

Interferon- α mediates human beta cell HLA class I overexpression, endoplasmic reticulum stress and apoptosis, three hallmarks of early human type 1 diabetes

Laura Marroqui¹ · Reinaldo S. Dos Santos¹ · Anne Op de beeck¹ ·
Alexandra Coomans de Brachène¹ · Lorella Marselli² · Piero Marchetti² ·
Decio L. Eizirik¹

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Abstract

Aims/hypothesis Three hallmarks of the pancreatic islets in early human type 1 diabetes are overexpression of HLA class I, endoplasmic reticulum (ER) stress and beta cell apoptosis. The mediators of these phenomena remain to be defined. The type I interferon IFN α is expressed in human islets from type 1 diabetes patients and mediates HLA class I overexpression. We presently evaluated the mechanisms involved in IFN α -induced HLA class I expression in human beta cells and determined whether this cytokine contributes to ER stress and apoptosis.

Methods IFN α -induced inflammation, ER stress and apoptosis were evaluated by RT-PCR, western blot, immunofluorescence and nuclear dyes, and proteins involved in type I interferon signalling were inhibited by small interfering RNAs. All experiments were performed in human islets or human EndoC- β H1 cells.

Results IFN α upregulates HLA class I, inflammation and ER stress markers in human beta cells via activation of the candidate gene *TYK2*, and the transcription factors signal transducer and activator of transcription 2 and IFN regulatory factor 9. Furthermore, it acts synergistically with IL-1 β to induce beta cell apoptosis.

Conclusions/interpretation The innate immune effects induced by IFN α may induce and amplify the adaptive immune response against human beta cells, indicating that IFN α has a central role in the early phases of diabetes.

Keywords Apoptosis · ER stress · IFN α · MHC class I · Pancreatic beta cells · Pancreatic islets · Type 1 diabetes · Type I IFN signalling

Laura Marroqui and Reinaldo S. Dos Santos contributed equally to the study.

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✉ Laura Marroqui
lmarroqu@ulb.ac.be

✉ Decio L. Eizirik
deizirik@ulb.ac.be

¹ ULB Center for Diabetes Research, Campus Erasme, Université Libre de Bruxelles, Route de Lennik, 808-CP618, B-1070 Brussels, Belgium

² Department of Clinical and Experimental Medicine, University of Pisa, Pisa, Italy

Abbreviations

ATF3	Activating transcription factor 3
BIP	Binding immunoglobulin protein
CHOP	C/EBP homologous protein
CXCL10	C-X-C motif chemokine ligand 10
eIF2	Eukaryotic initiation factor 2
ER	Endoplasmic reticulum
HO	Hoechst 33342
IFNAR1	IFN α / β receptor
IRF9	IFN regulatory factor 9
ISG	IFN-stimulated gene
JAK	Janus kinase
PI	Propidium iodide
PIC	Polyinosinic-polycytidylic acid
siCTRL	siRNA control
siRNA	Small interfering RNA
STAT	Signal transducer and activator of transcription
TYK2	Tyrosine kinase 2

TUDCA	Tauroursodeoxycholic acid
USP18	Ubiquitin-specific peptidase 18
XBP1	X-box binding protein 1
XBP1s	Spliced isoform of XBP1

Introduction

Three hallmarks of the pancreatic islets in early human type 1 diabetes and in mouse models of the disease are overexpression of HLA class I [1, 2], presence of markers of endoplasmic reticulum (ER) stress [3–5] and beta cell apoptosis [6]. The mediator(s) of these phenomena, however, remain(s) to be defined.

The cytokine IFN α , a member of the type I IFN family, is expressed in human islets from patients with type 1 diabetes [7–10] and in the pancreas of NOD mice [11–13]. Children genetically at risk for type 1 diabetes present a type I IFN-inducible transcriptional signature that precedes the development of autoantibodies [14, 15], and IFN α plays a major role as mediator of HLA class overexpression in human islet cells, a key event in early type 1 diabetes [16, 17]. Laser-captured islets obtained from living donors with recent onset type 1 diabetes showed a significant increase in nearly 50% of the IFN-stimulated genes (ISGs) evaluated [18]. Neutralisation of the IFN α / β receptor (IFNAR1) with monoclonal antibodies prevents diabetes in NOD mice [13, 19] and self-reactive antibodies targeting type I IFNs—particularly IFN α —are associated with protection against type 1 diabetes in patients with mutations in the thymus transcription factor autoimmune regulator (AIRE) [20]. It is surprising that very few studies have investigated the direct effects of this cytokine in pancreatic beta cells [21–23].

We presently tested the hypothesis that IFN α is a common mediator of HLA class I overexpression, ER stress and beta cell apoptosis in early type 1 diabetes, and evaluated the signal transduction mediating these effects. All experiments were performed in human pancreatic islets and the human insulin-producing cell line EndoC- β H1 [24], increasing their translational potential [25–28]. The data obtained indicate that IFN α is a crucial mediator of excessive inflammation and ER stress in the early steps of type 1 diabetes.

Methods

Culture of human EndoC- β H1 beta cells and human islets, and cell treatments The human beta cell line EndoC- β H1 (kindly provided by R. Scharfmann, Centre de Recherche de l'Institut du Cerveau et de la Moelle Épineuse, Paris, France) was cultured in Matrigel-fibronectin-coated plates [24]. MycoAlert Mycoplasma Detection kit (Lonza, Basel, Switzerland) was used to test for mycoplasma infection.

EndoC- β H1 cells have been shown to be free of mycoplasma infection.

Human islets from 12 non-diabetic organ donors (ESM Table 1) were isolated with the agreement of the local Ethical Committee in Pisa, Italy and sent to Brussels for experiments (see ESM Methods). Human islets or EndoC- β H1 cells were exposed to cytokines or other agents as described in ESM Methods [23, 26].

RNA interference Conditions for small interfering RNA (siRNA) transfection using Lipofectamine RNAiMAX lipid reagent (Invitrogen, Carlsbad, CA, USA) and optimal siRNA concentration (30 nmol/l) were established previously [29]. Allstars Negative Control siRNA (Qiagen, Venlo, the Netherlands) was used as a negative control (siCTRL); see ESM Methods for further details. siRNAs against *TYK2*, *STAT1*, *STAT2*, *IRF9*, *USP18*, *PTPN2*, and *CHOP* were used in this study (a list with further information is provided in ESM Table 2).

Assessment of cell viability Cell viability was determined after staining with the DNA-binding dyes Hoechst 33342 (HO) and propidium iodide (PI) as described [29, 30]. See ESM Methods.

mRNA extraction and real-time PCR Poly(A)⁺ mRNA extraction was performed using Dynabeads mRNA DIRECT kit (Invitrogen) in accordance with the manufacturer's instructions; reverse transcription was carried out as described [23]. Quantitative real-time PCR was performed using SYBR Green and the data were compared with a standard curve [31]. Expression values were corrected by the housekeeping gene β -actin, as its expression is not modified under the conditions used in this study [23] (data not shown). A list with the primers used in this study is provided in ESM Table 3.

Western blot analysis, immunofluorescence and flow cytometry Western blotting was performed as described [23]. Briefly, cells were washed with cold PBS and lysed in Laemmli or RIPA buffer. Immunoblot analysis was performed with antibodies against signal transducer and activator of transcription (STAT) 1–3 and their phosphorylated forms, MHC class I, interferon regulatory factor 9 (IRF9), activating transcription factor 3 (ATF3), X-box binding protein 1 (XBP1), phosphorylated eukaryotic initiation factor 2 (p-eIF2 α), binding immunoglobulin protein (BIP), insulin (all at 1:1000 dilution) and α -tubulin (1:5000; see ESM Table 4). Peroxidase-conjugated antibodies (1:5000) were used as secondary antibodies. SuperSignal West Femto chemiluminescent substrate (Thermo Scientific, Rockford, IL, USA) and ChemiDoc XRS+ (Bio-Rad Laboratories, Temse, Belgium) were used to detect bands and Image Lab software (version 3.0, Bio-

Rad Laboratories, Temse, Belgium) was used for densitometry analysis.

Immunofluorescence was performed as described [23]. Briefly, cells were plated on polylysine-coated coverslips, treated with intracellular IFN α for 24 h and fixed with 4% paraformaldehyde. Cells were permeabilised and incubated with rabbit anti-MHC class I (W6/32) (1:1000) or mouse monoclonal anti-insulin (1:1000). Alexa Fluor-conjugated secondary antibodies were used (see ESM Table 4). After nuclear staining with HO, coverslips were mounted with fluorescent mounting medium (Dako, Carpinteria, CA, USA) and immunofluorescence was visualised on a Zeiss microscope equipped with a camera (Zeiss-Vision, Munich, Germany). Images were acquired at $\times 20$ or $\times 40$ magnification and analysed using AxiVision software (version 4.7.2; Zeiss-Vision, Munich, Germany). Images (magnification $\times 20$) were quantified using FIJI software (version 2.0; <https://fiji.sc>) and calculated as (mean of fluorescence / number of cells) $\times 100$.

The same protocol used for immunofluorescence, but without permeabilisation, was used for flow cytometry. Cells were detached by a mild Accutase (Sigma-Aldrich, Schnellendorf, Germany) treatment and then suspended in 2% paraformaldehyde and EDTA-containing PBS and analysed by flow cytometry (FacsCanto; BD Biosciences, San Jose, CA, USA). Analysis was performed using FACSDiva software version 1.0 (BD Biosciences, San Jose, CA, USA). The cellular populations were selected based on size and cell granularity and were analysed for BV421 fluorescence.

Antibodies have been previously validated by our group.

Statistical analysis Data are shown as means \pm SEM or presented as box plots indicating lower quartile, median and higher quartile, with whiskers representing the range of the remaining data points. Comparisons were performed by two-tailed paired *t* test or by ANOVA followed by paired *t* test with Bonferroni correction, as indicated. Results with $p < 0.05$ were considered significant.

Results

IFN α activates STAT pathways that increase expression of MHC class I protein and inflammatory markers in human beta cells IFN α activated STAT1, STAT2 and STAT3; the maximum effect was observed at 1–2 h post treatment and returned to baseline by 24 h (Fig. 1a). At later time points (8 and 24 h), IFN α induced the expression of IRF9, an essential protein for IFN α signal transduction [32] (Fig. 1a), and of MHC class I expression at mRNA and protein levels in EndoC- β H1 cells and dispersed human islets (Fig. 1b–d, g). IFN α also increased, at 4–8 h, expression of mRNAs encoding for the chemokine C-X-C motif chemokine ligand

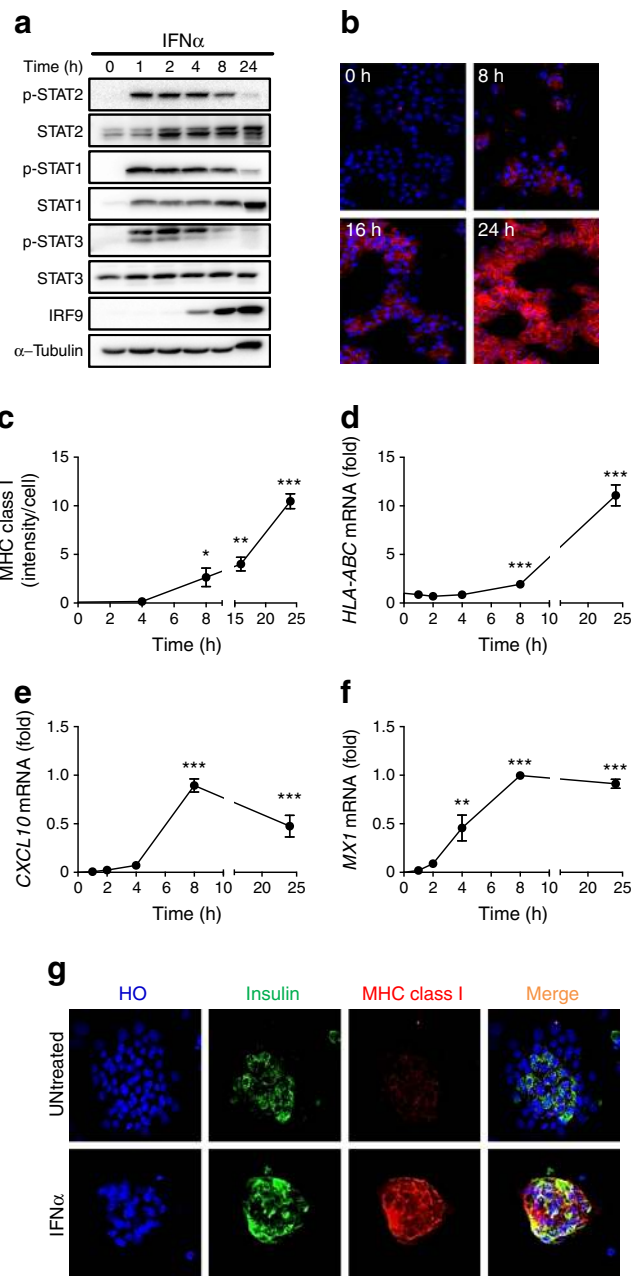


Fig. 1 IFN α activates STAT pathways and increases inflammatory and innate immune response markers and MHC class I expression in human beta cells. EndoC- β H1 (a–f) or dispersed human islets (g) were left untreated or were treated with IFN α (2000 U/ml) for 1–24 h (a–f) or 24 h (g). (a) Protein expression was measured by western blot. Images are representative of three independent experiments. (b, c) MHC class I protein expression was analysed by immunocytochemistry (ICC). Representative images of three independent experiments (magnification $\times 40$) (b) and quantification (c) is shown. (d–f) mRNA expression of *HLA-ABC* (d), *CXCL10* (e) and *MX1* (f) was analysed by RT-PCR and normalised by β -actin. In (d), values were normalised by control (0 h), considered as 1. In (e) and (f), values were normalised by the highest value of each experiment (considered as 1). Results are means \pm SEM of three to six independent experiments. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ vs untreated (ANOVA). (g) ICC of MHC class I (red), insulin (green) and HO (blue) was performed to confirm MHC class I expression in two dispersed human islet preparations (magnification $\times 40$)

10 (CXCL10) and the antiviral protein MX dynamin like GTPase 1 (MX1) (Fig. 1e, f).

IFN α and IFN γ similarly induced MHC class I expression (around 15-fold increase above basal), while IL-1 β showed no effect (ESM Fig. 1b, c) and polyinosinic-polycytidylic acid (PIC) only a twofold-increased effect. Expression of MHC class I was not associated with apoptosis in EndoC- β H1 cells (ESM Fig. 1a).

Flow cytometry analysis showed that IFN α induced a dose-dependent (starting at 2 U/ml) increase in MHC class I surface expression (ESM Fig. 2a–c) and *HLA-ABC* and *CXCL10* (ESM Fig. 2d, e) mRNA expression.

IFN α increases ER stress markers in human beta cells

Exposure of EndoC- β H1 cells to IFN α for 24 h upregulated expression of mRNAs and proteins for the following ER stress markers (an outline of the evaluated ER stress markers is depicted in Fig. 2a): DNA damage inducible transcript 3 (encoded by *CHOP*, also known as *DDIT3*) (Fig. 2b), ATF3 (Fig. 2c, f, h), heat shock protein family A (Hsp70) member 5

(encoded by *BIP*, also known as *HSPA5*) (Fig. 2d, f, i), spliced isoform of XBP1 (XBP1s, encoded by *XBP1s*; Fig. 2e, f, j) and phosphorylated eIF2 α (Fig. 2f, g). Expression of *CHOP* was also induced in EndoC- β H1 cells by 20–200 U/ml of IFN α (ESM Fig. 3). There was an early (1 h) and transitory increase in phosphorylated EIF2 α and its downstream protein ATF3 (Fig. 2f–h). In contrast, XBP1s and BIP protein expression was augmented after 4 h and remained increased until 24 h (Fig. 2f, i, j).

IFN α -induced inflammation and ER stress response is abolished by tyrosine kinase 2 knockdown in human beta cells

TYK2, a candidate gene for type 1 diabetes, contributes to the activation of the type I IFN pathway and regulation of MHC class I expression in human beta cells [23]. Tyrosine kinase 2 (TYK2) knockdown prevented IFN α -induced MHC class I protein expression in EndoC- β H1 cells (ESM Fig. 4a, c) and dispersed human islets (ESM Fig. 4b, d) and also partially or completely prevented induction of *CXCL10* (Fig. 3a, g), *MX1* (Fig. 3b, h) and the ER stress markers *CHOP*

Fig. 2 IFN α increases ER stress markers in EndoC- β H1 cells. (a)

Schematic representation of the ER stress markers measured. IRE1, inositol-requiring protein 1; PERK, protein kinase RNA-like endoplasmic reticulum kinase.

(b–j) EndoC- β H1 cells were left untreated or were treated with IFN α (2000 U/ml). mRNA expression of *CHOP* (b), *ATF3* (c), *BIP* (d) and *XBP1s* (e) was analysed by RT-PCR, normalised by β -actin and then by the highest value of each experiment (considered as 1). Protein expression was measured by western blotting and representative images of four independent experiments are shown (f). Densitometry results are shown for p-EIF2 α (g), ATF3 (h), BIP (i) and XBP1s (j). Values were normalised by α -tubulin (α -tub), and then by the highest value of each experiment (considered as 1). Results are means \pm SEM of four to six independent experiments. * p < 0.05, ** p < 0.01 and *** p < 0.001 vs untreated (ANOVA)

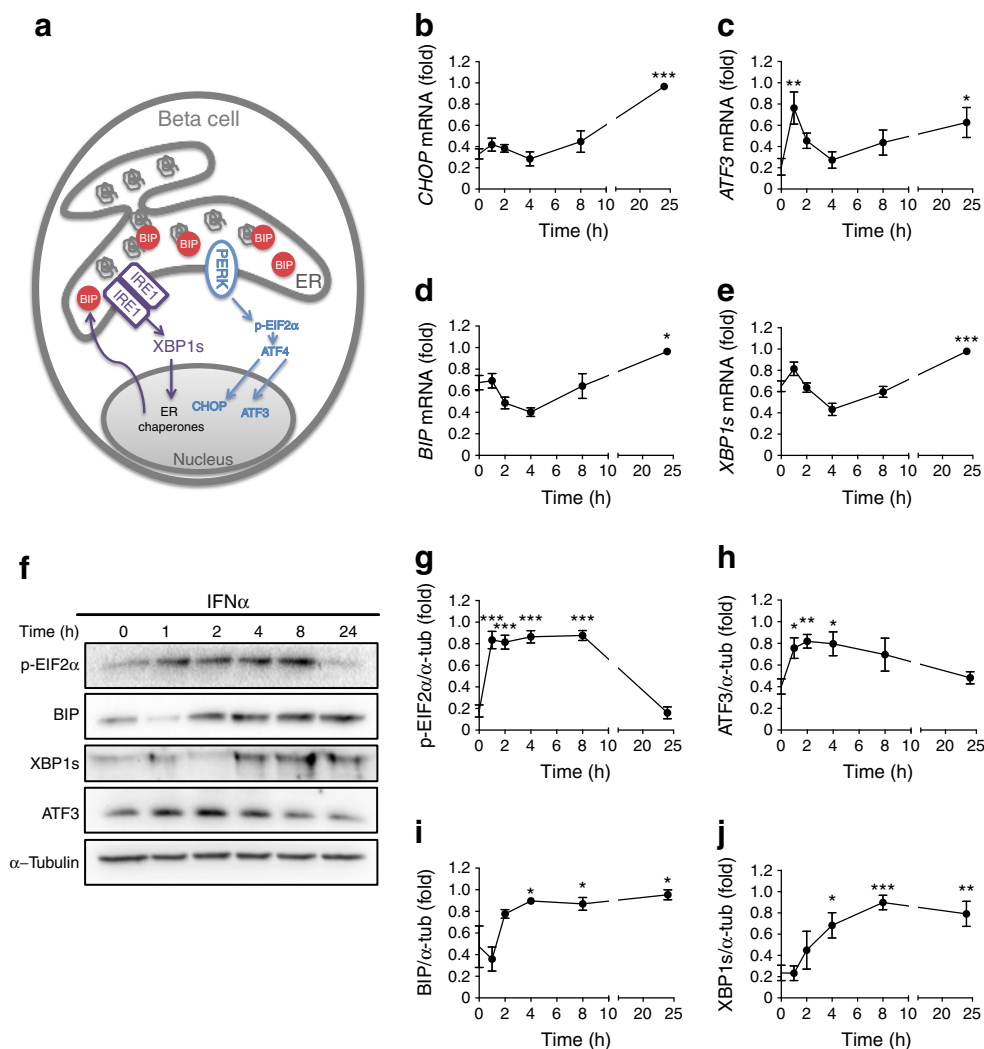
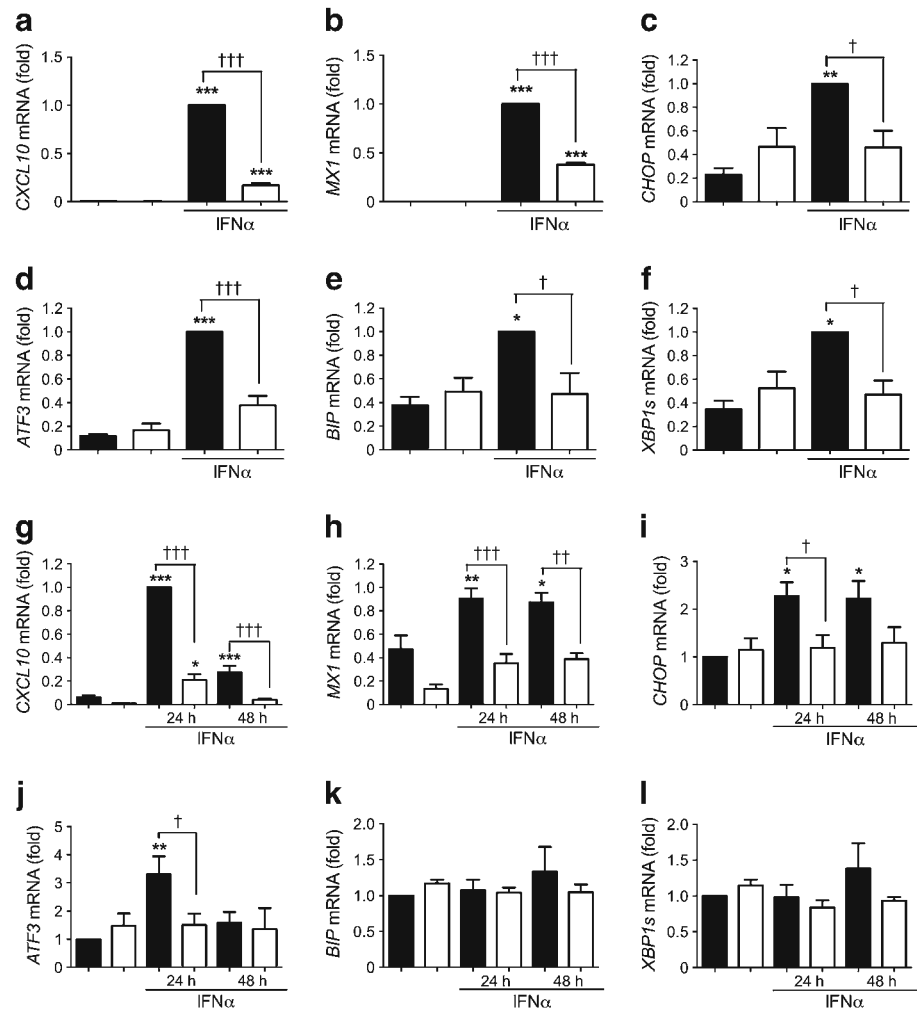


Fig. 3 TYK2 inhibition prevents IFN α -induced inflammation and ER stress markers in human beta cells. EndoC- β H1 cells (a–f) and dispersed human islets (g–l) were transfected with siCTRL (black bars) or with siRNA targeting *TYK2* (white bars). Cells were left untreated or were treated with IFN α (2000 U/ml) for 24 h (EndoC- β H1 cells) or 24 and 48 h (dispersed human islets). mRNA expression of *CXCL10* (a, g), *MX1* (b, h), *CHOP* (c, i), *ATF3* (d, j), *BIP* (e, k) and *XBP1s* (f, l) was analysed by RT-PCR and normalised by β -actin. In (a–h), values were normalised by the highest value of each experiment (considered as 1). In (i–l), values were normalised by siCTRL (black bars, non-treated), considered as 1. Results are means \pm SEM of three or four independent experiments. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ vs untreated and transfected with the same siRNA; † $p < 0.05$, †† $p < 0.01$ and ††† $p < 0.001$, as indicated by bars (ANOVA)



(Fig. 3c, i), *ATF3* (Fig. 3d, j) *BIP* (Fig. 3e, k) and *XBP1s* (Fig. 3f, l) by IFN α .

STAT2 and IRF9, but not STAT1, are important mediators of IFN α -induced inflammation and ER stress markers in human beta cells

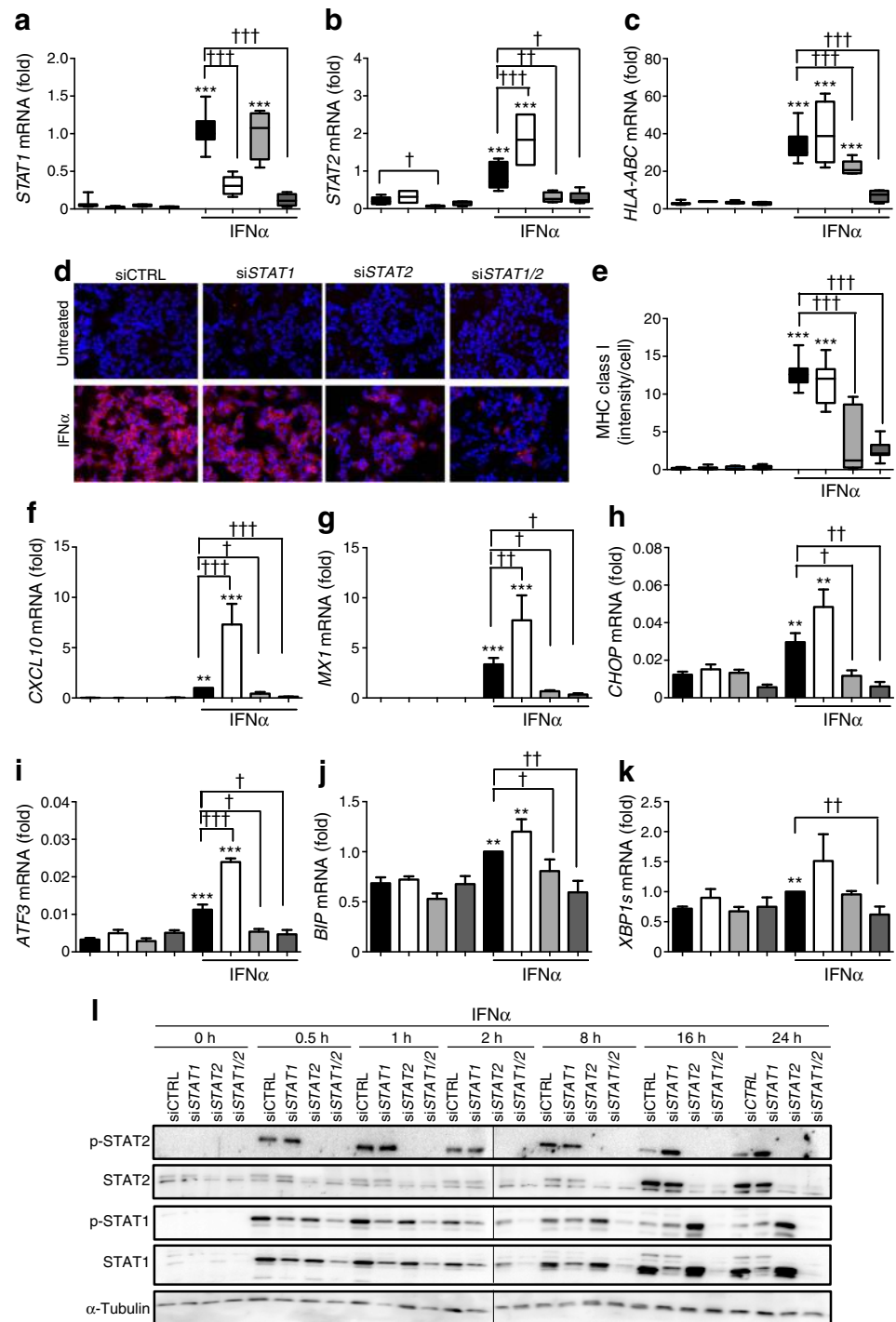
Type I IFN binding to its receptor activates TYK2, which in turn phosphorylates STAT1 and STAT2 [32]. Surprisingly, STAT1 knockdown ($\geq 70\%$ at mRNA and protein levels; Fig. 4a, l and ESM Fig. 5a, b) failed to decrease IFN α -induced MHC class I expression at mRNA or protein levels in EndoC- β H1 cells (Fig. 4c–e). This was paralleled by increased expression of *CXCL10* (Fig. 4f), *MX1* (Fig. 4g) and ER stress markers (Fig. 4h–k), especially *CHOP* and *ATF3*. STAT1 knockdown, however, prevented IFN γ -induced HLA class I and *CXCL10* expression (ESM Fig. 5d, e), indicating that the level of STAT1 inhibition reached was sufficient to block its downstream effects.

Knockdown of STAT2 and double knockdown of STAT1 and 2 ($\geq 60\%$ and $\geq 70\%$, respectively; Fig. 4a, b, l), almost completely prevented IFN α -induced expression of MHC class I (Fig. 4c–e), *CXCL10* (Fig. 4f) and *MX1* (Fig. 4g),

and ER stress markers (Fig. 4h–k). STAT2 knockdown led to STAT1 overactivation, and vice versa, suggesting a compensatory effect between them (Fig. 4l). This may explain the observed increment in *CXCL10* and *MX1* mRNA expression after STAT1 knockdown. These results were confirmed in dispersed human islets (Fig. 5), where only STAT2 knockdown ($\geq 80\%$ at mRNA and $\geq 50\%$ at protein level; Fig. 5b, i) decreased IFN α -induced inflammatory and ER stress markers (Fig. 5f–h), whereas STAT1 knockdown ($\geq 80\%$ at mRNA and protein levels; Fig. 5a, i) increased expression of *CXCL10* (Fig. 5d) and *MX1* (Fig. 5e) mRNA.

STAT2 dimerises with IRF9 in the absence of STAT1; this dimer subsequently binds to the IFN-stimulated response element (ISRE) and mediates downstream signal transduction [33]. Knockdown of IRF9 ($\geq 60\%$ at mRNA and $\geq 80\%$ at protein level; Fig. 6b, ESM Fig. 6) and double knockdown of STAT2 and IRF9 (Fig. 6a, b and ESM Fig. 6) decreased IFN α -induced *HLA-ABC*, *CXCL10* and *MX1* (Fig. 6c–e) and ER stress markers (Fig. 6f–i) mRNA levels to the same extent as STAT2 knockdown alone, indicating that both are part of the same signalling pathway. No change in apoptotic rate was

Fig. 4 Inhibition of STAT2 but not STAT1 prevents IFN α -induced inflammation and expression of ER stress markers and MHC class I in EndoC- β H1 cells. EndoC- β H1 cells were transfected with siCTRL (black bars) or with siRNAs targeting *STAT1* (white bars), *STAT2* (light-grey bars) and *STAT1/2* (dark-grey bars). Cells were left untreated or were treated with IFN α (2000 U/ml) for 24 h (a–k) or for different lengths of time (l). mRNA expression of *STAT1* (a), *STAT2* (b), *HLA-ABC* (c), *CXCL10* (f), *MX1* (g), *CHOP* (h), *ATF3* (i), *BIP* (j) and *XBP1s* (k) was analysed by RT-PCR and normalised by β -actin. In (f, j, k), values were normalised by siCTRL (black bars treated with IFN α), considered as 1. (d, e) immunocytochemistry (ICC) of MHC class I (red) and HO (blue) in EndoC- β H1 cells (magnification $\times 40$); representative images of four independent experiments (d) and quantification (e) are shown. Results are means \pm SEM of three or four independent experiments. $**p < 0.01$ and $***p < 0.001$ vs untreated and transfected with the same siRNA; $^\dagger p < 0.05$, $^\dagger\dagger p < 0.01$ and $^\dagger\dagger\dagger p < 0.001$, as indicated by bars (ANOVA). In (l), protein expression was measured by western blot; representative images of three independent experiments are shown



observed after knockdown of STAT1, STAT2 or IRF9 (or combinations of them) in EndoC- β H1 cells, or after knockdown of STAT1 or STAT2 in dispersed human islets treated (or not) with IFN α (data not shown).

Inhibition of ubiquitin-specific peptidase 18 overstimulates type I IFN signalling and increases ER stress and inflammation markers in beta cells Inhibition of ubiquitin-specific

peptidase 18 (USP18) induces beta cell inflammation and apoptosis upon IFN α treatment by exacerbating IFN-induced phosphorylation of STATs [34]. To determine whether this would also augment IFN-induced ER stress, we knocked down USP18 in EndoC- β H1 cells. *USP18*-silenced cells ($\geq 50\%$ at mRNA level; Fig. 7a) treated with IFN α had increased levels of p-STAT2 and p-STAT1 (five- and twofold, respectively) (Fig. 7b–d), higher prevalence of apoptosis

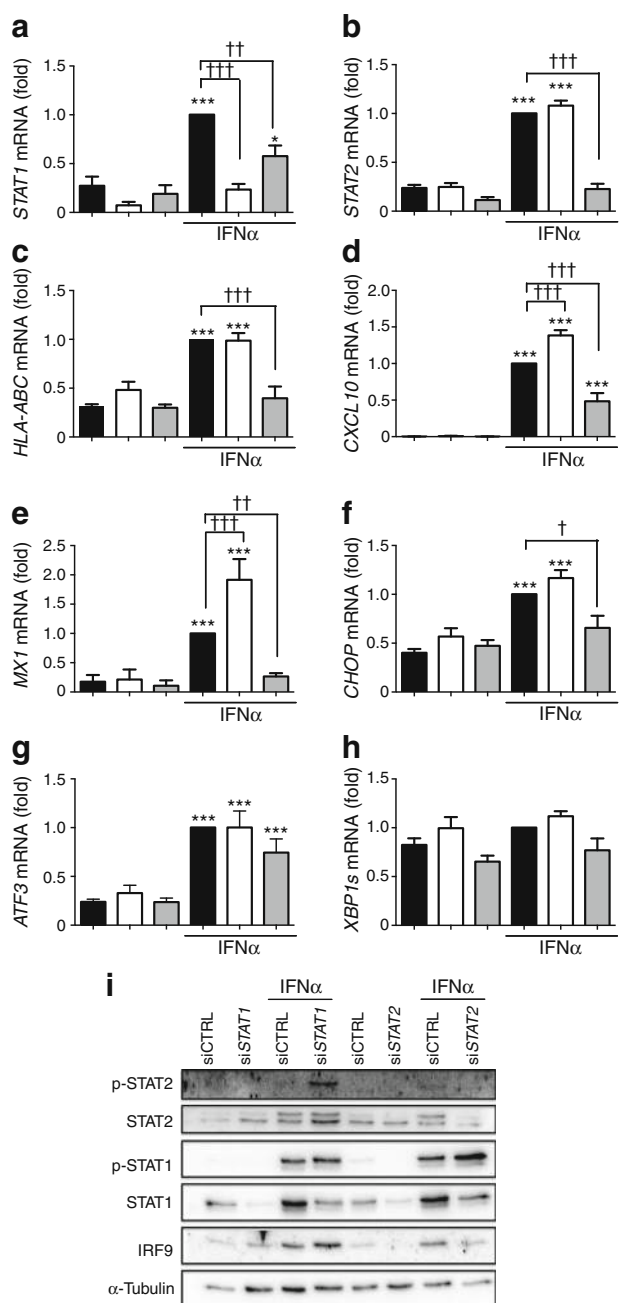


Fig. 5 Inhibition of STAT2 but not STAT1 prevents IFN α -induced inflammation and expression of ER stress markers in dispersed human islets. Dispersed human islets were transfected with siCTRL (black bars) or with siRNAs targeting *STAT1* (white bars) or *STAT2* (light-grey bars). Cells were left untreated or were treated with IFN α (2000 U/ml) for 24 h. (a–h) mRNA expression of *STAT1* (a), *STAT2* (b), *HLA-ABC* (c), *CXCL10* (d), *MX1* (e), *CHOP* (f), *ATF3* (g) and *XBP1s* (h) was analysed by RT-PCR and normalised by β -actin, and then normalised by siCTRL (black bars treated with IFN α), considered as 1. Results are means \pm SEM of four or five independent human islet preparations. * p < 0.05, ** p < 0.01 and *** p < 0.001 vs untreated and transfected with the same siRNA; † p < 0.05, †† p < 0.01 and ††† p < 0.001, as indicated by bars (ANOVA). (i) Protein expression was measured by western blot; representative images of four or five independent experiments are shown

(Fig. 7e) and increased mRNA expression of *HLA-ABC* (threefold, Fig. 7f), *CXCL10* (50-fold, Fig. 7g) and *MX1* (threefold, Fig. 7h) and at least threefold higher ER stress markers (Fig. 7i–l), compared with control cells.

On the other hand, *PTPN2* (a candidate gene for type 1 diabetes), which encodes protein tyrosine phosphatase, non-receptor type 2, a protein phosphatase that dephosphorylates members of the Janus kinase (JAK)/STAT family but not TYK2 or STAT2 [23], did not affect IFN α -induced *HLA-ABC*, inflammation and ER stress marker mRNA expression in EndoC- β H1 cells (ESM Fig. 7).

IFN α sensitises human beta cells against IL-1 β -induced apoptosis at least in part via increased ER stress

Mild ER stress increases the expression of inflammatory mediators and sensitises rat beta cells to IL-1 β -induced apoptosis [35]. As previously observed (ESM Fig. 1), neither IFN α nor IL-1 β alone induced apoptosis. The combination of both cytokines, however, increased cell death in a dose-dependent manner (Fig. 8a). IFN α alone, but not IL-1 β , induced the expression of *HLA-ABC*, *MX1* and *CXCL10* mRNA, even at low concentrations (200 U/ml) (Fig. 8b–d). The two cytokines only acted synergistically in the case of *CXCL10* (Fig. 8c). The combination of cytokines, mainly at their highest concentrations, led to a significant increase in ER stress markers when compared with IFN α or IL-1 β alone (Fig. 8e–h). Similarly to the observations made in EndoC- β H1 cells, treatment with IFN α or IL-1 β separately did not induce apoptosis in dispersed human islets, while treatment with IFN α plus IL-1 β doubled beta cell death after treatment for 24 h (Fig. 8i).

Silencing of *CHOP* or pre-treatment with the chemical chaperone tauroursodeoxycholic acid (TUDCA) partially prevented apoptosis induced by a combination of IFN α and IL-1 β in EndoC- β H1 cells, suggesting that IFN α + IL-1 β -induced apoptosis is at least in part mediated by ER stress (ESM Fig. 8).

Discussion

Type 1 diabetes is a multifactorial autoimmune disease in which an individual's genetic background interacts with environmental cues, leading to islet inflammation (insulinitis), amplification of this early innate immune response and, in some cases, transition to a long-term adaptive autoimmune attack against the beta cells [6]. The nature of the first components of the innate immune response and their contribution towards the transition to a full autoimmune response remains to be determined [6, 36].

Type I IFNs may be key links between environmental and genetic risk factors in type 1 diabetes and the triggering/amplification of insulinitis [36]. Thus, pathway analysis of type 1 diabetes candidate genes expressed in human pancreatic islets identified 'interferon signalling', 'role of JAK1, JAK2

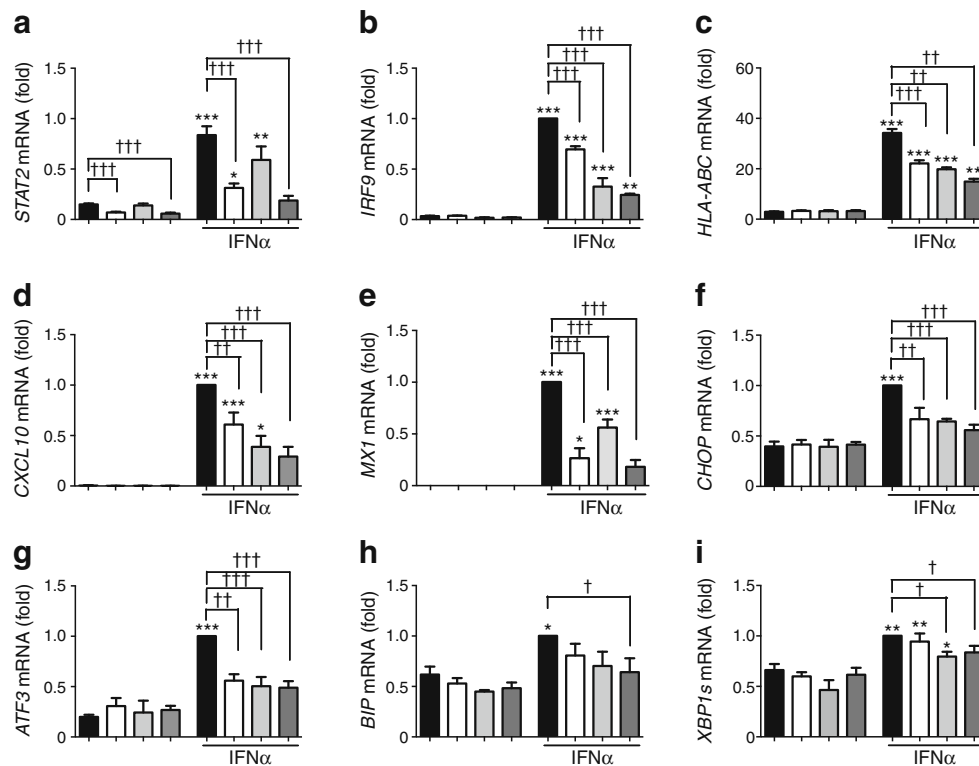


Fig. 6 Inhibition of IRF9 prevents IFN α -induced inflammation and expression of ER stress markers in EndoC- β H1 cells. EndoC- β H1 cells were transfected with siCTRL (black bars treated with IFN α), considered as 1. Results are means \pm SEM of three or four independent experiments. * p < 0.05, ** p < 0.01 and *** p < 0.001 vs untreated and transfected with the same siRNA; † p < 0.05, †† p < 0.01 and ††† p < 0.001, as indicated by bars (ANOVA)

analysed by RT-PCR and normalised by β -actin, and then normalised by siCTRL (black bars treated with IFN α), considered as 1. Results are means \pm SEM of three or four independent experiments. * p < 0.05, ** p < 0.01 and *** p < 0.001 vs untreated and transfected with the same siRNA; † p < 0.05, †† p < 0.01 and ††† p < 0.001, as indicated by bars (ANOVA)

and TYK2 in IFN signalling' and 'role of pattern recognition receptors in recognition of bacteria and virus' as the three top canonical pathways [23]. *TYK2*, a candidate gene for type 1 diabetes, regulates both PIC- (a mimic of double-stranded RNA produced during viral infection) and IFN α -induced MHC class I expression and inflammation in human beta cells [23]. Furthermore, histological analysis and other approaches have identified higher expression of type I IFNs in islets from patients with type 1 diabetes [7–10], and self-reactive antibodies targeting type I IFNs are associated with protection against type 1 diabetes in patients with an autoimmune syndrome [20].

Three hallmarks of the pancreatic islets in early human type 1 diabetes are overexpression of HLA class I [1, 2], ER stress [3, 37] and beta cell apoptosis [6], and we presently show that IFN α induces or contributes to these three phenomena in human beta cells.

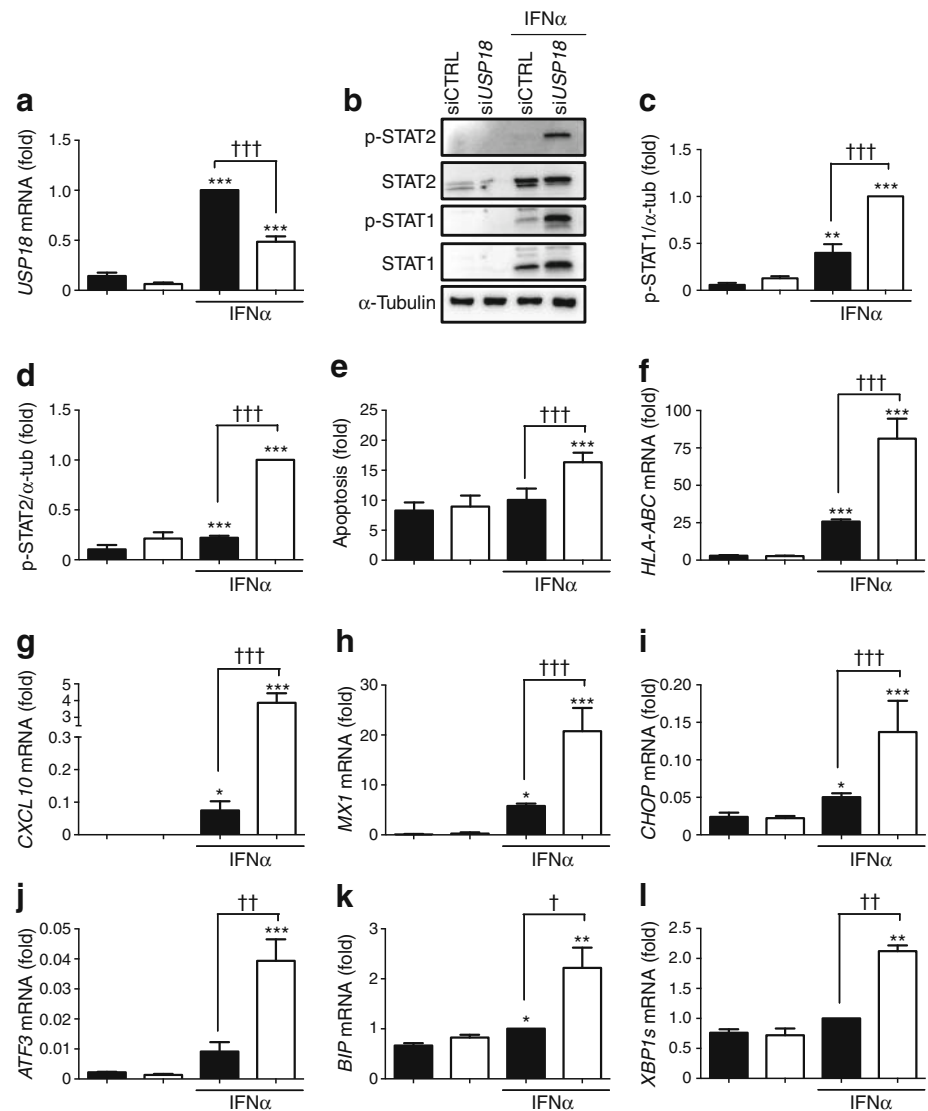
Combinations of the pro-inflammatory cytokines IL-1 β , TNF α and IFN γ , which probably appear later in the progression of islet inflammation as compared with IFN α , induce ER stress in beta cells. ER stress might contribute to the magnification of apoptotic pathways, exacerbation of inflammation and increased antigen presentation in the context of type 1

diabetes [26, 37, 38]. Here, we report for the first time that IFN α alone upregulates expression of several ER stress markers, including p-EIF2 α , XBP1s, BIP, C/EBP homologous protein (CHOP) and ATF3. Although not sufficient by itself to induce beta cell death, ER stress contributes to human beta cell death when these cells are exposed to IFN α plus IL-1 β , as demonstrated by the partial protection afforded by knockdown of the pro-apoptotic transcription factor CHOP or co-culture of the cells with the chemical chaperone TUDCA (present data). Importantly, TUDCA administration was found to provide protection against diabetes in two mouse models of the disease [5].

Data from other tissues have already indicated a possible crosstalk between type I IFNs and ER stress: ER stress enhances IFN β induction in PIC-treated macrophages [39] and potentiates PIC-induced expression of IFN β and other inflammatory cytokines in dendritic cells [40]. Furthermore, PIC-induced overexpression of ISGs triggers ER stress in HeLa cells [41].

To better understand the mechanisms underlying IFN α -mediated signalling in human beta cells, we silenced the different proteins involved in the type I IFN pathway (Fig. 8j), including TYK2, STATs, and IRF9. Inhibition of TYK2, a key

Fig. 7 Inhibition of USP18 overstimulates type I IFN signalling and increases ER stress and inflammation markers in EndoC- β H1 cells. EndoC- β H1 cells were transfected with siCTRL (black bars) or with an siRNA targeting *USP18* (si*USP18*, white bars). Cells were left untreated or were treated with IFN α (2000 U/ml) for 24 h. (a, f–l) mRNA levels of *USP18* (a), *HLA-ABC* (f), *CXCL10* (g), *MX1* (h), *CHOP* (i), *ATF3* (j), *BIP* (k) and *XBPIs* (l) were analysed by RT-PCR and normalised by β -actin. In (a), values were normalised by the highest value of each experiment (considered as 1). (b–d) Protein expression was measured by western blot. Representative images of three independent experiments are shown (b) and densitometry results are presented for p-STAT1 (c) and p-STAT2 (d). Values were normalised by α -tubulin (α -tub), and then by the highest value of each experiment (considered as 1). (e) Apoptosis was evaluated using HO/PI staining. Results are means \pm SEM of three to five independent experiments. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ vs untreated and transfected with the same siRNA; † $p < 0.05$, †† $p < 0.01$ and ††† $p < 0.001$, as indicated by bars (ANOVA)

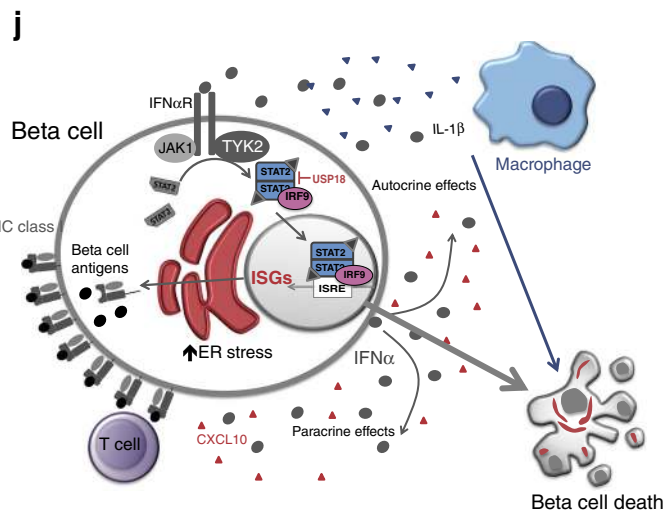
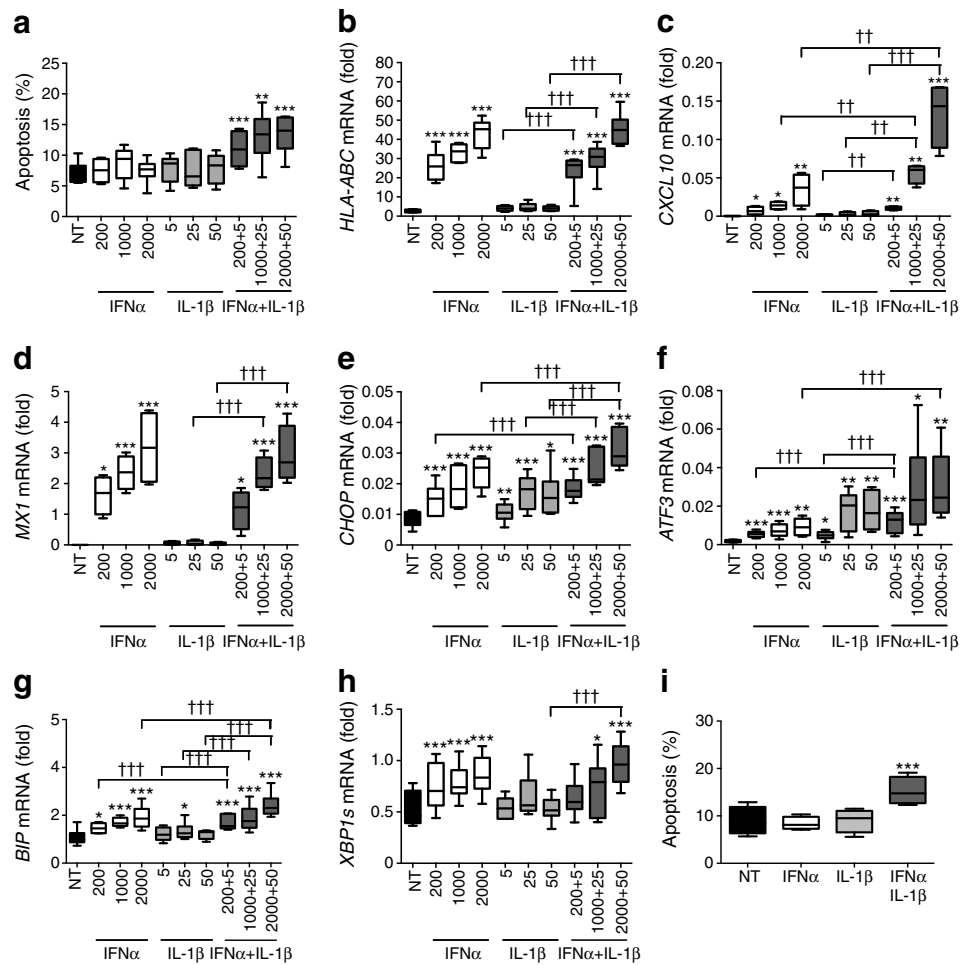


link between the type I IFN receptor and its downstream signalling, decreased IFN α -induced expression of ER stress marker genes, mainly *CHOP* and *ATF3*, and prevented *CXCL10* and *MX1* expression. Inhibition of the transcription factors STAT2 and IRF9 prevented IFN α -induced expression of HLA class I, and inflammation and ER stress markers, suggesting that these two proteins are critical for the activity of IFN α in beta cells. Unexpectedly, STAT1 inhibition not only failed to decrease the expression of these markers but also actually increased the expression of *CXCL10*, *MX1* and *ATF3*. *STAT1*-silenced cells presented higher p-STAT2 expression, suggesting a compensatory activation of STAT2 once STAT1 is blocked, apparently increasing markers of inflammation and ER stress. The same was the case when STAT2 was blocked: there was an increase in STAT1 activation but in this case upregulation of STAT1 did not prevent inhibition of the downstream effects of IFN α (Figs 3 and 4). Primary murine macrophages deficient in STAT1, when

exposed to *Legionella pneumophila* in the presence of type I IFNs, induce a complex between STAT2 and IRF9 that triggers a potent but delayed IFN response against the bacteria [33]. This indicates that both STATs are functionally redundant in macrophages [33]. Our present data, however, suggest that the roles of STAT1 and STAT2 are not redundant in beta cells, as the observed increase in STAT1 activation is not sufficient to compensate for the lack of STAT2.

A very recent study has shown that HLA hyperexpression is strongly correlated with STAT1 expression in beta cell-containing islets from type 1 diabetes patients [1]. STAT2 was not investigated in this study. The present data suggest that STAT1 is important for IFN γ -induced HLA class I upregulation, but not for IFN α , which acts mainly via STAT2. Collectively, these observations suggest that HLA overexpression in the islets of patients developing type 1 diabetes may be regulated by different mechanisms at different stages of the disease. In the early stage, IFN α -induced HLA upregulation may require mostly the

Fig. 8 IFN α acts synergistically with IL-1 β to induce beta cell apoptosis. **(a–h)** EndoC- β H1 cells were left untreated (black bars) or were treated with IFN α (white bars) (200, 1000 or 2000 U/ml), IL-1 β (light grey bars) (5, 25 or 50 U/ml) or a combination of both (dark grey bars) for 24 h. **(i)** Dispersed human islets were left untreated (black bars) or were treated with IFN α (white bars) (2000 U/ml), IL-1 β (light grey bars) (50 U/ml) or a combination of both (dark grey bars) for 24 h. Apoptosis was evaluated using HO/PI staining **(a, i)**. mRNA levels of *HLA-ABC* **(b)**, *CXCL10* **(c)**, *MX1* **(d)**, *CHOP* **(e)**, *ATF3* **(f)**, *BIP* **(g)** and *XBPIs* **(h)** were analysed by RT-PCR and normalised by β -actin. Results are means \pm SEM of four to six independent experiments. * p < 0.05, ** p < 0.01 and *** p < 0.001 vs untreated; †† p < 0.01 and ††† p < 0.001, as indicated by bars (ANOVA). **(j)** Proposed model for the role of IFN α in pancreatic beta cells. JAK1, Janus kinase 1; IFN α R, IFN α / β receptor



TYK2–STAT2–IRF9 axis, while at a later stage there may be a second wave of induction promoted by immune-cell-produced IFN γ via STAT1 and downstream mediators.

Taken together, the above observations indicate that the following steps are crucial for IFN α signalling in beta cells (Fig. 8j). Upon exposure to ‘danger signals’ (e.g. viral

infection or other exogenous or endogenous mediators that remain to be determined; [10, 36, 42, 43]), production and release of type I IFNs (IFN α / β) is triggered in beta cells and neighbour cells, exerting both autocrine and paracrine effects. IFN α binds to the IFNAR1 and activates the type I IFN pathway, in which the TYK2–STAT2–IRF9 axis plays a critical

role. This activation induces a massive expression of ISGs, hyperexpression of MHC class I proteins and an increase in the chemokine CXCL10. Conjunction of these factors with a genetic predisposition to type 1 diabetes (for instance, increased expression of TYK2) places the beta cells in a delicate situation: on one hand, the increase in CXCL10 expression attracts monocytes, T lymphocytes and natural killer cells [6], and on the other, upregulation of MHC class I and the putative induction of modified autoantigens by ER stress increases the efficiency of presentation of beta cell antigens to the immune cells [44]. All these effects may be secondary to local release of IFN α . Importantly, IFN α also sensitises beta cells to IL-1 β released by activated macrophages during early insulinitis, increasing beta cell death and consequent antigen presentation. This places IFN α as a central modulator of excessive inflammatory and ER stress responses in the early stages of type 1 diabetes, contributing to the progressive destruction of pancreatic beta cells and to the triggering of autoimmunity in genetically predisposed individuals. Thus, targeting IFN α in type 1 diabetes may be a promising adjuvant therapy in the very early stages of the disease.

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Contribution statement LM and RSDS contributed to the original idea and the design of the experiments, researched data, contributed to the discussion and wrote, revised and edited the manuscript. AODB, ACdB, LM and PM researched data and revised and edited the manuscript. DLE contributed to the original idea and the design and interpretation of the experiments, contributed to discussion and wrote, revised and edited the manuscript. All authors read and approved the manuscript, and gave informed consent for publication. LM and DLE are the guarantors of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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