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***DEXI*, a candidate gene for type 1 diabetes, modulates rat and human pancreatic beta cell inflammation via regulation of the type I IFN/STAT signaling pathway.**

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Tweet (Figure 7): The type 1 diabetes susceptibility gene *DEXI* modulates pancreatic beta cell inflammation via regulation of the type I IFN/STAT signalling pathway. #Dexi #T1D #type1IFNs #pancreaticbetacell. @biocrucesHRI @upvehu @ciberdem @rsdossantos13 @lmarroqui

ABSTRACT

Aims/hypothesis: The initial stages of type 1 diabetes are characterized by an aberrant islet inflammation that is in part regulated by the interaction between type 1 diabetes susceptibility genes and environmental factors. Chromosome 16p13 is associated with type 1 diabetes and *CLEC16A* has been considered the etiologic gene in the region. However, recent gene expression analysis indicates that SNPs in *CLEC16A* modulate the expression of a neighbouring gene with unknown function named *DEXI*. We presently evaluated the role of DEXI in beta cell responses to "danger signals" and determined the mechanisms involved.

Methods: Functional studies based on silencing or overexpression of DEXI were performed in rat and human pancreatic beta cells. Viral double-stranded RNA-driven beta cell inflammation and apoptosis were evaluated by RT-PCR, western blot and luciferase assays.

Results: DEXI-silenced beta cells exposed to double-stranded RNA (PIC; a by-product of viral replication) showed reduced STAT1 activation and lower production of pro-inflammatory chemokines that was preceded by a reduction in IFNβ expression. Exposure to PIC increased chromatin-bound DEXI and IFNβ promoter activity. This effect on IFNβ promoter was inhibited in DEXI-silenced beta cells, suggesting that DEXI is implicated in the regulation of IFNβ transcription. In a mirror image of knockdown experiments, DEXI overexpression led to increased STAT1 and pro-inflammatory chemokine expression.

Conclusions: These observations support *DEXI* as the aetiological gene in the type 1 diabetes-associated 16p13 genomic region and provide the first indication of a link

between this candidate gene and the regulation of local antiviral immune response in beta cells. Moreover, our results provide initial information on the function of *DEXI*.

KEYWORDS

Type 1 diabetes, pancreatic beta cell, susceptibility gene, inflammation, viral infection, type I IFNs, *DEXI*.

ABBREVIATIONS

CCL5: Chemokine (C-C motif) ligand 5

CVB5: Coxsackievirus B5

CXCL9: C-X-C motif chemokine 9

CXCL1: C-X-C motif chemokine 1

IFNAR: Interferon α/β receptor

ISRE: Interferon-stimulated response element

KD: Knockdown

PIC: Polyinosinic:polycytidylic acid

siRNA: Small interfering RNA

STAT1: Signal transducer and activator of transcription 1

RESEARCH IN CONTEXT

What is already known about this subject?

- Islet inflammation is partially regulated by the interaction between type 1 diabetes susceptibility genes and environmental factors.
- Type 1 diabetes-associated SNPs in *CLEC16*A correlate with differential expression of a neighbouring gene with unknown function named *DEXI*.

What is the key question?

- Is the type 1 diabetes -associated candidate gene *DEXI* implicated in pancreatic beta cell inflammation and death?

What are the new findings?

- The type 1 diabetes candidate gene *DEXI* is involved in virus-induced pancreatic beta cell inflammation via regulation of the type I IFN-STAT signalling pathway.
- DEXI is located in the chromatin of pancreatic beta cells and participates in the transcriptional regulation of virus-induced IFNβ expression.
- Our findings support *DEXI* as the aetiological gene for type 1 diabetes in chromosome 16p13 and provide initial insights on the function of this type 1 diabetes susceptibility gene.

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

- Molecular characterization of the interactions between predisposing genes and environmental triggers will allow to clarify the mechanisms by which a viral infection or other danger signals leads to type 1 diabetes, providing crucial information to develop therapeutic strategies to avoid the triggering of autoimmunity in genetically susceptible individuals.

INTRODUCTION

Type 1 diabetes is an autoimmune disease in which pancreatic beta cells are destroyed by the immune system leading to a progressive and severe insulin deficiency. The initial stages of the disease are characterized by an islet inflammation (insulitis) that is partially driven by a "dialog" between beta cells and the infiltrating immune cells [1]. Accumulating evidence suggest that the triggering of insulitis depends on the interaction between type 1 diabetes susceptibility genes [2] and environmental factors, such as viral infections [3,4]. During viral infections, pancreatic beta cells and other islet cells release chemokines and cytokines, including type I interferons (IFNs) [5,6], that contribute to attract and activate immune cells homing to the islets, contributing to type 1 diabetes pathogenesis [1].

Linkage and genome-wide association studies have identified 58 genomic regions showing evidence for type 1 diabetes association and about 50 genes have been suggested to be potential causal disease genes [7]. Pathway analysis and functional studies of type 1 diabetes-associated genes expressed in human pancreatic islets indicate that many of these type 1 diabetes-associated genes are involved in the regulation of antiviral responses and type I IFN signalling [2,8–12].

Functional characterization at the beta cell level of type 1 diabetes candidate genes related to innate immunity, e.g. *MDA5* [8], *PTPN2* [10] and the associated gene *USP18* [11], indicate a common theme, namely that polymorphisms leading to hyperactivation of innate immunity and inflammatory responses are usually associated with increased type 1 diabetes risk, while decreased responses in these pathways lowers the risk of developing type 1 diabetes [2].

The chromosome 16p13 region of the human genome has been associated with several autoimmune diseases, including type 1 diabetes [13], celiac disease [14], and multiple sclerosis [15]. This genomic region is dominated by the *CLEC16A* gene and the most highly disease-associated SNPs, rs12708716 and rs8062322, are located within its intron 19 [13–16]. *CLEC16A* has been considered the etiologic gene in the region, and experiments indicated that it regulates mitophagy in pancreatic beta cells [17]. Recent gene expression analysis followed by chromosome conformation capture experiments, however, indicated that intron 19 of *CLEC16A* behaves as a regulatory sequence that physically interacts with the promoter of a neighbouring gene named *DEXI* (dexamethasone-induced transcript), affecting its expression [18]. The presence of the risk allele in *CLEC16A* correlated with a decrease in *DEXI* expression, whereas the presence of the protective allele is associated with higher levels of *DEXI* expression in monocytes [18], thymus [19] and B lymphoblastoid cell lines [20]. These findings suggest that *DEXI* might be the main etiologic gene in the 16p13 region and may represent a novel and important pathway in the pathogenesis of autoimmune diseases.

DEXI is a gene that encodes a 95 amino acid protein highly conserved across several species [18]. *DEXI* was identified as a differentially expressed transcript in lung tissue of patients with emphysema [21]. The function of DEXI is unknown, but analysis of its protein sequence shows a central transmembrane domain and a repeating leucine zipper motif, which is a typical feature of many transcription factors and regulatory proteins [18].

Here we studied the *DEXI* role in the beta cell response to stresses relevant to type 1 diabetes to clarify its potential involvement in the pathogenesis of the disease at the beta cell level.

METHODS

Culture of INS-1E cells, human EndoC-βH1 cells, primary rat beta cells, and human islets, and collection of human biological samples

The INS-1E cell line (kindly provided by Dr. C. Wollheim, Centre Medical Universitaire, Geneva, Switzerland) was cultured in Roswell Park Memorial Institute 1640 GlutaMAX-I as previously described [22]. For small interfering (si)RNA and polyinosinic:polycytidylic acid (PIC) transfection, antibiotic-free medium was used.

The EndoC-βH1 human beta cell line (kindly provided by Dr. R. Scharfmann, Centre de Recherche de l'Institut du Cerveau et de la Moelle Épinière, Paris, France) was cultured in plates coated with Matrigel-fibronectin (100 mg/ml and 2 mg/ml, respectively) in low-glucose DMEM as previously described [23]. For siRNA and PIC transfection, BSA- and antibiotic-free medium was used.

INS-1E and EndoC-βH1 cells were free from Mycoplasma, as evaluated by the MycoAlert Mycoplasma Detection kit (Lonza, Basel, Switzerland). For the prevention of Mycoplasma contamination, Plasmocin Prophylactic (Invivogen, San Diego, CA, USA) was added to the culture medium on a regular basis.

Rat islets were isolated by collagenase digestion and handpicked under a stereomicroscope, and beta cells were fluorescence activated cell sorting (FACS)-purified and cultured as previously described [24] (see ESM methods for details). For siRNA and PIC transfection, BSA- and antibiotic-free medium was used.

Human islet isolation from nondiabetic organ donors was carried out according to the local Ethical Committee in the University of Pisa (Pisa, Italy) as previously described [25]. Human islets were then sent to Brussels and, after overnight recovery, dispersed and

cultured as described [12,22]. As determined by insulin immunocytochemistry [26], the percentage of beta cells in the human islet preparations was 53 ± 14% (n = 4). Of note, the human islet samples were previously described [12].

Duodenal biopsies from celiac patients and non-celiac donors were collected at the Cruces University Hospital, Pediatric Gastroenterology Unit (Barakaldo, Spain) (see ESM methods for details).

RNA interference

The siRNAs targeting rat and human *DEXI* used in this study are in ESM Table 1. The optimal siRNA concentration (30 nmol/l) and conditions for beta cell transfection were previously established [22,27]. Cells were transfected using the Lipofectamine RNAiMAX lipid reagent (Invitrogen, Carlsbad, CA, USA) as described [11]. After transfection, cells were cultured for 48 h recovery period and subsequently exposed to intracellular PIC, treated with pro-inflammatory cytokines interleukin (IL)-1β plus IFNγ, or infected with coxsackievirus B5 (CVB5).

Cell treatments

The synthetic viral dsRNA analogue PIC (InvivoGen) was used at the final concentration of 1 μg/ml as previously described [11]. PIC transfection was performed in the same conditions as described for siRNA but using Lipofectamine 2000 lipid reagent (Invitrogen).

Cells were treated with IL-1β plus IFNγ (10 and 100 U/ml, respectively) as described elsewhere [22,28] and infection of primary rat beta cells with CVB5 (multiplicity of infection, MOI, 1) was performed as before [5].

EndoC-βH1 cells were treated with the JAK inhibitor ruxolitinib (Cayman Chemicals, Ann Arbor, MI, USA) as previously described [29].

Assessment of cell viability

Cell viability was determined after incubation with the DNA-binding dyes propidium iodide (PI, 5 mg/ml; Sigma, St. Louis, MO, USA) and Hoechst 33342 (HO, 5 mg/ml) as described [10,28]. See ESM Methods.

Real Time PCR

RNA extraction was performed using NucleoSpin RNA Kit (Macherey Nagel, Düren Germany) and cDNA synthetized using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA). Real Time PCR was performed using TaqMan Gene Expression Assays (Thermo Fisher, Waltham, MA, USA) specific for rat and human DEXI, STAT1, CXCL1, CCL5, CXCL9 and IFNβ. Expression was corrected for the housekeeping genes glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) (in rat beta cells), *β-actin* (in human beta cells) and *RPLP0* (in intestinal biopsies). The presently utilized treatments do not affect expression of these housekeeping genes (data not shown). The TaqMan Gene Expression Assays (Thermo Fisher) used are listed in ESM Table 2. *DEXI* expression was also measured in a commercially available RNA panel set of different human tissues (Human total RNA master panel II, Clontech, France).

Western blotting

Cells were lysed in Laemmli buffer and the immunoblot analysis was performed with the antibodies listed in ESM Table 3. See ESM Methods.

ELISA

Supernatants from INS-1E and EndoC-βH1 cells were collected for determination of rat CCL5 and human IFNβ, respectively, using commercially available ELISA kits (R&D Systems, Abingdon, UK). ELISA was performed following the manufacturer's instructions.

Luciferase reporter assays

INS-1E cells were transfected with siRNAs as described above. After 12 h of recovery, cells were co-transfected with pRL-CMV encoding Renilla luciferase (Promega, Madison, WI, USA) and either a firefly luciferase promoter-reporter construct containing the four interferon-stimulated response element (ISRE) consensus sequences or the IFNβ promoter [30]. After 24 h of recovery and 16 h of PIC transfection, luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega) and corrected for the luciferase activity of the internal control plasmid, pRL-CMV.

Statistical analysis

Data are expressed as means ± SEM. A significant difference between experimental conditions was assessed by ANOVA followed by a paired Student' t test with Bonferroni correction. p values < 0.05 were considered statistically significant. Statistical analysis was performed using GraphPad Prism version 5 (GraphPad Software, La Jolla, CA, USA).

RESULTS

DEXI expression is upregulated by intracellular dsRNA in human and rodent pancreatic beta cells

We first evaluated the expression of *DEXI* mRNA in dispersed human islets and in the human beta cell line EndoC-βH1 compared with a set of human tissues (Fig. 1a). *DEXI* is expressed among several tissues with the highest expression values in heart, brain, kidney and liver and the lowest in lung. *DEXI* mRNA expression in dispersed human islets and in human beta cells was around two-fold higher than in tissues involved in immune response (*e.g.* thymus and spleen). This was confirmed in human islets studied by RNA sequencing [26], as compared to 16 control tissues using the Illumina BodyMap 2.0 dataset (ESM Fig. 1a). The pro-inflammatory cytokines IL-1β + IFNγ did not modify *DEXI* expression in rat and human beta cells (ESM Fig. 1b and 1c). In line with this, RNA sequencing of human islets exposed or not to IL-1β + IFNγ [8] showed similar *DEXI* expression in controls and cytokine-treated human islets, i.e. RPKM (reads per kilobase per million mapped reads) respectively 19 ± 1 and 23 ± 3 (means ± SEM of 5 samples) (ESM Fig. 1a). *DEXI* expression was also similar in intestinal biopsies of celiac patients at debut (on a gluten-containing diet, with celiac disease-associated antibodies, atrophy of intestinal villi and T lymphocyte infiltration), and the same patients in remission after being treated with gluten-free diet for >2 years (asymptomatic, antibody negative and normalized intestinal epithelium at that time) (ESM Fig. 1d). Altogether, these results suggest that inflammation by itself does not alter *DEXI* expression in pancreatic beta and intestinal cells.

It has been previously shown that PIC, a synthetic viral dsRNA analogue, induces beta cell inflammation and death [8,11,31]. Exposure of human beta cells to PIC led to a 74-

fold increase in *DEXI* mRNA expression after 2 h, increasing further at 8 h (179-fold) and slightly decreasing at 24 h (148-fold) (Fig. 1b). The effect of PIC in DEXI expression was confirmed at the protein level in EndoC-βH1 cells (Fig. 1c). Exposure to PIC for 24 h increased *Dexi* mRNA expression by around 2.3-fold in INS-1E cells (Fig. 1d) and FACS-purified primary rat beta cells (Fig. 1e).

Risk alleles of autoimmune-disease associated SNPs in *CLEC16A* gene correlate with decreased *DEXI* expression in several immune cells and tissues, including B cells, monocytes and thymus [18–20]; however, DEXI-CLEC16A eQTL seems to be tissue- and context-specific [18,32]. Available eQTL data from GTEx Portal showed no correlation between rs12708716 genotype and *DEXI* expression in whole pancreas (ESM Fig. 2a). SNPs in *CLEC16A* are also associated with celiac disease, another autoimmune disease that shares several candidate genes with type 1 diabetes. According to GTEx data, disease-associated protective allele (G) in rs12708716 correlates with a slight decrease in *DEXI* expression in small intestine (ESM Fig. 2b). These data were confirmed in intestinal biopsies of healthy donors, where biopsies from donors that were heterozygous for the protective allele (G) showed decreased *DEXI* expression when compared to biopsies from donors homozygous for the risk allele A (ESM Fig. 2c).

Conversely, CVB5 infection in beta cells downregulated *Dexi* expression by 40% (ESM Fig. 3a), suggesting the potential activation of a virus-related mechanism to downregulate antiviral host defence.

DEXI inhibition reduces viral dsRNA-induced pancreatic beta cell death

Intracellular treatment with PIC or infection with the diabetogenic CVB5 triggered beta cell death [5,33]. To examine the role of DEXI in PIC- and CVB5-induced beta cell apoptosis, two independent siRNAs were used to knockdown (KD) DEXI expression in

primary rat beta cells and in INS-1E cells. These different siRNAs inhibited *Dexi* mRNA expression by 50-80% in all the conditions tested (Fig. 2a and ESM Fig. 3a). PIC transfection for 24 h increased apoptosis by 23% in siCTRL-transfected primary rat beta cells, whereas DEXI inhibition reduced cell death in PIC-transfected cells by around 10% (Fig. 2b). Similarly, CVB5 infection for 24 h increased beta cell apoptosis compared to mock-infected INS-1E cells and DEXI inhibition reduced CVB5-induced apoptosis by around 50% (ESM Fig. 3b). The decrease in PIC-induced apoptosis after DEXI KD was confirmed by decreased caspase 3/7 activity in INS-1E cells using a luminescence-based assay (ESM Fig. 3c).

DEXI silencing partially inhibits PIC-induced STAT signalling pathway activation and decreases pro-inflammatory chemokine production in pancreatic beta cells

We next examined the effect of DEXI inhibition on the kinetics of PIC-induced STAT signalling activation. STAT1 phosphorylation was markedly enhanced in INS-1E cells exposed to PIC after 8 h and up to 24 h (Fig. 2c and ESM Fig. 4a). DEXI inhibition abrogated STAT1 phosphorylation 8 h after PIC transfection and this effect was prolonged up to 24 h (Fig. 2c and ESM Fig. 4a). Although total STAT1 protein expression did not differ between siCTRL- and siDEXI-transfected cells 24 h after PIC transfection, PIC-induced *Stat1* mRNA expression was significantly reduced in DEXI-inhibited INS-1E cells (Fig. 2d), suggesting a potential role of DEXI in the regulation of STAT1 signalling. As PIC exposure activates the expression of several pro-inflammatory genes regulated by ISRE [34], we assessed the activation of an ISRE reporter in DEXI-inhibited beta cells. As shown in Fig. 2e, PIC transfection induced ISRE reporter activity in siCTRL-transfected cells by 40-fold, whereas DEXI KD significantly inhibited ISRE activation (around 70% less activity).

To evaluate the role of DEXI in PIC-induced chemokine production, we analysed the mRNA expression of *CCL5* (chemokine (C-C motif) ligand 5), *CXCL9* (C-X-C motif chemokine 9) and *CXCL1* (C-X-C motif chemokine 1) in DEXI-silenced INS-1E cells (Fig. 3a-d), EndoC-βH1 cells (Fig. 3e-h), and FACS-purified primary rat beta cells (ESM Fig. 5). PIC treatment for 8 and 24 h induced *Ccl5*, *Cxcl9* and *Cxcl1* mRNA expression in siCTRL-transfected INS-1E cells, but DEXI inhibition partially prevented this effect at both time-points (Fig. 3a-d). In EndoC-βH1 cells, PIC transfection for 24 h significantly increased pro-inflammatory chemokine expression (Fig. 3e-h). The downregulation of *Ccl5* mRNA expression in DEXI-inhibited INS-1E cells was confirmed at the protein level, as DEXI-silenced cells secreted lower amounts of CCL5 compared with siCTRL-transfected cells after PIC transfection (ESM Fig. 6). In DEXI-silenced EndoC-βH1 cells, PIC-induced *CCL5*, *CXCL9* and *CXCL1* expression was decreased by 50-65%, 30-60% and 90%, respectively (Fig. 3e-h). In FACS-purified rat primary beta cells, PIC transfection increased *Ccl5*, *Cxcl9* and *Cxcl1* expression, but only *Cxcl9* expression was significantly decreased after DEXI inhibition (ESM Fig. 5).

In a mirror image of these experiments, DEXI upregulation in INS-1E cells using an overexpression vector (fold increase of 220-fold compared to pCMV-control-transfected cells) exacerbated PIC-induced STAT1, *Ccl5*, *Cxcl9* and *Cxcl1* mRNA expression by 25-40% (Fig. 4).

To confirm that DEXI affects PIC-induced pro-inflammatory chemokine expression through the modulation of the type IFN-STAT signalling pathway, we exposed DEXI-overexpressing EndoC-βH1 cells to PIC in the absence or presence of ruxolitinib, a JAK inhibitor. As shown in Figure 5, *DEXI* overexpression exacerbated PIC-induced *CCL5,*

CXCL9 and *CXCL1* mRNA expression, and this effect was counteracted by the presence of ruxolitinib.

Downregulation of STAT signalling pathway is preceded by reduced IFNβ expression in DEXI-silenced pancreatic beta cells

During viral infections, pancreatic beta cells release type I IFNs that can act in an autocrine way through the type I IFN receptor (IFNAR) to activate pro-inflammatory signalling pathways [11,12]. To evaluate the potential role of DEXI in PIC-induced type I IFN signalling pathway activation, we evaluated the expression of *IFNβ* in DEXI-silenced beta cells. *IFNβ* mRNA expression was upregulated after 24 h of PIC transfection in siCTRL-transfected beta cells, while DEXI inhibition decreased PIC-induced *IFNβ* expression, both in INS-1E and EndoC-βH1 cells (Fig. 6a, b).

To clarify whether DEXI modulates viral dsRNA-induced STAT signalling activation by regulating the early production of IFNβ, beta cells were exposed to PIC at different time-points. As shown in Fig. 6c, after 4 h of PIC transfection *IFNβ* expression was already upregulated in siCTRL-transfected INS-1E cells and inhibition of DEXI reduced this increase in *IFNβ* mRNA expression. The time-course experiment revealed two upregulation peaks of PIC-induced *Ifnβ* expression (8 and 24 h after PIC transfection), suggesting the presence of an early and late phase of type I IFN production in response to viral infections in beta cells. At both time-points (8 and 24 h), DEXI inhibition reduced *IFNβ* expression (Fig. 6a, b), confirming that downregulation of the STAT signalling pathway in DEXI-silenced beta cells is preceded by an inhibition of IFNβ production. In line with these results, DEXI-inhibited EndoC-βH1 cells showed reduced PIC-induced IFNβ release when compared to siCTRL-transfected cells (Fig. 6d).

DEXI is located in the chromatin of pancreatic beta cells and acts as a transcriptional regulator of IFNβ expression

The protein sequence of DEXI has been predicted to contain a repeating leucine pattern similar to a leucine zipper motif [18]. The leucine zipper motif is the simplest known protein-DNA recognition one, typical of a superfamily of transcription factors that include ATF, CREB and Maf families [35].

As the results described above suggest that DEXI regulates the transcription of IFNβ in pancreatic beta cells, we analysed the cellular localization of DEXI under basal condition and following 4 h of PIC exposure. DEXI was located in the chromatin of EndoC-βH1 cells before and after PIC transfection, although after 4 h of PIC transfection DEXI chromatin expression was slightly higher than in basal condition (Fig. 6e).

To determine whether DEXI could modulate the activation of the IFNβ promoter, we performed a luciferase reporter assay using an IFNβ promoter reporter in INS-1E cells. As shown in Fig. 6f, the IFNβ promoter reporter was activated 3-fold by PIC. DEXI inhibition decreased IFNβ promoter reporter activity by respectively 92% and 78% under basal condition and in PIC-transfected cells, suggesting that DEXI regulates IFNβ transcription.

DISCUSSION

The type 1 diabetes candidate gene *DEXI* encodes a 10 kDa protein with unknown function that was first identified as an upregulated transcript in lung tissue of patients with emphysema [21]. *DEXI* has not been positionally associated with type 1 diabetes, but the type 1 diabetes-associated intronic SNPs in the neighbouring gene *CLEC16A* correlate with differential expression of DEXI [18]. Thus, *DEXI* has emerged as a

potential candidate gene for type 1 diabetes and other autoimmune diseases in the 16p13 region of the human genome.

DEXI expression levels in relation to disease-associated SNPs in *CLEC16A* indicate that homozygotes for the risk alleles had lower expression of *DEXI*, whereas the heterozygotes and homozygotes for the other allele display higher *DEXI* expression in immune-related cells [18–20]. However, as described for other several eQTLs, it seems that the DEXI-CLEC16A eQTL is tissue- and context-specific [18,32], and the nature of this eQTL in pancreatic beta cells remains to be determined. Definition of eQTL signatures relevant for type 1 diabetes rely on performing genotyping and expression studies in beta cells from type 1 diabetes patients and respective controls, but the very limited access to these samples makes it difficult to perform conclusive studies.

In the present study we observed that *DEXI* is highly expressed in human pancreatic beta cells/islets when compared to other human tissues, including immune-related cells. Intracellular viral dsRNA upregulates DEXI expression in beta cells, suggesting that DEXI may play a relevant role in beta cell responses to danger signals.

Our results showed that CVB5 infection downregulates *Dexi* expression in pancreatic beta cells. This phenomenon is probably part of the strategy that viruses use to inhibit the antiviral response of the host cell and increase their own virulence. Indeed, several studies have demonstrated that viruses are able to inhibit the antiviral responses by inhibiting the activation of the IFN-induced JAK-STAT signalling pathway through different strategies [36,37].

Here we show that DEXI inhibition reduces viral dsRNA-induced pro-inflammatory chemokine production via downregulation of the type I IFN-STAT signalling pathway.

DEXI overexpression exacerbated PIC-induced STAT signalling and pro-inflammatory chemokine expression, suggesting that DEXI plays a pro-inflammatory role in response to viral infections at the beta cell level.

Decreased STAT1 signalling activation in DEXI-silenced beta cells is accompanied by a protective effect on PIC- and CVB5-induced beta cell apoptosis (present data). Previous studies have reported the implication of DEXI in the regulation of apoptotic pathways and showed that DEXI inhibition protects against camptothecin- or hypoxia-induced apoptosis in fibroblasts [38]. In pancreatic beta cells, the implication of DEXI in virus-induced apoptosis most probably rely on its effect on the STAT1 signalling pathway activation, which is a key pro-apoptotic pathway in beta cells [11,12,39].

Human and rodent beta cells express several pattern recognition receptors (PRRs) that recognize and respond to viral components, including toll-like receptor 3 (TLR3), melanoma differentiation-associated protein 5 (*MDA5*, a candidate gene for type 1 diabetes) and retinoic acid-inducible gene I (*RIG-I*) [8,31]. Upon recognition of viral dsRNA, PRR activation initiates several signal transduction cascades that include the JAK-STAT and the NF-κB-driven pathways [31]. Through activation of these pathways, viral dsRNA enhances the expression and release of pro-inflammatory chemokines in beta cells by promoting the production of type I IFNs, which, in an autocrine phase, activate the STAT signalling pathway, inducing local inflammation and contributing to beta cell apoptosis [10-12]. In line with these results, we observed that the decrease in STAT1 and pro-inflammatory chemokine expression in DEXI-inhibited cells is preceded by a decrease in *IFNβ* expression. Indeed, we already detected a decrease in *IFNβ* expression in DEXI-inhibited cells after 4 h of PIC transfection, which is 4 h before observing any increase in STAT1 phosphorylation and pro-inflammatory chemokine

production that starts after 8 h of PIC transfection. Type I IFNs, including IFNβ, are important mediators of antiviral responses that have been implicated in the early stages of type 1 diabetes development. Several studies have demonstrated the presence of type I IFNs in the islets and blood of individuals with type 1 diabetes and their relation with the presence of CVB infection [3,40,41]. The implication of type I IFNs in the pathogenesis of type 1 diabetes is also supported by studies in the NOD mice where type I IFNs play a crucial role in the initiation or acceleration of the autoimmune process [42]. Moreover, therapy with type I IFNs caused the development of type 1 diabetes in patients with hepatitis C, multiple sclerosis and melanoma [43–45], supporting the idea that type I IFNs may be implicated in the development of type 1 diabetes.

Our results demonstrate that DEXI participates in the transcriptional activation of IFNβ upon a viral infection in beta cells. Protein sequence analysis suggests that DEXI has a leucine zipper motif in its carboxy-terminal [21]. The leucine zipper motif contains a predicted kinase phosphorylation site, suggesting that DEXI interactions with itself or other proteins may be regulated via phosphorylation [21]. We observed that DEXI is bound to the chromatin and the amount of chromatin-associated DEXI is augmented after 4 h of PIC transfection, supporting the potential role of DEXI as a transcriptional regulator activated in response to danger signals in beta cells. Further studies are needed to clarify the mechanisms by which DEXI regulates IFNβ expression and to determine whether DEXI acts as transcriptional regulator by itself or in combination with other gene expression regulatory proteins.

In summary, the present study provides evidence that the type 1 diabetes candidate gene *DEXI* participates in the activation of the local antiviral immune response through regulation of the type I IFN-STAT signalling pathway in pancreatic beta cells (Fig. 7).

Our findings support *DEXI* as the etiological gene for type 1 diabetes in chromosome 16p13 and provide initial insights on the mechanisms involved. Further molecular characterization of the interactions between predisposing genes and environmental triggers will allow to clarify the mechanisms by which a viral infection or other danger signals leads to autoimmunity and type 1 diabetes in genetically susceptible individuals.

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DATA AVAILABILITY

No data are available.

DUALITY OF INTEREST

The authors declare that there is no duality of interest associated with this manuscript.

CONTRIBUTION STATEMENT

RSS, LM, TV, AOG, AJM and AC researched data, and revised and edited the manuscript. DLE contributed to the design and interpretation of the experiments, contributed to discussion, and wrote, revised and edited the manuscript. LC contributed to interpretation of the experiments, discussion and revised and edited the manuscript. IS contributed to the original idea, design and interpretation of experiments, researched data, contributed to discussion, and wrote, revised, and edited the manuscript. All authors have read and approved the manuscript and gave informed consent. IS is the guarantor 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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FIGURE LEGENDS

Figure 1. DEXI is expressed in pancreatic beta cells and its expression is upregulated by PIC. (a) DEXI mRNA expression was analysed in the human beta cell line EndoC-βH1, in dispersed human islets and in a set of human tissues (thymus, spleen, brain, heart, kidney, liver, lung, stomach, small intestine and colon). *DEXI* expression was determined by RT-PCR and normalized by the housekeeping gene β-actin. Results are means of three experimental replicates. (b, c) Human EndoC-βH1 cells, (d) INS-1E cells and (e) FACS-purified primary rat beta cells were left untreated (i.e. 0 h) or treated with intracellular PIC (1 µg/ml) for 2, 8 and 24 h (b-d) or 24 h (e). mRNA expression of *DEXI* and β-actin (b) or *Gapdh* (d, e) was assayed by RT-PCR and DEXI protein by Western blot (c). Results are means ± SEM of 3-4 independent experiments and the blot is representative of 4 independent experiments; $**p<0.01$ and $*p<0.05$ *versus* time 0 h; Student's *t* test.

Figure 2. DEXI inhibition reduces PIC-induced apoptosis and STAT1 signalling pathway activity in pancreatic beta cells. Primary rat beta cells (a, b) were transfected with siCTRL (white bars) or two independent siRNAs targeting *Dexi* (siDEXI#1 (dark grey bars) and siDEXI#2 (light grey bars)). After 48 h of recovery, cells were left untreated (NT) or treated with PIC (1µg/ml) for 24 h. (a) *Dexi* mRNA expression was assayed by RT-PCR and normalized by the housekeeping gene *Gapdh*. (b) Apoptosis was evaluated using HO/PI staining. Results are means ± SEM of 3-5 independent experiments; $***p<0.001$, $**p<0.01$ and $*p<0.05$ *versus* NT (i.e. not treated) and transfected with the same siRNA; $^{\dagger\dagger\dagger}p<0.001$ *versus* its respective siCTRL; ANOVA followed by Student's *t* test with Bonferroni correction. (c) INS-1E cells were transfected with siCTRL ("C") or two independent siRNAs targeting *Dexi* ("D1" and "D2"). After 48 h of recovery, cells were left untreated (0 h) or treated with intracellular PIC (1 µg/ml)

for 2, 4, 8, 16 or 24 h. Phospho-STAT1, total STAT1 and α-tubulin (used as loading control) were evaluated by Western blot. The results are representative of 3 independent experiments. (d) INS-1E cells were transfected with siCTRL (white bars) or two independent siRNAs targeting *Dexi* (siDEXI#1 (dark grey bars) and siDEXI#2 (light grey bars)). After 48 h of recovery, cells were left untreated (0 h) or treated with intracellular PIC (1 µg/ml) for 8 or 24 h. *Stat1* mRNA expression was assayed by RT-PCR and normalized by the housekeeping gene *Gapdh*. The results are means ± SEM of 5 independent experiments; ***p<0.001, **p<0.01 and *p<0.05 *versus* time 0 h (not treated) and transfected with the same siRNA; $^{###}p$<0.001 *versus* siCTRL treated with PIC (1 µg/ml) for 24 h; ANOVA followed by Student's *t* test with Bonferroni correction. (e) INS-1E cells were transfected with siCTRL (white bars), siDEXI#1 (dark grey bars) or siDEXI#2 (light grey bars) and co-transfected with an ISRE reporter construct plus a pRL-CMV plasmid (used as internal control). After 48 h of recovery, they were left untreated (0 h) or treated with PIC for 16 h and luciferase activities were measured. Results are means ± SEM of 4 independent experiments; ***p<0.001 *versus* untreated (i.e. time 0 h) and transfected with the same siRNA; $^{†††}p$<0.001 *versus* siCTRL treated with PIC (1µg/ml) for 16 h; ANOVA followed by Student's *t* test with Bonferroni correction.

Figure 3. DEXI inhibition reduces PIC-induced pro-inflammatory chemokine expression in rat and human beta cells. INS-1E (a-d) or EndoC-βH1 cells (E-H) were transfected with siCTRL (white bars) or two independent siRNAs targeting *DEXI* (siDEXI#1 (dark grey bars) and siDEXI#2 (light grey bars)). After 48 h of recovery, cells were left untreated (0 h) or treated with intracellular PIC (1µg/ml) for 8 or 24 h (in INS-1E cells) or for 24 h (in EndoC-βH1 cells). *Dexi* (a and e), *Ccl5* (b and f), *Cxcl9* (c and g) and *Cxcl1* (d and h) expression was assayed by RT-PCR and normalized by the

housekeeping genes *Gapdh* (a-d) or β-actin (e-h). The results are means ± SEM of 4-5 independent experiments; ***p<0.001, **p<0.01 and *p<0.05 *versus* time 0 h (i.e. not treated) and transfected with the same siRNA; $^{\dagger\dagger\dagger}p$<0.001, $^{\dagger\dagger}p$<0.01 and $^{\dagger}p$<0.05 *versus* its respective siCTRL; ANOVA followed by Student's *t* test with Bonferroni correction.

Figure 4. DEXI overexpression in pancreatic beta cells exacerbates PIC-induced STAT1 and chemokine expression. INS-1E cells were transfected with pCMV-Control (white bars) or with pCMV-DEXI (grey bars) and subsequently left untreated (0 h) or treated with intracellular PIC (1 µg/ml) for 24 h. *Dexi* mRNA (a) and protein (b) expression was determined by RT-PCR and Western blot, respectively. The results are means ± SEM of 3 independent experiments; **p<0.01 *versus* time 0 h (i.e. not treated) and transfected with the same plasmid; $^{\dagger\dagger\dagger}p$<0.001 *versus* its respective pCMV-Control; ANOVA followed by Student's *t* test with Bonferroni correction. (c-f) *Stat1*, *Ccl5*, *Cxcl1* and *Cxcl9* expression was assayed by RT-PCR and normalized by the housekeeping gene *Gapdh*. The results are means ± SEM of 3 independent experiments; ***p<0.001 and *p<0.05 *versus* untreated 0 h (i.e. not treated) and transfected with the same plasmid; $^{\dagger\dagger\dagger}p$<0.001, $^{\dagger\dagger}p$<0.01 and $^{\dagger}p$<0.05 *versus* its respective pCMV-Control; ANOVA followed by Student's *t* test followed with Bonferroni correction.

Figure 5. Inhibition of the STAT signaling pathway counteracts the effect of DEXI in PIC-induced pro-inflammatory chemokine expression. Human EndoC-βH1 cells were transfected with pCMV-Control (white bars) or with pCMV-DEXI (grey bars) and subsequently left untreated (NT), treated with intracellular PIC (1 µg/ml) for 24 h (PIC) or treated with PIC and ruxolitinib for 24 h (PIC + Inhib). *DEXI* (a), *CCL5* (b), *CXCL9* (c) and *CXCL1* (d) mRNA expression was measured by RT-PCR and normalized by the housekeeping gene β-actin. The results are means ± SEM of 3 independent experiments; ***p<0.001 and **p<0.01 *versus* NT and transfected with the same plasmid; $^{\dagger\dagger\dagger}p$<0.001

and $^{\dagger\dagger}p<0.01$ *versus* its respective pCMV-DEXI; ‡‡‡ $p<0.001$ *versus* PIC and transfected with the same plasmid; ANOVA followed by Student's *t* test followed with Bonferroni correction.

Figure 56. The downregulation of the STAT signalling pathway in DEXI-silenced beta cells is preceded by a decrease in IFNβ production. INS-1E (a) or EndoC-βH1 (b) cells were transfected with siCTRL (white bars) or with two independent siRNAs targeting *DEXI* (dark and light grey bars) and subsequently left untreated or treated with intracellular PIC (1 µg/ml) for 24 h. Expression of *IFN*β was assessed by RT-PCR and normalized by *Gapdh* (a) or β-actin (b). The results are means ± SEM of 4 independent experiments; ***$p<0.001$ and *$p<0.05$ *versus* time 0 h (i.e. not treated) and transfected with the same siRNA; $^{\dagger\dagger\dagger}p<0.001$ and $^{\dagger}p<0.05$ *versus* siCTRL treated with PIC (1 µg/ml) for 24 h; Student's *t* test followed by Bonferroni correction. (c) INS-1E cells were transfected with siCTRL (white dots) or with one siRNA targeting *Dexi* (black squares) and subsequently left untreated or treated with intracellular PIC (1 µg/ml) for 2, 4, 8, 16 and 24 h. Expression of *Ifn*β was assessed by RT-PCR and normalized by *Gapdh*. ***$p<0.001$ *versus* time 0 h (i.e. not treated) and transfected with the same siRNA; $^{\dagger\dagger\dagger}p<0.001$ and $^{\dagger\dagger}p<0.01$ *versus* siCTRL treated with PIC (1 µg/ml) at the same time-point; ANOVA followed by Student's *t* test with Bonferroni correction. (d) EndoC-bH1 cells were transfected with with siCTRL (white bars) or with two independent siRNAs targeting *DEXI* (dark and light grey bars) and subsequently left untreated or treated with intracellular PIC (1 µg/ml) for 24 h. IFNβ protein was assayed in cell supernatants by ELISA. The results are means ± SEM of 3 independent experiments; ***$p<0.001$ and *$p<0.05$ *versus* time 0 h (i.e. not treated) and transfected with the same siRNA; $^{\dagger}p<0.05$ *versus* siCTRL treated with PIC (1 µg/ml) for 24 h; ANOVA followed by Student's *t* test followed with Bonferroni correction. (e) EndoC-βH1 cells were exposed to intracellular

PIC (1 μg/ml) for 4 h and DEXI protein expression in different subcellular fractions was determined by Western blot. HSP90 (cytoplasm), HDAC1 (nuclear) and H3 (chromatin) protein expression was also determined by Western blot as subcellular fraction markers. The densitometry results for DEXI are means ± SEM of 3 independent experiments; *p<0.05: Student's t test. (f) INS-1E cells were transfected with siCTRL (white bars) or siRNA targeting *Dexi* (grey bars) and co-transfected with an IFNβ promoter reporter construct plus a pRL-CMV plasmid (used as internal control). After 48 h of recovery, they were left untreated or treated with PIC for 16 h and luciferase activities were measured. Results are means ± SEM of 4 independent experiments; ***p<0.001 *versus* time 0 h (i.e. not treated) and transfected with the same siRNA; $^{\dagger\dagger\dagger}p$<0.001 and $^{\dagger}p$<0.05 *versus* its respective siCTRL; ANOVA followed by Student's t test with Bonferroni correction.

Figure 7. A model for the role of DEXI in the modulation of virus-induced pro-inflammatory pathways in pancreatic beta cells. Upon recognition of dsRNA by the cytoplasmic receptors the antiviral response is activated in pancreatic beta cells, leading to the production of type I IFNs. Viral dsRNA upregulates chromatin-bound DEXI expression that acts as a transcriptional activator of IFNβ expression. Together with other pro-inflammatory mediators (*e.g.* chemokines), IFNβ is released by beta cells, and participate in the attraction of immune cells. At the same time, IFNβ acts in an autocrine way, activating the STAT signalling pathway, promoting the production of pro-inflammatory chemokines and increasing local inflammation. In genetically susceptible individuals, type 1 diabetes -associated SNPs may affect the function of *DEXI* leading to an excessive inflammatory response to viral infections that may contribute to local inflammation and eventual beta cell destruction.