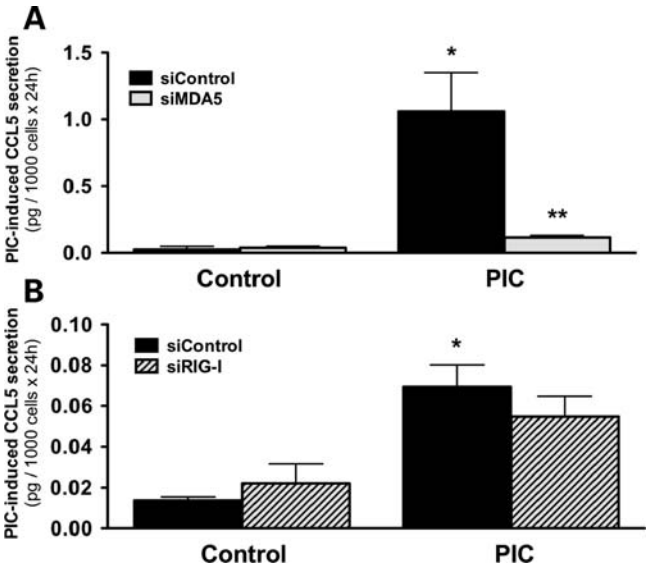

Figure 3. MDA5 but not RIG-I KD prevents PIC-induced CCL5 secretion in INS-1E cells. INS-1E cells were transfected with siControl (black bars), siMDA5 (**A**, grey bars) or siRIG-I (**B**, striped bars) and then treated as described in Figure 2. CCL5 secretion was evaluated in the supernatant by ELISA 24 h after internal PIC exposure (PIC1 for siRIG-I experiments and PIC2 for siMDA5 experiments), as described in Materials and Methods. Results are mean ± SEM of three to four independent experiments; $^{*}P < 0.01$ versus siControl, $^{**}P < 0.01$ versus siControl+PIC, ANOVA.

increased caspase-3 cleavage (Supplementary Material, Fig. S7) and aggravated PIC-induced apoptosis in both INS-1E cells and autofluorescence-activated cell sorting (FACS)-purified β-cells (Fig. 6C and D). These results were confirmed with a second siRNA against JunB (data not shown). To determine whether increase in cell death was NO- and ER stress dependent, we evaluated the mRNA expression of XBP1s, CHOP and iNOS, and nitrite production. There was no difference between expression of these markers in cells treated with siJunB or siControl plus PIC (data not shown), suggesting that JunB protects β-cells against PIC-induced apoptosis by an ER stress- and NO-independent mechanism that remains to be clarified.

JunB is one of the subunits of the transcription factor activator protein-1 (AP-1). Besides its role in the process of apoptosis, AP-1 also regulates the expression of cytokines and chemokines in other cell types (33). We did not, however, observe any effect of JunB KD on PIC-induced expression of cytokines and chemokines (Supplementary Material, Fig. S8).

PTPN2 modulates PIC-induced β-cell apoptosis

PIC induced expression of PTPN2 at both mRNA and protein levels in INS-1E cells and FACS-purified rat β-cells (Fig. 7 A, B and E). The upregulation of PTPN2 mRNA was significant after 6 h of PIC transfection and remained increased up to 24 h (Supplementary Material, Fig. S2B). A previously validated siRNA against PTPN2 (18) decreased its basal mRNA expression by >60 and >80% in INS-1E and primary β-cells, respectively, and blocked PTPN2 induction after PIC exposure in both cell types (Fig. 7A and B). Of relevance, PTPN2 KD sensitized INS-1E cells (Fig. 7C and F) and primary β-cells (Fig. 7D) to PIC-induced apoptosis, indicating a potential modulatory function for this candidate gene in β-cell death during viral infection. On the other hand, PTPN2 had no or mild effect on the expression of chemokines and cytokines following transfection with PIC (Supplementary Material, Fig. S9). These results were confirmed with a second siRNA against PTPN2 (data not shown).

DISCUSSION

We presently show that two candidate genes for T1D, namely MDA5 and PTPN2, modulate β-cell responses to dsRNA, a by-product of viral infection. The findings suggest that MDA5 controls the local expression of cytokines and chemokines during a viral infection, whereas PTPN2 might protect β-cells from virus-induced apoptosis.

Viruses are one of the putative environmental factors associated with T1D in genetically susceptible individuals (1,2). As the first line of defence against pathogens, non-immune cells (present data) and cells from the innate immune system detect viral infections by pattern-recognition receptors (PRRs) that identify pathogen-associated molecular patterns, such as dsRNAs. Both RNA and DNA viruses produce dsRNA as an intermediate product of their replication. Human and rodent pancreatic islets express PRRs for dsRNA, namely TLR3, RIG-I and MDA5 (17,26,34,35; present data). Studies in knockout animals demonstrated that the helicases RIG-I and MDA5 recognize different types of viruses, with MDA5 acting as the main receptor for cytoplasmic dsRNA in astrocytes (36) and β-cells (present data). Of particular relevance in the context of T1D, MDA5 recognizes members of the picornavirus family, including Coxsackievirus B4, Encephalomyocarditis virus and Theiler's virus (37). Coxsackievirus B4 was recently isolated from the pancreas of patients with new onset T1D (38,39), and polymorphisms leading to loss of function of MDA5 confer resistance against T1D (20).

To study the mechanisms by which changes in MDA5 function may contribute to insulitis and β-cell apoptosis, we combined the use of siRNA-mediated inhibition of MDA5 with transfection of β-cells with synthetic dsRNA (PIC). Decrease in the expression of MDA5 or the related helicase RIG-I did not protect β-cells against PIC-induced apoptosis, but KD of MDA5 inhibited expression of the inflammatory mediators CXCL10, CCL2, CCL5, IFN-β and IL15. The chemokine CXCL10 attracts monocytes, T lymphocytes and natural killer (NK) cells (40), and islet-specific expression of CXCL10 in a mouse model of autoimmune diabetes caused by viruses [rat insulin promotor (RIP)-LMCV] accelerates autoimmunity by enhancing the migration of antigen-specific lymphocytes (41). On the other hand, neutralization of CXCL10 (42) or its receptor (CXCR3) (43) prevents autoimmune disease in the same mouse model (RIP-LCMV). CCL2 attracts monocytes and T lymphocytes and may thus contribute to the migration of mononuclear cells to pancreatic islets in early T1D (44). CCL5 attracts activated T-cells, dendritic cells and monocytes during inflammation and early immune responses (45). Individuals with specific polymorphisms in this gene present lower

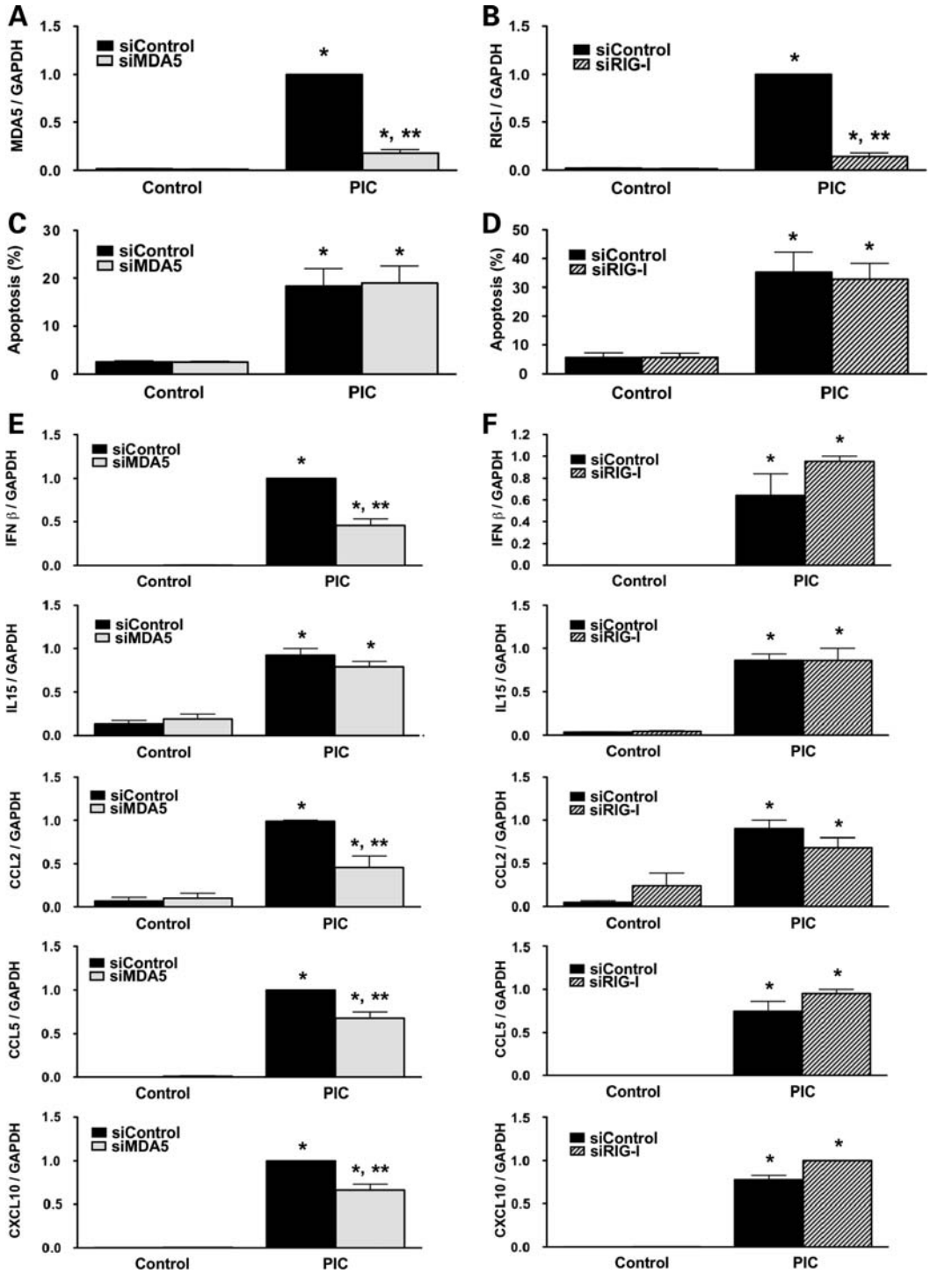

Figure 4. siMDA5 or siRIG-I prevent PIC-induced upregulation of cytokines and chemokines, but not apoptosis, in primary rat β-cells. FACS-purified rat β-cells were transfected with siControl (black bars), siMDA5 (grey bars) or siRIG-I (striped bars) as described in Materials and Methods. After 48 h of recovery, cells were left untreated or transfected with different PICs (PIC1 for siRIG-I experiments and PIC2 for siMDA5 experiments) for 24 h. (**A** and **B**) MDA5 and RIG-I mRNA expression was assayed by real-time RT–PCR and corrected for the housekeeping gene GAPDH. Results are mean ± SEM of four to six independent experiments; $^{*}P < 0.01$ versus siControl; $^{**}P < 0.01$ versus siControl+PIC, ANOVA. (**C** and **D**) Apoptosis was evaluated 24 h after PIC exposure using HO/PI staining. Results are mean ± SEM of three to five independent experiments; $^{*}P < 0.01$ versus siControl, ANOVA. (**E** and **F**) INF-β, IL15, CCL2, CCL5 and CXCL10 mRNA expression were assayed by real-time RT–PCR and corrected for the housekeeping gene GAPDH. Results are mean ± SEM of four to six independent experiments; $^{*}P < 0.01$ versus siControl; $^{**}P < 0.01$ versus siControl+PIC, ANOVA.

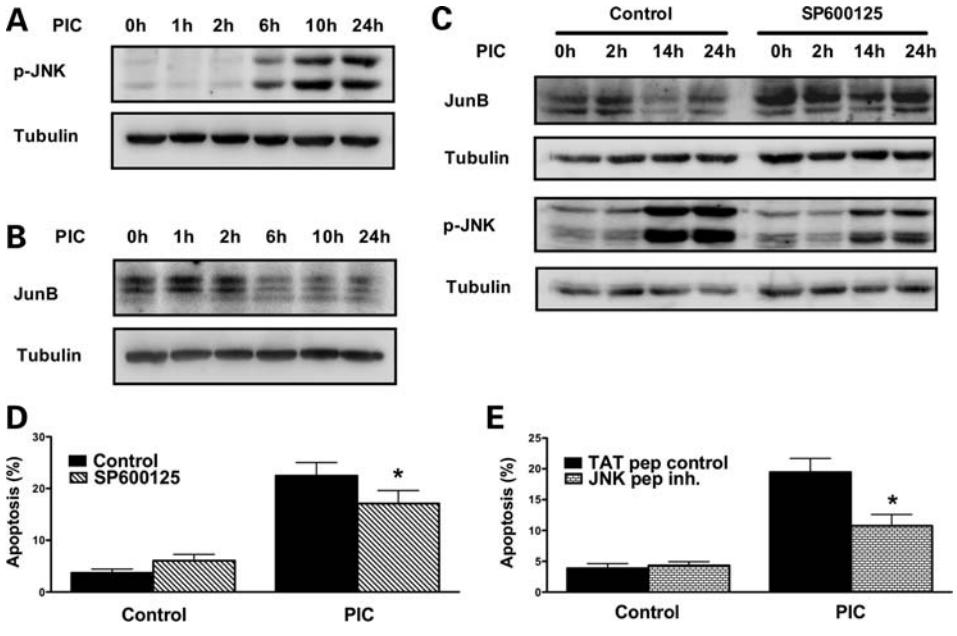

Figure 5. Inhibition of JNK activation partially prevents PIC-induced JunB degradation and apoptosis in INS-1E cells. INS-1E cells were transfected with PIC1 for the indicated time points. (**A** and **B**) Expression of phospho-JNK, JunB and α-tubulin were evaluated by western blot. Pictures are representative of three independent experiments. (**C**) INS-1E cells were pre-treated with 10 μM of the JNK inhibitor SP600125 for 4 h and then transfected with PIC before being retrieved for protein extraction at the indicated time points; SP600125 was maintained in the medium following PIC treatment. Phospho-JNK, JunB and α-tubulin protein were evaluated by western blot. Pictures are representative of three independent experiments. (**D** and **E**) INS-1E cells were treated with the JNK chemical inhibitor SP600125 (D) or the JNK peptide inhibitor (E) for 4 h, and then transfected with PIC plus SP600125 or JNK peptide inhibitor. Apoptosis was evaluated 24 h after PIC exposure using HO/PI staining. Results are mean ± SEM of four independent experiments; $^{*}P < 0.05$ versus siControl+PIC, paired *t*-test.

serum levels of CCL5 and may be protected against T1D (46). IL15 enhances the cytolytic function of T and NK cells and induces IFN-γ production by NK cells; it also functions as a potent growth factor of T-, B-, and NK cells (47). Studies in NOD mice have demonstrated elevated expression of CXCL10, CCL2 and IL15 mRNAs and/or protein in pancreatic islets during the prediabetic stage (1,48,49). The type I interferon IFN-β is a cytokine produced by most cells during viral infections, promoting the expression of diverse genes relevant for the antiviral response in target cells; and it also modulates the adaptive immune system by activating dendritic cells, T- and B-cells (23,50). Of relevance, type I IFNs have been detected in the islets of patients with recent onset T1D (51,52), and transgenic expression of IFN-β in mouse β-cells leads to diabetes (53). The present observation that MDA5 modulates dsRNA-induced expression of these cytokines and chemokines in INS-1E cells and primary β-cells reinforces the hypothesis that this candidate gene has a key role in the regulation of local islet inflammation during viral infection.

Since there was no protection against PIC-induced apoptosis after MDA5 and/or RIG-I KD in our model, we searched for alternative pathways that may explain the pro-apoptotic effects of intracellular PIC in β-cells. The main finding was that JNK, an activator of the transcription factor AP-1, contributes for apoptosis after PIC transfection, an effect mediated at least in part via JunB protein degradation. The role of JNK activation in PIC-induced apoptosis has been shown previously in HeLa cells (31), but to our knowledge there are no previous reports on the putative role for JunB degradation on this process. JunB degradation also plays a role in cytokine-induced β-cell apoptosis (29), emphasizing the relevant role of JunB in preserving β-cell viability. In the case of cytokine-triggered apoptosis, however, part of the protective effects of JunB are due to inhibition of NO formation and induction of ER stress (29). NO and ER stress do not play a major role for intracellular PIC-induced β-cell death (data not shown), suggesting that JunB acts by another mechanism in this context. The nature of this mechanism remains to be clarified.

As mentioned earlier, type I IFNs are important cytokines for the host immune response against viral infections (23). In addition to antiviral properties, these cytokines have the potential to induce apoptosis and/or promote systemic autoimmunity (50). To limit these effects of IFNs, organisms have developed several negative regulators of the IFN responses (54). One of these regulators is the phosphatase PTPN2, which prevents sustained IFN signalling by dephosphorylating JAKs and STATs (55). We have recently shown that KD of PTPN2 in pancreatic β-cells exposed to IFN-γ augments STAT1 signalling and increases apoptosis (18). In line with these findings, STAT1 knockout prevents both IL-1β+IFN-γ (56) and external dsRNA plus IFN-γ-induced apoptosis in β-cells (26) and abolishes the pro-apoptotic effect of PTPN2 KD in the presence of IFN-γ (18). We presently demonstrate that, similar to cytokines, PTPN2 KD exacerbates dsRNA-induced apoptosis, suggesting a potential interaction between an environmental agent (dsRNA, a viral by product) and a candidate gene for T1D (PTPN2). This type of interaction may explain why viral infections only cause diabetes in some individuals or mouse strains that overreact to the viral challenge. In line with this hypothesis, PIC only induces diabetes in C57BL/6 mice when

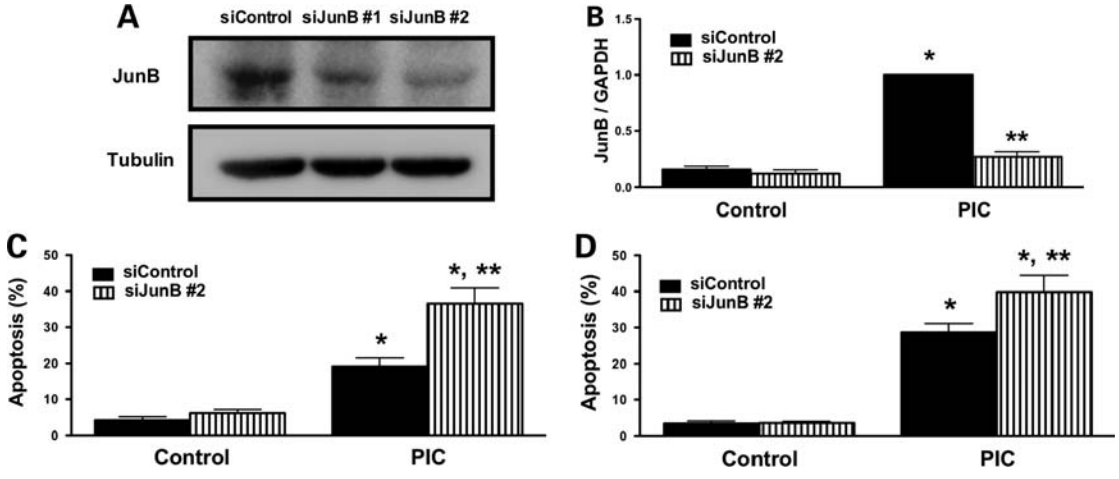

Figure 6. JunB KD augments PIC-induced β-cell death. INS-1E cells (**A**–**C**) and primary rat β-cells (**D**) were transfected with siControl, siJunB 1 or siJunB 2 and then treated as described in Figures 2 and 4. (A) JunB protein expression was evaluated after transfection with two specific siRNAs. Pictures are representative of three independent experiments. (B) PIC-induced JunB mRNA expression was blocked by siRNA against JunB. Results are mean ± SEM of three to four independent experiments; $^{*}P < 0.01$ versus siControl, $^{**}P < 0.01$ versus siControl+PIC, ANOVA. (C and D) Apoptosis was evaluated in INS-1E cells (C) or FACS-purified rat β-cells (D) 24 h after PIC exposure using HO/PI staining. Results are mean ± SEM of four to six independent experiments; $^{*}P < 0.01$ versus siControl, $^{**}P < 0.01$ versus siControl+PIC, ANOVA.

these mice are backcrossed onto strains that express the co-stimulatory molecule B7.1 in the islets (57). The B7.1 mice produce higher levels of type I IFNs than wt C57BL/6, and administration of an antibody against type I IFN prevents diabetes (57). The protective effects of PTPN2 against PIC-induced apoptosis seems, however, to be transitory or insufficient, since 12 h after PIC transfection β-cells start to die independent of the continuous increase in PTPN2 expression. These results are in line with previous studies showing that cytokine-induced increase in protective molecules such as SOCS-3 (58) or JunB (29) is not sufficient to prevent β-cell death induced by protracted pro-apoptotic stimulus.

MDA5 and PTPN2 are expressed also in other tissues, including in cells from the immune system (18,19). T1D is a chronic autoimmune disease in which macrophages, NK and T-cells play a crucial role in disease development (1). Thus, different levels of MDA5 and PTPN2 expression in immune cells may also play a role in the triggering and progression of insulitis. The relative contribution of candidate gene expression in immune cells or β-cells needs to be clarified by future studies using tissue-specific knockout models.

In conclusion, we demonstrate that two candidate genes for T1D, namely MDA5 and PTPN2, may contribute to the pathogenesis of diabetes by modifying the β-cell responses to a viral infection. MDA5 potentially modulates the cross-talk between β-cells and the innate/adaptive immune system through the local production of cytokines and chemokines, whereas PTPN2 may protect β-cells against apoptosis due to negative feedback on IFN signalling. Single or, most probably, combined genetically determined changes in the expression of these and other relevant genes in β-cells and the immune system may lead to an 'unlucky genetic combination', which causes, for instance, an exaggerated inflammatory response to a β-cell viral infection. If this response is coupled to defective β-cell protective mechanisms, it may result in excessive and/or protracted β-cell death, leading to amplification of insulitis and eventually causing enough destruction of β-cells to trigger clinical diabetes.

MATERIALS AND METHODS

Culture of primary FACS-purified rat β-cells and INS-1E cells

Male Wistar rats (Charles River Laboratories, Brussels, Belgium) were housed and used according to the guidelines of the Belgian Regulations for Animal Care. Rat islets were isolated by collagenase digestion followed by hand-picking under a stereomicroscope. For β-cell isolation, islets were dispersed and β-cells purified by FACS (FACSAria, BD Bioscience, San Jose, CA, USA) (26,59). The preparations used in the present study contained 89.4 ± 1.2% β-cells ($n = 22$). Purified β-cells were cultured for 2 days in Ham's F-10 medium containing 10 mM glucose, 2 mM glutamine, 50 μM 3-isobutyl-L-methylxanthine, 5% heat-inactivated fetal bovine serum (FBS), 0.5% charcoal-absorbed bovine serum albumin (BSA Fraction V, Boehringer, Indianapolis, IN, USA), 50 U/ml penicillin and 50 μg/ml streptomycin before use in subsequent experiments (26,60). During dsRNA and siRNAs transfection, cells were cultured in the same medium but without antibiotics or BSA.

The rat insulin-producing INS-1E cell line (a kind gift from Dr C. Wollheim, Centre Medical Universitaire, Geneva, Switzerland) was cultured in RPMI 1640 GlutaMAX-I, 5% FBS, 10 mM HEPES, 1 mM Na-pyruvate and 50 μM 2-mercaptoethanol (61). INS-1E cells (passages 57–75) were plated for 48 h prior to transfection.

RNA interference

The following siRNAs were used in this study: Allstars Negative Control siRNA (Qiagen, Venlo, Netherlands),

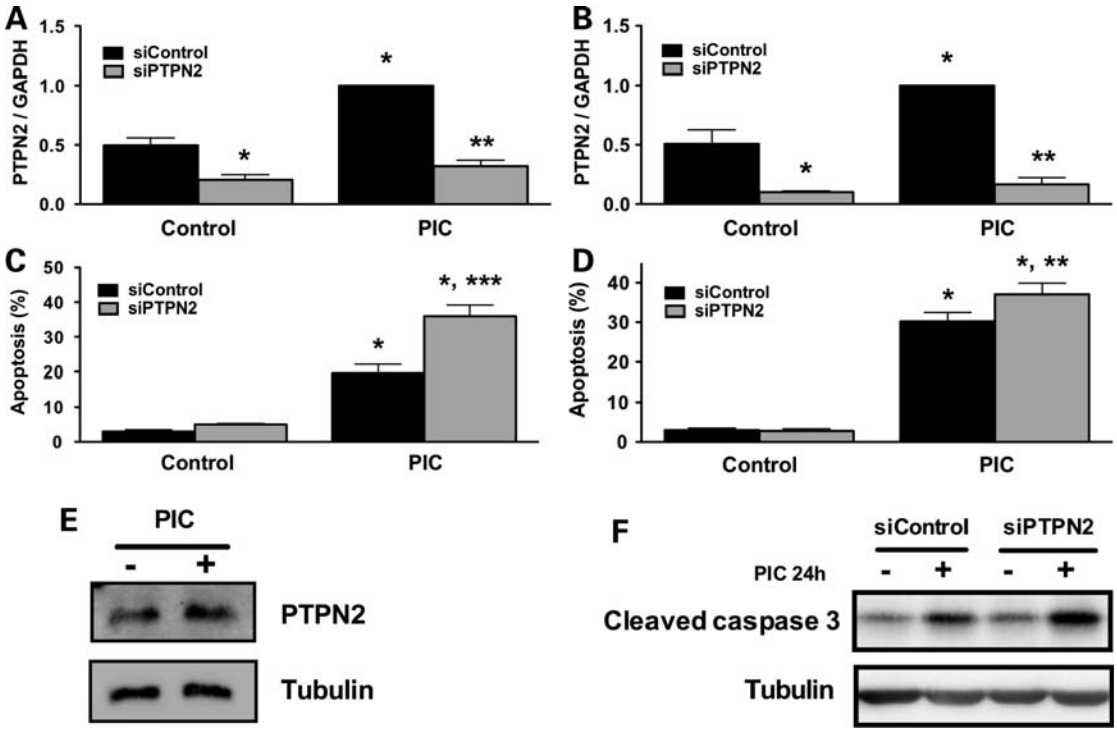

Figure 7. KD of PTPN2 sensitizes INS-1E cells and primary β-cells to PIC-induced apoptosis. INS-1E cells (**A**) or FACS-purified rat β-cells (**B**) were transfected with siControl (black bars) or siPTPN2 (grey bars) and 48 h after recovery exposed to PIC1 for 24 h. (A and B) PTPN2 mRNA expression was evaluated by real-time RT–PCR and corrected for the housekeeping gene GAPDH. Results are mean ± SEM of three to five independent experiments; $^{*}P < 0.01$ versus siControl, $^{**}P < 0.01$ versus siControl+PIC, ANOVA. (**C** and **D**) INS-1E cells (C) or primary β-cells (D) were transfected with siControl or siPTPN2 and 48 h after recovery exposed to internal PIC1 for 24 h. Apoptosis was evaluated using HO/PI staining. Results are mean ± SEM of six independent experiments; $^{*}P < 0.01$ versus siControl, $^{**}P < 0.05$ versus siControl+PIC, $^{***}P < 0.01$ versus siControl+PIC, ANOVA. (**E**) PTPN2 and α-tubulin protein expression was assessed by western blot in FACS-purified rat β-cells exposed to internal PIC1 for 24 h; the picture is representative of two independent experiments. (**F**) INS-1E cells were transfected with control siRNA, or with an siRNA targeting PTPN2 as described in Materials and Methods, and 48 h after recovery exposed to internal PIC1 for 24 h. Cleavage of caspase-3 was observed by western blot. The picture is representative of two independent experiments.

ON-TARGETplus rat MDA5 SMARTpool® (siMDA5 1; Thermo Scientific, Chicago, IL, USA), Silencer® Select Predesigned siRNA rat MDA5 (siMDA5 2; Applied Biosystems, Austin, TX, USA), Silencer® Select Custom Designed siRNA rat RIG-I (Applied Biosystems), ON-TARGETplus rat PTPN2 SMARTpool® (Thermo Scientific), Silencer® Select Predesigned siRNA rat PTPN2 (Applied Biosystems), rat JunB 1 and rat JunB 2 (Invitrogen, Carlsbad, CA, USA) and ON-TARGETplus rat PKR SMARTpool® (Thermo Scientific) (sequences are provided in Supplementary Material, Table S1). The optimal settings for the transfection of siRNAs in both INS-1E cells and primary FACS-purified rat β-cells were first established by using an FITC-coupled siRNA (siGLO Green Transfection Indicator, Thermo Scientific), and the transfection procedure performed using DharmaFECT 1 (Thermo Scientific) as described (18). The concentration of 30 nM was selected after dose–response studies (18; data not shown). Afterwards, cells were cultured for a 24–48 h recovery period and subsequently transfected with the synthetic dsRNA, PIC, as described in the following.

dsRNA transfection and JNK inhibition

The synthetic dsRNAs, PIC, were from Sigma-Aldrich (PIC1; St Louis, MO, USA) and Invivogen (PIC2; San Diego, CA, USA) and used at the final concentration of 1 μg/ml (28). All experiments were performed with intracellular PIC, obtained via cell transfection. For PIC transfection, the same conditions described for siRNA were utilized, except that the DharmaFECT:PIC ratio used was 0.8 and 1.25 μl of DharmaFECT to 5 μg of PIC for INS-1E cells and primary β-cells, respectively. Since it was demonstrated that helicases recognize dsRNA molecules on the basis of their length (21), a PIC with >2000 bp (PIC2) was used for experiments targeting MDA5, whereas all other experiments were done with PIC1, with <2000 bp, as in our previous studies (26,28).

The JNK inhibitor SP600125 (Sigma-Aldrich) was dissolved in DMSO and used at a concentration of 10 μM (24). The peptide JNK inhibitor D-TAT-JNKi (a kind gift of C. Bonny and M. Mathieu; XigenPharma, Lausanne, Switzerland) and the control peptide D-TAT were dissolved in culture medium and used at the concentration of 10 μM (32). Both inhibitors were added to the cell culture 4 h before PIC transfection and kept in the medium during PIC exposure.

Assessment of cell viability

The percentage of viable, apoptotic and necrotic cells was determined following 15 min of incubation with the DNA-binding dyes propidium iodide (PI, 5 μg/ml, Sigma) and Hoechst 33342 (HO, 5 μg/ml, Sigma). This method is

quantitative and has been validated for use in pancreatic β-cells and INS-1E cells by systematic comparison with electron microscopy, caspase-3 activation and DNA laddering (14,18,24,26,62). A minimum of 500 cells was counted in each experimental condition. Viability was evaluated by two independent observers, one of them being unaware of sample identity. The agreement between findings obtained by the two observers was >90%. Results are expressed as percentage of apoptosis and were calculated as number of apoptotic cells/total number of cells × 100. In some experiments, the presence of apoptosis was confirmed by western blot analysis of activated (cleaved) caspase-3 (see what follows).

mRNA extraction and real-time PCR

Poly(A)+ mRNA was isolated from INS-1E cells and rat primary β-cells using the Dynabeads mRNA DIRECT™ kit (Invitrogen, Paisley, UK), and reverse-transcribed as described previously (14,26,44). The real-time PCR amplification reaction was done as described (26,44), using SYBR Green and compared with a standard curve (63). Expression values were then corrected for the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and normalized by the highest value of each experiment considered 1; PIC treatment does not modify GAPDH expression in insulin-producing cells (64,65). The primer sequences for rat PTPN2, MDA5, RIG-I, PKR, INF-β, IL15, CCL2, CCL5, CXCL10, CHOP, XBP1-s, iNOS, JunB, GAPDH are described in Supplementary Material, Table S2. The cytokines and chemokines to be studied were selected on the basis of our previous array analysis (15) and studies focused (26,28,64,65) as representative of β-cell inflammatory responses to dsRNA.

Promoter studies

A total of 10^5 INS-1E cells were plated in 24-well plates and transfected with siRNAs against MDA5, RIG-I or control as described earlier. After 24 h of recovery, plasmid constructs containing the firefly luciferase gene under the control of either multiple copies of the NF-κB consensus sequence (BD Biosciences Clontech, Mountain View, CA USA) or the mouse IFN-β promoter (66) were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer instructions. Twelve hours later, cells were exposed overnight to internal PIC (1 μg/ml). Luciferase activities were assayed 24 h after PIC treatment using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) (26,65). Test values were corrected for the luciferase activity of the internal control plasmid, pRL-CMV, and shown as fold induction compared with siControl.

Assessment of CCL5 protein and nitrite production

After 24 h of PIC transfection, cell supernatants were retrieved and rat CCL5 was measured by enzyme-linked immunosorbent assay (Quantikine ELISA kit, R&D Systems, Minneapolis, MN, USA).

In some experiments, culture supernatants were also collected for nitrite determination (nitrite is a stable product of nitric oxide oxidation) at OD_{540nm} using the Griess reagent (67).

Western blot analysis

Cells were washed once with cold PBS and directly lysed with either Laemmli buffer (25 mM Tris–HCl, pH 6.8, 10% glycerol, 1% SDS, 350 mM 2-mercaptoethanol, 175 mM dithiothreitol, 0.005% bromophenol blue completed by a protease inhibitor cocktail—Roche Diagnostics, Vilvoorde, Belgium) or phospho lysis buffer (1% NP40, 25 mM Hepes, pH 7.8, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 50 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF and protease inhibitor cocktail). Lysates were then resolved by 8–12% SDS–PAGE and transferred to a nitrocellulose membrane. Immunoblot analysis was performed with antibodies targeting PTPN2 (R&D Systems), JunB, dsRNA-dependent protein kinase (PKR) (Santa Cruz Biotechnology, CA, USA), phospho-JNK, cleaved caspase-3 (Cell Signaling, Danvers, MA, USA) or α-tubulin (Sigma, Bornem, Belgium), used as the housekeeping protein. Horseradish peroxidase-conjugated donkey anti-rabbit or anti-mouse IgG was used as a secondary antibody (Lucron Bioproducts, De Pinte, Belgium). Immunoreactive bands were revealed using the SuperSignal West Femto chemiluminescent substrate (Thermo Scientific), detected using an LAS-3000 CCD camera and quantified with the Aida Analysis software (Fujifilm).

Statistical analysis

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

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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REFERENCES

1. Eizirik, D.L., Colli, M.L. and Ortis, F. (2009) The role of inflammation in insulitis and β-cell loss in type 1 diabetes. *Nat. Rev. Endocrinol.*, **5**, 219–226.
2. Filippi, C.M. and von Herrath, M.G. (2008) Viral trigger for type 1 diabetes: pros and cons. *Diabetes*, **57**, 2863–2871.
3. Harrison, L.C., Honeyman, M.C., Morahan, G., Wentworth, J.M., Elkassaby, S., Colman, P.G. and Fourlanos, S. (2008) Type 1 diabetes: lessons for other autoimmune diseases? *J. Autoimmun.*, **31**, 306–310.
4. Peng, H. and Hagopian, W. (2006) Environmental factors in the development of type 1 diabetes. *Rev. Endocr. Metab. Disord.*, **7**, 149–162.
5. Hardy, J. and Singleton, A. (2009) Genomewide association studies and human disease. *N. Engl. J. Med.*, **360**, 1759–1768.
6. Klareskog, L., Catrina, A.I. and Paget, S. (2009) Rheumatoid arthritis. *Lancet*, **373**, 659–672.
7. Lempainen, J., Vaarala, O., Makela, M., Veijola, R., Simell, O., Knip, M., Hermann, R. and Ilonen, J. (2009) Interplay between PTPN22 C1858T polymorphism and cow's milk formula exposure in type 1 diabetes. *J. Autoimmun.*, **33**, 155–164.
8. Concannon, P., Rich, S.S. and Nepom, G.T. (2009) Genetics of type 1A diabetes. *N. Engl. J. Med.*, **360**, 1646–1654.
9. Burton, P.R., Clayton, D.G., Cardon, L.R., Craddock, N., Deloukas, P., Duncanson, A., Kwiatkowski, D.P., McCarthy, M.I., Ouwehand, W.H., Samani, N.J. *et al.* (2007) Association scan of 14,500 nonsynonymous SNPs in four diseases identifies autoimmunity variants. *Nat. Genet.*, **39**, 1329–1337.
10. Cooper, J.D., Smyth, D.J., Smiles, A.M., Plagnol, V., Walker, N.M., Allen, J.E., Downes, K., Barrett, J.C., Healy, B.C., Mychaleckyj, J.C. *et al.* (2008) Meta-analysis of genome-wide association study data identifies additional type 1 diabetes risk loci. *Nat. Genet.*, **40**, 1399–1401.
11. Smyth, D.J., Cooper, J.D., Bailey, R., Field, S., Burren, O., Smink, L.J., Guja, C., Ionescu-Tirgoviste, C., Widmer, B., Dunger, D.B. *et al.* (2006) A genome-wide association study of nonsynonymous SNPs identifies a type 1 diabetes locus in the interferon-induced helicase (IFIH1) region. *Nat. Genet.*, **38**, 617–619.
12. Todd, J.A., Walker, N.M., Cooper, J.D., Smyth, D.J., Downes, K., Plagnol, V., Bailey, R., Nejentsev, S., Field, S.F., Payne, F. *et al.* (2007) Robust associations of four new chromosome regions from genome-wide analyses of type 1 diabetes. *Nat. Genet.*, **39**, 857–864.
13. Cardozo, A.K., Kruhoffer, M., Leeman, R., Orntoft, T. and Eizirik, D.L. (2001) Identification of novel cytokine-induced genes in pancreatic β-cells by high-density oligonucleotide arrays. *Diabetes*, **50**, 909–920.
14. Kutlu, B., Cardozo, A.K., Darville, M.I., Kruhoffer, M., Magnusson, N., Orntoft, T. and Eizirik, D.L. (2003) Discovery of gene networks regulating cytokine-induced dysfunction and apoptosis in insulin-producing INS-1 cells. *Diabetes*, **52**, 2701–2719.
15. Rasschaert, J., Liu, D., Kutlu, B., Cardozo, A.K., Kruhoffer, M., Orntoft, T.F. and Eizirik, D.L. (2003) Global profiling of double stranded RNA- and IFN-γ-induced genes in rat pancreatic β-cells. *Diabetologia*, **46**, 1641–1657.
16. Magnusson, N.E., Cardozo, A.K., Kruhoffer, M., Eizirik, D.L., Orntoft, T.F. and Jensen, J.L. (2005) Construction and validation of the APOCHIP, a spotted oligo-microarray for the study of β-cell apoptosis. *BMC Bioinformatics*, **6**, 311.
17. Ylipaasto, P., Kutlu, B., Rasilainen, S., Rasschaert, J., Salmela, K., Teerijoki, H., Korsgren, O., Lahesmaa, R., Hovi, T., Eizirik, D.L. *et al.* (2005) Global profiling of coxsackievirus- and cytokine-induced gene expression in human pancreatic islets. *Diabetologia*, **48**, 1510–1522.
18. Moore, F., Colli, M.L., Cnop, M., Esteve, M.I., Cardozo, A.K., Cunha, D.A., Bugliani, M., Marchetti, P. and Eizirik, D.L. (2009) PTPN2, a candidate gene for type 1 diabetes, modulates interferon-γ-induced pancreatic β-cell apoptosis. *Diabetes*, **58**, 1283–1291.
19. Kumar, H., Kawai, T. and Akira, S. (2009) Pathogen recognition in the innate immune response. *Biochem. J.*, **420**, 1–16.
20. Nejentsev, S., Walker, N., Riches, D., Egholm, M. and Todd, J.A. (2009) Rare variants of IFIH1, a gene implicated in antiviral responses, protect against type 1 diabetes. *Science*, **324**, 387–389.
21. Kato, H., Takeuchi, O., Mikamo-Satoh, E., Hirai, R., Kawai, T., Matsushita, K., Hiiragi, A., Dermody, T.S., Fujita, T. and Akira, S. (2008) Length-dependent recognition of double-stranded ribonucleic acids by retinoic acid-inducible gene-I and melanoma differentiation-associated gene 5. *J. Exp. Med.*, **205**, 1601–1610.
22. Marcus, P.I. (1983) Interferon induction by viruses: one molecule of dsRNA as the threshold for interferon induction. *Interferon*, **5**, 115–180.
23. Randall, R.E. and Goodbourn, S. (2008) Interferons and viruses: an interplay between induction, signalling, antiviral responses and virus countermeasures. *J. Gen. Virol.*, **89**, 1–47.
24. Cunha, D.A., Hekerman, P., Ladriere, L., Bazarra-Castro, A., Ortis, F., Wakeham, M.C., Moore, F., Rasschaert, J., Cardozo, A.K., Bellomo, E. *et al.* (2008) Initiation and execution of lipotoxic ER stress in pancreatic β-cells. *J. Cell. Sci.*, **121**, 2308–2318.
25. Barton, G.M. (2008) A calculated response: control of inflammation by the innate immune system. *J. Clin. Invest.*, **118**, 413–420.
26. Rasschaert, J., Ladriere, L., Urbain, M., Dogusan, Z., Katabua, B., Sato, S., Akira, S., Gysemans, C., Mathieu, C. and Eizirik, D.L. (2005) Toll-like receptor 3 and STAT-1 contribute to double-stranded RNA+ interferon-γ-induced apoptosis in primary pancreatic β-cells. *J. Biol. Chem.*, **280**, 33984–33991.
27. Scarim, A.L., Arnush, M., Blair, L.A., Concepcion, J., Heitmeier, M.R., Scheuner, D., Kaufman, R.J., Ryerse, J., Buller, R.M. and Corbett, J.A. (2001) Mechanisms of β-cell death in response to double-stranded (ds) RNA and interferon-γ: dsRNA-dependent protein kinase apoptosis and nitric oxide-dependent necrosis. *Am. J. Pathol.*, **159**, 273–283.
28. Dogusan, Z., Garcia, M., Flamez, D., Alexopoulou, L., Goldman, M., Gysemans, C., Mathieu, C., Libert, C., Eizirik, D.L. and Rasschaert, J. (2008) Double-stranded RNA induces pancreatic β-cell apoptosis by activation of the TLR3 and IRF-3 pathways. *Diabetes*, **57**, 1236–1245.
29. Gurzov, E.N., Ortis, F., Bakiri, L., Wagner, E.F. and Eizirik, D.L. (2008) JunB inhibits ER stress and apoptosis in pancreatic β-cells. *PLoS ONE*, **3**, e3030.
30. Gao, M., Labuda, T., Xia, Y., Gallagher, E., Fang, D., Liu, Y.C. and Karin, M. (2004) Jun turnover is controlled through JNK-dependent phosphorylation of the E3 ligase Itch. *Science*, **306**, 271–275.
31. Matsumoto, S., Miyagishi, M., Akashi, H., Nagai, R. and Taira, K. (2005) Analysis of double-stranded RNA-induced apoptosis pathways using interferon-response noninducible small interfering RNA expression vector library. *J. Biol. Chem.*, **280**, 25687–25696.
32. Borsello, T., Clarke, P.G., Hirt, L., Vercelli, A., Repici, M., Schorderet, D.F., Bogousslavsky, J. and Bonny, C. (2003) A peptide inhibitor of c-Jun N-terminal kinase protects against excitotoxicity and cerebral ischemia. *Nat. Med.*, **9**, 1180–1186.
33. Shaulian, E. and Karin, M. (2002) AP-1 as a regulator of cell life and death. *Nat. Cell. Biol.*, **4**, E131–E136.
34. Wen, L., Peng, J., Li, Z. and Wong, F.S. (2004) The effect of innate immunity on autoimmune diabetes and the expression of toll-like receptors on pancreatic islets. *J. Immunol.*, **172**, 3173–3180.
35. Hultcrantz, M., Huhn, M.H., Wolf, M., Olsson, A., Jacobson, S., Williams, B.R., Korsgren, O. and Flodstrom-Tullberg, M. (2007) Interferons induce an antiviral state in human pancreatic islet cells. *Virology*, **367**, 92–101.
36. De Miranda, J., Yaddanapudi, K., Hornig, M. and Lipkin, W.I. (2009) Astrocytes recognize intracellular polyinosinic–polycytidylic acid via MDA-5. *FASEB J.*, **23**, 1064–1071.
37. Kato, H., Takeuchi, O., Sato, S., Yoneyama, M., Yamamoto, M., Matsui, K., Uematsu, S., Jung, A., Kawai, T., Ishii, K.J. *et al.* (2006) Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses. *Nature*, **441**, 101–105.
38. Dotta, F., Censini, S., van Halteren, A.G., Marselli, L., Masini, M., Dionisi, S., Mosca, F., Boggi, U., Muda, A.O., Prato, S.D. *et al.* (2007) Coxsackie B4 virus infection of β-cells and natural killer cell insulitis in

recent-onset type 1 diabetic patients. *Proc. Natl Acad. Sci. USA*, **104**, 5115–5120.
39. Richardson, S.J., Willcox, A., Bone, A.J., Foulis, A.K. and Morgan, N.G. (2009) The prevalence of enteroviral capsid protein vp1 immunostaining in pancreatic islets in human type 1 diabetes. *Diabetologia*, **52**, 1143–1151.
40. Rotondi, M., Chiovato, L., Romagnani, S., Serio, M. and Romagnani, P. (2007) Role of chemokines in endocrine autoimmune diseases. *Endocr. Rev.*, **28**, 492–520.
41. Rhode, A., Pauza, M.E., Barral, A.M., Rodrigo, E., Oldstone, M.B., von Herrath, M.G. and Christen, U. (2005) Islet-specific expression of CXCL10 causes spontaneous islet infiltration and accelerates diabetes development. *J. Immunol.*, **175**, 3516–3524.
42. Christen, U., McGavern, D.B., Luster, A.D., von Herrath, M.G. and Oldstone, M.B. (2003) Among CXCR3 chemokines, IFN-γ-inducible protein of 10 kDa (CXC chemokine ligand (CXCL) 10) but not monokine induced by IFN-γ (CXCL9) imprints a pattern for the subsequent development of autoimmune disease. *J. Immunol.*, **171**, 6838–6845.
43. Frigerio, S., Junt, T., Lu, B., Gerard, C., Zumsteg, U., Hollander, G.A. and Piali, L. (2002) β-Cells are responsible for CXCR3-mediated T-cell infiltration in insulitis. *Nat. Med.*, **8**, 1414–1420.
44. Chen, M.C., Proost, P., Gysemans, C., Mathieu, C. and Eizirik, D.L. (2001) Monocyte chemoattractant protein-1 is expressed in pancreatic islets from prediabetic NOD mice and in interleukin-1β-exposed human and rat islet cells. *Diabetologia*, **44**, 325–332.
45. Schall, T.J., Bacon, K., Toy, K.J. and Goeddel, D.V. (1990) Selective attraction of monocytes and T lymphocytes of the memory phenotype by cytokine RANTES. *Nature*, **347**, 669–671.
46. Zhernakova, A., Alizadeh, B.Z., Eerligh, P., Hanifi-Moghaddam, P., Schloot, N.C., Diosdado, B., Wijmenga, C., Roep, B.O. and Koeleman, B.P. (2006) Genetic variants of RANTES are associated with serum RANTES level and protection for type 1 diabetes. *Genes Immun.*, **7**, 544–549.
47. Waldmann, T.A. (2006) The biology of interleukin-2 and interleukin-15: implications for cancer therapy and vaccine design. *Nat. Rev. Immunol.*, **6**, 595–601.
48. Cardozo, A.K., Proost, P., Gysemans, C., Chen, M.C., Mathieu, C. and Eizirik, D.L. (2003) IL-1β and IFN-γ induce the expression of diverse chemokines and IL-15 in human and rat pancreatic islet cells, and in islets from pre-diabetic NOD mice. *Diabetologia*, **46**, 255–266.
49. Martin, A.P., Rankin, S., Pitchford, S., Charo, I.F., Furtado, G.C. and Lira, S.A. (2008) Increased expression of CCL2 in insulin-producing cells of transgenic mice promotes mobilization of myeloid cells from the bone marrow, marked insulitis and diabetes. *Diabetes*, **57**, 3025–3033.
50. Baccala, R., Hoebe, K., Kono, D.H., Beutler, B. and Theofilopoulos, A.N. (2007) TLR-dependent and TLR-independent pathways of type I interferon induction in systemic autoimmunity. *Nat. Med.*, **13**, 543–551.
51. Stewart, T.A., Hultgren, B., Huang, X., Pitts-Meek, S., Hully, J. and MacLachlan, N.J. (1993) Induction of type I diabetes by interferon-α in transgenic mice. *Science*, **260**, 1942–1946.
52. Huang, X., Yuang, J., Goddard, A., Foulis, A., James, R.F., Lernmark, A., Pujol-Borrell, R., Rabinovitch, A., Somoza, N. and Stewart, T.A. (1995) Interferon expression in the pancreases of patients with type I diabetes. *Diabetes*, **44**, 658–664.
53. Pelegrin, M., Devedjian, J.C., Costa, C., Visa, J., Solanes, G., Pujol, A., Asins, G., Valera, A. and Bosch, F. (1998) Evidence from transgenic mice that interferon-β may be involved in the onset of diabetes mellitus. *J. Biol. Chem.*, **273**, 12332–12340.
54. Greenhalgh, C.J. and Hilton, D.J. (2001) Negative regulation of cytokine signaling. *J. Leukoc. Biol.*, **70**, 348–356.
55. Zhu, W., Mustelin, T. and David, M. (2002) Arginine methylation of STAT1 regulates its dephosphorylation by T cell protein tyrosine phosphatase. *J. Biol. Chem.*, **277**, 35787–35790.
56. Gysemans, C.A., Ladriere, L., Callewaert, H., Rasschaert, J., Flamez, D., Levy, D.E., Matthys, P., Eizirik, D.L. and Mathieu, C. (2005) Disruption of the γ-interferon signaling pathway at the level of signal transducer and activator of transcription-1 prevents immune destruction of β-cells. *Diabetes*, **54**, 2396–2403.
57. Devendra, D., Jasinski, J., Melanitou, E., Nakayama, M., Li, M., Hensley, B., Paronen, J., Moriyama, H., Miao, D., Eisenbarth, G.S. *et al.* (2005) Interferon-α as a mediator of polyinosinic:polycytidylic acid-induced type 1 diabetes. *Diabetes*, **54**, 2549–2556.
58. Karlsen, A.E., Ronn, S.G., Lindberg, K., Johannesen, J., Galsgaard, E.D., Pociot, F., Nielsen, J.H., Mandrup-Poulsen, T., Nerup, J. and Billestrup, N. (2001) Suppressor of cytokine signaling 3 (SOCS-3) protects β-cells against interleukin-1β- and interferon-γ-mediated toxicity. *Proc. Natl Acad. Sci. USA*, **98**, 12191–12196.
59. Pipeleers, D.G., in't Veld, P.A., Van de Winkel, M., Maes, E., Schuit, F.C. and Gepts, W. (1985) A new *in vitro* model for the study of pancreatic α and β cells. *Endocrinology*, **117**, 806–816.
60. Ling, Z., Hannaert, J.C. and Pipeleers, D. (1994) Effect of nutrients, hormones and serum on survival of rat islet β-cells in culture. *Diabetologia*, **37**, 15–21.
61. Asfari, M., Janjic, D., Meda, P., Li, G., Halban, P.A. and Wollheim, C.B. (1992) Establishment of 2-mercaptoethanol-dependent differentiated insulin-secreting cell lines. *Endocrinology*, **130**, 167–178.
62. Hoorens, A., Van de Casteele, M., Kloppel, G. and Pipeleers, D. (1996) Glucose promotes survival of rat pancreatic β-cells by activating synthesis of proteins which suppress a constitutive apoptotic program. *J. Clin. Invest.*, **98**, 1568–1574.
63. Overbergh, L., Valckx, D., Waer, M. and Mathieu, C. (1999) Quantification of murine cytokine mRNAs using real time quantitative reverse transcriptase PCR. *Cytokine*, **11**, 305–312.
64. Liu, D., Darville, M. and Eizirik, D.L. (2001) Double-stranded ribonucleic acid (RNA) induces β-cell Fas messenger RNA expression and increases cytokine-induced β-cell apoptosis. *Endocrinology*, **142**, 2593–2599.
65. Liu, D., Cardozo, A.K., Darville, M.I. and Eizirik, D.L. (2002) Double-stranded RNA cooperates with interferon-γ and IL-1β to induce both chemokine expression and nuclear factor-kB-dependent apoptosis in pancreatic β-cells: potential mechanisms for viral-induced insulitis and β-cell death in type 1 diabetes mellitus. *Endocrinology*, **143**, 1225–1234.
66. Sato, M., Suemori, H., Hata, N., Asagiri, M., Ogasawara, K., Nakao, K., Nakaya, T., Katsuki, M., Noguchi, S., Tanaka, N. *et al.* (2000) Distinct and essential roles of transcription factors IRF-3 and IRF-7 in response to viruses for IFN-α/β gene induction. *Immunity*, **13**, 539–548.
67. Green, L.C., Wagner, D.A., Glogowski, J., Skipper, P.L., Wishnok, J.S. and Tannenbaum, S.R. (1982) Analysis of nitrate, nitrite, and nitrate in biological fluids. *Anal. Biochem.*, **126**, 131–138.