Differential regulation of pro-inflammatory cytokine signalling by protein tyrosine phosphatases in pancreatic β -cells

William J Stanley^{1,2}, Prerak M Trivedi^{1,2}, Andrew P Sutherland¹, Helen E Thomas^{1,2} and Esteban N Gurzov^{1,2,3}

¹St. Vincent's Institute of Medical Research, Melbourne, Australia

²Department of Medicine, St. Vincent's Hospital, The University of Melbourne, Melbourne, Australia

³ULB Center for Diabetes Research, Universite Libre de Bruxelles (ULB), Brussels, Belgium

Correspondence
should be addressed
to E N Gurzov

Email
esteban.gurzov@unimelb.edu.au

Abstract

Type 1 diabetes (T1D) is characterized by the destruction of insulin-producing β -cells by immune cells in the pancreas. Pro-inflammatory including TNF- α , IFN- γ and IL-1 β are released in the islet during the autoimmune assault and signal in β -cells through phosphorylation cascades, resulting in pro-apoptotic gene expression and eventually β -cell death. Protein tyrosine phosphatases (PTPs) are a family of enzymes that regulate phosphorylative signalling and are associated with the development of T1D. Here, we observed expression of PTPN6 and PTPN1 in human islets and islets from non-obese diabetic (NOD) mice. To clarify the role of these PTPs in β -cells/islets, we took advantage of CRISPR/Cas9 technology and pharmacological approaches to inactivate both proteins. We identify PTPN6 as a negative regulator of TNF- α -induced β -cell death, through JNK-dependent BCL-2 protein degradation. In contrast, PTPN1 acts as a positive regulator of IFN- γ -induced STAT1-dependent gene expression, which enhanced autoimmune destruction of β -cells. Importantly, PTPN1 inactivation by pharmacological modulation protects β -cells and primary mouse islets from cytokine-mediated cell death. Thus, our data point to a non-redundant effect of PTP regulation of cytokine signalling in β -cells in autoimmune diabetes.

Key Words

- ▶ pancreatic β -cells
- ▶ protein tyrosine phosphatases
- ▶ PTPN1
- ▶ PTPN6
- ▶ cytokines
- ▶ inflammation

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Introduction

Type 1 diabetes (T1D) is an organ-specific autoimmune disease in which self-reactive T-cells destroy insulin-producing β -cells in the pancreas. Cytokines, in particular tumour necrosis factor (TNF)- α , interferon (IFN)- γ and interleukin (IL)-1 β are released by immune cells in the islet microenvironment and can promote β -cell destruction by inducing pro-apoptotic gene expression and stimulating post-translational modification and degradation of

anti-apoptotic BCL-2 proteins (Thomas *et al.* 2006, Gurzov & Eizirik 2011, Moore *et al.* 2011). Pro-apoptotic gene expression occurs by cytokine-stimulated cell signalling cascades that activate a variety of kinases and transcription factors, predominantly by phosphorylation events.

In β -cells, both IL-1 β and TNF- α stimulation activates downstream mitogen-activated kinase proteins (MAPKs) by dual tyrosine and threonine phosphorylation and the

NF- κ B transcription factor by phosphorylation-dependent release from I κ B α (Ortis *et al.* 2012). The extent of NF- κ B activation, however, is more prominent with IL-1 β than with TNF- α (Ortis *et al.* 2008, 2010, 2012). Cytokine-stimulated MAPKs, in particular c-Jun N-terminal kinases (JNKs), are associated with the induction of apoptosis in β -cells (Thomas *et al.* 2006, Gurzov *et al.* 2009). Inhibiting JNK activity has been shown to protect β -cell lines from apoptosis induction *in vitro* (Bonny *et al.* 2001, Gurzov *et al.* 2009). Anti-apoptotic BCL-2 proteins, such as BCL-2 and MCL-1, are substrates of JNK and upon extended phosphorylation of their BH3-binding domains, these proteins are altered in their capacity to bind and inhibit pro-apoptotic BH3-only proteins or targeted for degradation by the proteasome, which predisposes cells to death by apoptosis (Inoshita *et al.* 2002, Wei *et al.* 2008a,b, Wang *et al.* 2014). IL-1 β and TNF- α induce chemokine and inducible nitric oxide synthase (iNOS) expression in islets, and this effect is potentiated by IFN- γ , which contribute to mouse β -cell dysfunction and death (Chong *et al.* 2002, Gurzov *et al.* 2009).

IFN- γ activates the STAT1 transcription factor by tyrosine phosphorylation (Y701), facilitated by receptor-linked Janus kinases (JAKs). Once activated, STAT1 dimers translocate to the nucleus and induce gamma-activated site (GAS) gene expression (Moore *et al.* 2011), which includes chemokines, BH3-only apoptotic proteins and the major histocompatibility complex (MHC-I), important for diabetes development in the NOD mouse model (Serreze *et al.* 1994, Hamilton-Williams *et al.* 2003). Indeed, inhibition of STAT1 activity in NOD mice has been shown to protect from insulinitis and diabetes development (Chong *et al.* 2004, Kim *et al.* 2007, Trivedi *et al.* 2017).

Protein tyrosine phosphatases (PTPs) are a superfamily of enzymes that regulate tyrosine phosphorylation-dependent signalling. These enzymes are among the highest frequency risk alleles genetically associated with T1D, as shown by genome wide association studies (reviewed in Gurzov *et al.* 2015). PTPN2 (TCPTP) has been shown to have a pivotal role in IFN- γ -induced death of β -cells by negatively regulating STAT1-induced BCL-2-like protein 11 (BIM) expression (Moore *et al.* 2009, Santin *et al.* 2011). However, the role of PTPs, other than PTPN2, in cytokine signalling regulation remained unknown. Previously, we reported that PTPN2 and PTPN6 (SHP-1) are inactivated by inflammation-dependent oxidation in NOD mouse islets (Stanley *et al.* 2015). We and others have shown that cytokines produced by immune cells within the islet directly affect β -cells, resulting in their destruction. Cytokine signalling is tightly regulated

by PTPs and suppressors of cytokine signalling (SOCS) proteins. Inactivation of cytokine signalling in β -cells can reduce autoimmune diabetes (Chong *et al.* 2004, Kim *et al.* 2007, Moore *et al.* 2009, 2011, Santin *et al.* 2011, Trivedi *et al.* 2017), therefore, understanding the key players in regulation of these pathways is important and may result in the development of therapies to protect β -cells. In this study, we characterize a novel negative regulatory function of PTPN6 in TNF- α -induced death in β -cells by JNK signalling, as well as a positive regulatory role for PTPN1 (opposite to PTPN2) in IFN- γ -induced STAT1 activation and downstream gene expression.

Materials and methods

Mice, cell culture and treatments

NOD/Lt, NOD.PI and NOD8.3 mice were bred and maintained at St Vincent's Institute. Experiments were approved by the institutional animal ethics committee. NOD/Lt mice spontaneously develop insulinitis and autoimmune diabetes and are used as a model to study T1D (Makino *et al.* 1980). NOD.PI mice expressing proinsulin II under control of the MHC class II (I-E $_{\alpha}^*$) promoter do not develop diabetes due to immune tolerance development in the thymus (French *et al.* 1997). As these mice do not develop immune infiltrate in islets but otherwise have a normal immune system, they are used as negative controls to NOD/Lt mice in our biochemical studies. NOD8.3 mice expressing T-cell receptor $\alpha\beta$ rearrangements recognizing the H2K d restricted peptide (VYLKTNVFL) from islet-specific glucose 6-phosphatase catalytic subunit-related protein (IGRP) develop β -cell-specific CD8 $^+$ T-cells used in our ^{51}Cr assay (Verdaguer *et al.* 1997). Mouse islets were isolated as described previously (Thomas *et al.* 2006). Islets post isolation were cultured in CMRL (Life Technologies) supplemented with 10% FCS. Human pancreata were obtained, with informed consent from next-of-kin, from non-diabetic, heart-beating, brain-dead donors by the Australian Islet Transplant Consortium and approved by the human ethics committees of the hospitals involved and the Australian Red Cross. Human islets were isolated as described previously (Campbell *et al.* 2012). Insulin-producing MIN6 and NIT-1 cell lines were cultured in DMEM (Life Technologies) supplemented with 10% FCS. Cytokine treatments were performed in serum free media and concentrations were selected based on previous time course and dose-dependent studies (Thomas *et al.* 2006, Stanley *et al.* 2015). NIT-1 cells were treated with 10ng/mL of recombinant TNF- α (In Vitro Technologies, Melbourne, Australia) and/or 10U/mL of recombinant IFN- γ (Australian

Biosearch, Perth, Australia). Islets and MIN6 cells were treated with 100 ng/mL of recombinant TNF- α and/or 100 U/mL of recombinant IFN- γ . MIN6 and NIT-1 were treated with 10 μ M of the PTPN1 inhibitor (CAS 765317-72-4, Merck Millipore) or NSC 87877 (CAS 56932-43-5, Merck Millipore). Isolated islets were treated with 100 μ M of the PTPN1 inhibitor.

CRISPR/Cas9 deletion of protein tyrosine phosphatases

To delete PTPN6 or PTPN1 from NIT-1 cells, we employed a CRISPR/Cas9 exon deletion strategy incorporating the Cas9 D10A nickase mutant and four exon flanking guide RNA (gRNA) sequences to minimise off-target effects (Cong & Zhang 2015). After primer annealing, double-stranded oligonucleotide sequences containing gRNAs (sequences in [Supplementary Table 1](#), see section on [Supplementary data](#) given at the end of this article, BioRad) were cloned into pX335-U6-Chimeric BB-CBh-hSpCas9n(D10A), a gift from Feng Zhang (Addgene plasmid #42335). Chemically competent *Stbl3 E. coli* (ThermoFisher) were transformed by heat shock and grown on LB plates containing ampicillin (50 μ g/mL). Surviving colonies were then grown overnight in LB media and plasmid DNA purified using the AccuPrep Nano-Plus Plasmid Mini Extraction Kit (Bioneer, Daejeon, Republic of Korea) and validated by Sanger sequencing at the Australian Genome Research Facility. The four targeting vectors were then transiently cotransfected with pEGFP-puro, a gift from Michael McVoy (Addgene plasmid # 45561) using Lipofectamine 2000 (ThermoFisher) and transfected cells were selected with DMEM containing puromycin (10 μ g/mL) for 48 h. Gene-deficient cell lines were then identified via limiting dilution single cell cloning and screening by immunoblot for PTP expression.

Immunoblotting

Islets and cells were washed with PBS and lysed with radio-immunoprecipitation assay (RIPA) buffer. Lysates were then denatured at 100°C and resolved by 12–20% SDS-PAGE gel and transferred to a nitrocellulose membrane and blocked with 10% skim milk powder in TBST. Membranes were then incubated with primary antibody in 10% foetal bovine serum overnight at 4°C and secondary antibody in 10% skim milk powder for 1 h at room temperature. Membranes were washed with TBST between blocking and staining. HRP complexes were then activated with enhanced chemiluminescence and visualised on X-ray film (Fujifilm). Densitometry quantification was performed with ImageJ software. The list of antibodies used is provided in [Supplementary Table 2](#).

Cell viability

Cell viability was evaluated by either Hoechst-33342 and propidium iodide DNA staining or Nicoletti DNA fragmentation analysis (McKenzie *et al.* 2006). Cells were stained with Hoechst-33342 (1 μ g/mL, Sigma Aldrich) and propidium iodide (1 μ g/mL, Invitrogen) and the percentage cell death was evaluated by counting a minimum of 2000 cells in each condition.

mRNA extraction and real-time PCR

mRNA was extracted from cells and islets using a Isolate II RNA Micro Kit (Bioline, London, UK) and reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Real-Time PCR was performed using the AmpiTaq Gold Kit (Applied Biosystems). RNA analysis was performed with the ddCT method using β -actin as an internal control. The list of primers is provided in [Supplementary Table 3](#).

Flow cytometry

MHC-I expression was measured on MIN6 and NIT-1 cells with biotinylated H2D^b- and H2K^d-specific MHC-I antibodies ([Supplementary Table 2](#)), respectively, followed by FITC-conjugated streptavidin. NOD PI islets were dispersed into single cells and MHC-I was measured on β -cells using the H2K^d-specific MHC-I antibody with an APC conjugate and auto fluorescence to identify β -cells. Propidium iodide was used to exclude non-viable cells from analysis. Data were collected on a BDFortessa and subsequently analysed on FlowJo software.

⁵¹Cr release assay

Activated CD8⁺ T-cells recognizing the beta-cell antigen, islet-specific glucose 6-phosphatase catalytic subunit-related protein (IGRP) were generated as described previously (Dudek *et al.* 2006, Wali *et al.* 2015). These were then co-cultured overnight with NOD/Lt islets (10 islets/well, uniform shape and size) loaded with 200 μ Ci ⁵¹Cr. Medium alone and 2% Triton X-100 were considered as spontaneous and total cell lysis, respectively. The following day, ⁵¹Cr release from the harvested supernatant was measured using a gamma counter (PerkinElmer). Specific ⁵¹Cr release was measured using the following equation: percent lysis = (test counts per minute – spontaneous counts per minute) / (total counts per minute – spontaneous counts per minute) $\times$ 100.

Statistical analysis

Data are the mean $\pm$ S.E.M. of three to eight independent experiments. Comparisons between groups were made by one-way ANOVA with Tukey correction or by unpaired T-test when appropriate. Analysis was performed using version 7 of GraphPad Prism Software.

Results

PTPN6 and PTPN1 are expressed in human and NOD mouse islets

We have previously shown that PTPN6 and PTPN1 are expressed in the pancreas of diabetic NOD mice, and global PTP inactivation enhanced cytokine-induced islet death (Stanley *et al.* 2015). In the present study, we aimed to clarify their specific roles in cytokine signalling regulation in β -cells. As a first step, we evaluated whether PTPN6 and PTPN1 are present in islets from NOD mice and NOD PI mice, which do not develop insulinitis or diabetes due to immune tolerance to proinsulin (French *et al.* 1997). Immunoblot showed that PTPN6 and PTPN1 were expressed in both diabetic NOD and non-diabetic NOD PI islets (Fig. 1A and B). Consistent with this result, we observed the expression of PTPN6 and PTPN1 in human islet samples from 6 non-diabetic organ donors (Fig. 1C). Moreover, both proteins are expressed in FACS-purified human and rat primary β -cells (Villate *et al.* 2014,

Segerstolpe *et al.* 2016). Thus, we developed knockout models of PTPN6 and PTPN1 in the NIT-1 β -cell line to determine their roles in cytokine signalling regulation. PTPN6 and PTPN1-deficient NIT-1 cells were generated using CRISPR/Cas9 technology, as well as NIT-1 cells transfected with the empty vector to act as control. The targeting strategy was based on previously generated knockout mice, where PTPN1 exons 6–8 (Bence *et al.* 2006) and PTPN6 exons 5–10 were targeted (Pao *et al.* 2007) (Fig. 1D and E). Immunoblot confirmed the development of both PTPN6- and PTPN1-deficient NIT-1 cells with no protein of interest detected in comparison to the vector control (Fig. 1D and E).

PTPN6 inactivation enhances TNF- α induced JNK phosphorylation and β -cell death

To study if PTPN6 regulates pro-inflammatory cytokine-induced apoptosis of β -cells (Eizirik *et al.* 2009). PTPN6-deficient and control NIT-1 cells were treated with combinations of TNF- α , IFN- γ and IL-1 β and cell death was evaluated. Deficiency of PTPN6 did not affect viability in cells treated with IFN- γ , IL-1 β or combination of both cytokines (Fig. 2A, B, C and D and Supplementary Fig. 1A and B). Moreover, deficiency of PTPN6, or culture of cells with a dual specific inhibitor for PTPN6 and PTPN11, NSC 87877 (Chen *et al.* 2006) did not affect IFN- γ -induced STAT1 phosphorylation (Supplementary Fig. 1C, D and E).

Figure 1

Expression of PTPN6 and PTPN1 in human and murine islets and development of PTPN6- and PTPN1-deficient NIT-1 cells. (A and B) Western blot demonstrate that PTPN6 (A) and PTPN1 (B) are expressed in NOD/Lt and NOD PI mouse islets. The intensity values of the proteins were corrected by the values of the β -actin loading control and are shown in arbitrary units (A.U.). Results are means $\pm$ S.E.M. of four independent experiments. (C) PTPN6 and PTPN1 expression in human islets. Samples were acquired from six brain-dead non-diabetic organ donors and subjected to Western blotting with antibodies detecting PTPN6 and PTPN1 or α -tubulin as loading control. (D and E) Schematic of the CRISPR/Cas9 deletion strategy for PTPN6 (D) and PTPN1 (E). Western blot demonstrate deletion of PTPN6 and PTPN1 from NIT-1 cells, respectively. Results are the means of four/five independent experiments.

TNF- α has been shown previously to induce cell death in NIT-1 cells (Thomas *et al.* 2006). Interestingly, NIT-1 cells deficient in PTPN6 had enhanced cell death levels when treated with TNF- α alone or combined with IFN- γ /IL-1 β , in comparison to the vector control (Fig. 2A, B, C and D and Supplementary Fig. 1B). Consistent with this, NIT-1

cells treated with the PTPN6 inhibitor and TNF- α had enhanced cell death as measured by DNA fragmentation analysis (Supplementary Fig. 1F).

We next evaluated whether TNF- α -induced MAPK phosphorylation was affected by PTPN6 deficiency. We focused on the MAPKs JNK and p38 as these are activated

Figure 2

PTPN6 inactivation enhances TNF- α -induced NIT-1 cell death by regulating JNK signalling and BCL-2 and MCL-1 degradation. (A, B, C and D) PTPN6 negatively regulates TNF- α -induced cell death of NIT-1 cells. (A and B) PTPN6-knockout NIT-1 cells were treated with TNF- α and IFN- γ for 48h and cell death was assessed by the ratio of Hoechst-33342 (blue, viable) to propidium iodide (red, dead) nuclei. Scale bar represents 10 μ m. (C and D) DNA fragmentation of PTPN6 KO NIT-1 cells was assessed by Nicoletti staining of single cell suspensions and quantification of the sub-G1 phase of the cell cycle after 48-h cytokine treatment. Results are means $\pm$ S.E.M. of five independent experiments, * P < 0.05, ** P < 0.01. (E, F and G) PTPN6 negatively regulates TNF- α -induced JNK signalling and BCL-2 and MCL-1 degradation in NIT-1 cells. PTPN6 KO NIT-1 cells were treated with a time course of TNF- α for 24h and subjected to Western blotting for p-JNK (E), BCL-2 (F), MCL-1 (G) and β -actin as loading control. Quantification below represents p-JNK, BCL-2 and MCL-1 densitometry after correction for β -actin. Results are means $\pm$ S.E.M. of four to six independent experiments, * P < 0.05, ** P < 0.01, *** P < 0.001. A full colour version of this figure is available at <http://dx.doi.org/10.1530/JME-17-0089>.

by tyrosine phosphorylation and are implicated in the cytokine-induced death of β -cells (Gurzov *et al.* 2009). PTPN6-knockout NIT-1 cells were treated with TNF- α and the kinetics and magnitude of TNF- α -induced MAPK phosphorylation were evaluated. Immunoblot showed that JNK phosphorylation was enhanced in PTPN6-deficient cells (Fig. 2E), whereas p38 phosphorylation was not affected (Supplementary Fig. 1G). IL-1 β also induces JNK and c-Jun phosphorylation; thus, the kinetics of IL-1 β -induced MAPK signalling were evaluated. Interestingly, deficiency of PTPN6 partially reduced IL-1 β -induced JNK phosphorylation at early time points, but did not affect the downstream activation of c-Jun (Supplementary Fig. 1H and I).

TNF- α regulates anti-apoptotic BCL-2 protein levels in a variety of cell types altering the susceptibility of cells to apoptotic stimuli (Tamani *et al.* 1999, Wei *et al.* 2008a,b). The BCL-2 proteins BCL-2, MCL-1 and BCL-XL are substrates of JNK (Kharbanda *et al.* 2000, Inoshita *et al.* 2002, Wei *et al.* 2008a,b, Wang *et al.* 2014), where JNK phosphorylates their BH3-binding domains. Phosphorylation leads to either inability to bind BH3-only apoptotic proteins or targets them for degradation, which ultimately frees BH3-only proteins and enhances the susceptibility of the cell to apoptosis. Immunoblotting was used to elucidate whether enhanced TNF- α -induced JNK phosphorylation in PTPN6-deficient cells altered the expression of anti-apoptotic BCL-2 proteins. BCL-2 levels were enhanced after 24h of TNF- α treatment in PTPN6-expressing but not in PTPN6-deficient cells (Fig. 2F). Moreover, MCL-1 expression was reduced after 24h of treatment in wild-type cells and was further reduced in PTPN6-deficient cells (Fig. 2G). BCL-XL expression was not affected by TNF- α treatment or PTPN6 deficiency (not shown). Taken together, these results demonstrate that the deletion of PTPN6 in β -cells enhances TNF- α -induced JNK phosphorylation and cell death.

PTPN1 inactivation decreases cytokine-induced β -cell death

Next, we examined whether PTPN1 regulates cytokine-induced apoptosis of β -cells. PTPN1-deficient and control cells were treated with combinations of IL-1 β , IFN- γ and TNF- α and cell death levels evaluated. Treatment with IL-1 β or IFN- γ alone did not affect cell viability (Supplementary Fig. 3A and B); however, PTPN1-deficient cells had reduced cell death when treated with TNF- α or combination of cytokines (Fig. 3A, B, C and D and Supplementary

Fig. 3B). To verify that PTPN1 inactivation reduced TNF- α - and IFN- γ -induced cell death, a PTPN1 specific inhibitor was utilized (Wiesmann *et al.* 2004). MIN6 or NIT-1 cells were treated with the inhibitor and cytokines, and cell death levels were evaluated. Consistent with PTPN1-deficient NIT-1 cells, both lines treated with the inhibitor were significantly protected from cell death induced by TNF- α and IFN- γ , but were not protected from TNF- α -induced death alone (Fig. 3E and H). PTPN1 deficiency ablated induction of JNK, p38 and c-Jun phosphorylation by TNF- α in comparison to control cells (Supplementary Fig. 2A, B and C). In line with these results, the PTPN1 inhibitor only partially reduced phosphorylation of MAPKs (Supplementary Fig. 2D and E), suggesting that TNF- α -induced death is MAPK dependent. PTPN1 deficiency significantly reduced both IL-1 β -induced JNK and c-Jun phosphorylation (Supplementary Fig. 3C and D). We examined whether downstream iNOS and chemokine expression was affected with PTPN1 inhibition in NOD PI islets. Interestingly, PTPN1, iNOS and chemokine gene expression was significantly reduced by PTPN1 inhibition in IL-1 β -treated islets (Supplementary Fig. 3E).

TNF- α - and IFN- γ -induced β -cell death is attributed to the activation of the intrinsic apoptotic cascade, which results in the cleavage of the final effector caspase-3 (Barthson *et al.* 2011). We treated PTPN1 knockout and control cells with TNF- α and IFN- γ and performed immunoblotting for caspase-3 cleavage. Consistent with the decrease in cell death, caspase-3 cleavage was reduced in PTPN1-deficient cells in comparison to control cells (Fig. 3I). Reduced cytokine-induced caspase-3 cleavage was confirmed with the PTPN1 inhibitor in NIT-1 cells (Fig. 3J).

PTPN1 inactivation reduces IFN- γ -induced gene expression and T-cell-mediated death of mouse islets

To assess the impact of PTPN1 deficiency in IFN- γ signalling, we studied the signal transduction induced by IFN- γ in β -cells. PTPN1 knockout and control cells were treated with IFN- γ and STAT1 phosphorylation was evaluated. Immunoblot showed that PTPN1 deficiency reduced IFN- γ -induced STAT1 phosphorylation over the 24-h time course in comparison to control cells, as well as in both MIN6 and NIT-1 cells when treated with the PTPN1 inhibitor (Fig. 4A, B and C). To observe if this effect was conserved in islets, we isolated NOD PI mouse islets and treated them with the PTPN1 inhibitor and IFN- γ . Immunoblot confirmed that inhibition of PTPN1

Figure 3

PTPN1 inactivation protects β -cell lines from TNF- α - and IFN- γ -induced apoptosis. (A, B, C, D, E, F, G and H) PTPN1 deletion/inhibition reduces cytokine-induced death/DNA fragmentation of β -cell lines. PTPN1 KO NIT-1 cells (A and B) and PTPN1 inhibited MIN6 cells (E and F) were treated with TNF- α and IFN- γ (24 h for MIN6 cells and 48 h for NIT-1 cells) and cell death was assessed by the ratio of Hoechst-33342 (blue, viable) to propidium iodide (red, dead) nuclei. Scale bar represents 10 μ m. DNA fragmentation of PTPN1 KO NIT-1 cells (C and D) and PTPN1 inhibited NIT-1 cells (G and H) was assessed by Nicoletti staining of single cell suspensions and quantification of the sub-G1 phase of the cell cycle after 48 h cytokine treatment. Results are means $\pm$ S.E.M. of four to eight independent experiments, * P < 0.05, ** P < 0.01, *** P < 0.001. (I and J) PTPN1 deletion/inhibition reduces cytokine-induced caspase-3 cleavage. PTPN1 knockout NIT-1 cells (I) and PTPN1 inhibited NIT-1 cells (J) were treated with TNF- α and IFN- γ for 24 h and subjected to Western blotting for cleaved caspase-3 and β -actin as loading control. Quantification to the right of (I) and A.U. below (J) represent cleaved caspase 3 densitometry after correction for β -actin. Results are means $\pm$ S.E.M. of three independent experiments, *** P < 0.001. A full colour version of this figure is available at <http://dx.doi.org/10.1530/JME-17-0089>.

reduced STAT1 phosphorylation by ~50% in contrast to the vehicle-treated control islets (Fig. 4D).

IFN- γ -induced STAT1 signalling results in gene transcription of numerous pro-apoptotic genes (Moore *et al.* 2011). Strikingly, PTPN1 inhibition reduced expression of chemokines and iNOS in IFN- γ -treated NOD PI islets (Fig. 4E). Real-time qPCR confirmed that this effect was independent of PTPN2 and STAT1 expression

as both the vehicle control and inhibitor treated groups had comparable expression levels. IFN- γ induces STAT1-dependent MHC-I expression on β -cells and the presence of this antigen presentation complex is critical for autoimmune CD8⁺ T-cell destruction of β -cells in NOD mice (Serreze *et al.* 1994). We evaluated whether PTPN1 inactivation altered IFN- γ -induced MHC-I expression on β -cells. NIT-1 cells, MIN6 cells and isolated NOD PI

Figure 4

PTPN1 inactivation reduces IFN- γ -induced gene expression. (A, B and C) PTPN1 positively regulates IFN- γ signalling in β -cell lines. PTPN1 knockout NIT-1 cells (A) or MIN6 (B) and NIT-1 (C) cells inhibited for PTPN1 were treated with a time course of IFN- γ for 24h and subjected to Western blotting for p-STAT1, PTPN1 and β -actin as loading control. Quantification below represents p-STAT1 densitometry after correction for β -actin. Results are means $\pm$ s.e.m. of four to seven independent experiments, * P < 0.05, ** P < 0.01, *** P < 0.001. (D and E) PTPN1 positively regulates IFN- γ -induced STAT1 phosphorylation and downstream gene expression in primary islets. NOD PI islets inhibited for PTPN1 were treated with IFN- γ for 8h and subjected to Western blotting for p-STAT1 (D) and quantitative PCR for IFN- γ -induced gene expression (E). Quantification below represents p-STAT1 densitometry after correction for β -actin and expression profiles below represent gene expression relative to β -actin. Results are means $\pm$ s.e.m. of four independent experiments, * P < 0.05, *** P < 0.001.

Figure 5

PTPN1 inactivation reduces IFN- γ -induced MHC-I expression and protects islets from autoimmune destruction. (A, B and C) PTPN1 positively regulates IFN- γ -induced MHC-I expression on β -cell lines and primary islets. PTPN1 inhibited MIN6 cells (A), NIT-1 cells (B) and NOD PI islets (C) were treated with IFN- γ (24h for MIN6 and NIT-1 cells, 48h for NOD PI islets) and MHC-I expression was assessed by fluorescently activated cell sorting. Quantifications represent the Mean Fluorescent Intensity (MFI) of MHC-I. Results are means $\pm$ S.E.M. of four independent experiments, ** P < 0.01, *** P < 0.001. (D) PTPN1 inhibition protects islets from autoimmune destruction by NOD8.3 CD8 $^{+}$ T-cells. Islets were isolated from 4 to 6 week-old NOD/Lt mice and were treated with the PTPN1 inhibitor (100 μ M) or vehicle overnight and were then loaded with 51 Cr. These islets were then co-cultured with activated NOD8.3 CD8 $^{+}$ T-cells overnight at an effector:target ratio of 20:1. Result is representative of two independent experiments and shows 51 Cr release measurement in triplicate.

islets were treated with IFN- γ and the vehicle control or PTPN1 inhibitor and flow cytometry was performed to measure MHC-I expression. IFN- γ -induced the expression of MHC-I on all cell types and addition of the inhibitor reduced expression by ~50% on MIN6 and NIT-1 cells and ~20% on the islets (Fig. 5A, B and C). Moreover, NOD islets treated with the PTPN1 inhibitor were protected from NOD8.3 T-cell-induced destruction in comparison to control islets, as measured by specific 51 Cr release (Fig. 5D). Our results demonstrate that PTPN1 regulates IFN- γ signalling in β -cells and islets and has potential as a therapeutic target for novel anti-inflammatory drugs.

Discussion

Pancreatic β -cells are sensitive to the cytotoxic effects of TNF- α , IFN- γ and IL-1 β through activation of the intrinsic apoptotic cascade (Thomas *et al.* 2006, Gurzov *et al.* 2009, Gurzov & Eizirik 2011, Moore *et al.* 2011, Santin *et al.* 2011). PTPN2 was the first example of PTP inactivation sensitizing β -cells to death through hyperactivation of IFN- γ -induced STAT1-dependent BIM induction (Moore *et al.* 2009, Santin *et al.* 2011). In addition, we showed that chronic oxidative stress developed during insulinitis in the NOD mouse inactivated PTPN2 and PTPN6 and enhanced IFN- γ -dependent STAT1 signalling, culminating in islet death by BIM induction (Stanley *et al.* 2015). These previous studies showed a link for *in vivo* PTP inactivation enhancing the sensitivity of β -cells to cytokine-induced destruction. In the present study, we demonstrated that PTPN6 and PTPN1 have an important role in cytokine signalling regulation. Thus, PTPN6 negatively regulates

the TNF- α pathway and PTPN1 positively affects IFN- γ signalling. Furthermore, we showed that inactivation of each PTP could either predispose or protect β -cells to death.

Studies related to the role of PTPN6 in T1D are limited to nephropathy development (Denhez *et al.* 2015). PTPN6-deficient mice (motheaten mice) display a prominent autoimmune phenotype with development of granulocytic skin lesions, arthritis, multiple autoantibodies and succumb to lethal pneumonitis at the age of 2–3 weeks (Green & Shultz 1975, Abram *et al.* 2013). The motheaten viable mouse, which expresses PTPN6 with ~50% reduced catalytic activity, display increased levels of TNF- α in the lung and serum (Thrall *et al.* 1997). When treated with a soluble TNF- α receptor 1 (sTNFR1), these mice display a 2-fold increase in lifespan (Su *et al.* 1998), highlighting the requirement for TNF- α in the pathogenesis of the autoimmunity caused by loss of PTPN6. Here, we describe a mechanism by which PTPN6 inactivation promotes TNF- α -induced β -cell death by hyperactivation of JNK and degradation of BCL-2 and MCL-1. PTPN6 has previously been shown to negatively regulate JNK phosphorylation induced by 4-hydroxy-2-nonenal in HBE1 cells (Rinna & Forman 2008) as well as JNK-dependent IGF-1-induced MCF-7 breast cancer cell proliferation (Amin *et al.* 2011). JNK activity has also been linked to β -cell death in various *in vitro* studies using IL-1 β (Bonny *et al.* 2001, Gurzov *et al.* 2009). However, the activation of IL-1 β -induced JNK was slightly lower in PTPN6-deficient cells, which suggests that the role of PTPN6 in JNK signalling regulation is cytokine dependent. Inactivation of JNK2 also protects NOD.SCID mice from diabetes development by adoptive transfer of

diabetic NOD splenocytes (Jaeschke *et al.* 2005), linking JNK to autoimmune-dependent β -cell death *in vivo*. Our findings provide a mechanistic link to the motheaten mouse, where PTPN6 inactivation by oxidation during autoimmune pathologies, such as T1D, may lead to the hyperactivation of JNK in response to TNF- α culminating in enhanced cell death and autoimmunity.

PTPN1 activity is well studied in peripheral and pancreatic metabolic signalling in type 2 diabetes. Thus, PTPN1 is a negative regulator of insulin signalling in the liver and muscle, as well as leptin signalling in the hypothalamus (reviewed in Feldhammer *et al.* 2013). Moreover, mice deficient for PTPN1 showed a higher β -cell area, higher β -cell proliferation and a lower β -cell apoptosis induced by different stimuli (Fernandez-Ruiz *et al.* 2014). This has made it a potential target for treating type 2 diabetes (Krishnan *et al.* 2014). Macrovascular and microvascular complications of T1D, such as retinopathy, neuropathy and nephropathy can occur with chronic vascular inflammation attributed to high concentration of glucose in the blood. Inhibition of PTPN1 improved vascular relaxation and decreased endothelial dysfunction by restoring COX-2 expression and enhancing AKT expression in mice and human aortic endothelial cells (Herren *et al.* 2010, Herre *et al.* 2015). These studies show that targeting PTPN1 could be clinically beneficial for cardiovascular health and to enhance insulin signalling in patients with T1D.

Here, we describe a novel role for PTPN1 in the positive control of IFN- γ signalling and cytokine-induced cell death, opposite to PTPN2. Activation of the STAT1 transcription factor by IFNs in β -cells has been shown to be critical for the pathogenesis of autoimmune diabetes in the NOD mouse. Indeed, STAT1-deficient NOD mice and NOD mice that overexpress SOCS-1 in β -cells, a potent negative regulation of STAT1 signalling, are protected from insulinitis and diabetes development (Chong *et al.* 2004, Kim *et al.* 2007). Inhibiting JAK1 and JAK2, which activate STAT1 in response to IFN- γ , has also been shown to protect and reverse diabetes development in the NOD mouse (Trivedi *et al.* 2017). The insulinitis reduction in these models is attributed to reduced chemokine production and MHC-I expression on β -cells (Sarkar *et al.* 2012, Trivedi *et al.* 2016, 2017), masking them from autoreactive immune cells. We have shown that PTPN1 inactivation decreased IFN- γ -induced STAT1 phosphorylation and transcription of downstream genes, which enhanced islet viability when co-cultured with autoreactive CD8⁺ T-cells. PTPN1 has been described to negatively regulate

IFN- α -induced STAT1 and STAT3 signalling in renal mesangial cells (Zhang *et al.* 2015), which signals through similar JAKs to IFN- γ (JAK1/Tyk2 and JAK1/JAK2, respectively). PTPN1 can also negatively regulate leptin signalling through dephosphorylation of JAK2 and STAT3 (Lund *et al.* 2005). This previous work and the present study suggest that regulation of cytokine signalling by PTPs is cell type specific. For example, PTPN22 is reported to negatively regulate IFN- α -induced STAT1 phosphorylation in haematopoietic progenitor cells (Holmes *et al.* 2015), whilst positively regulating the same signalling pathway in P14 CD8⁺ T-cells (Jofra *et al.* 2017). We also show that PTPN1 positively regulates IL-1 β -induced MAPK signalling and downstream iNOS and chemokine expression, which contribute to β -cell dysfunction (Thomas *et al.* 2006, Gurzov *et al.* 2009).

PTPs were thought to have generalized and redundant house-keeping activity when they were first characterized (Gurzov *et al.* 2015). However, it is now clear that these proteins have non-redundant roles in both positive and negative regulation of multiple cell signalling pathways. Future studies are required to identify the specific targets of PTPN1 and PTPN6 in β -cells, to fully clarify the mechanism of action. We presently demonstrated that PTPN1 inactivation is cytoprotective to β -cells when stimulated with IFN- γ , TNF- α and IL-1 β . It would be desirable to identify the substrate of PTPN1 and elucidate whether overexpression or specific stimulation have potential to further protect β -cells from cytotoxic stimuli in the context of inflammation and T1D. Moreover, our findings could be translated to islet transplantation, as islet graft rejection requires IFN- γ /STAT1 (Kim *et al.* 2007, Yi *et al.* 2012). Thus, PTPN1 inhibition, by limiting IFN- γ signalling *in situ*, may also be beneficial to transplant recipients.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/JME-17-0089>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

W J Stanley researched data; designed experiments and reviewed, edited, and wrote the manuscript. P M Trivedi, A P Sutherland and H E Thomas contributed to experimental design, researched data and reviewed and edited the manuscript. E N Gurzov researched data, contributed to experimental design and discussion and reviewed and edited the manuscript.

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