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EXTENDED ARTICLE

Interferons are key cytokines acting on pancreatic islets in type 1 diabetes

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Abstract

Aims/hypothesis The proinflammatory cytokines IFN-α, IFN-γ, IL-1β and TNF-α may contribute to innate and adaptive immune responses during insulitis in type 1 diabetes and therefore represent attractive therapeutic targets to protect beta cells. However, the specific role of each of these cytokines individually on pancreatic beta cells remains unknown.

Methods We used deep RNA-seq analysis, followed by extensive confirmation experiments based on reverse transcription-quantitative PCR (RT-qPCR), western blot, histology and use of siRNAs, to characterise the response of human pancreatic beta cells to each cytokine individually and compared the signatures obtained with those present in islets of individuals affected by type 1 diabetes.

Results IFN-α and IFN-γ had a greater impact on the beta cell transcriptome when compared with IL-1β and TNF-α. The IFN-induced gene signatures have a strong correlation with those observed in beta cells from individuals with type 1 diabetes, and the level of expression of specific IFN-stimulated genes is positively correlated with proteins present in islets of these individuals, regulating beta cell responses to 'danger signals' such as viral infections. Zinc finger NFX1-type containing 1 (ZNFX1), a double-stranded RNA sensor, was identified as highly induced by IFNs and shown to play a key role in the antiviral response in beta cells.

Conclusions/interpretation These data suggest that IFN-α and IFN-γ are key cytokines at the islet level in human type 1 diabetes, contributing to the triggering and amplification of autoimmunity.

Keywords IFN-α · IFN-γ · IL-1β · Pancreatic beta cells · TNF-α · Type 1 diabetes · ZNFX1

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Research in context

What is already known about this subject?

- Proinflammatory cytokines from innate and adaptive immunity are more abundant in islets or sera of individuals affected by type 1 diabetes than in non-diabetic individuals
- There is currently no therapy to stop the progressive beta cell loss in type 1 diabetes
- Clinical trials targeting some of these cytokines have already been performed with different outcomes

What is the key question?

- What are the specific roles of individual proinflammatory cytokines on human beta cells?

What are the new findings?

- IFN-α and IFN-γ are drivers of the beta cell inflammatory response in type 1 diabetes
- IFN-α and IFN-γ, but less so IL-1β or TNF-α, induce gene signatures that closely correlate to the gene signature present in beta cells of individuals with type 1 diabetes
- ZNFX1 is a novel mediator of the innate immune response in the early stages of type 1 diabetes

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

- IFN-α and IFN-γ seem to be important cytokines for type 1 diabetes progression at the islet level. Targeting the downstream signalling of these cytokines in beta cells (and potentially in the immune system) may contribute to arrest or delay the autoimmune attack on beta cells

Abbreviations

AAB1+	One islet-specific autoantibody positive
AAB2+	Two or more islet-specific autoantibodies positive
BACH2	Basic leucine zipper transcription factor 2
CXCL10	CXC motif chemokine ligand 10
DGE	Differential gene expression
dIF	Differential isoform fraction
dsRNA	Double-stranded RNA
DTU	Differential transcript usage
ER	Endoplasmic reticulum
fGSEA	Fast pre-ranked Gene set enrichment Analysis
GSEA	Gene set enrichment analysis
HPAP	Human Pancreas Analysis Program
ICI	Insulin-containing islet
IDI	Insulin-deficient islet
IL-1R	IL-1 receptor
IRF	IFN regulatory factor
ISG	IFN-stimulated gene
JAK	Janus kinase
KEGG	Kyoto Encyclopedia of Genes and Genomes
LARP1	La ribonucleoprotein 1
PDL1	Programmed Cell Death 1 Ligand 1
PIC	Polyinosinic-polycytidylic acid
RRHO	Rank–rank hypergeometric overlap
RT-qPCR	Reverse transcription-quantitative PCR
STAT	Signal transducer and activator of transcription
TYK2	Tyrosine kinase 2
ZNFX1	Zinc finger NFX1-type containing 1

Introduction

Type 1 diabetes is caused by severe insulin deficiency due to an immune-mediated assault on the pancreatic beta cells, leading to islet inflammation (insulitis) and loss of beta cells in genetically susceptible individuals submitted to environmental triggers [1, 2]. There is still no cure for type 1 diabetes and the standard treatment relies on lifelong exogenous insulin administration [3].

Cytokines are signalling molecules that play a central role in type 1 diabetes by controlling innate and adaptative immune responses and thus impacting on the initiation and amplification of beta cell autoimmunity [1, 2, 4]. Specifically, the proinflammatory cytokines IFN-α, IFN-γ, IL-1β and TNF-α are produced mainly by islet-infiltrating immune cells and affect beta cell function and survival, contributing to type 1 diabetes development [4–7].

IFN-α, a type I IFN cytokine secreted by microbially infected cells, initiates the innate immune response. IFN-α is expressed in islets from individuals with type 1 diabetes and its signalling is a key component of the early stages of

type 1 diabetes, as there is evidence for a type I IFN signature before the onset of the disease [1, 8]. This cytokine induces endoplasmic reticulum (ER) stress, insulitis and overexpression of HLA class I in human beta cells, three hallmarks of type 1 diabetes [6, 9, 10] that contribute to beta cell recognition by the invading $CD8^+$ T cells and their eventual death [1]. IFN-α signals via Janus kinase (JAK) 1/tyrosine kinase 2 (TYK2) and signal transducer and activator of transcription (STAT) 1/STAT2/IFN regulatory factor (IRF) 9 (or IRF7), triggering the expression of IFN-stimulated genes (ISGs), including HLAs and chemokines, among others [11]. Interestingly, *TYK2* is a susceptibility gene for type 1 diabetes and single nucleotide polymorphisms that decrease *TYK2* expression have a protective effect against the disease [12].

IFN-γ is secreted by adaptive immune cells, such as $CD4^+$ and $CD8^+$ T cells, and signals via JAK1/JAK2/STAT1 to regulate the expression of many genes, including the transcription factors of the IRF family [13, 14]. An IFN-γ signature was found in young islet autoantibody-positive individuals [15]. Blocking IFN-γ in NOD mice, either by using specific anti-IFN-γ antibodies or soluble IFN-γ receptors [16], decreases the incidence of diabetes and prevents disease transfer by splenocytes from hyperglycaemic NOD donor mice [17]. Since IFN-γ acts on both beta cells and immune cells, the protective effect of IFN-γ blockade is probably due to an action on these different cells. IFNs also induce protective mechanisms in beta cells, such as programmed cell death 1 ligand 1 (PDL1; encoded by *PDL1*, also known as *CD274*) and suppressor of cytokine signalling 1 (SOCS1) overexpression [1, 6, 18], which may explain why some models of IFN-γ deletion in NOD mice reduce beta cell antigen presentation and the ability of $CD8^+$ T cells to recognise and kill them, but does not completely prevent diabetes [18]. IFN-γ alone does not induce beta cell apoptosis in vitro, but its combination with either IL-1β or TNF-α causes beta cell death [1, 7, 19].

IL-1β is a powerful proinflammatory cytokine secreted by monocytes, macrophages and dendritic cells during inflammation and host response to infections, and serum levels of IL-1β are higher in individuals with type 1 diabetes when compared with non-diabetic individuals [19]. IL-1β, together with IFN-γ, induces ER stress and the unfolded protein response in both rodent and human islets [1, 20]. IL-1β also acts synergistically with IFN-α to induce human beta cell apoptosis [9]. TNF-α is also produced by immune cells, including macrophages and dendritic cells, and elevated levels of TNF-α are found in the plasma and the islets of individuals with type 1 diabetes. A significant association between the plasma levels of TNF-α and glycaemic control in individuals with type 1 diabetes has been observed [15].

Cytokines participate in an effective immune response to different pathogens, but their dysregulation contributes to the development and progression of type 1 diabetes and other autoimmune diseases, making them attractive immunotherapeutic targets [12, 21]. Until now, most studies evaluating the impact of proinflammatory cytokines on human pancreatic beta cells in the context of type 1 diabetes used combinations of two to four proinflammatory cytokines to simulate the inflammatory environment in type 1 diabetes. To identify key targets for therapy, however, it is crucial to clarify the role and relevance of each individual cytokine. Having this in mind, we performed deep RNA-seq analysis on human insulin-producing EndoC-βH1 cells treated with each individual cytokine (IFN-α, IFN-γ, IL-1β and TNF-α) alone and investigated the main molecular pathways activated by these four cytokines. Our data indicate a predominant role for the IFNs, both IFN-α and IFN-γ, in the pathogenic response observed at the beta cell level in type 1 diabetes as compared with a milder impact of IL-1β and TNF-α. Therefore, we suggest that future studies and clinical trials dealing with the role of beta cells in the pathogenesis of type 1 diabetes might focus on IFNs.

Methods

Culture of EndoC-βH1 cells and human islets The human insulin-secreting EndoC-βH1 cells were provided by R. Scharfmann (Institut Cochin, Université Paris Descartes, Paris, France) [22] and cultured as previously described (see details in the electronic supplementary material [ESM] Methods) [20].

Human islets were isolated from non-diabetic organ donors by collagenase digestion and density gradient purification [23, 24] and cultured as previously described [6, 20]. Randomisation was not carried out in these in vitro experiments.

RNA-seq and analysis After treatment with the respective cytokines for 24 h, total RNA was isolated from EndoC-βH1 cells using the RNeasy Plus Micro Kit (Qiagen, Venlo, the Netherlands), according to the recommended protocol. For all the samples, a minimum of 500 ng of high-quality RNA with RNA integrity number (RIN) >9 (determined using Agilent Bioanalyzer 2100) was used for the library preparation. The RNA-seq was performed on a NovaSeq 6000 (Eurofins Genomics Europe Sequencing, Konstanz, Germany). Analysis was performed as described in the ESM Methods.

Gene set enrichment and rank–rank hypergeometric overlap analysis Gene set enrichment analysis (GSEA) was done using the previously generated differential gene expression (DGE) analysis (ESM Methods). The enrichment or depletion in metabolic pathways was assessed with fast preranked GSEA (fGSEA) [25] using the Kyoto Encyclopedia

of Genes and Genomes (KEGG) [26] and REACTOME [27] databases as references.

Rank–rank hypergeometric overlap (RRHO) test has been used to evaluate the level of agreement between two ranked lists [28, 29]. Briefly, RRHO was used to evaluate the concordance between gene expression signatures of the different cytokines (IFN-α, IFN-γ, IL-1β and TNF-α), and also between each cytokine and the gene expression signature observed by RNA-seq on FACS-purified beta cells from individuals with type 1 diabetes [30].

Cell treatment and small RNA interference EndoC-βH1 cells and dispersed human islets were exposed or not to cytokines and treated as described in the ESM Methods.

The percentage of apoptotic EndoC-βH1 cells and dispersed human islets was determined using fluorescence microscopy after 15 min of incubation with the DNA binding dyes Hoechst 33342 (10 µg/ml, Sigma-Aldrich, St Louis, MO, USA) and propidium iodide (10 µg/ml, Sigma-Aldrich) [31, 32]. The cell counting was performed by two observers, one of them unaware of the identity of the samples, and the agreement between them was >90%. The results are expressed as percentage of apoptosis (number of apoptotic cells/total number of cells).

mRNA and protein extraction, quantitative real-time PCR, validation of the differential transcript usage, protein extraction, western blotting analysis and ELISA After the respective treatments, Poly(A)+ mRNA was isolated using the Dynabeads mRNA DIRECT Kit (Invitrogen, Carlsbad, CA, USA), and reverse transcription-quantitative PCR (RT-qPCR) and validation of the differential transcript usage (DTU) were performed as described in the ESM Methods.

The methods used for protein extraction, western blotting analysis and ELISA are described in the ESM Methods.

Human Pancreas Analysis Program single-cell RNA-seq data analyses Raw FASTQ files of single-cell RNA-seq data from human pancreatic islets were downloaded from the Human Pancreas Analysis Program (HPAP) (https://hpap.pmacs.upenn.edu), which includes samples from non-diabetic donors, individuals presenting one islet-specific autoantibody (AAB1+), individuals presenting two or more islet-specific autoantibodies (AAB2+) and donors with type 1 diabetes. The analysis was performed as previously described by our group [33] and is described in the ESM Methods.

DTU DTU analysis measures the proportional differences in the expressed transcript composition of a gene, and the relative contribution of these transcripts for the expression of that specific gene. DTU analysis was performed using the R library IsoformSwitchAnalyzeR 1.12.0 [34] (https://bioconductor.org/packages/release/bioc/html/IsoformSwitchAnalyzeR.html) following the pipeline instructions, and using DEXSEq 1.36.0 to test differential isoform fraction (dIF). The files used as input in the *isoformSwitchAnalysisPart2* function were generated externally by running Coding Potential Calculator 2, Pfam, Signal peptide (SignalIP 6.0) and IUPred2A, as recommended in the pipeline. We selected all the isoforms with overall false discovery rate (OFDR) <0.05, and |dIF >0.05|.

Human islets for microtissue production at InSphero, culture, treatment and analysis Human islets for microtissue production were acquired and processed as described in the ESM Methods.

Pancreatic samples and immunofluorescence Pancreatic tissue from the Exeter Archival Diabetes Biobank (https://pancreatlas.org/datasets/960/overview) was used in this study. This biobank contains paraffin-embedded human pancreas samples recovered from people who died between 1940 and 1995 with recent-onset type 1 diabetes, and from individuals without diabetes. Ethical approval for research with the Biobank was granted by the West of Scotland Research Ethics Service (REC reference 15-WS-0258). Immunostaining was performed on 4-µm-thick sections of tissue. Immunofluorescence is described in the ESM Methods.

Statistical analysis and data deposition The data are shown as means ± SEM and details of statistical analysis and data deposition are provided in the ESM Methods. A normality test was performed to assess Gaussian distribution and outliers were detected and eliminated using the outliers removal test Grubbs (α=0.05). The excluded outliers were as follows: Fig. 2i—PD-L1 expression in human islets, we excluded one experiment per group (not paired) after outlier analysis; Fig. 7l— HLA-ABC expression, we excluded one experiment (paired) after outlier analysis. In both cases the values excluded pointed in the same direction as the retained experiments but had much higher values (outlier) than in all the other experiments.

Results

IFN-α, IFN-γ, IL-1β and TNF-α have distinct effects on human pancreatic beta cells Exposure to each individual cytokine for 24 h did not induce apoptosis in EndoC-βH1 cells or dispersed human islets (Fig. 1a,c). This aligns with our previous findings indicating that even longer exposure of primary human islets to individual cytokines does not induce apoptosis [1, 7, 31]. However, a 48 h exposure of EndoC-βH1 cells to IFN-α or IFN-γ induced a mild increase (16–35%) in cell death (Fig. 1b).

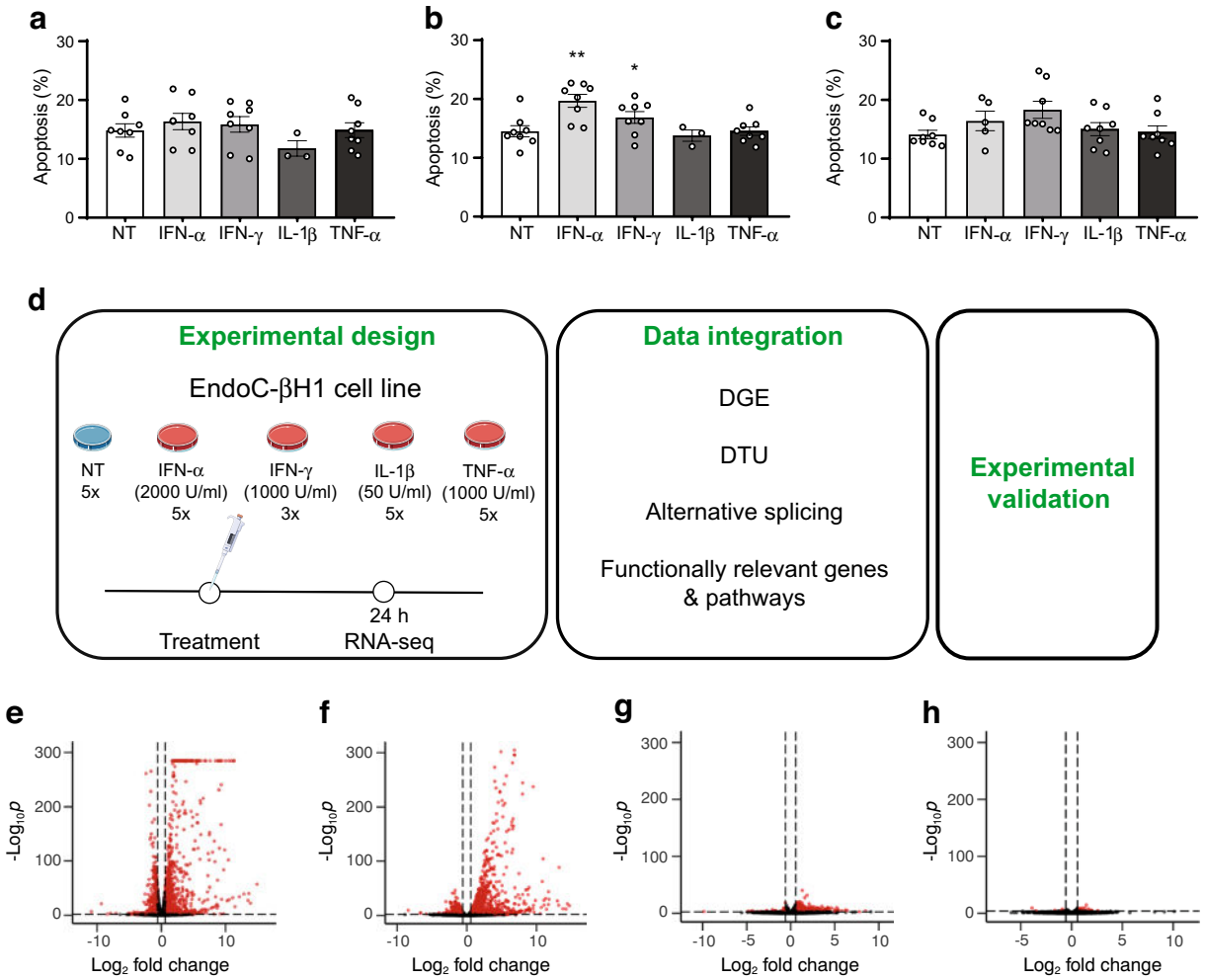


Fig. 1 IFN-α, IFN-γ, IL-1β and TNF-α have distinct effects on human pancreatic beta cells. EndoC-βH1 cells and dispersed human islets were treated with IFN-α (2000 U/ml), IFN-γ (1000 U/ml), IL-1β (50 U/ml) or TNF-α (1000 U/ml). (**a**) Apoptosis was evaluated by Hoechst 33342 + propidium iodide after 24 h (**a**) and 48 h (**b**) in EndoC-βH1 cells (n=3–8), and after 24 h in dispersed human islets (n=6–9) (**c**). Results are expressed as mean ± SEM. *p<0.05, **p<0.01 vs NT (ANOVA). (**d**) Experimental design for the RNA-seq experiments. EndoC-βH1 cells were treated with IFN-α, IFN-γ, IL-1β or TNF-α and RNA-seq performed after 24 h of treatment (n=5 for all conditions, except for IFN-γ: n=3). The data analysis and integration were performed as described in the Methods section. (**e**–**h**) Volcano plots for the differentially expressed genes in EndoC-βH1 cells after the respective treatments. The horizontal dashed line delimits the genes with an adjusted p value <0.05. The vertical dashed lines delimit the genes with $|\log_2$ fold change$|$ >0.58. NT, non-treated

RRHO analysis to compare the ranked gene expression of previous RNA-seq data from EndoC-βH1 cells and human islets exposed to either IFN-α or IFN-γ + IL-1β [35] showed a similar gene signature between EndoC-βH1 cells and human islets (ESM Fig. 1), indicating that EndoC-βH1 cells are a good model to investigate the effects of cytokines on human beta cells. Therefore, we treated EndoC-βH1 cells for 24 h with IFN-γ, IL-1β or TNF-α individually to perform deep RNA-seq analysis (>200 million reads) (Fig. 1d). Our group has previously published an IFN-α RNA-seq dataset [6, 33] which was re-analysed here using the pipeline described in the ESM Methods. We have previously shown that most IFN-α-stimulated genes are modified after 24 h of stimulation [33] and thus performed the present experiments at 24 h to avoid possible changes associated with the apoptosis seen at later time points.

RNA-seq analysis showed that IFN-α, IFN-γ, IL-1β and TNF-α have distinct impacts on gene expression in EndoC-βH1 cells (Fig. 1e–h, ESM Table 1), with both IFN-α and IFN-γ (Fig. 1e,f) modifying a larger number of genes than IL-1β or TNF-α (Fig. 1g,h). IFN-α and IFN-γ induced the upregulation of 4323 and 2018 genes, respectively, and the downregulation of 4010 and 1516 genes (ESM Table 1). Treatment with IL-1β

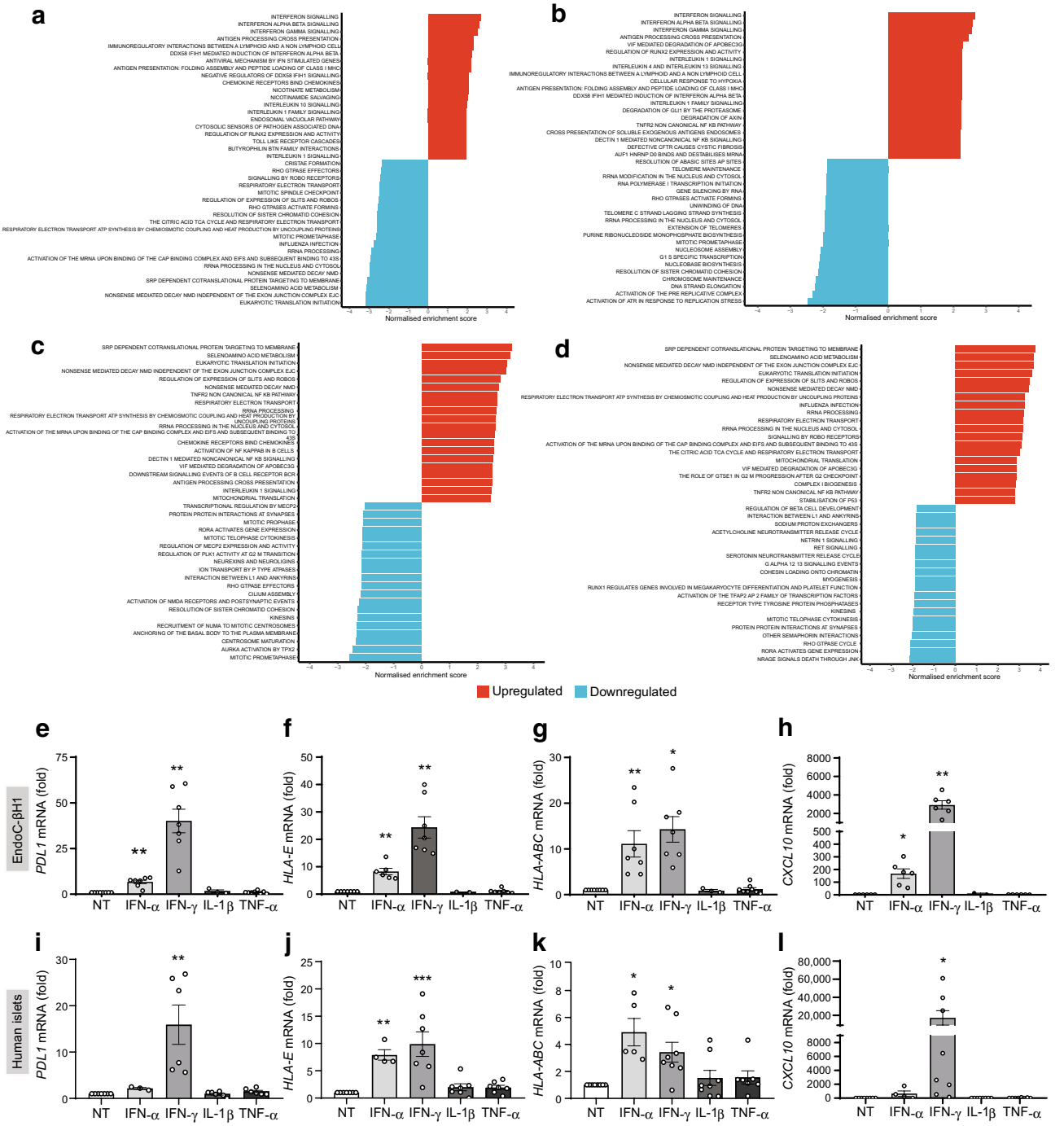


Fig. 2 IFN-α and IFN-γ induce a more marked immune-related response in human beta cells than IL-1β and TNF-α. (**a**–**d**) Results from the DGE analysis were input in fGSEA. The horizontal bar plots show the significantly enriched pathways for IFN-α (**a**), IFN-γ (**b**), IL-1β (**c**) and TNF-α (**d**). The upregulated pathways are represented in red and the downregulated pathways in blue. (**e**–**l**) EndoC-βH1 cells (**e**–**h**) and dispersed human islets (**i**–**l**) were left untreated (NT, non-treated) or exposed to IFN-α (2000 U/ml), IFN-γ (1000 U/ml), IL-1β (50 U/ml) or TNF-α (1000 U/ml) for 24 h. mRNA expression of *PDL1* (**e**, **i**), *HLA-E* (**f**, **j**), *HLA-ABC* (**g**, **k**) and *CXCL10* (**h**, **l**) was measured by RT-qPCR and normalised by the geometric mean of *ACTB* and *VAPA* and then by the NT value considered as 1. Results are expressed as mean ± SEM of 2–7 independent experiments. *$p<0.05$, **$p<0.01$, ***$p<0.001$ vs NT (ANOVA)

induced the upregulation of 556 genes and the downregulation of 550 genes (ESM Table 1). By contrast, TNF-α induced the upregulation of only 49 genes, and the downregulation of only 20 genes (ESM Table 1). Of note, we confirmed that our treatment with TNF-α worked by measuring *TNFAIP3* (also known as *A20*)

expression as a marker of TNF-α activity [36] (ESM Fig. 2). All genes differentially expressed after treatment with the respective proinflammatory cytokines are listed in ESM Data 1.

The analysis of the expression of the different cytokine receptors under basal condition, based on previous and present RNA-seq of EndoC-βH1 cells, dispersed human islets and FACS-purified beta cells from donors with and without type 1 diabetes, indicates that all receptors are expressed in human beta cells but with a predominance of the receptors for IFNs (*IFNAR1*, *IFNAR2*, *IFNGR1* and *IFNGR2*) and TNF-α (*TNFRSF1A*) as compared with IL-1β (*IL1R1* and *IL1R2*) receptors (ESM Fig. 3). This is in marked contrast with observations in rat FACS-purified beta cells, where expression of the IL-1 receptor (IL-1R) is nearly 15-fold higher than IFN receptors [37].

IFN-α and IFN-γ induce a more marked immune-related response in human beta cells than IL-1β or TNF-α Using GSEA based on the KEGG and REACTOME databases (Fig. 2a–d), we re-analysed the IFN-α dataset and, in line with our previous findings [6], observed an enrichment in IFN signalling-related pathways, antigen processing and presentation, and antiviral response among the upregulated pathways, while there was depletion in oxidative phosphorylation and mitochondrial function among the downregulated pathways (Fig. 2a).

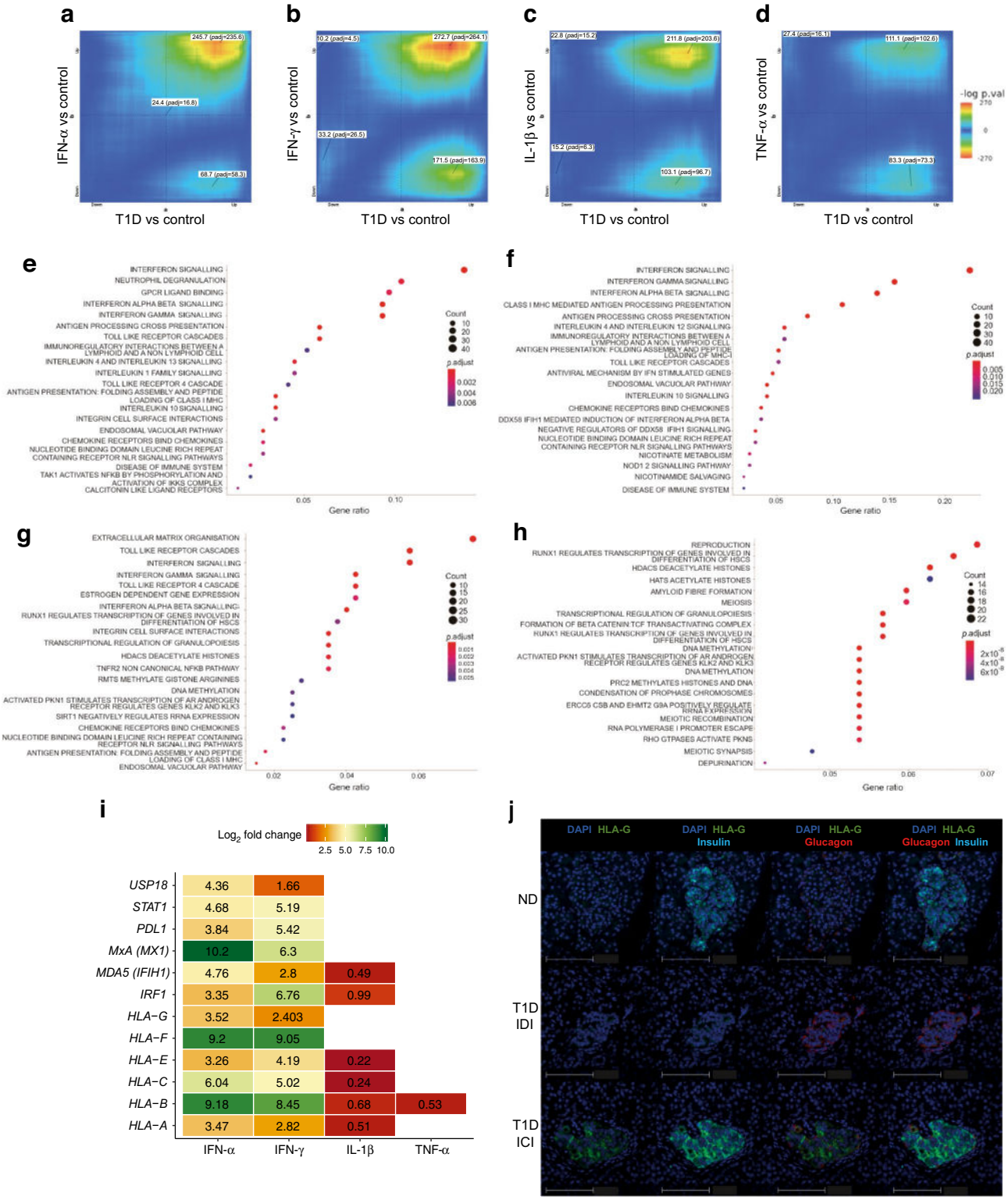
GSEA of the IFN-γ-upregulated genes showed enrichment in IFN signalling-related pathways, antigen processing and presentation, and assembly and peptide loading of HLA class I molecules. Analysis of the downregulated genes revealed a depletion in chromosome maintenance, DNA replication and RNA processing-related pathways (Fig. 2b). IL-1β-upregulated genes were involved in translation regulation, nonsense-mediated decay, IL-1 signalling and NF-κB signalling, while the downregulated genes were involved in adherent junctions, cilium structure and assembly, and global DNA packaging (Fig. 2c). GSEA performed on the TNF-α dataset showed an over-representation of nonsense-mediated decay, eukaryotic translation and RNA processing-related pathways among the upregulated genes. The downregulated genes were enriched in pathways related to beta cell development and different signalling pathways (Fig. 2d).

Together, these data indicate that IFN-α and IFN-γ are the main drivers of the immune-related response in human beta cells. Based on the concept that a key role for cytokines is to render the beta cells more visible to the $CD8^+$ T cells [38–42], the main mediators of beta cell death, we focused on further validation for genes involved in beta cell recognition by the $CD8^+$ T cells and their homing in the islets. We analysed the impact of the cytokines on the expression of the immune-related genes *HLA-ABC* and *CXCL10* (which favour beta cell antigen presentation and $CD8^+$ T cell homing), as well as *PDL1* and *HLA-E* (which may hamper beta cell antigen presentation), all four previously shown to be more expressed in pancreatic beta cells or serum of individuals with type 1 diabetes as compared with non-diabetic individuals [6, 43–45]. IFN-α induced the expression of these four genes in EndoC-βH1 cells (Fig. 2e–h), as previously described [6, 9, 33, 46], and *HLA-E*, *HLA-ABC* and *CXCL10* in dispersed human islets (Fig. 2j–l) [6]. Interestingly, IFN-γ also upregulated these four genes in both EndoC-βH1 cells and dispersed human islets (Fig. 2e–l). By contrast, neither IL-1β nor TNF-α had any effect on the expression of these genes (Fig. 2e–l). Of note, we did not observe any correlation between the purity of the presently used human islet preparations and IL-1β-induced gene expression in human islets (ESM Fig. 4). Furthermore, we did not find a correlation between IL-1R expression and expression of genes representative of the IL-1β signature in human islets exposed or not to IFN-γ + IL-1β [35] (ESM Fig. 5). This indicates that the lack of response to IL-1β observed in Fig. 2i–l cannot be attributed to the purity of the preparations or *IL1R* expression.

The effect of cytokines was confirmed in human islet microtissues where only IFN-γ at different concentrations (alone or mixed with IL-1β and TNF-α), but not IL-1β or TNF-α alone, induced HLA class I upregulation after 1, 4 and 7 days of treatment (ESM Fig. 6a–c). Of note, only the mixture of these three cytokines induced cell death and even a 7 day exposure to individual cytokines failed to induce beta cell death in human islet microtissues (ESM Fig. 6d–f).

We next evaluated the expression of selected transcription factors known to regulate gene expression mediated by the different cytokines (ESM Fig. 7a). Apart from NF-κB-related transcription factors induced by the four cytokines, the expression of the other transcription factors was induced more strongly by IFN-α and IFN-γ than by IL-1β and TNF-α, with a marked induction of *STAT1* [6]. IFN-α and IFN-γ upregulated most IRF family members, with *IRF1* [6], *IRF7* and *IRF9* being most induced (ESM Fig. 7a). We validated *IRF7* and *IRF9* induction by IFN-α in EndoC-βH1 cells and showed that the silencing of these transcription factors repressed IFN-α-induced *HLA-ABC* (only for *IRF7*), *CXCL10*, *PDL1*, *HLA-E* and *MX1* expression (ESM Fig. 7b–m).

IFN-α and IFN-γ induce a gene signature comparable to that observed in beta cells from individuals affected by type 1 diabetes RRHO analysis between the different pairs of proinflammatory cytokines (ESM Fig. 8a) showed that IFN-α and IFN-γ share many differentially expressed genes, with commonly upregulated genes involved in pathways related mainly to IFN signalling and antigen processing and presentation (ESM Fig. 8b). However, there was no enrichment



for the commonly downregulated genes. We also observed a close correlation between genes modulated by IL-1β and TNF-α (ESM Fig. 8a), which is in line with the central role for NF-κB in signalling of both cytokines. These results complement the GSEA (Fig. 2a–d) as RRHO compares the results from two differential expression analyses, retrieves the shared genes going in a similar (or opposite) direction and then identifies the pathways that are enriched in these sets of genes, sometimes highlighting pathways that were not unravelled by comparisons based on GSEA results alone.

◂**Fig. 3** IFN-α and IFN-γ induce a gene signature similar to the one observed in beta cells from individuals with type 1 diabetes. (**a**–**d**) RRHO algorithm was conducted for each pairwise analysis. The genes were ranked according to their fold change. The significance of overlap between genes commonly upregulated (top-right quadrant), commonly downregulated (bottom-left quadrant), upregulated by the proinflammatory cytokine and downregulated in FACS-purified beta cells (top-left quadrant) and downregulated by the proinflammatory cytokine and upregulated in FACS-purified beta cells (bottom-right quadrant) is represented by the level map. The colours represent the −log(adjusted *p* values). For comparison purposes, all the level maps are plotted using the same scale, which corresponds to the highest scale number when no scale restriction is applied. (**e**–**h**) Pathway enrichment analysis for the commonly upregulated genes between IFN-α (**e**), IFN-γ (**f**), IL-1β (**g**) and TNF-α (**h**) and type 1 diabetes samples was done using gProfiler. (**i**) Heatmap showing statistically significant (adjusted *p* value <0.05) gene expression changes (in $\log_2$ fold change), after the treatment of EndoC-βH1 cells with IFN-α, IFN-γ, IL-1β or TNF-α for 24 h, of genes known to be altered in type 1 diabetes pancreatic samples. (**j**) Immunohistochemistry of pancreatic sections stained for HLA-G (green), glucagon (red) and insulin (cyan) from non-diabetic (ND), type 1 diabetes IDI (T1D IDI) and type 1 diabetes ICI (T1D ICI) donors (scale bar, 100 μm)

We did not find any significantly enriched pathways for the other pairs of cytokines.

Using the same RRHO approach, we evaluated the similarities between the gene signatures induced by individual cytokines and those observed in FACS-purified beta cells from individuals with type 1 diabetes compared with control individuals (Fig. 3a–d). There was a predominance of commonly upregulated genes between the different proinflammatory cytokines and type 1 diabetes samples, with a closer correlation between IFNs (IFN-α and IFN-γ) and type 1 diabetes samples (Fig. 3a,b). For these two cytokines, the commonly upregulated genes were related to IFN signalling, antigen processing and presentation, chemokine-related signalling and IL signalling (Fig. 3e,f). For the commonly upregulated genes between type 1 diabetes and IL-1β, there was an enrichment for IFN-related pathways, and pathways related to extracellular matrix organisation, histone acetylation and DNA methylation (Fig. 3g). Regarding the commonly upregulated genes between TNF-α and type 1 diabetes samples, there were pathways related to chromatin modifications and DNA methylation (Fig. 3h).

We selected 12 genes related to the immune and antiviral response that are highly expressed at the protein level in pancreatic islets from donors with type 1 diabetes when compared with control individuals [43, 44, 47–49], and investigated the impact of the different cytokines on their expression (Fig. 3i). Both IFNs induced a significant upregulation of all 12 genes, while IL-1β and TNF-α had only a mild impact (6/12 and 1/12 genes induced by these two cytokines, respectively, with a low fold change compared with IFNs). We validated the expression of HLA-G in human pancreatic islets, observing that it was more expressed in insulin-containing islets (ICIs) of individuals with type 1 diabetes compared with insulin-deficient islets (IDIs) of donors with type 1 diabetes or islets from control individuals (Fig. 3j), as previously described [47]. Further examples of elevated expression of these proteins in type 1 diabetes islets are shown in ESM Fig. 9, to provide additional context and examples for the selection of the key molecules that we explored.

We next evaluated the presence of an ISG signature (defined as described in the ESM Methods) in beta cells obtained from non-diabetic control individuals, AAB1+ or AAB2+ individuals or donors with type 1 diabetes and analysed by single-cell RNA-seq. There was an increase in the ISG score that positively correlated with the natural evolution of the disease, i.e. the lowest ISG score was observed in beta cells from non-diabetic individuals and individuals with one autoantibody, but ISG score increased in individuals with two autoantibodies and reached the highest level in beta cells from individuals affected by type 1 diabetes (Fig. 4a). We did a similar analysis for a presently generated IL-1β signature (defined as described in the ESM Methods) but did not observe differences between non-diabetic control individuals, AAB1+ or AAB2+ individuals or donors with type 1 diabetes (ESM Fig. 10).

Analyses of bulk RNA-seq data of FACS-purified beta cells from non-diabetic individuals and donors with type 1 diabetes again indicated a significantly higher ISG score in beta cells isolated from individuals with type 1 diabetes as compared with non-diabetic individuals (nearly threefold increase; Fig. 4b), confirming the key role for IFNs in human type 1 diabetes.

The expression of type 1 diabetes candidate genes is preferentially affected by IFN-α and IFN-γ Genome-wide association studies have identified more than 60 loci that influence type 1 diabetes risk and many susceptibility genes for type 1 diabetes have been identified in these regions [50]. Since more than 60% of the type 1 diabetes candidate genes are expressed in pancreatic beta cells [51], we investigated their regulation by the different individual cytokines and observed that they were mainly regulated by IFN-α and IFN-γ (51/66 and 41/66, respectively) as compared with IL-1β and TNF-α (18/66 and 1/66, respectively) (Fig. 5a).

In a previous study, we found that the expression of basic leucine zipper transcription factor 2 (*BACH2*), one of the candidate genes for type 1 diabetes, was significantly induced by IFN-γ + IL-1β in human beta cells and that *BACH2* silencing enhanced IFN-γ + IL-1β-induced beta cell apoptosis [52]. In the present study, we observed that both IFN-γ and IL-1β, studied separately, as well as IFN-α, induced the expression of *BACH2* in EndoC-βH1 cells, while TNF-α had no effect (Fig. 5a,b). By contrast, none of these cytokines induced the expression of the *BACH2* paralog, *BACH1* (Fig. 5c). *BACH2* silencing exacerbated all

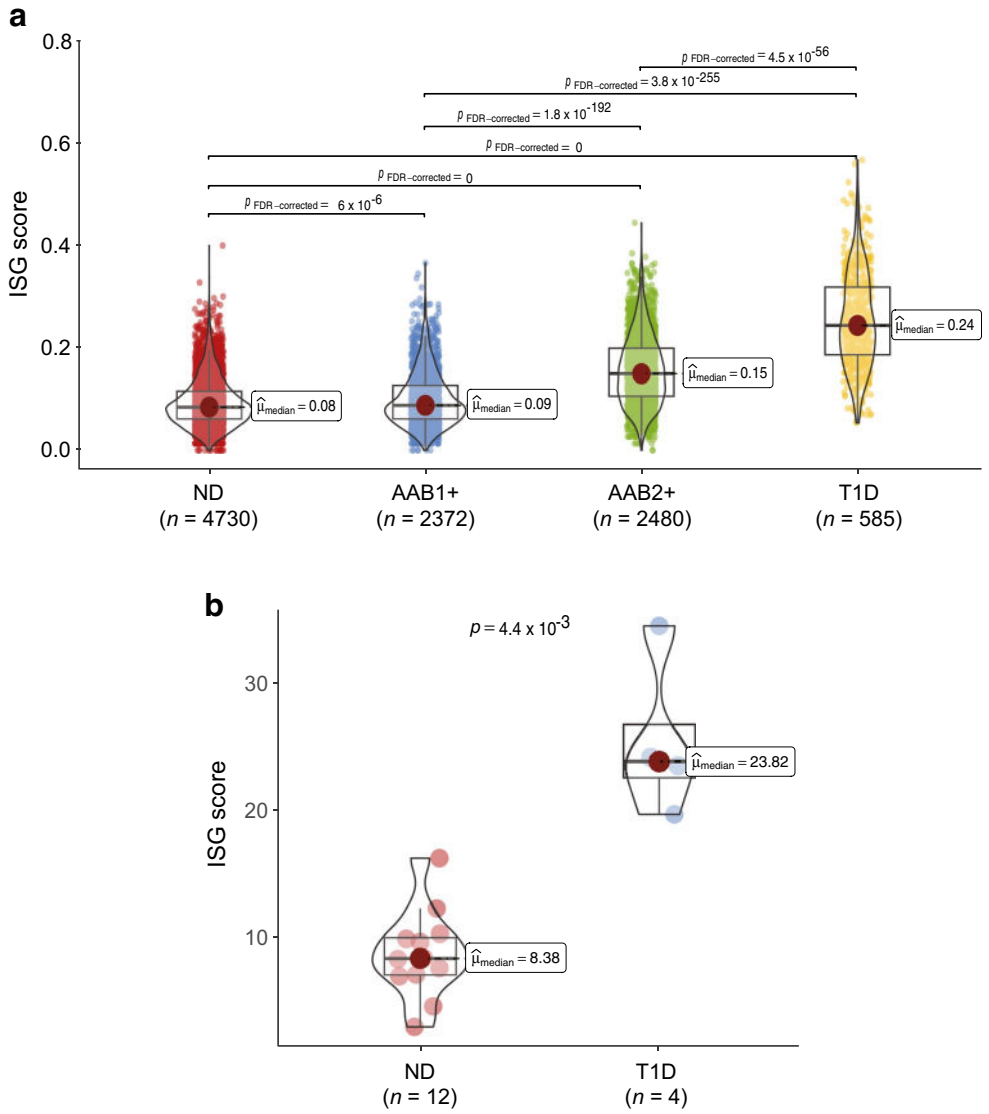


Fig. 4 Beta cells from autoantibody-positive individuals and individuals with type 1 diabetes present an elevated ISG score. (**a**) An ISG score (described in the ESM Methods) was calculated for beta cells from single-cell RNA-seq data of non-diabetic donors (ND, *n*=15), AAB1+ donors (*n*=8), AAB2+ donors (*n*=2) and donors with type 1 diabetes (T1D, *n*=9) obtained from HPAP (https://hpap.pmacs.upenn.edu). (**b**) An ISG score (see the ESM Methods) was calculated from bulk RNA-seq data of FACS-purified beta cells of ND (*n*=12) and T1D (*n*=4) donors [30]. The ISG score was calculated as the average expression of the defined ISGs in each cell for (**a**) and each donor for (**b**). Box limits correspond to the 25th and 75th percentiles, bold lines represent the median score; *p* values were determined by the Dunn test for multiple group comparisons in (**a**) and by the two-tailed Mann–Whitney *U* test for (**b**); *p* values were adjusted for multiple comparisons by 'FDR' for (**a**). FDR, false discovery rate; ISG, IFN-stimulated gene

individual cytokine- and IFN-γ + IL-1β-induced beta cell apoptosis, supporting a key role for this candidate gene in the survival of human beta cells exposed to a proinflammatory environment (Fig. 5d,e).

The expression of antiviral and immune-related genes in beta cells is mainly regulated by IFNs Pancreatic beta cells of individuals with type 1 diabetes have a strong antiviral and inflammatory gene signature [1, 10] and we next investigated the impact of the different cytokines on the expression of 17 genes related to these pathways. Most of these genes were modified by IFN-α (15/17) and IFN-γ (12/17), while IL-1β had a limited effect (4/17 genes modified), and TNF-α did not affect the expression of these genes (Fig. 6a).

Recent findings in other cell types suggest that the *ZNFX1* gene, one of the most IFN-induced antiviral genes in the present analysis, is of particular interest for the early antiviral response [53]. We analysed some of our previous RNA-seq

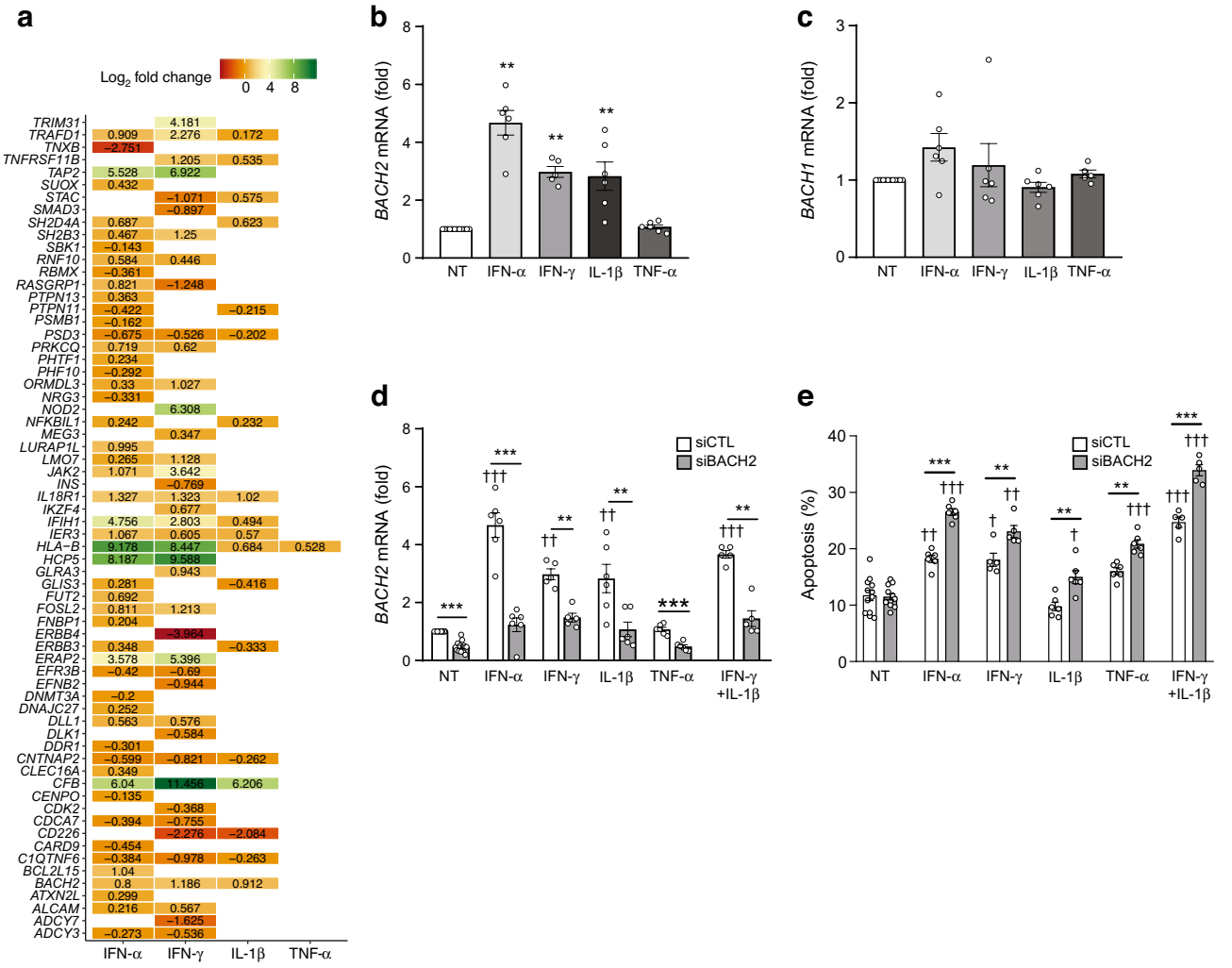


Fig. 5 The expression of type 1 diabetes candidate genes is more affected by IFN-α and IFN-γ than by IL-1β and TNF-α in human beta cells. (**a**) Heatmap showing statistically significant (adjusted *p* value <0.05) gene expression changes (log_2 fold change) of type 1 diabetes candidate genes after treatment with the indicated proinflammatory cytokines. (**b**, **c**) EndoC-βH1 cells were left untreated (NT, non-treated) or treated with IFN-α (2000 U/ml), IFN-γ (1000 U/ml), IL-1β (50 U/ml) or TNF-α (1000 U/ml for 48 h. (**d**, **e**) EndoC-βH1 cells were transfected with an siRNA control (siCTL, white bars) or an siRNA targeting *BACH2* (siBACH2, grey bars). The cells were left untreated (NT) or treated with the respective proinflammatory cytokines (at the same concentrations as for **b** and **c**, with the addition of: IFN-γ + IL-1β [1000 U/ml and 50 U/ml, respectively]) for 48 h. *BACH2* (**b**, **d**) and *BACH1* (**c**) mRNA expression was evaluated by RT-qPCR and normalised by the geometric mean of *ACTB* and *VAPA* and then by the value of NT considered as 1. (**e**) The impact of *BACH2* silencing on EndoC-βH1 cell apoptosis was evaluated by direct cell counting after Hoechst 33342 + propidium iodide staining. Results are expressed as mean ± SEM of 5–6 independent experiments. $^{**}p<0.01$ and $^{***}p<0.001$ vs NT (**b**) or siCTL treated at the same time point with IFN-α, IFN-γ or TNF-α (**d**, **e**) and $^{\dagger}p<0.05$, $^{\dagger\dagger}p<0.01$ and $^{\dagger\dagger\dagger}p<0.001$ vs NT (ANOVA)

datasets and confirmed *ZNFX1* upregulation in EndoC-βH1 cells (Fig. 6b) and dispersed human islets (Fig. 6c) exposed to IFN-α for different time points, starting at 2 h. *ZNFX1* expression is slightly upregulated in FACS-purified beta cells from individuals with type 1 diabetes as compared with non-diabetic individuals (Fig. 6d) but does not reach significance (adjusted *p* value=0.70). For comparison, we also evaluated the expression of two other well-known [53] double-stranded RNA (dsRNA) sensors, namely *MDA5* (also known as *IFIH1*) and *RIG-I* (also known as *DDX58*), involved in the antiviral response of human beta cells. Both genes followed the same pattern of induction as *ZNFX1* in EndoC-βH1 cells (ESM Fig. 11a,e) and dispersed human islets (ESM Fig. 11b,f) exposed to IFN-α. There was a significant induction as early as 2 h post-stimulation, reaching a peak after 8 h of treatment. We validated *ZNFX1*, *MDA5* and *RIG-I* induction by IFN-α in EndoC-βH1 cells (Fig. 6e–g, ESM Fig. 11c, g) and showed that *ZNFX1* upregulation was repressed by the JAK1/2 inhibitor baricitinib (Fig. 6h) or by the silencing of *STAT2*, but not of *STAT1* (Fig. 6i). IFN-α also

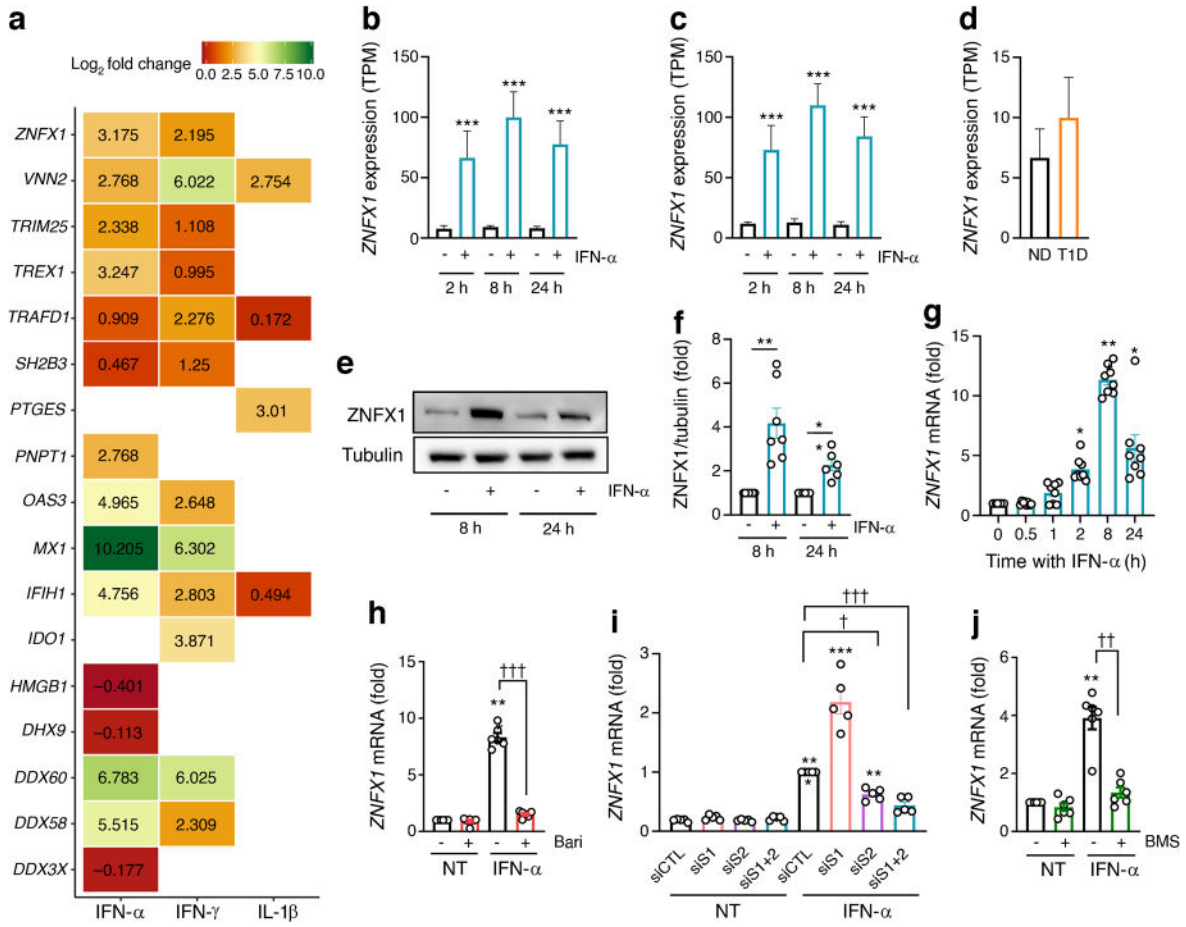


Fig. 6 The expression of antiviral and immune-related genes in beta cells is mainly regulated by IFN-α and IFN-γ. (**a**) Heatmap showing statistically significant (adjusted *p* value <0.05) gene expression changes (in $\log_2$ fold change) of immune-related genes and antiviral response-related genes after the treatment of EndoC-βH1 cells with IFN-α, IFN-γ or IL-1β for 24 h (TNF-α did not induce any of these genes and is thus not shown here). (**b**–**d**) *ZNFX1* expression from RNA-seq data of EndoC-βH1 cells (n=5) (**b**) and human islets (n=5) (**c**) exposed to IFN-α for different time points, and FACS-purified beta cells from non-diabetic (ND) individuals or individuals with type 1 diabetes (T1D) [30] (**d**). EndoC-βH1 cells (**e**–**i**) or dispersed human islets (**j**) were exposed to IFN-α (2000 U/ml) alone (**e**–**g**) or in combination with siRNAs (**i**) or inhibitors (**h**, **j**). EndoC-βH1 cells (**h**) and dispersed human islets (**j**) were pre-treated for 2 h with baricitinib (Bari, 4 μmol/l) or BMS-986165 (BMS, 0.3 μmol/l), respectively, and then IFN-α was added for an additional 24 h. (**i**) EndoC-βH1 cells were transfected with an siRNA control (siCTL: black outline) or with an siRNA targeting *STAT1* (siS1: pink outline), *STAT2* (siS2: purple outline) or both (siS1+2: blue outline), and then treated with IFN-α for 24 h. (**e**) Protein expression was measured by western blot and representative images of 6 (24 h) or 7 (8 h) independent experiments are shown. Densitometry results are shown for ZNFX1 (**f**). mRNA expression of *ZNFX1* (**g**–**j**) was analysed by RT-qPCR and normalised by the geometric mean of *ACTB* and *VAPA* or *ACTB* and *GAPDH* and then by the control value considered as 1. Results are expressed as mean ± SEM of 5–8 independent experiments. *p<0.05, **p<0.01 and ***p<0.001 vs non-treated condition and $^{\dagger}p$<0.05, $^{\dagger\dagger}p$<0.01 and $^{\dagger\dagger\dagger}p$<0.001 as indicated (ANOVA)

significantly induced *ZNFX1* expression in dispersed human islets in a TYK2-dependent manner as observed with the use of the TYK2 inhibitor BMS-986165 (Fig. 6j). We next analysed *ZNFX1* expression in beta cells obtained from single-cell RNA-seq data of non-diabetic individuals, AAB1+ and AAB2+ individuals and donors with type 1 diabetes (ESM Fig. 12). There was a gradual increase in the percentage of cells expressing *ZNFX1* and in the average expression in beta cells, from the non-diabetic individuals to the AAB2+ individuals and the donors with type 1 diabetes. There was also an increase in *RIG-I* and *MDA5* expression in beta cells from donors with type 1 diabetes, as observed in human beta cells exposed to IFN-α (Fig. 6a, ESM Figs. 11, 12).

***ZNFX1* is involved in the antiviral response of human pancreatic beta cells** To evaluate the potential antiviral role of *ZNFX1* in EndoC-βH1 cells, we first tested its regulation by the dsRNA analogue polyinosinic-polycytidylic acid (poly-IC or PIC), used to mimic a viral infection. Treatment with PIC significantly increased *ZNFX1* expression both at the protein level after 48 h (Fig. 7a,b) and at the mRNA level after 24 h (Fig. 7c).

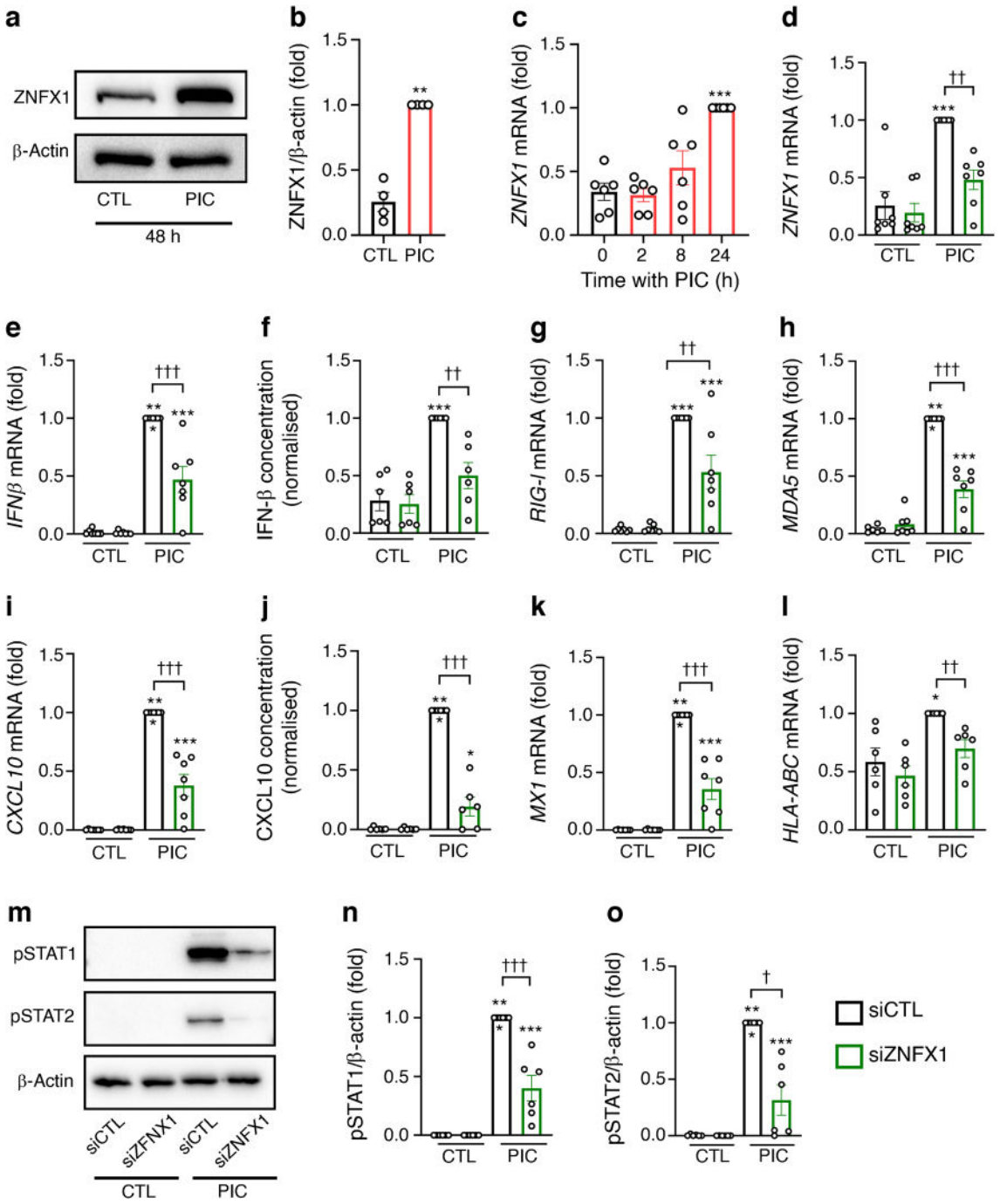


Fig. 7 *ZNFX1* knock-down prevents the human beta cell response to dsRNA (PIC). EndoC-βH1 cells were transfected with PIC (1 μg/ml) for 48 h (**a**, **b**) or for 2, 8 and 24 h (**c**); or they were transfected with an siRNA control (siCTL: black outline) or with the siRNA#1 targeting *ZNFX1* (siZNFX1: green outline) for 72 h prior to transfection with PIC for an additional 24 h (**d**–**o**). Protein expression was measured by western blot and representative images of four (**a**) or six (**m**) independent experiments are shown. Densitometry results are shown for ZNFX1 (**b**), pSTAT1 (phospho-STAT1 Tyr701) (**n**) and pSTAT2 (phospho-STAT2 Tyr690) (**o**) with the values normalised by β-actin. The mRNA expression of *ZNFX1* (**d**), *IFNβ* (**e**), *RIG-I* (**g**), *MDA5* (**h**), *CXCL10* (**i**), *MX1* (**k**) and *HLA-ABC* (**l**) was analysed by RT-qPCR and the values normalised by *ACTB*. The release of IFN-β (**f**) and CXCL10 (**j**) to the culture medium (by 35,000 cells/200 μl) was determined by ELISA. In all experiments the values were normalised by the condition PIC 48 h (**b**) PIC 24 h (**c**) or siCTL + PIC (**d**–**o**) considered as 1. Results are mean ± SEM of 6–7 (RT-qPCR) or 6 (ELISA) independent experiments. *$p<0.05$, **$p<0.01$ and ***$p<0.001$ vs control (CTL or siCTL + CTL); $^{\dagger}p<0.05$, $^{\dagger\dagger}p<0.01$ and $^{\dagger\dagger\dagger}p<0.001$ vs siCTL + PIC (ANOVA). CTL, control

MDA5 and *RIG-I* were also significantly induced after 24 h of exposure to PIC (ESM Fig. 11d,h). To assess the functional role of zinc finger NFX1-type containing 1 (ZNFX1) in the antiviral response against PIC, we first validated two different siRNAs targeting *ZNFX1* that partially reduced its expression (ESM Fig. 13a–c). Partial *ZNFX1* silencing reduced PIC-induced *IFNβ* (a central player of the innate immune and antiviral response) expression (ESM Fig. 13d). Furthermore, in subsequent experiments we observed that *ZNFX1* silencing (Fig. 7d) reduced not only PIC-induced *IFNβ* expression (Fig. 7e) but also IFN-β secretion in the cell supernatant (Fig. 7f). In addition, PIC-induced *RIG-I*, *MDA5*, *CXCL10*, *MX1* and *HLA-ABC* expression, as well as CXC motif chemokine ligand 10 (CXCL10) secretion, was repressed by *ZNFX1* silencing (Fig. 7g–l). The phosphorylation of STAT1 and STAT2, the two key transcription factors activated by type I IFN, was also significantly reduced following *ZNFX1* knock-down (Fig. 7m–o). Altogether, these data identified *ZNFX1* as a novel important player of the antiviral response in human beta cells.

The proinflammatory cytokines change the alternative splicing landscape of human beta cells Modifications that occur at the transcript level may be overlooked when analysing global DGE only. To address this potential gap, we used transcript-level analysis to identify isoform switches [34], i.e. preferentially expressed isoforms in the non-treated condition that switch to another preferentially expressed isoform following cytokine treatment. DTU analysis showed that IFN-α and IFN-γ modified more transcripts than IL-1β and TNF-α (Fig. 8a–d), with each dot representing a different transcript plotted according to the difference in inclusion fraction (i.e. the ratio between the isoform expression and the gene expression) between the non-treated and the cytokine-treated conditions. Some genes that were not differentially expressed in the global analysis had differences in terms of DTU (represented in yellow in the volcano plot in Fig. 8a–d). This observation was particularly relevant for TNF-α. Indeed, while this cytokine modified the expression of only 69 genes (ESM Table 1), it induced a switch in isoform usage in 40 additional genes that were not differentially expressed, adding another level of gene regulation (Fig. 8d). We validated the DTU analysis for *CD47* (also known as *IAP*) and *LARP1* by RT-PCR in EndoC-βH1 cells and dispersed human islets. Visualisation plots reporting structural and functional information about the transcript isoforms produced by these genes, DGE and DTU are shown in Fig. 8e,f. The DTU analysis indicated that the four *CD47* isoforms expressed in EndoC-βH1 cells were affected differently by TNF-α treatment. The canonical isoform ENST00000355354.13 (CD47-201, mentioned as 54.13) and the non-coding transcript ENST0000000644850.1 (CD47-206) were unchanged, but there was a switch of isoform usage between the two others: the coding isoform ENST00000361309.6 (CD47-202, mentioned as 09.6) was decreased by TNF-α, while the non-coding isoform ENST0000000517766.5 (CD47-205) was increased, resulting in no change in the total gene expression (Fig. 8e). Using primers that allow us to discriminate between two different isoforms, we observed a significantly reduced proportion of the isoform 09.6 relative to the canonical one (54.13) in EndoC-βH1 cells treated with TNF-α (Fig. 8g), but not in dispersed human islets (Fig. 8h). The DTU analysis of *LARP1* isoforms (Fig. 8f) indicated that TNF-α increased the usage of the canonical isoform ENST00000336314.9 (La ribonucleoprotein 1 [LARP1]-201, mentioned as 14.9) while decreasing the usage of the coding isoform ENST00000518297.6 (LARP1-204, mentioned as 97.6). These findings were validated in EndoC-βH1 cells and dispersed human islets after TNF-α treatment (Fig. 8i,j). Altogether, these data indicate that cytokines can also affect beta cell gene expression via a switch in isoform usage, possibly leading to a change in function.

Discussion

We identified IFN-α and IFN-γ as the two key proinflammatory cytokines for the transcriptomic modulation of beta cells in the context of type 1 diabetes, showing that they induce a gene signature that closely correlates with the gene signature observed in beta cells from individuals affected by type 1 diabetes. A good understanding of the action of the main proinflammatory cytokines present in inflamed islets is crucial to identify the relevant therapeutic targets to preserve beta cell mass, and also to define experimental conditions to better model the human disease using human tissues.

IFN-α is found in beta cells of individuals with type 1 diabetes [54] and a type I IFN gene signature is present in individuals at risk of developing type 1 diabetes, even before the development of autoantibodies [8]. We observed that IFN-α induces the expression of all the genes encoding proteins previously shown to be overexpressed in pancreatic islets of individuals affected by type 1 diabetes. IFN-α also modulates the expression of most of the candidate genes for type 1 diabetes and triggers a strong antiviral and inflammatory response in human beta cells. As the disease progresses, there is a transition from innate to adaptive immunity with the local release of proinflammatory cytokines, such as IFN-γ, and chemokines, such as CXCL10, mostly by the immune cells attracted to the islets in response to the initial inflammatory response [1]. Our GSEA showed that exposure of human beta cells to IFN-α and IFN-γ, but not IL-1β and TNF-α, induces an enrichment in genes involved in antigen presentation and attraction of immune cells, particularly HLA class I and related mRNAs. By doing so, they initiate

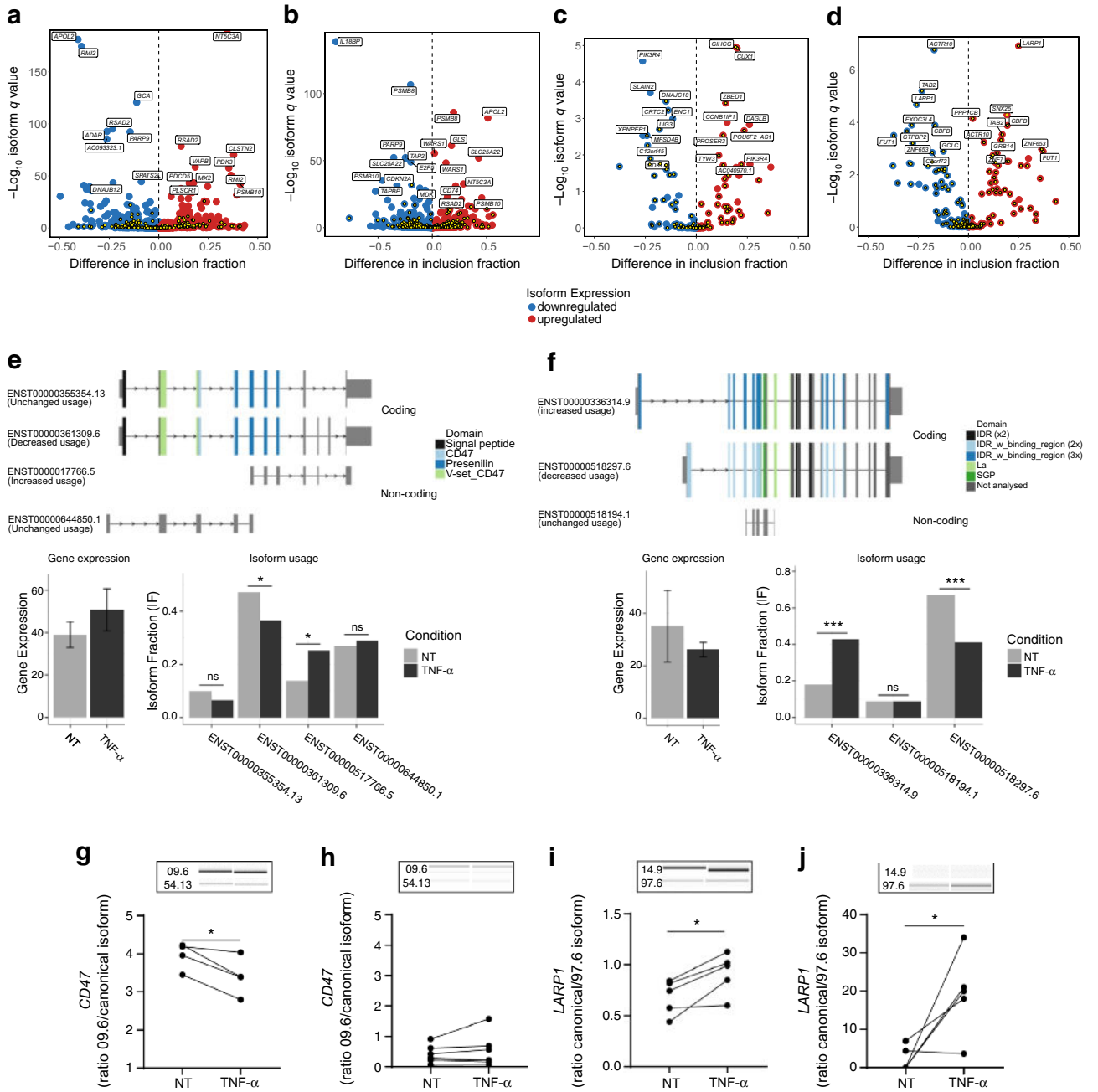


Fig. 8 IFN-α, IFN-γ, IL-1β and TNF-α induce DTU independently of total gene expression. (**a**–**d**) Volcano plots showing the genes with DTU after exposure to IFN-α (**a**), IFN-γ (**b**), IL-1β (**c**) or TNF-α (**d**) for 24 h. Each dot corresponds to a different transcript; the *x*-axis represents the difference in inclusion fraction measured as the ratio between the isoform expression and the gene expression, while the *y*-axis represents the isoform *q* value ($-\log_{10}$). Blue circles correspond to the downregulated transcripts and red circles to the upregulated ones. The yellow dots indicate differentially used transcripts from which the corresponding gene is not differentially expressed under the same experimental conditions. (**e**) *CD47* isoforms with intron−exon structures of each transcript. The bar plots represent the gene and transcript isoform expression of *CD47* comparing non-treated (NT) and TNF-α treatment (24 h). (**g**, **h**) Validation of the *CD47* isoform usage in EndoC-βH1 cells (**g**) and dispersed human islets (**h**); results were expressed as a ratio between ENST00000361309.6 and ENST00000355354.13 isoforms. (**f**) *LARP1* isoforms with intron–exon structures of each transcript. The bar plots represent the gene and transcript isoform expression of *LARP1* comparing NT and TNF-α treatment (24 h). (**i**, **j**) Validation of the *LARP1* isoform usage in EndoC-βH1 cells (**i**) and dispersed human islets (**j**); results were expressed as a ratio between ENST00000336314.9 and ENST00000518297.6 isoforms. All transcript isoforms were identified using Ensembl ID. cDNA was amplified by RT-PCR using primers located in the upstream and downstream exons of the splicing event and the product was evaluated using a Bioanalyzer 2100. (**g**, **h**, **i**, **j**) n=4–5 (EndoC-βH1) and n=5–6 (human islets). Two-sided paired t test, *p<0.05 and ***p<0.001 vs NT. IDR, intrinsically disordered region; La, La domain containing protein; NS, not significant; SGP, sulphur globule protein; V-set CD47, N-terminal domain mediates binding to CD47

(IFN-α) and then amplify (IFN-γ) a dialogue between beta cells and the immune cells that contributes to the transition and amplification of the adaptive immune response against the beta cells. The role of IFN-α and IFN-γ in the progression of the disease is further reinforced by the gradual increase in the ISG score that we observed from non-diabetic individuals to donors with type 1 diabetes, passing through individuals with AAB2+.

Considering the key roles for IFN-α and IFN-γ in the deleterious dialogue between the immune system and beta cells, targeting these cytokines for therapy may be of interest, with the caveat that these cytokines also induce protective effects (e.g. *PDL1*, *HLA-E* and *HLA-G* overexpression) that should ideally be preserved.

The candidate gene *TYK2* is an important mediator of IFN-α signalling, acting directly downstream of its receptor to transduce cell signalling in response to type I IFN stimulation. Polymorphisms in the *TYK2* gene confer protection against several autoimmune diseases, including type 1 diabetes, and it has been suggested that *TYK2* might be a good therapeutic target for type 1 diabetes [12]. Our group and others previously validated in vitro the use of different TYK2 inhibitors in EndoC-βH1 cells, dispersed human islets and human stem cell-derived beta-like cells to block IFN-α signalling and also reduce T cell-mediated cytotoxicity [55, 56]. JAK inhibitors can block the signalling of cytokines that function via the JAK–STAT pathway, including IFN-α and IFN-γ. Therefore, many JAK inhibitors have been developed (with different selectivity for the four JAK molecules: JAK1, JAK2, JAK3 and TYK2) and studied in the context of type 1 diabetes. Baricitinib, a JAK1/2 inhibitor, protects human beta cells against cytokine-induced apoptosis and HLA class I upregulation [6]. Importantly, a recent phase 2 clinical trial involving individuals with recent-onset type 1 diabetes showed that baricitinib preserves the capacity of beta cells to secrete insulin after 48 weeks of treatment [57].

Studies in rodents indicated an important role for IL-1β in the destruction of beta cells but clinical trials with monoclonal anti-IL-1β antibody (canakinumab) or IL1R antagonist (anakinra) failed to preserve beta cell function in individuals with type 1 diabetes (although a subset of anakinra-treated participants had an increase in C-peptide after 9 months of treatment compared with placebo), suggesting that targeting IL-1β is not effective as single immunomodulatory therapy for type 1 diabetes [58]. Considering the data from the present study, this absence of effect is not surprising since human beta cells, but not rat beta cells, have a much higher expression of IFN receptors than of IL-1Rs. On the other hand, golimumab, a monoclonal anti-TNF-α antibody already approved to treat other autoimmune diseases, has been tested with more success in a phase 2 clinical trial in children and young adults with recent-onset type 1 diabetes. Golimumab responders had better preservation of C-peptide production and glycaemic control than individuals who received a placebo, and this positive result was still present after 1 year off-therapy [59, 60]. A small pilot trial using another TNF-α blocker, etanercept, also suggested a beneficial effect for the preservation of beta cell function in paediatric patients with newly diagnosed type 1 diabetes [61]. However, this protective effect of etanercept was not confirmed in another study [62]. This contrasts with the limited impact of TNF-α on human beta cell gene expression (present data). We speculate that possible explanations for this dichotomy are: (1) the main role of TNF-α is on the immune system, and not on the beta cells [63, 64]; (2) TNF-α has a more prominent effect on alternative splicing, rather than on the regulation of gene expression; (3) TNF-α signalling is associated with deleterious polymorphisms, as recently shown for the type 1 diabetes candidate gene *PTPN2* [7], which may contribute to beta cell death only in a specific cohort of individuals carrying these polymorphisms; (4) TNF-α blockers prevent the synergistic deleterious effect observed in vitro between TNF-α and other cytokines such as IFN-γ and IL-1β. Additional studies are now required to distinguish between these possibilities.

Our data also indicate that many candidate genes for type 1 diabetes are regulated by IFN-α and IFN-γ while TNF-α and IL-1β had a more limited impact. Candidate genes are important components of the development and progression of type 1 diabetes and may explain why different individuals exposed to the same triggering factors (for example, viral infections and/or proinflammatory cytokines) respond differently. It is therefore relevant to evaluate the crosstalk between candidate genes and cytokines that are present in the inflamed islets during type 1 diabetes. We showed that inhibition of the candidate gene *BACH2* decreases beta cell viability in the presence of the different proinflammatory cytokines tested. A recent publication, based on observations that *BACH2* inhibition in models of type 2 diabetes increases nuclear factor erythroid-2-related factor-2 (NRF2)-dependent antioxidant response genes, decreases beta cell damage and increases beta cell function [65], suggested that inhibition of *BACH2* could be a potential pharmacological intervention in type 2 diabetes. Considering our previous [52] and present data suggesting a deleterious impact of *BACH2* silencing on human beta cells exposed to proinflammatory cytokines, we are concerned that eventual pharmacological interventions to inhibit *BACH2* as proposed in reference [65] will actually increase beta cell susceptibility to inflammation in both type 1 and type 2 diabetes.

The present findings indicate that IFN-α also induces a strong antiviral gene signature in human beta cells. Epidemiological and clinical studies, and experimental data, support the involvement of viral infections, particularly by the enterovirus coxsackievirus B, in the pathogenesis of type 1 diabetes [66, 67]. The cytosolic viral dsRNA sensor *MDA5*

is a candidate gene for type 1 diabetes that is expressed in islet cells of individuals with type 1 diabetes [49, 68] and, together with *RIG-I*, another viral dsRNA sensor, plays an important role in viral detection in beta cells and subsequent induction of type I IFN [67, 69]. These two genes are mainly induced by IFN-α and IFN-γ in human beta cells (present data), with also a mild effect of IL-1β on *MDA5* expression. Besides these two genes, we identified *ZNFX1* [53] as a new beta cell viral dsRNA sensor, potentially acting in the early stages of viral infections (or exposure to other danger signals) to induce a type I IFN response. *ZNFX1* is induced by IFN-α and IFN-γ and by the mimic of viral infection PIC in human beta cells. Gene silencing of *ZNFX1* indicates that it acts as a viral dsRNA sensor in beta cells exposed to PIC, leading to the production of IFN-β and the induction of downstream ISGs, including the two other viral sensors *MDA5* and *RIG-I*. ZNFX1 seems thus to be a critical regulator of the IFN-mediated defence responses in human beta cells and could be an important player in the initiation of the innate immune response in the early stages of type 1 diabetes.

We have previously studied the impact of proinflammatory cytokines on alternative splicing and on how this can affect beta cell function and survival and their recognition by the immune system in the context of type 1 diabetes [1, 4, 6, 33, 35, 70]. We have shown that cytokines induce a switch in preferentially used transcripts for some genes without alteration of the total gene expression in human beta cells. We were particularly interested in TNF-α in this context because this cytokine induces limited changes in total gene expression (69 genes) while inducing changes at the transcript level (40 additional genes impacted). From the list of genes that we identified in our DTU analysis, we validated *CD47*, a gene that results in a membrane protein expressed in several cell types and is well known for providing a 'don't eat me' signal via binding to signal regulatory protein α (SIRPα) on macrophages, preventing phagocytosis [71]. Interestingly, *CD47* is strongly expressed in human beta cells and regulates their viability; *CD47* expression is altered in type 1 diabetes with differences observed between putative type 1 diabetes endotypes [72]. In the present study, we observed that although TNF-α did not alter *CD47* total gene expression, there was a reduced expression of a protein-coding transcript counterbalanced by the increase in a non-coding transcript. The functional consequences of the DTU observed for *CD47* and other genes will require further studies to evaluate their potential relevance in type 1 diabetes.

A limitation of the present study is that, in vivo, cytokines operate in concert, with synergistic and antagonistic interactions between pro- and anti-inflammatory cytokines determining the fate of the target cells. Thus, the present reductionist approach of aiming to identify the relative importance of cytokines in type 1 diabetes pathogenesis by adding them individually in vitro, taken out of the cytokine-network context, is probably an over-simplification of the complex in vivo situation. However, the close correlation presently observed between IFN-induced gene signatures on human beta cells in vitro as compared with the signatures observed in the islet cells of individuals affected by type 1 diabetes supports, at least in part, the relevance of IFNs for pancreatic beta cell outcome in type 1 diabetes.

In conclusion, we have applied a deep RNA-seq approach to study the individual impacts of the four major proinflammatory cytokines involved in type 1 diabetes, IFN-α, IFN-γ, IL-1β and TNF-α, on the human beta cell transcriptome. Our data identified IFN-α and IFN-γ as key drivers of the beta cell inflammatory response during insulitis, inducing a gene signature that strongly correlates with the transcriptomic profile of beta cells from individuals with type 1 diabetes. Therefore, therapies that aim to protect beta cells from an immune attack should focus on these cytokines and on their downstream signalling to modulate the dialogue between beta cells and immune cells and break the deleterious circle of immune amplification and beta cell death.

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Data availability All data are available in the main text or the electronic supplementary material (ESM). They are available from the corresponding authors upon reasonable request. All newly generated RNA-seq data that support the findings of the present study have been deposited at GEO under accession code GSE235683.

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Contribution statement ACdB, MIA and FS contributed to the original idea; to the design, performance and interpretation of the experiments; and to the investigation and formal analysis; and wrote, revised and edited the manuscript. PLZ performed and analysed experiments, and wrote, revised and edited the manuscript. AC, BMdS, SJR, NGM, ARR, SM-C, XY, AOdB, SS, SJ, ACT and BY performed and analysed experiments. FP, JK-C, EM, MN, LM and PM contributed with material and acquisition of data. DLE contributed to the original idea and the design, supervision and interpretation of the experiments, and wrote and revised the manuscript. All authors have read and approved the final version of the manuscript. ACdB, MIA and DLE are the guarantors of this work and, as such, have 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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