STAT1 IS A MASTER REGULATOR OF PANCREATIC BETA CELLS
APOPTOSIS AND ISLET INFLAMMATION

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Cytokines produced by islet-infiltrating immune cells induce β-cell apoptosis in type 1 diabetes. The IFN-γ-regulated transcription factors STAT1/IRF-1 have apparently divergent effects on β-cells. Thus, STAT1 promotes apoptosis and inflammation while IRF-1 down-regulates inflammatory mediators. To understand the molecular basis for these differential outcomes within a single signal transduction pathway, we presently characterized the gene networks regulated by STAT1 and IRF-1 in β-cells. This was done by using siRNA approaches coupled to microarray analysis of insulin-producing cells exposed or not to IL-1β + IFN-γ. Relevant microarray findings were further studied in INS-1E cells and primary rat β-cells. STAT1, but not IRF-1, mediates the cytokine-induced loss of differentiated β-cell phenotype, as indicated by decreased insulin, Pdx1, MafA and Glut2. Furthermore, STAT1 regulates cytokine-induced apoptosis via up-regulation of the pro-apoptotic protein DP5. STAT1 and IRF-1 have opposite effects on cytokine-induced chemokine production, with IRF-1 exerting negative feedback inhibition on STAT1 and downstream chemokine expression. The present study elucidates the transcriptional networks through which the IFN-γ/STAT1/IRF-1 axis controls β-cell function/differentiation, demise and islet inflammation.

Stressful signals are sensed by cells, which respond via the up- or down-regulation of relevant genes and proteins. This process can be divided in two broad steps: first, cells encode the stress signals internally by modulating the expression, activation and localization of transcription factors and second, these transcription factors trigger the expression of key downstream genes that mediate diverse cellular responses to the stress (1,2). Signalling events occurring inside the pancreatic β-cells and triggered by diverse stressful mediators are decisive for the survival or death of these cells in type 1 diabetes (T1D) (3). T1D is an inflammatory disorder characterized by infiltration of autoreactive immune cells in pancreatic islets (a process termed insulitis) and by selective destruction of insulin-producing β-cells (4). β-cell death in T1D occurs through apoptosis induced by a deadly “dialogue” between β-cells and infiltrating immune cells, where the pro-inflammatory cytokines interleukin(IL)-1β, interferon(IFN)-γ and tumor necrosis factor(TNF)-α produced by infiltrating macrophages and T cells play a key role (5-8).

We have previously shown that pancreatic β-cells respond to the pro-inflammatory cytokines IL-1β, TNF-α and IFN-γ by modifying the expression of complex gene networks under the regulation of at least two master transcription factors, namely NF-κB and STAT1 (3,5). Inhibition of NF-κB expression in vitro or in vivo prevents cytokine-induced β-cell apoptosis (9,10), and array analysis of IL-1β + IFN-γ-treated β-cells in the context of NF-κB blockade allowed us to identify several key mediators of β-cell dysfunction and death, including endoplasmic reticulum (ER) stress-related factors (11-13). Such information would be of particular interest for STAT1-regulated genes, since the IFN-γ-induced transcription factors STAT1 and downstream IRF-1 have been reported to exert opposite effects in β-cells. Thus, while STAT1 blockade prevents cytokine-induced β-cell death in vitro and diabetes induced by multiple-low doses of streptozotocin (14,15), IRF-1 deficiency does not protect β-cells against cytokines in vitro. On the contrary, it actually exacerbates local production of
chemokines, islet graft infiltration and rejection in NOD mice (16). These observations suggest that while STAT1 regulates genes triggering β-cell death and inflammation, IRF-1 may regulate putative anti-inflammatory genes. The nature of these genes, however, remains to be clarified. IRF-1 is induced by IFN-γ through binding of STAT1 to the IRF-1 promoter, but other transcription factors, such as NF-κB, may also induce its expression (17,18).

To characterize the broad network of genes regulated by STAT1 and IRF-1, we first validated siRNAs targeting each of these transcription factors, and then coupled RNA interference to global evaluation of gene expression. This was done by the use of microarray analysis of insulin-producing INS-1E cells exposed or not to pro-inflammatory cytokines. These microarray findings were subsequently confirmed in INS-1E and primary rat β-cells, and the function of novel STAT1- and IRF-1-dependent genes characterized by RNA interference and additional functional studies. We observed that STAT1, but not IRF-1, up-regulates the pro-apoptotic protein Death Protein 5 (DP5/Hrk) and the synthesis of several pro-inflammatory chemokines, including the CXCR3 ligands CXCL9, 10 and 11, while downregulating genes involved in β-cell differentiation and function. On the other hand, IRF-1 induces many genes independently of STAT1, including IL-6RA, Pkia or TAPBP. Interestingly, our data highlighted a novel role for IRF-1 in providing a negative feedback on STAT1-driven chemokine production through the induction of the regulatory protein SOCS-1. As a whole, these findings allow us to propose a unifying hypothesis explaining the effects of the transcription factors STAT1 and IRF-1 on β-cells.

**Experimental Procedures**

**RNA interference-** The siRNAs used in this study are BLOCK-iT Stealth™ Select siRNA (Invitrogen, Paisley, UK) and had the following sequences: siSTAT1 5'-CCGUAGACUCGACUACCAGAUU-3'; siSTAT1 #2 5'-CAGGCUUGGUAUCCAGACUUU-3'; siIRF-1 5'-GCCUCAUGGCAGUAAUCCUUG-3'; siIRF-1 #2 5'-CCUGGCUAGAGAUGCAUUU-3'; siSOCS-1 5'-CCGGUACUCCGACUACCAGAGU-3'; siSOCS-1 #2 5'-GAGAACCUGGCACGCUUCCUUA-3'.

Allstars Negative Control siRNA was used for control-transfected conditions (Qiagen, Venlo, The Netherlands, sequence not provided). The concentration of siRNA used for cell transfection (30 nM) was selected based on dose-response studies (19 & Suppl. Fig. 2). DharmaFECT 1 (Thermo Scientific) and Lipofectamine 2000 (Invitrogen) lipid reagents were used for siRNA transfection (19). The efficiency of transfection and the results are similar for both lipid reagents. After transfection, cells were cultured for a 24h recovery period and subsequently exposed to cytokines.

**Cell treatment and NO measurement-** The following cytokine concentrations were used, based on previous dose–response experiments (6,20): recombinant human IL-1β (specific activity (SA) 1.8x10^7 U/mg; a kind gift from C. W. Reimolds, National Cancer Institute, Bethesda, MD, USA) at 10 U/ml; recombinant rat IFN-γ (SA: 2x10^7 U/mg; R&D Systems, Abingdon, UK) at 100 U/ml; recombinant murine TNF-α (SA: 2x10^8 U/mg; Innogenetics, Gent, Belgium) at 1.000 U/ml. Culture supernatants were collected for nitrite determination (nitrite is a stable product of NO oxidation) at OD_540nm using the Griess method.

**Microarray data analysis-** Total RNA was isolated from INS-1E using the RNeasy Mini Kit (Qiagen). Aminoally lantisense-cRNA (aa-cRNA) was obtained, coupled to either fluorophore indocarbocyanine (Cy3) or indodicarbocyanine (Cy5) (Amersham, Diegem, Belgium) and prepared for hybridization (21). The gene expression profiling study was performed using the GeneChip® Rat Genome 230 2.0 arrays (Affymetrix) containing >31,000 probesets, and covering over 28,000 well-defined rat genes. Gene expression values were calculated from the Affymetrix Cel files using Robust Multichip Average (RMA) algorithm (22) implemented in the ArrayAssist® Expression Software (Stratagene). Only probesets detected as “present” by the Affymetrix MAS5 normalization algorithm in at least 2 out of 8 conditions for at least 1 time point were considered as “present” and used for further analysis. The RMA normalized intensities corresponding to the 21,569 filtered probesets were then imported to the Bioconductor free
software (www.bioconductor.org) where a robust Welch’s t-test (based on robust estimators of central tendency and dispersion namely the median and the inter-quartile range) was applied to compare each experimental condition at different time points (2, 12 or 24h) to the corresponding control. Probesets were considered as differentially expressed between two conditions if they had a mean fold change (up or down) > 1.5 and a p value <0.02. Lists of cytokine-modified probesets at each time point included those which were significantly modified when comparing both cytokine-treated to untreated untransfected INS-1E cells and cytokine-treated siControl-transfected to untreated siControl-transfected INS-1E cells. Cytokine-modified probesets were considered as STAT1-dependent when they were significantly modified when comparing cytokine-treated siSTAT1-transfected and cytokine-treated siControl-transfected samples. Cytokine-regulated and IRF1-dependent probesets were defined in the same way, by comparing cytokine-treated siIRF1-transfected cells to cytokine-treated siControl-transfected INS1-E cells at each time point. The complete array data will be deposited after publication of this article in our “Beta Cell Gene Expression Bank” (http://betacellgenebank.ulb.ac.be).

Western blots- Cells were washed, lysed, resolved by 8-10% SDS-PAGE and transferred to a nitrocellulose membrane as described (19). The antibodies used were: anti-STAT1 (sc-346) and anti-IRF1 (sc-640) from Santa Cruz Biotechnology (Santa Cruz, CA., U.S.A); anti-phospho-STAT1 (Y701 - #9171) from Cell Signalling (Danvers, MA, USA); anti PTPN2 (clone 252294) from R&D Systems (Abingdon, UK) and anti-α-tubulin. (T9026) from Sigma (Bornem, Belgium). HRP-conjugated anti-rabbit or anti-mouse IgG (Lucron Bioproducts, De Pinte, Belgium) were used as secondary antibodies. Immune-reactive bands were revealed using the SuperSignal West Femto chemiluminescent substrate (Thermo Scientific), detected using a LAS-3000 CCD camera and quantified with the Aida Analysis software (Fujifilm).

Culture of primary FACS-sorted rat β-cells and INS-1E cells- Male Wistar rats (Charles River Laboratories, Brussels, Belgium) were housed and used according to the guidelines of the Belgian Regulations for Animal Care. Islets were isolated by collagenase digestion and hand picked under a stereomicroscope. β-cells were purified by autofluorescence-activated cell sorting (FACSaria, BD Bioscience, San Jose, CA, USA) (50). The preparations contained 90.4 ± 3.2% β-cells (n=9). β-cells were cultured for 2 days in Ham’s F-10 medium containing 10 mM glucose, 2 mM glutamax, 50 µM 3-isobutyl-1-methylxanthine, 5% FBS, 0.5% charcoal-absorbed BSA (Boehringer, Indianapolis, IN, USA), 50 U/ml penicillin and 50 µg/ml streptomycin (50). During cytokine exposure, cells were cultured in the same medium but without serum. The rat insulin-producing INS-1E cell line (a kind gift from Dr. C. Wollheim, Centre Medical Universitaire, Geneva, Switzerland) was cultured as previously described (20).

Infection with recombinant adenoviruses- Cells were left uninfected or infected either with Ad-Luc (luciferase-expressing virus) or Ad-srIκB (a virus expressing an NF-κB super repressor protein) (23). Cells were infected for 2 h at 37°C with a multiplicity of infection (MOI) of 10. The MOI was selected based on lowest toxicity by viral infection combined with highest blockade of NF-κB activation. After infection (24h), cells were treated with cytokines. We have previously shown that infection of β-cells with Ad-srIκB at the MOI used in the present study does not change its function (23).

mRNA extraction and real time PCR- Poly(A)+ mRNA was isolated from INS-1E cells or rat primary β-cells using the Dynabeads mRNA DIRECT™ kit (Invitrogen), and reverse transcribed as previously described (50). The real time PCR amplification reaction was done as described (50), using SYBR Green and compared to a standard curve. Expression values were corrected for the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH); we have previously shown that cytokines do not modify GAPDH expression (19). The primers used in this study are listed below (sequence 5’-3’): rat GAPDH Std: F: ATGACTTCTACCCACCAGCAAG, R: TGTTGAGGAGATGCTCAGTG (975 bp); rat GAPDH RT: F: AGTTCAAGGGCAGCTCAGCA, R: TACTCAGCACCAGCATCACC (118 bp); rat STAT1 Std: F: CCTCTTCCACCCACGCAAG, R: ACTGCCAACAGTCAGTG (596 bp); rat STAT1 RT: F: TGAGTTCCAGACACTGACAGC, R: AGGTGGTCTCAAGGCTCAATCACC (102 bp); rat IRF-1 Std: F:
Assessment of cell viability- The percentage of viable, apoptotic and necrotic cells was determined following a 15 minute incubation with the DNA-binding dyes Propidium Iodide (PI, 5 µg/ml, Sigma) and Hoechst 33342 (HO, 5 µg/ml, Sigma) (50). A minimum of 500 cells was counted in each experimental condition. Viability was evaluated by two independent observers, one of them being unaware of sample identity. The agreement between findings obtained by the two observers was >90%. Results are expressed as percent apoptosis, calculated as (number of apoptotic cells / total number of cells) x 100. Apoptosis was confirmed in some experiments by the Cell Death Detection ELISAplus kit (Roche Diagnostics, Vilvoorde, Belgium), which detects cytoplasmic fragmented DNA.

Promoter reporter assays- INS-1E cells were transfected as previously described (10) with pRL-CMV encoding Renilla luciferase (Promega) and either a luciferase promoter-reporter construct containing 5 NF-κB consensus binding sites (NFAκB reporter) or 3 GAS consensus sequences (STAT1 reporter) or the fragment (+1568/+81) from the rat DP5 promoter (28). Luciferase activities were assayed after 24h of cytokine treatment (20).

Overexpression of rat STAT1 and rat IRF-1- The expression vectors pCMV-STAT1 (Imagene, Germany) and pCMV-IRF-1 (Thermo Scientific) were transfected in INS-1E cells using Lipofectamine 2000 (Invitrogen) as previously described (10). After overnight incubation, the medium was changed and cells were exposed to cytokines as indicated.

Statistical analysis- Data are presented as mean ± SEM. Comparisons were performed by two-tailed paired Student’s t-test or by ANOVA followed by Student’s t test with Bonferroni correction. A p value <0.05 was considered statistically significant.

RESULTS

STAT1 knockdown protects INS-1E and primary β-cells against cytokine-induced apoptosis. Transfection of siRNAs targeting STAT1 and IRF-1 (siSTAT1 and siIRF-1) respectively inhibited IL-1β + IFN-γ-induced STAT1 and IRF-1 induction by 89% and 72% (Fig. 1A, 1B & 1C). STAT1 silencing also inhibited cytokine-induced IRF-1 expression, which was expected since IRF-1 is a downstream
target of STAT1 (Fig. 1A & 1C). An irrelevant siRNA used as control (siCtrl) did not modify IRF-1- or STAT1 expressions. STAT1 silencing potently inhibited IL-1β + IFN-γ-induced apoptosis after 12 and 24h, while neither siCtrl-nor siIRF-1 protected INS-1E cells (Fig. 1D). This was confirmed by a second method that detects cytoplasmatic fragmented DNA (Fig. 1E). Both IRF-1 and STAT1 silencing decreased nitric oxide (NO) production by 38-48% and 27-35% after respectively 12 and 24h of treatment (Fig. 1F).

Transfection of siSTAT1 and siIRF-1 in primary β-cell respectively decreased cytokine-induced STAT1 and IRF-1 expressions by 72-87% and 70-89% (Suppl. Fig. 1A & 1B – online appendix). STAT1 silencing did not affect IRF-1 expression in primary β-cells (Suppl. Fig. 1B), suggesting that IRF-1 expression is less dependent on STAT1 in primary cells. NF-κB also contributed to cytokine-induced IRF-1 up-regulation in β-cells, as the addition of IL-1β or TNF-α (both NF-κB activators) augmented IFN-γ-induced IRF-1 induction in INS-1E cells (Suppl. Fig. 1C-D – online appendix), while NF-κB blockade using a super-repressor IκBα (23) inhibited cytokine-induced IRF-1 expression (Suppl. Fig. 1E – online appendix). STAT1 knockdown also protected primary β-cells against IL-1β + IFN-γ-induced apoptosis, while both siIRF-1 and siSTAT1 inhibited cytokine-induced NO production (Fig. 1G & 1H). This suggests that protection induced by STAT1 knockdown is at least in part independent on NO production. Similar findings were observed in INS-1E cells using additional siRNAs for each target gene (siIRF-1 #2 and siSTAT1 #2 – Suppl. Fig. 2 – online appendix), with siSTAT1 #1 and #2 protecting INS-1E cells against both IL-1β + IFN-γ and TNF-α + IFN-γ-induced apoptosis (Suppl. Fig. 2).

Analysis of gene networks and pathways regulated by IRF-1 and STAT1 in INS-1E cells. INS-1E cells were left untransfected or transfected with siCtrl, siIRF-1 or siSTAT1, and subsequently treated with IL-1β + IFN-γ for 2, 12 or 24h (Fig. 2A). In the microarray analysis, 21,569 probesets corresponding to 10,874 annotated genes were detected as present. The complete list of probesets present for each time point is provided as Suppl. Tables 2, 3 & 4 (online appendix). Less than 0.5% of the genes were differentially regulated between the untransfected and siCtrl-transfected conditions, suggesting that the transfection per se had only minor influence on the cells (Fig. 2E). STAT1 silencing affected the expression of 2.6-2.9% of the genes in untreated cells, suggesting a role for STAT1 in the control of basal β-cell function (Suppl. Table 5 – online appendix). Cytokine treatment in untransfected and siCtrl-transfected conditions regulated respectively 21-24% and 14-18% of the genes at 12 and 24h (Fig. 2E & Suppl. Tables 3, 4), while only about 5% of the genes were regulated by cytokines at 2h (Fig. 2E and Suppl. Table 2). At 12h, around 50% of the cytokine-regulated genes were at least partially modified by IRF-1 or STAT1 knockdown (Fig. 2C), while at 24h, 42% and 58% of the cytokine-regulated genes were respectively IRF-1- or STAT1-dependent (Fig. 2D). The effects of siSTAT1 and siIRF-1 on cytokine-induced genes showed only partial superposition, with 18-33% of the genes regulated by only IRF-1 or STAT1 at 24h (Fig. 2D). Analysis using the IPA software revealed that functions associated with cell cycle and development, inflammatory response, and endocrine systems disorder were significantly affected by STAT1 silencing (Suppl. Fig. 3A – online appendix). Interestingly, cytokine-regulated functions such as cell death, antimicrobial response, inflammatory response and antigen presentation were affected by IRF-1 deficiency, suggesting an inhibitory role of IRF-1 in these processes (Suppl. Fig. 3A). Canonical pathways regulated by cytokines, including HMGB1, NF-κB and IL-17 signalling, death receptor and apoptosis signalling were significantly altered by STAT1 knockdown (Suppl. Fig. 3B – online appendix). Suppl. Table 1 (online appendix) represents selected genes with a putative role in β-cell function/dysfunction and death as classified by one of us (D.L.E.) using a previously described method (24). Based on both the “hand-made” and unbiased analysis, we selected three pathways of high relevance in the context of T1D, namely β-cell function/differentiation, apoptosis and inflammation, for additional studies.

STAT1 silencing partially prevents cytokine-induced downregulation of genes involved in β-cell function and differentiation. Exposure of β-cell to cytokines decreases the expression of genes involved in β-cell differentiation and function (24,25). The present microarray analysis confirmed these findings, with genes such as insulin, Glut2, glucokinase, Pdx1, MafA and others being significantly...
downregulated after 12 and 24h of IL-1β + IFN-γ exposure (Fig. 3A-D). Importantly, STAT1 silencing partially prevented the downregulation of several of these genes, including insulin, Glut2, glucokinase, proconvertase 1 and 3 (Fig. 3A & 3B), and of all the transcription factors involved in β-cell differentiation (Fig. 3C & 3D). IRF-1 knockdown prevented the downregulation of proconvertase 1, glucokinase, Pdx1 and Nkx2.2 at 12h of cytokine (Fig. 3A & 3C) but this protective effect was lost at 24h (Fig. 3B & 3D). These results were mostly confirmed by real time PCR for selected genes (insulin 1, Glut2, Pdx1 and MafA) in INS-1E cells (Fig. 3E) and primary β-cells (Fig. 3F). This suggests that STAT1 mediates at least in part the deleterious effects of cytokines on β-cell differentiation during inflammation.

**Induction of the pro-apoptotic protein DP5** is partially prevented after STAT1 knockdown. DP5 (Hrk) is a pro-apoptotic BH3-only member of the Bel2 family (26) which plays a central role in cytokine-induced β-cell apoptosis (27). The microarray data demonstrated that DP5 induction was hampered by IRF-1 and STAT1 knockdown at 12h of cytokine treatment (Suppl. Table 1). Real time PCR analysis in INS-1E cells and primary β-cells confirmed that STAT1 silencing prevented DP5 induction at 12 and 24h of cytokine treatment (Fig. 4A & 4B), while siIRF-1 effect was only transient. Using a luciferase reporter containing the promoter sequence from the rat DP5 gene (28), we observed that STAT1 silencing completely abolished cytokine-induced DP5 promoter expression, while it was induced by 2-fold in untransfected, siCtrl- or siIRF-1-transfected cells (Fig. 4C). We then tested whether STAT1 overexpression affects cytokine-induced DP5 up-regulation. The transfection of a rat STAT1 expression vector in INS-1E cells resulted in increased STAT1 expression at both mRNA and protein levels (Suppl. Fig. 4A & 4B), and exacerbated DP5 induction after 16-24h of IL-1β + IFN-γ treatment (Suppl. Fig. 4C). These findings suggest that STAT1 participates in cytokine-dependent DP5 up-regulation, a key mechanism of cytokine-induced β-cell apoptosis (27). Of note, parallel knockdown of STAT1 and DP5 by specific siRNAs induced a more marked inhibition of cytokine-induced apoptosis than DP5 or STAT1 silencing alone (Suppl. Fig 4D & 4E). This suggests that STAT1 regulates other pro-apoptotic signals besides DP5.

**IRF-1 provides a negative feedback on cytokine-induced chemokine production.** During islet inflammation, β-cells produce chemokines that further attract immune cells (7). Our microarray data demonstrated that IL-1β + IFN-γ exposure induced the early production of CXCL1, 2, 9, 10, 11 and CCL20 (Fig. 5A, for statistical analysis see Suppl. Table 1). STAT1 silencing downregulated the production of all chemokines at 12- and/or 24h of cytokine treatment (Fig. 5A), while IRF-1 silencing exacerbated the production of CXCL9 and 11 after 12h, and of all chemokines after 24h (Fig. 5A). This was confirmed by real time PCR experiments in cytokine-treated INS-1E cells and primary β-cells for the chemokines CXCL1, 9, 10 and CCL20 (Fig. 5B & C). Again, IRF-1 silencing exacerbated cytokine-induced CXCL1, 9 and 10. This indicates that IRF-1 may provide a negative feedback on STAT1-driven chemokine production. To further investigate this phenomenon, we evaluated the influence of IRF-1 knockdown on STAT1 activation. IRF-1 expression was induced by cytokines until 8h and it was silenced by the two siIRF-1 (Fig. 6A). STAT1 phosphorylation was equally induced in all conditions at 2 and 4h, but remained up-regulated until 16- and 24h of cytokine exposure in IRF-1-silenced cells (Fig. 6A & Suppl. Fig 5A). This effect was independent of the total STAT1 content, and on the activity of the nuclear phosphatase PTPN2, previously reported by our group to regulate IFN-γ-induced STAT1 expression of SOCS1, a negative regulator of STAT1 previously reported to be induced by IRF-1 in fibroblasts (29). The array data indicated that SOCS1 expression was dependent on IRF-1 and STAT1 at 2h, and mostly STAT1-dependent at later time points (Suppl. Tables 2, 3 & 4 – online appendix). Real time PCR experiments showed that SOCS1 induction reflected the cytokine-induced IRF-1 up-regulation in INS-1E cells, reaching a peak at 4h and then slowly decreasing.
until 24h (Fig. 6C & 6D). IRF-1 knockdown decreased cytokine-induced SOCS-1 expression at all time points, suggesting that IRF-1 contributes to SOCS-1 expression in β-cells (Fig. 6D). To confirm these findings, we transfected INS-1E cells with an expression vector for rat IRF-1, resulting in increased basal- and cytokine-induced IRF-1 expression in these cells (Fig. 6E & Suppl. Fig. 5C). Overexpression of IRF-1 exacerbated cytokine-driven SOCS-1 induction (Fig. 6F), while significantly inhibiting the up-regulation of STAT1 and the chemokines CXCL1 and 9 after IL-1β + IFN-γ treatment (Suppl. Fig. 5D-F). To confirm the role of SOCS-1 for these inhibitory effects of IRF-1, we performed siRNA-mediated SOCS-1 silencing in INS-1E cells. As shown in Fig. 6G, the two SOCS-1-targeting siRNAs inhibited cytokine-induced SOCS-1 expression by 61-64% and, importantly, prolonged STAT1 phosphorylation as compared to siCtrl-transfected cells after 16- and 24h of IL-1β + IFN-γ treatment (Suppl. Fig. 5G). Moreover, SOCS-1 silencing by both siRNAs significantly augmented cytokine-induced CXCL1, 9 and 10 mRNA synthesis after 24h of exposure (Fig. 6H-J). Collectively, these data suggest that IRF-1 inhibition exacerbates cytokine-induced chemokine production in β-cells through a prolonged STAT1 activation resulting from a deficient SOCS-1 induction in IRF-1-silenced cells. These observations are summarized in Fig. 6K, which represents the proposed negative regulatory feedback loop by which IRF-1 modulates STAT1 activation.

**DISCUSSION**

Data-driven models based on array analysis identify large numbers of potential correlations, making it difficult to perturb each individual pathway experimentally. One alternative, followed in the present study, is to target key transcription factors regulating relevant gene networks. Cytokine-induced STAT1 activation in β-cells is associated with the induction of apoptosis and diabetes progression in murine models of T1D (14,15,30), while the STAT1 downstream transcription factor IRF-1 may decrease islet inflammation without directly regulating β-cell death (16,31). In order to explain these apparently divergent effects, we have presently combined siRNA-mediated STAT1- or IRF-1 silencing with global gene expression profiling. The siRNAs utilized (two independent ones for each target gene) were validated by showing their inhibitory effect of the target genes, by the protection they induce against cytokine-triggered β-cell death, and by the good agreement between their effects and the observed effects in islets from STAT1 and IRF-1 KO mice (15,16). Importantly, the non-specific siRNA used as control did not affect β-cell viability (present data; 19) or function (32); and had only minimal effect (~0.5%) on gene expression, as evaluated by array analysis (present data). Using this well controlled approach, we observed that cytokine-induced STAT1 and IRF-1 expression regulate gene networks associated with cell cycle, signal transduction, apoptosis, ER stress and inflammation in β-cells (a list of some of the key regulated genes is shown in Fig. 7). IRF-1 silencing affected the expression of nearly 800 cytokine-induced genes independently of STAT1 expression (Suppl. Table 3 and Fig. 7); this is somewhat surprising since IRF-1 is usually considered a downstream transcription factor of STAT1 (17). We presently show that early transcriptional control of IRF-1 expression in β-cells is also dependent on NF-κB (that is rapidly activated by IL-1β or TNF-α in β-cells (10)), as NF-κB blockade inhibits cytokine-induced IRF-1 up-regulation (Suppl. Fig. 1 + Suppl. Tables 2, 3 & 4). Moreover, IL-1β + IFN-γ-induced IRF-1 expression was not decreased by STAT1 silencing in primary rat β-cells (Suppl. Fig. 1B), confirming that IRF-1 may act independently of STAT1.

Detailed examination of the array data, either manually or by unbiased analysis using the IPA software, indicated three key pathways for β-cell survival and function, and local inflammation, potentially modulated by STAT1 and, for one of them, also by IRF-1. The first pathway is related to β-cell function and differentiation. Loss of a differentiated β-cell phenotype occurs during insulitis (5), and our group has recently reported that exposure of primary β-cells to IL-1β + IFN-γ or TNF-α + IFN-γ downregulates several genes involved in β-cell differentiated functions (e.g. insulin, glucokinase, Glut2, pro-hormone convertases, etc) as well as many transcription factors involved in the differentiation and maintenance of β-cell phenotype (e.g. Pdx1, MafA, Nkx2.2, etc) (24). Recent evidence points to the central role of IFN-γ in vivo in this inhibitory effect of inflammation in BB rat β-cells (33). The present array confirmed these findings and indicated that STAT1, but not IRF-1, silencing partially protects β-cells against the “de-
differentiating” effects of pro-inflammatory cytokines. This is in line with observations in other tissues, suggesting that STAT1 inhibits the differentiation of osteoblasts, myoblasts and human adipocytes (34-36).

It has been reported that re-expressing a combination of three key developmental transcription factors (Ngn3, Pdx1 and MafA) in adult mouse pancreas reprograms pancreatic exocrine cells into cells that closely resemble β-cells (25). People expected to benefit most from reprogramming of pancreatic exocrine cells to β-cells are patients with T1D. Insulin epitopes are targets of the immune assault in T1D (37) and new insulin-producing cells will be recognized and attacked by the immune system (38). Our present and previous data (39) suggest that immune mediators of insulitis such as cytokines can “push back” newly developed β-cells into a de-differentiated state. Unless novel strategies are found to prevent these cytokine effects, β-cell reprogramming will remain an interesting research finding with limited translational potential. The present data indicate that STAT1 is a promising target for this approach, and it will be of high interest to test whether combinations of key developmental regulators (25) with blockers of STAT1 (present data) or NF-κB (3,9) restore and maintain β-cell mass in animal models of autoimmune diabetes.

The second pathway studied is related to β-cell apoptosis, and focused on DP5. DP5 (Hrk) is a pro-apoptotic BH3-only member of the Bcl-2 family, playing a crucial role for apoptosis in neurons (40-42). Recent work from our laboratory established that DP5 is central for cytokine- and ER-stress-induced β-cell death (27). The present data demonstrate that silencing of STAT1, but not IRF-1, prevents to a large extent cytokine-induced DP5 mRNA up-regulation in β-cells, while STAT1 overexpression results in exacerbated DP5 induction upon IL-1β + IFN-γ exposure. This is probably a transcriptional effect, since siSTAT1 (but not IRF-1) also prevents cytokine-induced activation of a DP5 reporter promoter. However, we found no putative binding sites for STAT1 (GAS sequences) in the promoter region of the rat DP5 gene (data not shown), suggesting that STAT1 regulates DP5 expression in an indirect way. Collectively, these observations are in line with the present and previous observations that inhibition of STAT1 activity, but not of IRF-1, protects β-cells against cytokine-induced apoptosis (Fig. 1) (15,16), suggesting that up-regulation of DP5 is an important mechanism by which STAT1 lead to β-cell apoptosis. Of note, several other apoptosis-related genes such as Puma, CHOP (Ddit3), Bax, Bid and caspases 4, 7 and 12 were also differentially regulated by cytokines following STAT1 knockdown (Suppl. Table 1) and may contribute for cytokine-induced β-cell apoptosis.

The third pathway identified as STAT1/IRF-1-modulated is related to islet inflammation. During insulitis, locally produced cytokines both contribute to β-cell apoptosis (5,7) and stimulate the production of several chemokines by β-cells, further recruiting activated immune cells to the site of inflammation (7,43). This local production of chemokines may be crucial in early T1D, as transgenic expression of CCL2 in β-cells induces spontaneous diabetes (44), while KO of CXCR3 delays diabetes by preventing attraction of CXCR3-expressing T-cells (45). Furthermore, recent findings in new-onset T1D patients show islet expression of CXCL10, while infiltrating lymphocytes expressed its receptor CXCR3 (46). Our present findings indicate that STAT1 partially regulates cytokine-induced secretion of several chemokines by β-cells, including CXCL9, CXCL10, CXCL11 and CCL20. These results are in agreement with our previous observations that islets from STAT1 KO mice have decreased production of CXCL10 upon cytokine exposure in vitro and in vivo (15). In contrast to the inhibitory effect of STAT1 silencing, IRF-1 inhibition exacerbated cytokine-induced chemokine production in β-cells, especially at late time points (12h and particularly 24h), while IRF-1 overexpression hampered STAT1 induction and chemokine production after cytokine exposure (Suppl. Fig. 5). These data provide a molecular explanation for our previous in vivo observations in mice, which showed increased primary non-function and rejection of grafted IRF-1−/− islets, which was accompanied by augmented infiltration by macrophages and T cells (16). We thus suggest that IRF-1 provides a negative feedback on STAT1-induced chemokine production, which is probably mediated via SOCS-1 up-regulation and STAT1 dephosphorylation. Indeed, SOCS-1 silencing also prolongs STAT1 phosphorylation and exacerbates CXCL1, 9 and 10 production in INS-1E cells (Fig.
6). Transgenic expression of SOCS-1 in β-cells reduces diabetes development in NOD mice (47) and protects β-cells against the deleterious effects of infiltrating CD8+ T cells in the same model (48), reinforcing the role of SOCS-1 downstream of IRF-1. Interestingly, a similar role has been described for STAT3 in myeloid cells, where IFN-α-induced STAT3 expression represses STAT1-driven CXCL9 and CXCL10 induction through heterodimerization and suppression of formation of STAT1 homodimers (49). This mechanism, and the presently described IRF-1-mediated negative feedback on STAT1, are probably part of elaborate “defense” mechanisms utilized by long-lived cells, such as pancreatic β-cells, to downregulate local inflammation and thus limit tissue damage (7).

Analysis of the array data indicates that the negative feedback by IRF-1 on STAT1 activity is mostly restricted to up-regulation of chemokines; additional studies are required to clarify the mechanisms for this specificity.

In conclusion, we have presently combined RNA interference and array analysis to clarify the gene networks regulated by the IFN-γ-STAT1-IRF-1 pathway in β-cells. This enabled the identification of three key pathways that may play a role for loss of functional β-cell mass in T1D: 1. β-cell de-differentiation, an effect mediated by STAT1; 2. β-cell apoptosis, an effect mediated by STAT1 via DP5 up-regulation; 3. Induction and modulation of chemokine production, an effect mediated by STAT1 but with IRF-1 providing a negative feedback through SOCS-1 induction. This and the discovery of a large number of additional STAT1- and IRF-1-regulated genes in β-cells, broads our understanding of β-cell dysfunction and death, and opens new possibilities for prevention of loss of functional β-cell mass in early T1D.

REFERENCES


FOOTNOTES

This work was supported by grants from the Fonds National de la Recherche Scientifique (FNRS - FRSM) Belgium, the Communauté Française de Belgique – Actions de Recherche Concertées (ARC), the European Union (NAIMIT, Ref. Health-F2009-241447; in the Framework Programme 7 of the European Community) and the Belgium Program on Interuniversity Poles of Attraction initiated by the Belgium State (IUAP P6/40). F.M. is the recipient of a Post-Doctoral Fellowship from FNRS, Belgium. M.L.C. is the recipient of a scholarship from CAPES (Brazilian Coordination for the Improvement of Higher Education Personnel). E.N.G. is supported by an EMBO long-term Fellowship. We thank G. Vandenbroeck, R. Makhnas, A. M. Musuaya, S. Mertens, M.A. Neef, M. Urbain and M. Pangerl from the Laboratory of Experimental Medicine, ULB, for excellent technical support.

FIGURE LEGENDS

Fig. 1. siRNA-mediated STAT1 knockdown protects INS-1E and primary rat \( \beta \)-cells against cytokine-induced apoptosis. (A-F) Insulin-producing INS-1E cells were left untransfected (NT), or transfected with 30 nM of siCtrl, siIRF-1 or siSTAT1. After 24h of recovery, cells were left untreated or exposed to IL-1\( \beta \) 10 U/ml + IFN-\( \gamma \) 100 U/ml for 12 or 24h as indicated. (A) STAT1, IRF-1 and \( \alpha \)-tubulin protein expression were evaluated by Western blot. (B-C) Mean optical density measurements of STAT1 and IRF-1 Western blots corrected for protein loading by \( \alpha \)-tubulin. Results are mean fold variation \( \pm \) SEM of 5 independent experiments. (D-E) Apoptosis was evaluated using (D) HO/PI staining and (E) Cell Death Detection ELISAplus kit. (F) Nitrite concentrations in supernatants were evaluated as described in Methods. Results are mean \( \pm \) SEM of 5 independent experiments. (G-H) Primary FACSA sorting rat \( \beta \)-cells were cultured for 2 days and then left untransfected or transfected with 30 nM of siCtrl, siIRF-1 or siSTAT1 as indicated. After 24h of recovery, cells were left untreated or exposed to IL-1\( \beta \) 10 U/ml + IFN-\( \gamma \) 100 U/ml for 24h as indicated. (G) Apoptosis was evaluated using HO/PI staining. (H) Nitrite concentrations in supernatants. Results are mean \( \pm \) SEM of 5 independent experiments. *: \( p<0.05 \), **: \( p<0.01 \) and ***: \( p<0.001 \) vs untreated (i.e. not treated with cytokines) or untreated transfected with the same siRNA; §§: \( p<0.01 \) and §§§: \( p<0.001 \) vs NT & siCtrl treated with cytokines at the same time point, ANOVA followed by Student’s \( t \) test with Bonferroni correction.

Fig. 2. Analysis of gene networks regulated by cytokines, IRF-1 and STAT1 in INS-1E cells. INS-1E cells were left untransfected, or transfected with 30 nM of siCtrl, siIRF-1 or siSTAT1 as in Figure 1. After 24h of recovery, cells were left untreated, or exposed to IL-1\( \beta \) 10 U/ml + IFN-\( \gamma \) 100 U/ml for 2, 12 or 24h as indicated, before being harvested for RNA extraction and array analysis. (A) Schematic representation of the microarray conditions (3 independent experiments); (B-D) Ven diagrams showing the number of \( \beta \)-cell genes whose expression was modified by cytokines and that were either STAT1-dependent or IRF-1-dependent after 2h (B), 12h (C) or 24h (D) of cytokine exposure; (E) Mean
percentage of probesets considered as present in the 3 microarray experiments that were differentially regulated by STAT1A/IRFA1 silencing and/or cytokine treatment at 2h (white bars), 12h (black bars) or 24h (hatched bars) of cytokine treatment in the different conditions indicated. Results of three independent array experiments were analyzed. mRNA expression was considered as modified by cytokines when \( p < 0.02 \) and fold change >1.5 as compared to untransfected cells not treated with cytokines (Untransfected) or siCtrl-transfected cells not transfected with cytokines (siCtrl).

Fig. 3. STAT1 silencing partially prevents cytokine-induced downregulation of genes involved in β−cell differentiation and function. INS-1E cells (A-E) or primary FACS-purified rat β-cells (F) were left untransfected (NT), or transfected with 30 nM of siCtrl, siIRFA1 or siSTAT1. After 24h of recovery post-transfection, cells were left untreated or exposed to IL-1β 10 U/ml + IFN-γ 100 U/ml for 2, 12 or 24h as indicated. Expression of genes related to β−cell function (A-B) or regulatory transcription factors (C-D) were analyzed by microarray. Results represent the mean fold variations ± SEM of the genes as compared to untreated controls after 12h (A & C) or 24h (B & D) of cytokine treatment (n=3). Statistical analyses for the represented genes are described in Suppl. Table 1. (E) Independent confirmation experiments in INS-1E cells: Ins1, Glut2, Pdx1 and MafA mRNA expressions were assayed by real time RT-PCR and normalized for the housekeeping gene GAPDH. Results are mean ± SEM of 4 independent experiments. (F) Confirmation experiments in primary rat β-cells: Ins1, Glut2, Pdx1 and MafA mRNA expressions were assayed by real time RT-PCR and normalized for the housekeeping gene GAPDH. Results are mean ± SEM of 5 independent experiments. *: \( p < 0.05 \), **: \( p < 0.01 \) and ***: \( p < 0.001 \) vs untreated (i.e. not treated with cytokines) or untreated transfected with the same siRNA; §: \( p < 0.05 \), §§: \( p < 0.01 \) and §§§: \( p < 0.001 \) vs NT & siCtrl treated with cytokines at the same time point, ANOVA followed by Student’s \( t \) test with Bonferroni correction.

Fig. 4. Induction of the pro-apoptotic protein DP5 is partially prevented after STAT1 knockdown. INS-1E cells (A) or primary FACS-sorted rat β-cells (B) were transfected and treated as described in Figure 1. (A & B) DP5 mRNA expression was assayed by real time RT-PCR and normalized for the housekeeping gene GAPDH. Results are mean ± SEM of 4 (A) or 5 (B) independent experiments. (C) INS-1E cells were co-transfected with the DP5 promoter luciferase reporter and the control pRL-CMV alone (NT) or in combination with siCtrl, siIRFA1 or siSTAT1. After 1 day of recovery, cells were left untreated or exposed to IL-1β 10 U/ml + IFN-γ 100 U/ml for 24h as indicated. Results are mean ± SEM of 5 independent experiments and represent fold variation as compared to untreated control condition. *: \( p < 0.05 \), **: \( p < 0.01 \) and ***: \( p < 0.001 \) vs untreated (i.e. not treated with cytokines) or untreated transfected with the same siRNA; §: \( p < 0.05 \), §§: \( p < 0.01 \) and §§§: \( p < 0.001 \) vs NT & siCtrl treated with cytokines at the same time point, ANOVA followed by Student’s \( t \) test with Bonferroni correction.

Fig. 5. IRFA1 provides a negative feedback on cytokine-induced chemokine production. INS-1E cells (A & B) or primary FACS-sorted rat β-cells (C) were left untransfected (NT), or transfected with 30 nM of siCtrl, siIRFA1 or siSTAT1. After 24h of recovery, cells were left untreated or exposed to IL-1β 10 U/ml + IFN-γ 100 U/ml for 2, 12 or 24h as indicated. (A) Expression of the chemokines CXCL10, CXCL9, CXCL1, CXCL11, CXCL2 and CCL20 were analyzed by microarray. Results represent the mean fold variations ± SEM of the genes as compared to untreated controls at the same time points (n=3). Statistical analyses for the represented genes are described in Suppl. Table 1. (B & C) Confirmation experiments in INS-1E cells (B) and primary rat β-cells (C): CXCL10, CXCL9, CXCL1 and CCL20 mRNA expressions were assayed by real time RT-PCR and normalized for the housekeeping gene GAPDH. Results are mean ± SEM of 4-5 independent experiments. *: \( p < 0.05 \), **: \( p < 0.01 \) and ***: \( p < 0.001 \) vs untreated (i.e. not treated with cytokines) or untreated transfected with the same siRNA; §: \( p < 0.05 \), §§: \( p < 0.01 \) and §§§: \( p < 0.001 \) vs NT & siCtrl treated with cytokines at the same time point, ANOVA followed by Student’s \( t \) test with Bonferroni correction.

Fig. 6. IRF-1 hampers STAT1 activation through the induction of SOCS-1. (A, C, D) INS-1E cells were left untransfected (NT), or transfected with 30 nM of siCtrl, siIRF-1 or siIRF-1 #2. After 24h
of recovery, cells were left untreated or exposed to IL-1β 10 U/ml + IFN-γ 100 U/ml for 2, 4, 8, 16 or 24h as indicated. (A) Phospho-STAT1, total STAT1, IRF-1, PTPN2 and α-tubulin protein expressions were evaluated by Western blot. Pictures are representative of 5 independent experiments. (B) INS-1E cells were co-transfected with a STAT1 reporter + pRL-CMV alone (NT) or in combination with siCtrl, siIRF-1 or siIRF-1 #2. After 1 day of recovery, cells were left untreated or exposed to IL-1β 10 U/ml + IFN-γ 100 U/ml for 24h as indicated. Results are mean Relative Luciferase Unit (R.L.U.) ± SEM of 5 independent experiments. (C-D) IRF-1 and SOCS-1 mRNA expressions were assayed by real time RT-PCR and normalized for the housekeeping gene GAPDH. Results are mean ± SEM of 4 independent experiments. (E-F) INS-1E cells were transfected with pCMV-Ctrl or pCMV-IRF-1. After overnight incubation, the cells were left untreated (time 0) or exposed to IL-1β 10 U/ml + IFN-γ 100 U/ml for 2, 4, 8 16 or 24h as indicated. Results are mean ± SEM of 4 independent experiments. (E) IRF-1 and α-tubulin protein expressions were evaluated by Western blot in untreated cells 24h after transfection. Pictures are representative of 4 independent experiments. (F) SOCS-1 mRNA expression was assayed by real time RT-PCR and normalized for the housekeeping gene GAPDH. Results are mean ± SEM of 4 independent experiments. (G-J) INS-1E cells were transfected with 30 nM of siCtrl, siSOCS-1 or siSOCS-1 #2. After 24h of recovery, cells were left untreated or exposed to IL-1β 10 U/ml + IFN-γ 100 U/ml for 24h as indicated. SOCS-1, CXCL1, CXCL9 and CXCL10 mRNA expressions were assayed by real time RT-PCR and normalized for the housekeeping gene GAPDH. Results are mean ± SEM of 4 independent experiments. *: p<0.05, **: p<0.01 and ***: p<0.001 vs respective untreated control; §: p<0.05, §§: p<0.01 and §§§: p<0.001 vs respective control treated with cytokines at the same time point, ANOVA followed by Student’s t test with Bonferroni correction. (K) Schematic representation of the suggested regulatory loop controlled by IRF-1 in β-cells. IFN-γ binding to its receptor induces Jak-mediated STAT1 phosphorylation and dimerization (1) and its subsequent migration to the nucleus (2), where it stimulates the transcription of many genes, including chemokines and IRF-1. Once synthesized in the cytoplasm (3), IRF-1 also migrates to the nucleus (4) to stimulate the transcription of several genes including SOCS-1 (5). SOCS-1 may then interfere with Jak-mediated STAT1 phosphorylation (6), hence hampering STAT1 activation over time. In the absence of IRF-1 signalling, the defective SOCS-1 induction allows prolonged Jak-mediated STAT1 phosphorylation (7) and sustained STAT1 activity (8).

Fig. 7. Schematic representation of selected cytokine-dependent genes differentially regulated by the transcription factors STAT1 (left panel), IRF-1 (right panel) or both STAT1 and IRF-1 (center panel). Of note, some genes (e.g. chemokines) are regulated, at least in part, in opposite direction by STAT1 and IRF-1.
Figure 1

A) Untreated and IL-1β + IFN-γ treated cells were analyzed by western blotting for STAT1 and IRF-1 expression. The blots were normalized to α-tubulin. 

B) Apoptosis analysis was performed for each condition. The data is represented as mean ± SD. 

C) Nitrite production was measured by the Griess reaction. The data is represented as mean ± SD. 

D) The concentration of STAT1/Tubulin and IRF-1/Tubulin was determined by densitometry. The data is represented as mean ± SD.
Figure 2

A

1. Untransfected
2. Untransfected
3. siCtrl
4. siCtrl
5. siIRF-1
6. siIRF-1
7. siSTAT1
8. siSTAT1

1. 24 h
2. 2, 12 or 24 h
3. Untreated
4. Untreated
5. Untreated
6. Untreated
7. Untreated
8. Untreated

RNA extraction
RNA extraction
RNA extraction
RNA extraction
RNA extraction
RNA extraction
RNA extraction
RNA extraction

B

Cytokine & STAT1-dependent
Cytokine & IRF-1-dependent

96 32 119

2h

C

Cytokine & STAT1-dependent
Cytokine & IRF-1-dependent

758 1216 797

12h

D

Cytokine & STAT1-dependent
Cytokine & IRF-1-dependent

884 640 470

24h

E

% present probesets

0 5 10 15 20 25

siCtrl vs Untransfected
siIRF-1 vs siCtrl
siSTAT1 vs siCtrl
Cyt. vs Untransfected
Cyt. siCtrl vs siCtrl
Cyt. siIRF-1 vs siCtrl
Cyt. siSTAT1 vs siCtrl

2h IL-1β+IFN-γ treatment
12h IL-1β+IFN-γ treatment
24h IL-1β+IFN-γ treatment
Figure 4

A

B

C

Fold variation

0 0.5 1 1.5 2 2.5

0 12 24

ILD1 + IFN treatment (h)

Fold variation

0 12 24

ILD1 + IFN treatment (h)

Fold variation

0 12 24

ILD1 + IFN treatment (h)
Figure 5

A) CXCL10, CXCL9, CXCL1, CCL20, CXCL2, CXCL10 (fold variation), CXCL11 (fold variation)

B) CXCL10, CXCL9, CXCL1, CCL20

C) CXCL10, CXCL9, CXCL1, CCL20

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J. Biol. Chem. published online October 27, 2010 originally published online October 27, 2010

Access the most updated version of this article at doi: 10.1074/jbc.M110.162131

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