ARTICLE

Inhibition of the type 1 diabetes candidate gene *PTPN2* aggravates TNF-α-induced human beta cell dysfunction and death

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Abstract

Aims/hypothesis TNF-α plays a role in pancreatic beta cell loss in type 1 diabetes mellitus. In clinical interventions, TNF-α inhibition preserves C-peptide levels in early type 1 diabetes. In this study we evaluated the crosstalk of TNF-α, as compared with type I IFNs, with the type 1 diabetes candidate gene *PTPN2* (encoding protein tyrosine phosphatase non-receptor type 2 [PTPN2]) in human beta cells.

Methods EndoC-βH1 cells, dispersed human pancreatic islets or induced pluripotent stem cell (iPSC)-derived islet-like cells were transfected with siRNAs targeting various genes (siCTRL, siPTPN2, siJNK1, siJNK3 or siBIM). Cells were treated for 48 h with IFN-α (2000 U/ml) or TNF-α (1000 U/ml). Cell death was evaluated using Hoechst 33342 and propidium iodide staining. mRNA levels were assessed by quantitative reverse transcription PCR (qRT-PCR) and protein expression by immunoblot.

Results *PTPN2* silencing sensitised beta cells to cytotoxicity induced by IFN-α and/or TNF-α by 20–50%, depending on the human cell model utilised; there was no potentiation between the cytokines. We silenced c-Jun N-terminal kinase (JNK)1 or Bcl-2-like protein 2 (BIM), and this abolished the proapoptotic effects of IFN-α, TNF-α or the combination of both after PTPN2 inhibition. We further observed that PTPN2 silencing increased TNF-α-induced JNK1 and BIM phosphorylation and that JNK3 is necessary for beta cell resistance to IFN-α cytotoxicity.

Conclusions/interpretation We show that the type 1 diabetes candidate gene *PTPN2* is a key regulator of the deleterious effects of TNF-α in human beta cells. It is conceivable that people with type 1 diabetes carrying risk-associated *PTPN2* polymorphisms may particularly benefit from therapies inhibiting TNF-α.

Abbreviations

BIM	Bcl-2-like protein 2
iPSC	Induced pluripotent stem cell
JNK	c-Jun N-terminal kinase
qRT-PCR	Quantitative reverse transcription PCR
MKK7	Mitogen-activated protein kinase kinase 7
PTPN2	Protein tyrosine phosphatase non-receptor type 2
STAT	Signal transducer and activator of transcription

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Introduction

Type 1 diabetes is a complex chronic autoimmune disease during which pancreatic beta cells are progressively attacked by the immune system. The pathophysiological mechanisms that trigger type 1 diabetes are multifactorial and poorly understood. The risk of developing type 1 diabetes involves around 50% heritable genetic factors [1, 2], suggesting that environmental and (epi)genetic factors impact disease susceptibility. In recent years, genome-wide association studies

Research in context

What is already known about this subject?

- Type 1 diabetes is being diagnosed at progressively younger ages, suggesting that the autoimmune process may start early in beta cell development

- The type 1 diabetes candidate gene *PTPN2* protects beta cells from IFNs in a proinflammatory context

- TNF-α levels have been associated with an aggressive phenotype of early-onset type 1 diabetes

What is the key question?

- Does the presence of a *PTPN2* risk allele sensitise beta cells to the cytotoxic effects of TNF-α?

What are the new findings?

- PTPN2 directly interacts with JNK1 in human beta cells and its silencing increases JNK1 phosphorylation fourfold after TNF-α treatment

- PTPN2 silencing increases the cytotoxic effects of TNF-α in mature (adult human pancreatic islets) and less mature (induced pluripotent stem cell-derived islet-like and EndoC-βH1 cells) beta cells

- The cytotoxic effects of TNF-α or IFN-α in PTPN2-silenced cells were abolished by silencing JNK1 or BIM, pointing to an unexpected common pathway for signalling by both cytokines

How might this impact on clinical practice in the foreseeable future?

- Individuals with type 1 diabetes carrying risk-associated *PTPN2* polymorphisms may particularly benefit from TNF-α inhibitory therapies

(GWAS) helped to identify nearly 80 loci associated with a risk of developing type 1 diabetes [3]. Candidate genes such as *IFIH1* and *BACH2*, which play a role in triggering apoptosis in beta cells, or *GLIS3*, related to beta cell phenotype and susceptibility to cytokine attack [4, 5], illustrate the complexity of the disease, where a SNP can contribute to the development and/or acceleration of type 1 diabetes. Another susceptibility gene identified in these studies is *PTPN2*, which encodes protein tyrosine phosphatase non-receptor type 2 (PTPN2), a tyrosine phosphatase with multiple targets implicated in the control of beta cell physiology, survival and expansion [6–8]. PTPN2 targets include Janus kinases (JAKs), signal transducer and activator of transcription (STAT) and EGF receptor [6]. PTPN2 modulates early immune responses in beta cells [9], including viral and type I and II IFN responses, protecting the beta cells from excessive cytotoxic signalling in a proinflammatory context. *PTPN2* was also identified as a candidate gene for other autoimmune diseases such as Crohn's disease [10] and rheumatoid arthritis [11]. *PTPN2* disease-related polymorphisms cause partial loss of function or decreased protein expression [12–14]. Collectively, these observations point to the role of this phosphatase in controlling the immune response.

Type 1 diabetes is diagnosed at progressively younger ages [15], suggesting that the autoimmune process may start early in life, when beta cell development and maturation is not yet complete, and indicating an important role for the innate immune system and early-response cytokines such as IFNs or TNF-α in beta cell failure.

We were further studying the impact of PTPN2 on IFN signalling [9], using TNF-α as a negative control based on our previous findings that TNF-α mediates its deleterious effects on beta cells via NF-κB [16, 17] and should thus not be affected by knockdown of PTPN2. To our surprise, we observed an important augmentation of TNF-α deleterious effects following PTPN2 knockdown. TNF-α plays an important role in the immune system and its levels are associated with the onset of autoimmune diseases such as rheumatoid arthritis, inflammatory bowel disease or psoriatic arthritis; TNF-α inhibitors are used to treat these diseases [18]. One of these inhibitors used to treat rheumatoid arthritis is golimumab, a human IgG1-κ monoclonal antibody that forms high-affinity, stable complexes with both the soluble and transmembrane bioactive forms of human TNF-α, thus preventing the binding of TNF-α to its receptors [19]. Golimumab was evaluated in a placebo-controlled clinical trial in children and young adults with newly diagnosed type 1 diabetes [20]. Patients who received golimumab showed preserved endogenous insulin production and less exogenous insulin needs, suggesting an improvement in beta cell health and confirming the relevance of TNF-α in the pathogenesis of type 1 diabetes. Furthermore, a recent study by Achenbach et al, using a classification and regression tree analysis, distributed two cohorts of patients with diabetes diagnosed before

age 20 years into seven beta cell autoantibody-positive and three autoantibody-negative subgroups [21]. A TNF-α inflammatory signature was enriched in the youngest autoantibody-positive groups and in patients with the lowest C-peptide levels, suggesting that TNF-α levels correlate with an aggressive phenotype of early-onset type 1 diabetes.

Based on this and on our serendipitous finding that PTPN2 knockdown increases TNF-α effects on beta cells, in this study we evaluated the effects of TNF-α, as compared with IFN-α, in different models of human pancreatic beta cells silenced for the tyrosine phosphatase PTPN2.

Methods

Culture of human EndoC-βH1 cells and human pancreatic islets The human pancreatic beta cell line EndoC-βH1 was kindly provided by R Scharfmann (Cochin Institute, France) [22]. Cells were cultured in Matrigel–fibronectin-coated plates as previously described [23]. EndoC-βH1 cells were free of mycoplasma infection, as determined by monthly testing using the MycoAlert Mycoplasma Detection kit (Lonza, Basel, Switzerland).

Human pancreatic islets from eight non-diabetic organ donors were isolated in Pisa, Italy, before November 2021, with written consent from donors' next-of-kin and approval of the local ethics committee, following a previously described protocol [24, 25].

Differentiation of induced pluripotent stem cells into islet-like cells To gain knowledge on the crosstalk between immune mediators, a candidate gene and maturing beta cells, we took advantage of beta-like cells derived from induced pluripotent stem cells (iPSCs). These cells already express *INS* and *PDX1* mRNAs and receptors to proinflammatory cytokines, but are not yet fully mature, providing an interesting model to study the impact of cytokines and candidate genes on developing beta cells [26–28]. The human iPSC line 1023A was kindly provided by DM Egli (Columbia University, USA). The differentiation of iPSCs into islet-like cells was approved by the Ethics Committee of the Erasmus Hospital, Université Libre de Bruxelles, reference P2019/498. iPSCs were cultured in Matrigel-coated plates (Corning, NY, USA) in E8 medium (Invitrogen Life Technologies, Paisley, UK) and passaged with 0.5 mmol/l EDTA (Invitrogen Life Technologies) twice per week. Cell quality and pluripotency were monitored using the MycoAlert Mycoplasma Detection kit for mycoplasma infection, cell karyotyping (Bio.be, Belgium) for chromosomal abnormalities and immunocytochemical staining for pluripotency markers as previously described [29]. For beta cell differentiation we used a seven-step protocol previously

published by our group [30, 31]. Differentiation efficiency was monitored by immunocytochemistry as described in electronic supplementary material (ESM) Fig. 1a,b.

Once the differentiation was completed, cell aggregates were dispersed, seeded on Matrigel-coated culture plates and cultured in HAM's F-10 medium (Thermo Fisher Scientific, Waltham, MA, USA) containing 2% fatty acid-free BSA (Roche, Basel, Switzerland), 2 mmol/l GlutaMAX (Thermo Fisher Scientific) and 100 U/ml penicillin–streptomycin (Thermo Fisher Scientific) for exposure to cytokines and/or siRNA as described [29].

RNA interference Dispersed human pancreatic islets or dispersed iPSC-derived islet-like cells were transfected overnight with 30 nmol/l siRNA; the medium was changed and cells were left to recover for 24 h. Transfection was performed using previously validated siRNAs targeting *PNPT2* (siPTPN2; 5′-CACA AAGGAGTTACATCTTAA-3′; 1027415; Qiagen, Venlo, the Netherlands) [32], *JNK1* (also known as *MAPK8*; siJNK1; 5′-GGGCCUACAGAGAGCUAGUUCUUAU-3′; MAPK8HSS108547, Thermo Fisher Scientific) [33], *BIM* (siBIM; 5′-CACGAAUGGUUAUCUUACGACUGUU-3′; 10620318, Thermo Fisher Scientific) [5] and *JNK3* (also known as *MAPK10*; siJNK3; 5′-TCCATATGTGGTGACACGTTA-3′; 1027415; Qiagen) (validated in this study), using Lipofectamine RNAiMax (Invitrogen) as described [6]. For experiments with double or triple transfection, we mixed 30 nmol/l of each siRNA. Allstars Negative Control siRNA (siCTRL; Qiagen) was used as a negative control.

Exposure to cytokines After 24 h recovery from silencing, cells were left untreated (NT) or treated for 24 h or 48 h with IFN-α (2000 U/ml; Peprotech, London, UK), TNF-α (1000 U/ml; Peprotech), IFN-α+TNF-α (2000 and 1000 U/ml, respectively), thapsigargin (1 μmol/l; Sigma Aldrich, MO, USA) or brefeldin A (0.025 μg/ml; Sigma Aldrich). For protein phosphorylation studies, we first performed a time course analysis and then selected 1 h as the optimal time point for cytokine exposure in subsequent experiments. c-Jun N-terminal kinase (JNK) was chemically inhibited using SP600125 (Selleck Chemicals, Germany). SP600125 optimal concentration to inhibit JNK1 in EndoC-βH1 cells, i.e. 20 μmol/l, was determined by a dose–response study shown in ESM Fig. 2. For the subsequent experiments, cells were pre-treated with 20 μmol/l SP600125 for 1 h and then treated with IFN-α or TNF-α in the continued presence of 20 μmol/l SP600125 for 48 h.

Immunocytochemistry Dispersed iPSC-derived islet-like cells were fixed in 4% paraformaldehyde for 15–20 min, permeabilised with 0.5% triton-X100 in PBS, blocked with UltraV block (Thermo Fisher Scientific) for 10 min and then

incubated with primary antibodies diluted in 0.1% Tween in PBS overnight at 4°C. Cells were then washed with PBS and incubated with secondary antibodies diluted in 0.1% Tween in PBS. Samples were mounted with Vectashield with DAPI (Vector Laboratories, Newark, CA, USA) and covered with glass coverslips. The antibodies used in the study are listed in ESM Table 1.

Cell death assays Cell death was detected by fluorescence microscopy after staining with the DNA binding dyes Hoechst 33342 (5 μg/ml, Sigma Aldrich) and propidium iodide (5 μg/ml, Sigma Aldrich) [6, 34]. Cell death was determined in at least 500 cells by two observers, one of them unaware of the experimental conditions. Caspase 3/7 activity was determined by Caspase-Glo 3/7 Assay System (Promega, WI, USA). Whole cleaved caspase 3 was detected by immunoblot and normalised to total procaspase 3 expression.

Static glucose-stimulated insulin secretion EndoC-βH1 cells were washed and incubated for 2 h in a 2.8 mmol/l glucose cell medium; washed in PBS and pre-incubated for 1h in glucose-free Krebs buffer (Univercell Biosolutions, Toulouse, France). Then, the cell medium was changed for Krebs buffer containing 0 mmol/l glucose, 20 mmol/l glucose or 20 mmol/l glucose plus 10 μmol/l forskolin and incubated for 40 min. The supernatant was collected for human insulin measurement by ELISA (Mercodia, Uppsala, Sweden). Cellular insulin was extracted using acid ethanol (0.18 mol/l hydrochloric acid in 95% ethanol) and quantified by ELISA (Mercodia). Insulin secretion and total insulin content were normalised to total protein content, measured by protein assay dye (Bio-Rad Laboratories, CA, USA).

Immunoblot Total protein was extracted using Laemmli or RIPA buffer supplemented with phosphatase and protease inhibitors (Roche) and separated on 10% SDS–PAGE. The nitrocellulose membranes were probed using specific primary antibodies diluted 1:1000 in TBST (TBS, 0.1% Tween 20) with 5% BSA. After overnight incubation at 4°C, membranes were probed for 1 h at room temperature with peroxidase-conjugated secondary antibodies diluted 1:5000 in TBST with 5% BSA. Detection of immunoreactive bands was performed using chemiluminescent substrate (SuperSignal West Femto, Thermo Fisher Scientific) using a Bio-Rad ChemiDoc XRS+ system (Bio-Rad Laboratories). The densitometric values were quantified by ImageLab software version 6.1 (Bio-Rad Laboratories, RRID:SCR_014210) and normalised to GAPDH or the respective total protein forms, after background subtraction. Antibodies are listed in ESM Table 1.

Co-immunoprecipitation EndoC-βH1 cells were mechanically lysed with a syringe in PBS containing 5 mmol/l EDTA

Fig. 1 PTPN2 regulates TNF-α-induced JNK1 phosphorylation. (**a**) ▶ EndoC-βH1 cells were left untreated (0) or treated for 10 min, 30 min, 1 h, 4 h, 8 h or 24 h with IFN-α (2000 U/ml) or TNF-α (1000 U/ml). P-JNK1, JNK1 and GAPDH protein levels were analysed by immunoblot. P-JNK1 bands were quantified by densitometry and normalised to JNK1. Results are means ± SEM of three independent experiments and presented as fold-variation compared with untreated cells, considered as 1. **$p<0.01$ vs IFN-α non-treated control; $^{††}p<0.01$ vs TNF-α non-treated control; ANOVA. (**b**) EndoC-βH1 cells were transfected with siCTRL or siPTPN2. After 48 h of recovery the cells were left untreated (0) or treated for 1 h, 4 h, 8 h or 24 h with TNF-α (1000 U/ml); and for 1 h with IFN-α (2000 U/ml). PTPN2, P-STAT1, P-STAT2, STAT1, STAT2 and GAPDH protein expression were analysed by immunoblot. P-STAT1 and P-STAT2 bands were quantified by densitometry and normalised to their respective total protein forms. Results are means ± SEM of three independent experiments and presented as fold-variation compared with siCTRL-untreated cells, considered as 1. **$p<0.01$ vs siCTRL; t test. (**c**) EndoC-βH1 cells were transfected with siCTRL or siPTPN2. After 48 h of recovery cells were left untreated (0) or treated for 1 h, 4 h, 8 h or 24 h with TNF-α (1000 U/ml). PTPN2, P-JNK1, JNK1 and GAPDH protein expression were analysed by immunoblot. P-JNK1 bands were quantified by densitometry and normalised to JNK1. Results are means ± SEM of three independent experiments and presented as fold-variation compared with siCTRL-untreated cells, considered as 1. *$p<0.05$ vs siCTRL; ANOVA. (**d**) EndoC-βH1 cells were left untreated (NT) or treated for 1 h with IFN-α (2000 U/ml) or TNF-α (1000 U/ml); PTPN2 was then immunoprecipitated. PTPN2, STAT1, JNK1 and GAPDH protein levels were analysed by immunoblot. GAPDH and mouse IgG immunoprecipitation were used as negative controls. Images are representative of three independent experiments. (**e**) EndoC-βH1 cells were transfected with siCTRL or siPTPN2. After 48 h of recovery cells were left untreated (NT) or treated for 1h with TNF-α (1000 U/ml). PTPN2, P-JNK1, JNK1, P-BIM, BIM, P-MKK7, MKK7 and GAPDH protein expression were analysed by immunoblot. P-JNK1, P-BIM and P-MKK7 bands were quantified by densitometry and normalised to their respective total protein forms or to GAPDH for PTPN2. Results are means ± SEM of five or six independent experiments and presented as fold-variation compared with siCTRL-untreated cells, considered as 1. **$p<0.01$, ***$p<0.001$ vs siCTRL; ANOVA. EL, extra-large; IP, immunoprecipitate; L, large; S, short

with protease inhibitors and incubated on a shaker at 4°C for 3 h. Lysates were cleared by centrifugation at 12,000 g for 30 min at 4°C and immediately subjected to PTPN2 or IgG immunoprecipitation overnight on a shaker at 4°C by the addition of 2 μg of primary antibody or immunoglobulin. Pierce Protein Magnetic Beads (Thermo Fisher Scientific), previously saturated with 2% BSA, were then added for 2 h on a shaker at 4°C, followed by four washes with lysis buffer. Proteins were eluted in DTT-free Laemmli buffer by heating for 10 min at 50 °C. 100 mmol/l DTT was then added and protein complexes were analysed by immunoblot. Antibodies are listed in ESM Table 1.

Real-time PCR Poly(A)+ mRNA was isolated using the Dynabeads mRNA DIRECT kit (Invitrogen) according to the manufacturer's instructions. mRNA molecules were
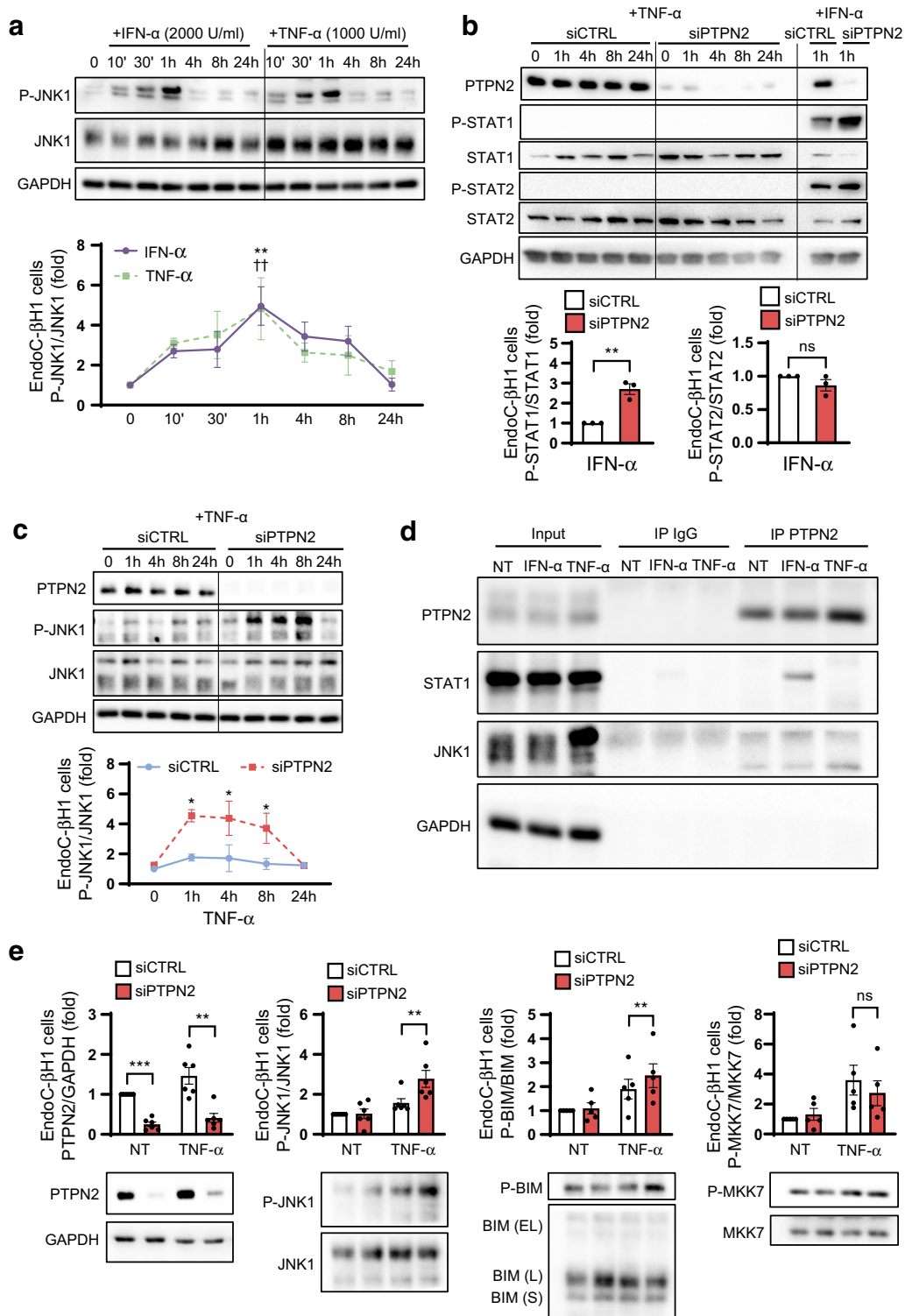
recovered in Tris–HCl elution solution and reverse transcription was performed using the Reverse Transcriptase Core kit (Eurogentec, Liège, Belgium) according to the manufacturer's instructions. The quantitative reverse transcription PCR (qRT-PCR) amplification was conducted using IQ SYBR Green Supermix (Bio-Rad Laboratories). The PCR product concentration was calculated as copies per μl using the standard curve method [35] and gene expression was normalised to the geometric mean of the reference genes *ACTB* and *GAPDH*. Primers are listed in ESM Table 2.

Statistics Data were analysed by unpaired t test, one-way or two-way ANOVA (corrected for repeated measures if required) followed by Bonferroni multiple comparisons tests as required, using GraphPad Prism 8 software (CA, USA). Results are presented as mean ± SEM. $p<0.05$ was considered statistically significant. In each experiment, $n=1$ is considered to correspond to one independent biological observation, i.e. EndoC-βH1 cells from different passages, human pancreatic islets from different donors, or iPSC-derived islet-like cells from different differentiations. To reduce variability between independent experiments, for some techniques, e.g. real-time PCR or immunoblot, each independent experiment was normalised against its respective siCTRL.

Results

PTPN2 regulates JNK1 phosphorylation Activation of the JNK pathway can lead to deleterious effects in cells [36]. We previously reported that interferons modulate the JNK pathway in PTPN2-silenced beta cells, leading to cell death [9]. A time course analysis of TNF-α or IFN-α-induced JNK1 phosphorylation in EndoC-βH1 cells (Fig. 1a) showed maximal induction at 1 h, the time point used in subsequent experiments to evaluate JNK activation. Next, EndoC-βH1 cells were silenced for PTPN2 using siPTPN2 and treated with TNF-α for different times. There was >90% inhibition of PTPN2 protein by the siRNA, and this led to fourfold higher P-JNK1 expression in TNF-treated cells (Fig. 1c). PTPN2 knockdown increased STAT-1 but not STAT2 phosphorylation in cells exposed to IFN-α; TNF-α treatment did not induce STAT1 or STAT2 phosphorylation (Fig. 1b). One-hour treatment with TNF-α significantly increased JNK1 and BIM phosphorylation in PTPN2-silenced cells while no changes were detected in the upstream protein mitogen-activated protein kinase kinase 7 (MKK7, Fig. 1e). The total expression of the different BIM isoforms (extra-large, EL; large, L; or short, S) was not affected. PTPN2 co-immunoprecipitated with JNK1 in EndoC-βH1 cells treated with TNF-α or IFN-α and with STAT1 in cells treated with IFN-α (Fig. 1d).

TNF-α has deleterious effects in beta cells silenced for PTPN2 Efficient PTPN2 silencing in EndoC-βH1 cells (Fig. 2a) potentiated cell death by 18% ($p<0.01$), 24% ($p<0.01$) and 25% ($p<0.001$) upon 48 h treatment with, respectively, IFN-α, TNF-α and the combination of IFN-α +TNF-α (Fig. 2b, ESM Fig. 3a). These results were confirmed by using three different and independent beta cell death measurements: Hoechst and propidium iodide staining (Fig. 2b, ESM Fig.

3a), Caspase-Glo 3/7 Assay System (ESM Fig. 3b) and cleaved caspase 3 protein expression (ESM Fig. 3c). Similarly, in dispersed human pancreatic islets, PTPN2 silencing (Fig. 2c) increased cell death by 50% for IFN-α, 50% for TNF-α and 42% for IFN-α +TNF-α (Fig. 2d). Finally, in iPSC-derived islet-like cells silenced for PTPN2 (Fig. 2e), there was an increase in cell death of 45% for IFN-α treatment, 44% for TNF-α and 43% for the cytokine combination (Fig. 2f). In non-silenced cells, dispersed adult human pancreatic islets were more resistant to cell death both under basal conditions and following cytokine exposure in comparison with the other beta cell models (ESM Fig. 4). There was no potentiating effect when we combined the two cytokines, suggesting that IFN-α and TNF-α act at least in part through similar downstream mechanisms of cell death, although there were divergences in the effects of the two cytokines on the induction of P-STAT1/2 (Fig. 1b) and the chemokine *CXCL10* (ESM Fig. 5). To rule out a nonspecific effect of PTPN2 silencing on beta cell death, we treated PTPN2-silenced EndoC-βH1 and iPSC-derived islet-like cells (ESM Fig. 6a,c) for 24 h with the endoplasmic reticulum stress inducers brefeldin A and thapsigargin [37]. Under these conditions, there were no changes in cell viability with PTPN2 inhibition (ESM Fig. 6b,d). In parallel, 24 h exposure of the PTPN2-silenced cells to IFN-α and/or TNF-α again led to increased cell death as described above.

PTPN2 silencing impairs *PDX1* expression and insulin release after exposure to TNF-α EndoC-βH1 cells, dispersed human pancreatic islets and iPSC-derived islet-like cells showed a decrease in *PDX1* (Fig. 3a) and *INS* (Fig. 3b) expression in cells silenced for PTPN2 and exposed to TNF-α. This effect was also detected in EndoC-βH1 cells exposed to IFN-α, but the presence of this cytokine did not affect *PDX1* and *INS* expression in the other beta cell models. Insulin release by EndoC-βH1 cells silenced for PTPN2 and exposed to TNF-α was inhibited following stimulation with high glucose plus forskolin (Fig. 3c) and these cells also had decreased insulin content (Fig. 3d).

BIM silencing preserves beta cell viability after IFN-α or TNF-α treatment We have previously shown that inhibiting the BH3-only protein BIM in beta cells silenced for PTPN2 prevents IFN-α-induced cell death [9]. To test if this was also the case for TNF-α, we performed double silencing for PTPN2 and BIM in EndoC-βH1 cells (Fig. 4a,c) and iPSC-derived islet-like cells (Fig. 4b,d), observing a complete inhibition of IFN-α- and/or TNF-α-induced cytotoxicity (Fig. 4e,f).

JNK1 silencing preserves beta cell viability in PTPN2 silenced cells exposed to IFN-α or TNF-α As shown in Fig. 1c, PTPN2

Fig. 2 PTPN2 silencing increases beta cell death after exposure to IFN-α, TNF-α or their combination. EndoC-βH1 cells, dispersed human pancreatic islets and iPSC-derived islet-like cells were transfected with siCTRL (white bars) or siPTPN2 (red bars). After 48 h of recovery cells were left untreated (NT) or treated for 48 h with IFN-α (2000 U/ml), TNF-α (1000 U/ml) or IFN-α+ TNF-α (2000 and 1000 U/ml, respectively). (**a**, **c**, **e**) PTPN2 silencing was confirmed by qRT-PCR in EndoC-βH1 cells (**a**), dispersed human pancreatic islets (**c**) and iPSC-derived islet-like cells (**e**). mRNA expression was normalised to the geometric mean of *ACTB* and *GAPDH* and presented as fold-variation compared with siCTRL-untreated cells, considered as 1. Results are means ± SEM of six to eight independent experiments. **p<0.01 and ***p<0.001 vs siCTRL treated with IFN-α, TNF-α or IFN-α+TNF-α. (**b**, **d**, **f**) Cell death was evaluated using Hoechst and propidium iodide staining in EndoC-βH1 cells (**b**), dispersed human pancreatic islets (**d**) and iPSC-derived islet-like cells (**f**). Results are means ± SEM of six to eight independent experiments. **p<0.01 and ***p<0.001 vs siCTRL treated with IFN-α, TNF-α or IFN-α+ TNF-α; $^{†}p$<0.05, $^{††}p$<0.01 and $^{†††}p$<0.001 siCTRL or siPTPN2 vs their respective untreated control; ANOVA
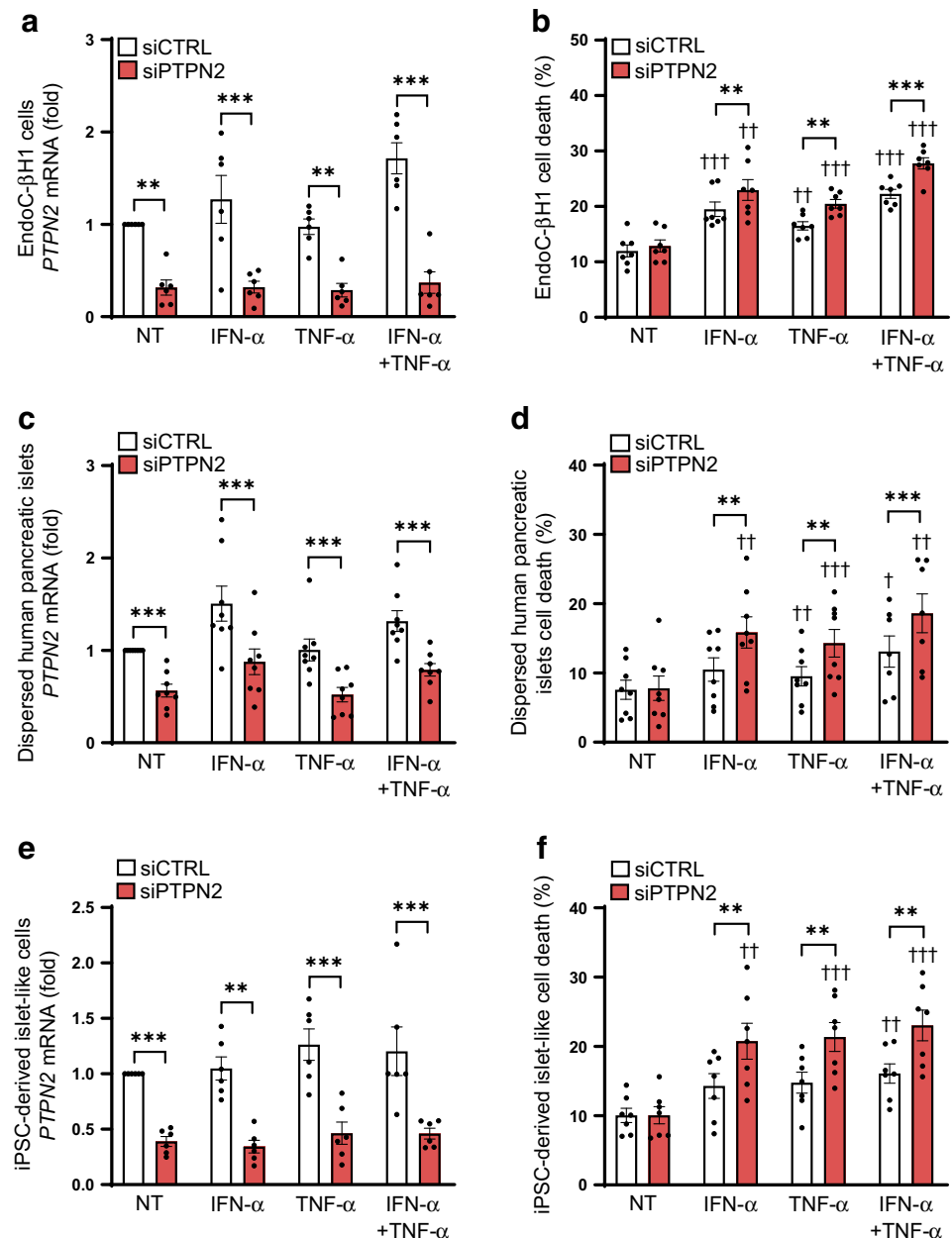
regulates JNK1 phosphorylation. To further analyse the role for JNK1 in beta cell death, we performed double silencing of PTPN2 and JNK1 in EndoC-βH1 cells (ESM Fig. 7a,b) or iPSC-derived islet-like cells (ESM Fig. 7c,d). JNK1 silencing prevented the cytotoxic effects of IFN-α or TNF-α treatment (Fig. 5a,b). We next blocked JNK1 activation in EndoC-βH1 cells or iPSC-derived islet-like cells silenced for PTPN2 (ESM Fig. 7e,f) using the JNK inhibitor SP600125. Chemical JNK inhibition preserved beta cell viability after TNF-α treatment in both cell models (Fig. 5c,d) but did not

inhibit IFN-α cytotoxicity in cells silenced or not for PTPN2, showing a surprising dissociation between genetic and chemical JNK inhibition.

JNK3 activation is essential for beta cell response to IFN-α
SP600125 inactivates not only JNK1, but also JNK2 and JNK3. We hypothesised that JNK3 could be involved in the discrepancy between the effects of the siRNA targeting *JNK1* (Fig. 5a,b) and the chemical JNK inhibitor (Fig. 5c,d). In
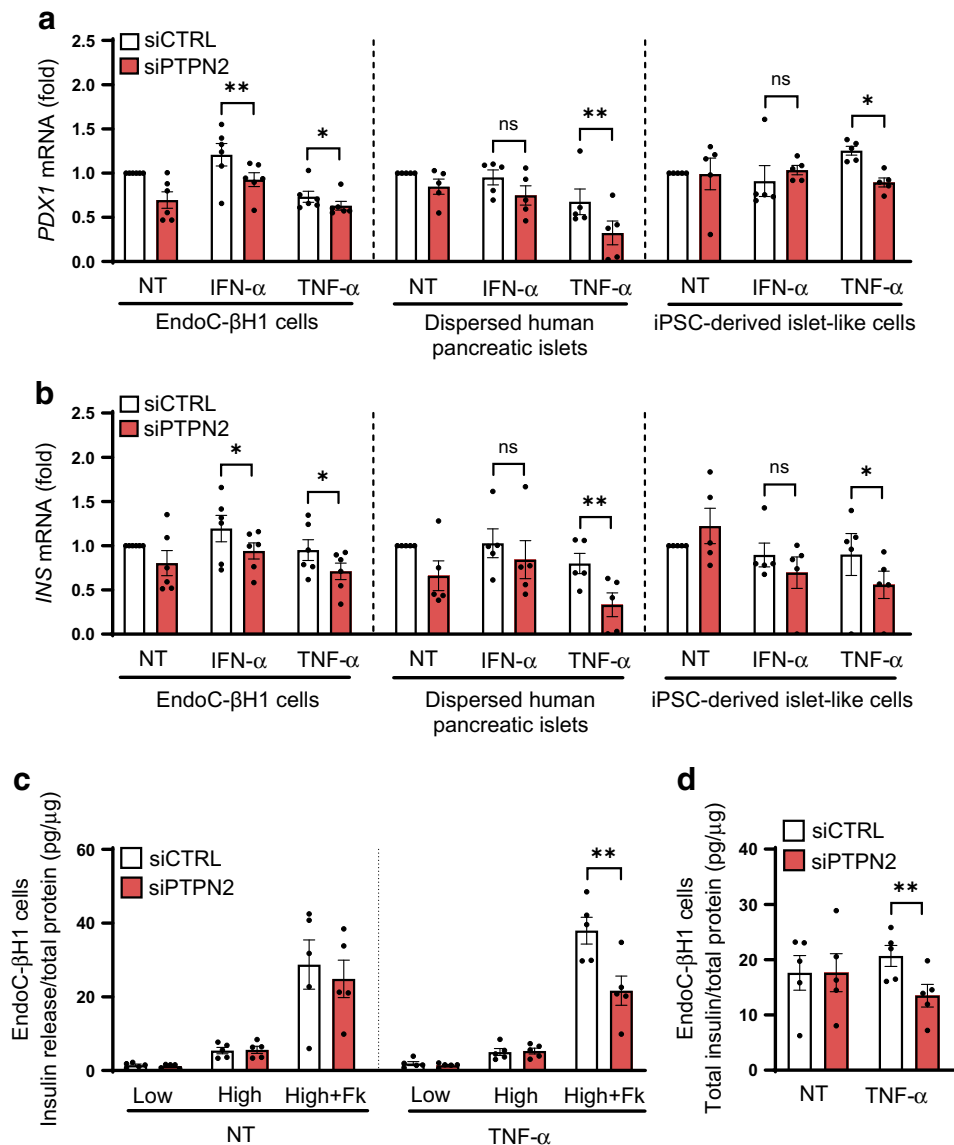
Fig. 3 PTPN2 silencing impairs *PDX1* expression and insulin release after exposure to TNF-α. (**a**, **b**) EndoC-βH1 cells, dispersed human pancreatic islets and iPSC-derived islet-like cells were transfected with siCTRL (white bars) or siPTPN2 (red bars). After 48 h of recovery cells were left untreated (NT) or treated for 48 h with IFN-α (2000 U/ml), TNF-α (1000 U/ml) or IFN-α+TNF-α (2000 and 1000 U/ml, respectively). PTPN2 silencing had been confirmed by qRT-PCR in EndoC-βH1 cells (Fig. 2a), dispersed human pancreatic islets (Fig. 2c) and iPSC-derived islet-like cells (Fig. 2e). *PDX1* (**a**) and *INS* (**b**) expression was evaluated by qRT-PCR. mRNA expression was normalised to the geometric mean of *ACTB* and *GAPDH* and presented as fold-variation compared with siCTRL-untreated cells, considered as 1. Results are means ± SEM of five or six independent experiments. *$p<0.05$ and **$p<0.01$ vs siCTRL treated with IFN-α or TNF-α; ANOVA. (**c**, **d**) EndoC-βH1 cells were transfected with siCTRL (white bars) or siPTPN2 (red bars). After 48 h of recovery cells were left untreated (NT) or treated for 48 h with TNF-α (1000 U/ml). After that, cells were exposed to low glucose (0 mmol/l), high (20 mmol/l) glucose or high glucose plus forskolin (Fk, 10 μmol/l). Insulin levels were measured by ELISA. Insulin release (**c**) and total insulin content (**d**) were normalised to total protein content. Results are means ± SEM of five independent experiments. **$p<0.01$ vs siCTRL; ANOVA

EndoC-βH1 cells, IFN-α decreased *JNK1* and *JNK2* expression by 12% and 18%, respectively, while *JNK3* expression was induced by 73% (ESM Fig. 8a). To assess the role of JNK3 in beta cell protection, we silenced JNK3 in EndoC-βH1 cells (ESM Fig. 8b) and observed that JNK3-silenced cells were significantly more sensitive to IFN-α (44% increase in cell death vs non-silenced cells, ESM Fig. 8c). Triple silencing of PTPN2 (Fig. 6a), JNK1 (Fig. 6b) and JNK3 (Fig. 6c) in EndoC-βH1 cells induced more cell death (Fig. 6d) in comparison with cells double silenced for PTPN2 and JNK1 (103%; $p<0.01$). These observations indicate a protective role for JNK3 against IFN-α toxicity that is lost during treatment with the chemical JNK inhibitor SP600125.

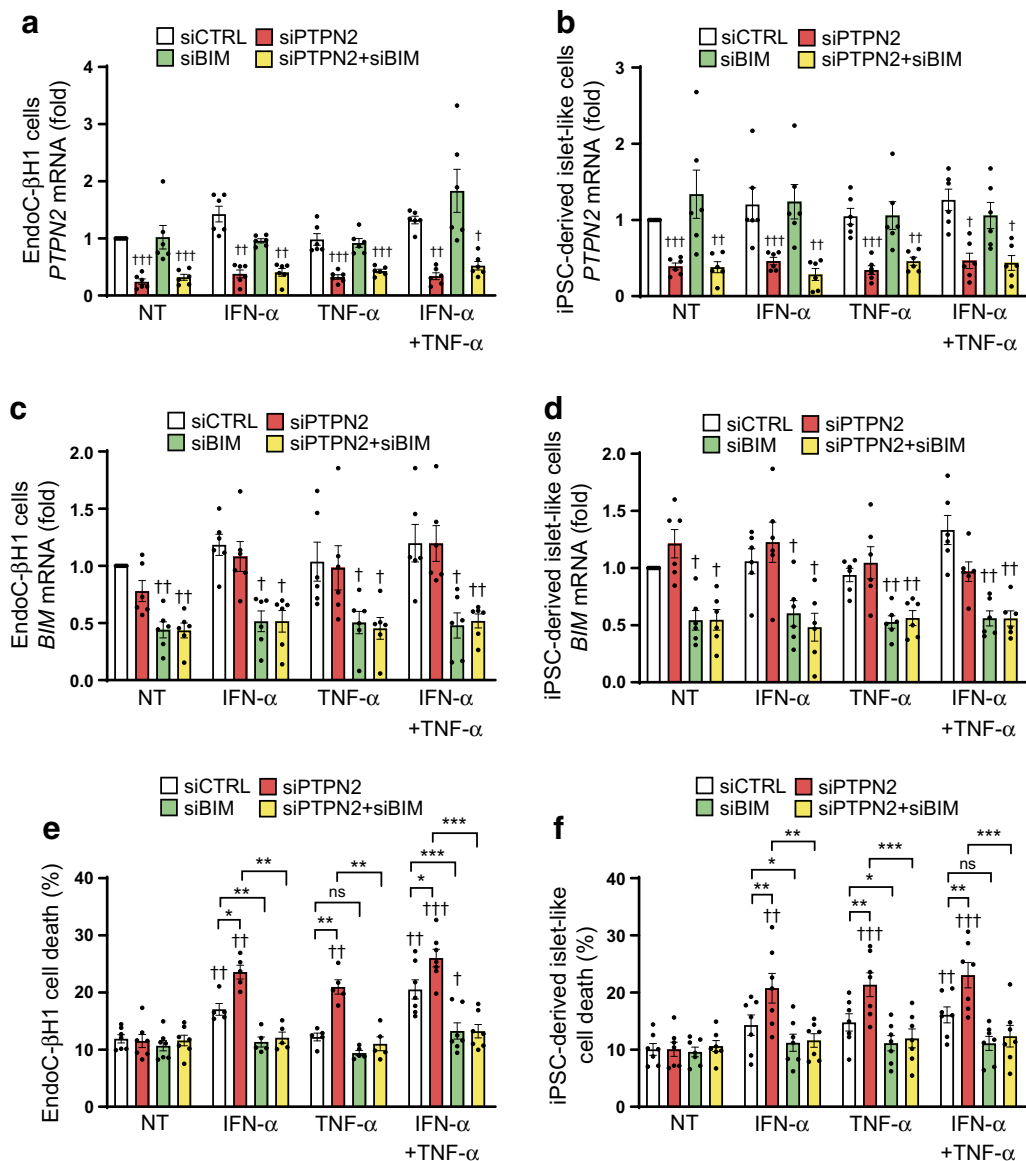**Fig. 4** BIM silencing preserves beta cell viability in cells silenced for PTPN2 and then exposed to IFN-α or TNF-α. EndoC-βH1 cells and iPSC-derived islet-like cells were transfected with siCTRL (white bars), siPTPN2 (red bars), siBIM (green bars) or siPTPN2+siBIM (yellow bars). After 48 h of recovery cells were left untreated (NT) or treated for 48 h with IFN-α (2000 U/ml), TNF-α (1000 U/ml) or IFN-α+TNF-α (2000 and 1000 U/ml, respectively). (**a**–**d**) PTPN2 and BIM silencing was confirmed by qRT-PCR for EndoC-βH1 cells (**a**, **c**) and iPSC-derived islet-like cells (**b**, **d**). mRNA expression was normalised to the geometric mean of *ACTB* and *GAPDH* and presented as fold-variation compared with siCTRL-untreated cells, considered as 1. (**e**, **f**) Cell death was evaluated using Hoechst and propidium iodide staining for EndoC-βH1 cells (**e**) and iPSC-derived islet-like cells (**f**). Results are means ± SEM of five to seven independent experiments. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ vs siCTRL treated with IFN-α, TNF-α or IFN-α+TNF-α; †$p < 0.05$, ††$p < 0.01$ and †††$p < 0.001$ siCTRL, siPTPN2, siBIM or siPTPN2+siBIM vs their respective untreated control; ANOVA

Discussion

In the present study we show that 'early-response' cytokines IFN-α and TNF-α have deleterious effects on human beta cells at different stages of development and differentiation, from immature cells (iPSC-derived islet-like cells and the EndoC-βH1 cell model) to fully mature adult cells (human pancreatic islets), and that this is aggravated when expression of the type 1 diabetes candidate gene *PTPN2* is diminished. We demonstrate that PTPN2 confers protection against both IFN-α and TNF-α exposure, showing an unexpected common downstream signalling pathway between the two cytokines. Inhibition of PTPN2 also impairs *PDX1* expression and insulin production after exposure to TNF-α. By silencing BIM or JNK1 together with PTPN2, we observed beta cell protection against cytokine-induced damage, revealing that
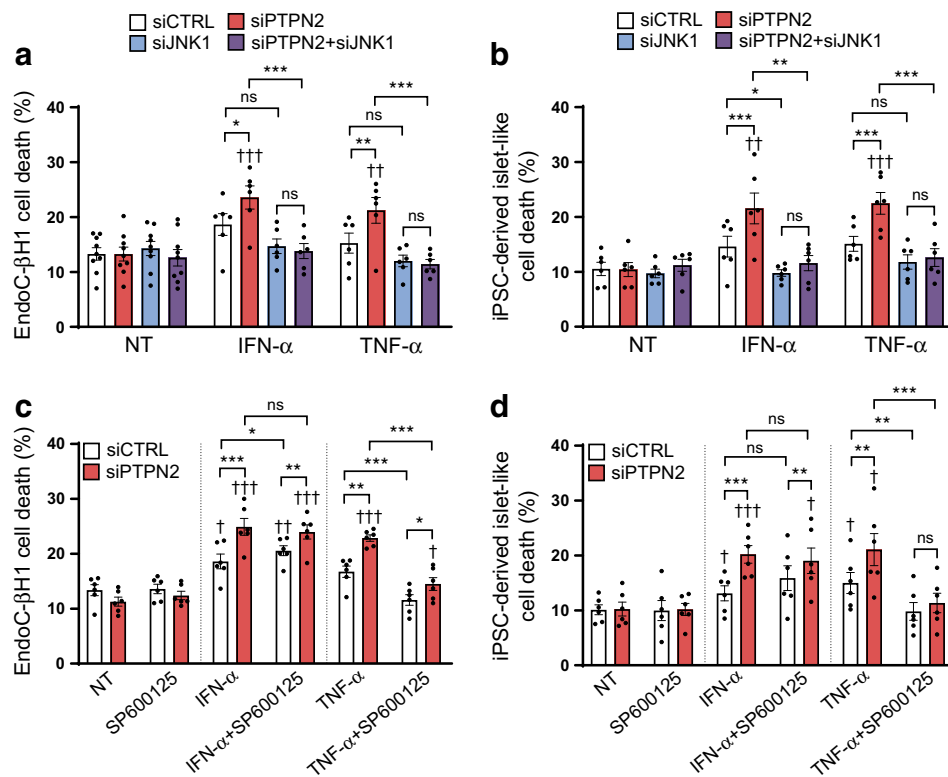
Fig. 5 JNK1 silencing preserves beta cell viability in cells silenced for PTPN2 after IFN-α or TNF-α treatment, but a chemical JNK inhibitor does not prevent IFN-α-induced cell death. (**a**, **b**) EndoC-βH1 cells and iPSC-derived islet-like cells were transfected with siCTRL (white bars), siPTPN2 (red bars), siJNK1 (blue bars) or siPTPN2+siJNK1 (purple bars). After 24 h of recovery, cells were left untreated (NT) or treated for 48 h with IFN-α (2000 U/ml) or TNF-α (1000 U/ml). Cell death was evaluated using Hoechst and propidium iodide staining for EndoC-βH1 cells (**a**) and iPSC-derived islet-like cells (**b**). Results are means ± SEM of six independent experiments. $*p<0.05$, $**p<0.01$ and $***p<0.001$ vs siCTRL treated with IFN-α or TNF-α; $^{††}p<0.01$ and $^{†††}p<0.001$ siCTRL, siPTPN2, siJNK1 or siPTPN2+siJNK1 vs their respective untreated control; ANOVA. (**c**, **d**) EndoC-βH1 cells and iPSC-derived islet-like cells were transfected with siCTRL (white bars) or siPTPN2 (red bars). After 24 h of recovery, cells were left untreated (NT) or treated for 48 h with SP600125 (20 μmol/l), IFN-α (2000 U/ml), IFN-α+SP600125, TNF-α (1000 U/ml) or TNF-α+SP600125. Cell death was evaluated using Hoechst and propidium iodide staining for EndoC-βH1 cells (**c**) and iPSC-derived islet-like cells (**d**). Results are means ± SEM of six independent experiments. $*p<0.05$, $**p<0.01$ and $***p<0.001$ vs siCTRL treated with IFN-α, IFN-α+SP600125, TNF-α or TNF-α+SP600125; $^{†}p<0.05$, $^{††}p<0.01$ and $^{†††}p<0.001$ siCTRL or siPTPN2 vs their respective untreated control; ANOVA

the cytotoxic activity is in both cases mediated by the activation of the intrinsic mitochondrial pathway of cell death. We also show that JNK1 phosphorylation is increased in PTPN2-silenced cells treated with TNF-α, demonstrating that PTPN2 can directly regulate JNK1 activation in beta cells, as it was previously described for myeloid cells [38].

Recent studies have shown that TNF-α is associated with an aggressive phenotype of early-onset type 1 diabetes [21] and may represent a marker of disease progression [39]. Of particular relevance, clinical trials targeting TNF-α delayed the progressive loss of C-peptide in recent onset type 1 diabetes [20]. *PTPN2* risk alleles, such as the intronic type 1 diabetes-associated SNP rs1893217, have been shown to cause a decrease in PTPN2 protein expression in CD4+ memory cells, suggesting that PTPN2 loss or reduced function may confer disease susceptibility by sensitising beta cells to immune-mediated cell death [12]. The key role of PTPN2 in maintaining a physiological immune response is highlighted

by the fact that PTPN2-deficient mice die a few weeks after birth due to systemic inflammation and severe colitis [40], and that several *PTPN2* SNPs are associated with other autoimmune disorders such as rheumatoid arthritis [11] or Crohn's disease [10]. Of note, patients affected by these diseases may benefit from anti-TNF-α therapies [41, 42]. PTPN2 interacts with another type 1 diabetes candidate gene, namely *BACH2*: the lack of BACH2 leads to inhibition of cytokine-induced PTPN2 expression, thus augmenting JNK1 and BIM deleterious effects and consequent beta cell death [5]. These findings suggest that the combination of risk alleles, in crosstalk with cytokines such as IFN-α or TNF-α, could confer a higher susceptibility to type 1 diabetes and/or contribute to an accelerated evolution from autoimmunity (as identified by islet autoantibodies) to overt diabetes. This hypothesis, however, remains to be tested in future studies.

For this study, we used siRNAs that cause a decrease in PTPN2 expression but not its total loss of function. The
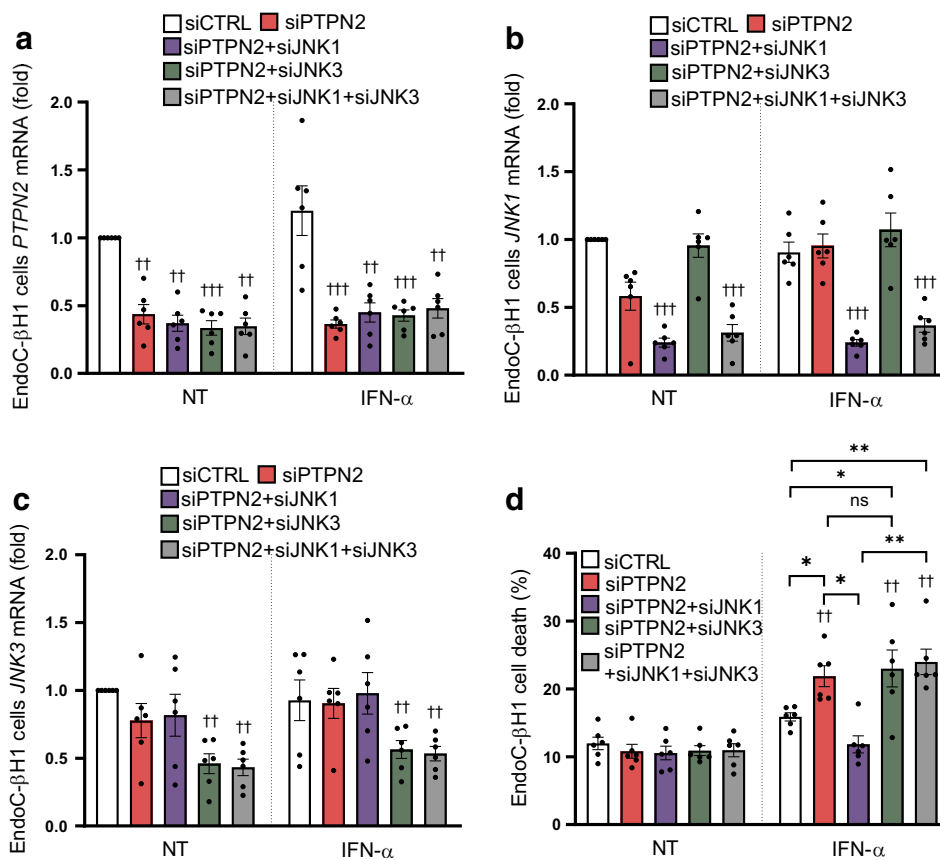
Fig. 6 JNK3 silencing hampers beta cell viability in cells silenced for PTPN2 and exposed to IFN-α. (**a–d**) EndoC-βH1 cells were transfected with siCTRL (white bars), siPTPN2 (red bars), siPTPN2+siJNK1 (purple bars), siPTPN2+siJNK3 (dark green bars) or siPTPN2+siJNK1+siJNK3 (grey bars). After 48 h of recovery cells were left untreated (NT) or treated for 48 h with IFN-α (2000 U/ml). (**a–c**) PTPN2 (**a**), JNK1 (**b**) and JNK3 (**c**) silencing was confirmed by qRT-PCR. mRNA expression was normalised to the geometric mean of *ACTB* and *GAPDH* and presented as fold-variation compared with siCTRL-untreated cells, considered as 1. (**d**) Cell death was evaluated using Hoechst and propidium iodide staining. Results are means ± SEM of six independent experiments. *$p<0.05$ and **$p<0.01$ vs siCTRL treated with IFN-α. ††$p<0.01$ and †††$p<0.001$ siCTRL, siPTPN2, siPTPN2+siJNK1, siPTPN2+siJNK3 or siPTPN2+siJNK1+siJNK3 vs their respective untreated control; ANOVA

complete lack of PTPN2 function, despite its attractiveness for functional studies, does not clearly match the type 1 diabetes context, characterised by a partial decrease in PTPN2 function. Indeed, complete loss of function of PTPN2 is rare in humans and this deletion—both in human and in mouse models—causes very early-onset inflammatory bowel disease and a strong impairment in intestinal function [43].

Interestingly, JNK3 was identified in this study as an important regulator of the deleterious effects of IFN-α in human beta cells. While JNK1 and JNK2 are expressed in most tissues, JNK3 expression is restricted to brain, heart, testes and beta cells [44]. Previous observations showed that JNK3 protects rat beta cells against apoptosis induced by a combination of IL-1β, TNF-α and IFN-γ, mainly through maintenance of the insulin receptor substrate 2 (IRS2)/AKT2 signalling pathway [45, 46]. Interestingly, JNK3 is required for the protective effects of the GLP-1 analogue exendin 4 against cytokine-induced rat beta cell death [47]. The present findings show that JNK3 also has a protective role against IFN-α-induced human beta cell

death, particularly in the context of decreased expression of the candidate gene *PTPN2*.

A potential limitation in this study, regarding the use of iPSC-derived islet-like cells and human islet preparations, is the presence of other cell types besides beta cells that could contribute to the observed phenotype. Of note, the results obtained with these cellular models were validated by using the pure beta cell model EndoC-βH1. This, and the observed inhibition of the beta cell-specific genes *PDX1* and *INS* in the different models studied following PTPN2 inhibition and TNF-α exposure, supports a key contribution by beta cells for the observed phenotypes.

In conclusion, several studies have shown that TNF-α plays a role in the pathogenesis of human type 1 diabetes. Here we demonstrate that the phosphatase PTPN2 is an important regulator of the deleterious effects of TNF-α in human beta cells. *PTPN2* is a candidate gene for type 1 diabetes, and it is conceivable that patients carrying risk-associated *PTPN2* polymorphisms may particularly benefit from therapies targeting TNF-α.

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