Diabetologia (2009) 52:281–288
DOI 10.1007/s00125-008-1199-1

ARTICLE

IL-1β-induced chemokine and *Fas* expression are inhibited by suppressor of cytokine signalling-3 in insulin-producing cells

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Received: 5 June 2008 / Accepted: 13 October 2008 / Published online: 11 November 2008
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Abstract
Aims/hypothesis Chemokines recruit activated immune cells to sites of inflammation and are important mediators of insulitis. Activation of the pro-apoptotic receptor Fas leads to apoptosis-mediated death of the *Fas*-expressing cell. The pro-inflammatory cytokines IL-1β and IFN-γ regulate the transcription of genes encoding the Fas receptor and several chemokines. We have previously shown that suppressor of cytokine signalling (SOCS)-3 inhibits IL-1β- and IFN-γ-induced nitric oxide production in a beta cell line. The aim of this study was to investigate whether SOCS-3 can influence cytokine-induced *Fas* and chemokine expression in beta cells.
Methods Using a beta cell line with inducible *Socs3* expression or primary neonatal rat islet cells transduced with a *Socs3*-encoding adenovirus, we employed real-time RT-PCR analysis to investigate whether SOCS-3 affects cytokine-induced chemokine and *Fas* mRNA expression. The ability of SOCS-3 to influence the activity of cytokine-responsive *Fas* and *Mcp-1* (also known as *Ccl2*) promoters was measured by reporter analysis.
Results IL-1β induced a time-dependent increase in *Mcp-1* and *Mip-2* (also known as *Cxcl2*) mRNA expression after 6 h of stimulation in insulinoma (INS)-1 and neonatal rat islet cells. This induction was inhibited when *Socs3* was expressed in the cells. In INS-1 cells, IL-1β + IFN-γ induced a tenfold and eightfold increase of *Fas* mRNA expression after 6 and 24 h, respectively. This induction was inhibited at both time-points when expression of *Socs3* was induced. In promoter studies SOCS-3 significantly inhibited the cytokine-induced activity of *Mcp-1* and *Fas* promoter constructs.
Conclusions/interpretation SOCS-3 inhibits the expression of cytokine-induced chemokine and death-receptor *Fas* mRNA.

Keywords Autoimmunity · Chemokine · Diabetes · IL-1 · Inflammation · NF-kappaB · Signalling · SOCS

Abbreviations

CIS	cytokine-inducible SH2-containing protein
FASL	fas ligand
GFP	green fluorescent protein
INS	insulinoma
MCP	monocyte chemoattractant protein
MIP-2	macrophage inflammatory protein-2
NFκB	nuclear factor-κB
SOCS	suppressor of cytokine signalling
STAT	signal transducers and activators of transcription

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Introduction

Type 1 diabetes mellitus is an immune-mediated disease caused by an inflammatory reaction in the pancreatic islets of Langerhans. IL-1β and IFN-γ are pro-inflammatory cytokines implicated in the inflammatory reaction causing beta cell death [1]. IL-1β activates the transcription factor nuclear factor kappa B (NFκB) and the mitogen-activated protein kinases extracellular signal-regulated kinase, p38 and c-Jun N-terminal kinase [2, 3]. IFN-γ activates the transcription factor signal transducers and activators of transcription (STAT)-1 [4]. These transcription factors and mitogen-activated kinases induce the expression of several pro-apoptotic genes, among them genes encoding small chemotactic cytokines (chemokines) [5], that enhance the inflammatory response by recruiting immune-cells to sites of injury or inflammation [6, 7]. Chemokines thereby play an important role in the development of the inflammatory infiltrate in insulitis in the early stages of diabetes [8].

Chemokines can be divided into four subfamilies based on their structure and function. The CC- and CXC-families of chemokines are the largest and the most thoroughly investigated groups. Both sub-families carry four cysteines that are paired by disulphide bonds [9, 10]. In the CC-subfamily of chemokines, the cysteines are adjacent to each other, whereas in the CXC family they are separated by a single amino acid [10]. The CC-family of chemokines includes monocyte chemoattractant protein (MCP)-1 and macrophage inflammatory protein-3α (ST-38). Both MCP-1 and ST-38 are known to attract monocytes, T cells and natural killer cells, and are upregulated in rat beta cells by cytokines [11, 12].

The chemokine macrophage inflammatory protein-2 (MIP-2) belongs to the family of CXC-chemokines and is involved in the attraction of monocytes, T cells, natural killer cells and basophils. Together with the chemokine interferon inducible protein-10 (MOB-1) of the same family, MIP-2 is also upregulated in beta cells upon cytokine-exposure as shown in array studies [12, 13]. Transcription of the genes encoding the chemokines mentioned above is dependent on the transcription factor NFκB [14–17].

Fas (CD95) is a cell-death receptor, which induces apoptosis through an intracellular death domain upon binding by the Fas ligand (FasL) [18, 19]. FasL is mainly expressed on the surface of activated T cells, but is also constitutively expressed by beta cells. FasL belongs to the TNF family of membrane-associated cytokines and Fas is a member of the TNF receptor family [20, 21]. Binding of the ligand to its receptor leads to activation of caspase-8, which subsequently cleaves procaspase-3, resulting in completion of the cell-death programme [22].

In beta cells cytokines induce upregulation of Fas, making the beta cells susceptible to apoptosis upon interaction with *FasL*-expressing T cells as well as neighbouring beta cells [19, 23]. One study showed that reduced T cell *FasL* expression prevents diabetes in NOD mice [24]. Several similar studies have focused on the importance of Fas in beta cell death and the development of diabetes [25, 26]. On the other hand, evidence against a role of Fas has also been presented [27, 28], leaving much scope for debate in the field [4].

Cytokines convey their biological information to target cells by binding to cell-surface receptors, thereby activating intracellular signal transduction cascades that induce changes in transcription. However, negative regulators that ensure an appropriate cellular and physiological response to cytokine stimulation are needed to withstand deleterious effects of cytokine signalling. The suppressor of cytokine signalling (SOCS) proteins constitute one such family of negative regulators of cytokine signal transduction that has been shown to downregulate cytokine signalling [29]. The SOCS family consists of eight members, all of which are intracellular proteins: SOCS-1 to -7 and cytokine-inducible SH2-containing protein (CIS). All eight contain a central SH2 domain (of approximately 95 amino acids) and a SOCS-box, i.e. a conserved carboxyl-terminal domain.

Transcripts encoding SOCS-1 to SOCS-3 and CIS are normally present at low or undetectable levels in resting cells, but can rapidly be induced by a broad spectrum of cytokines and other factors [30]. Transcription of *Socs* genes is induced by the STAT and NFκB transcription factors upon cytokine stimulation, and the SOCS proteins thus generated subsequently inhibit the same pathway that initiated their production. Thus, it is generally accepted that SOCS proteins act in a negative feedback-loop that attenuates cytokine-induced signalling [31].

Previous work has shown that SOCS-3 inhibits IL-1β- and IFN-γ-induced nitric oxide production and apoptosis in the beta cell line insulinoma (INS)-1 [32] through specific inhibition of IL-1β-induced TGF-β activated kinase activity [33]. Furthermore, characterisation of the gene expression profiles in these cells showed that IL-1β-induced pro-apoptotic genes were inhibited by SOCS-3 [13].

In the present study we have investigated whether SOCS-3 affects cytokine-induced beta cell chemokine and *Fas* expression, thereby potentially reducing the inflammatory response to beta cells and the susceptibility to T cell-mediated killing.

Methods

Cell culture and cytokine exposure The generation of the *Socs3*-inducible cell line INS-r3#2 has been described previously [34]. Cells were cultured in RPMI-1640 with glutamax-I (Gibco BRL, Paisley, Scotland, UK) supplemented with 10% (vol./vol.) heat-inactivated TeT System

Approved FCS (CLONTECH, Palo Alto, CA, USA), penicillin, streptomycin, 50 μmol/l β-mercaptoethanol (Sigma Aldrich, St Louis, MO, USA), hygromycin (100 μg/ml) and geneticin (100 μg/ml; Gibco BRL). For analysis of the effect of SOCS-3, doxycycline (Sigma Aldrich) was added and the cells cultured for 24 h to allow *Socs3* expression. Neonatal rat islets were isolated and single cells were prepared by trypsin treatment as described [35]. Cells (150,000/well) were cultured in extracellular matrix coated 6-well dishes (Biological Industries, Kibbutz Beit, Haemek, Israel) using RPMI 1640 with glutamax-I supplemented with 10% (vol./vol.) heat-inactivated FCS and 1% (wt/vol.) penicillin and streptomycin as described [35]. Recombinant mouse IL-1β was obtained from BD Pharmingen (#554577; San Diego, CA, USA). Recombinant rat IFN-γ was obtained from R&D Systems (585-IF; R&D Systems, Foster City, CA, USA).

Analysis of chemokine and Fas mRNA expression using real-time PCR For the analysis of chemokine mRNA-expression in INS-1 cells, cells were seeded in six-well plates. The following day 1 μg/ml doxycycline was added to half of the wells, and cells were incubated in medium containing 0.5% (vol./vol.) FCS and cultured overnight. After 48 h cells were exposed to either 150 pg/ml IL-1β for 1, 2, 4, 6, 8, 16 or 24 h or to varying amounts of IL-1β (18.75 pg/ml–2.4 ng/ml) for 6 h. RNA extraction was performed by the TRIzol method (GibcoBRL, Invitrogen, Carlsbad, CA, USA). For analysis of primary rat islet cells, monolayers were transduced with adenovirus encoding Luciferase or SOCS-3 at a concentration of 5×10^8 plaque forming units/ml [36]. The virus titre used was selected by transduction of islet cells with a green fluorescent protein (GFP)-encoding adenovirus and using a concentration giving >95% GFP-positive cells. After 2 days the cells were stimulated with 150 pg/ml IL-1β for 6 h and RNA was isolated. cDNA synthesis was performed by TaqMan Reverse Transcription Reagents (808-0234; Applied Biosystems, Foster City, CA, USA) using 200 ng RNA. Each cDNA sample was subjected to two individual PCR analyses using primer-pairs for the gene in question and *Sp-1* for the internal control. Primers used for the reaction were: *Mcp-1* (also known as *Ccl2*): forward primer: 5′-ATC TGT GCT GAC CCC AAT AAG G-3′, reverse primer: 5′-CAC TTG GTT CTG GTC CAG TTT TC-3′; rat *Icam1*: forward primer: 5′-GCT CAC CTT TAG CAG CTC AAC A-3′, reverse primer: 5′-GTG GAG GCA TGC AGG GAT T-3′; *Cx3cl1*: forward primer: 5′-ACT TGC ACA GCC CAG ATC ATT-3′, reverse primer: 5′-CTG CGC TCT CAG ATG TAG GAA A-3′; *St-38* (also known as *Ccl20*): forward primer: 5′-GCT TAC CTC TGC AGC CAG TCA-3′, reverse primer: 5′-TGT ACG TGA GGC AGC AGT CAA-3′; *Mip2* (also known as *Cxcl2*): forward primer: 5′-GGA AGA ACA TGG GCT CCT GTA C-3′, reverse primer: 5′-TTC CTG GGT GCA GTT TGT TTC-3′; *Mob-1* (also known as *Cxcl10*): forward primer: 5′-TCC CAC TAC AGC GTG ATG GA-3′, reverse primer: 5′-GCC TTG CTG CTG GAG TTA CTT T-3′; and *Sp-1*: forward primer: 5′-GGC TAC CCC TAC CTC AAA GG-3′, reverse primer: 5′-CAC AAC ATA CTG CCC ACC AG-3′ (DNA Technology, Århus, Denmark). Each reaction was amplified in a PCR Mastermix supplemented with the DNA binding dye SYBR Green (Applied Biosystems). The samples were run on ABI PRISM 7900 HT Taqman (Applied Biosystems) according to the following program: 95°C 10 min (95°C 15 s, 60°C 1 min)× 40; 95°C 15 s, 60°C 15 s, 95°C 15 s, 4°C hold.

For data analysis the ΔΔC(T) method was applied as described previously [37].

For assessment of *Fas* mRNA expression, cells were cultured in 60 mm dishes, 1×10^6 cells/3 ml medium. The next day 1 μg/ml doxycycline was added to the relevant cultures and after 24 h cells were exposed to 150 pg/ml or 1 ng/ml of IL-1β or a combination of IL-1β and IFN-γ (150 pg/ml IL-1β, 10 ng/ml IFN-γ,) for 6 or 24 h. Primers used for this PCR reaction were: forward primer: 5′-TGC ACC TCG TGT GGA CTT GA-3′, reverse primer: 5′-GGA ACT TTG TTT CTT GCA TT′-3 (DNA Technology, Århus, Denmark). *Sp-1* was used as internal control as described above.

Transient transfection and Luciferase assay INS-r3#2 cells were seeded in 24-well dishes. After 2 days cells were transiently transfected using SuperFect (Qiagen, Valencia, CA, USA) with a total of 2 μg DNA (0.2 μg of an internal control [pRL-*TK*]; Promega, Madison, WI, USA), 0.8 μg empty vector (pcDNA; Invitrogen, Carlsbad, CA, USA) and 1 μg promoter construct (pGL-*Fas* or p-*Mcp-1* [14]). After 4 h the transfection mixture was removed and the cells were incubated in the presence or absence of 1 μg/ml doxycycline. After 24 h, cells were exposed to cytokines for the times indicated and Luciferase assay was performed according to the manufacturer's instructions using the Dual-Luciferase Reporter Assay System (Promega). Luciferase activity was measured using a Luminometer (Berthold Technologies, Bad Wildbad, Germany).

Statistical analysis All values are presented as means± SEM. Statistical analysis was done by a Student's *t* test. Significance was assumed at a p value of less than 0.05.

Results

The effect of SOCS-3 on IL-1β-induced expression of chemokines To determine whether the upregulation of chemokines by IL-1β was inhibited by SOCS-3, expression

of six chemokine mRNAs was investigated. INS-r3#2 cells were cultured in the presence or absence of doxycycline for 24 h to induce *Socs3* expression and subsequently exposed to 150 pg/ml IL-1β for 6 h. Chemokine mRNA expression level was measured by real-time PCR. All investigated chemokines were upregulated following IL-1β stimulation for 6 h (Fig. 1a). The fold induction by IL-1β varied from 3.5-fold for *Cx3cl1* to about 100-fold for *St-38*. *Socs3* significantly inhibited IL-1β-induced expression of the chemokines *Mcp-1* and *Mip-2*, but not of *Cx3-c1*, *Mob-1*, rat *Icam1* or *St-38*. In order to test whether the observed effects of IL-1β and SOCS-3 on chemokine expression could also be observed in primary islet cells, primary neonatal rat islet cultures were transduced with recombinant adenovirus encoding SOCS-3. Neonatal rat islets were used because of the high efficiency of adenoviral transduction. The level of *Socs3* mRNA expression achieved using adenoviral transduction was found to be two to three times higher than doxycycline-inducible systems. We analysed the expression levels of the three chemokines showing the highest induction by IL-1β in INS-1 cells. These chemokines *Mcp-2*, *Mip-2* and *St-38* were all induced by IL-1β in primary neonatal rat islet cells, with induction being reduced in cells producing SOCS-3 by 60% (*Mcp-2*), 75% (*Mip-2*) and 70% (*St-38*) (Fig. 1b) compared with primary cells transduced with a control Luciferase-expressing adenovirus. Next we analysed the effect of SOCS-3 on IL-1β-induced chemokines in INS-1 cells at different time points and concentrations. However, only data from chemokines that were inhibited by SOCS-3 are shown.

SOCS-3 inhibits IL-1β-induced Mip-2 mRNA expression in a time- and dose-dependent manner In time-response experiments (Fig. 2a), transient *Mip-2* induction by IL-1β was observed with mRNA-expression increasing by a maximum of 14-fold after 6 h, returning to threefold after 16 to 24 h. SOCS-3 significantly inhibited *Mip-2* expression after 4, 6, 8 and 24 h of IL-1β exposure. Maximal inhibition (26%) was observed after 8 h. A dose-dependent increase in *Mip-2* mRNA expression was observed (Fig. 2b) at all concentrations of IL-1β tested, with the maximum increase (70-fold) occurring at 1.2 ng/ml of IL-1β. SOCS-3 significantly inhibited *Mip-2* expression in response to 18.75, 150 and 300 pg/ml, and 1.2 and 2.4 ng/ml of IL-1β, with maximal inhibition (45%) being registered at 18.75 pg/ml IL-1β.

SOCS-3 inhibits IL-1-induced Mcp-1 mRNA expression in a time- and dose-dependent manner IL-1β induced transient expression of *Mcp-1* (Fig. 3a). A 46-fold maximal increase in stimulation was reached after 6 h. SOCS-3 inhibited IL-1β-stimulated *Mcp-1* mRNA expression at all time points except 2, 16 and 24 h. Maximum inhibition (86%) was seen at 1 h. In dose–response experiments IL-1β concentrations above 37.5 pg/ml significantly induced *Mcp-1* mRNA expression (Fig. 3b) with a maximum induction of 160-fold at 2.4 ng/ml. SOCS-3 significantly inhibited expression of *Mcp-1* mRNA at all concentrations except 37.5 pg/ml. The greatest inhibition (95%) was observed at 18.75 pg/ml.

SOCS-3 inhibits cytokine-induced Mcp-1 promotor activity in INS-1 cells To investigate whether the regulation of chemokine mRNA expression by SOCS-3 was exerted at the transcriptional level, the ability of SOCS-3 to influence the activity of a cytokine-responsive *Mcp-1* promoter construct was analysed. The chemokine *Mcp-1* promoter was chosen for these experiments because of the marked induction of mRNA by IL-1β. When cells were exposed for 6 h to 150 pg/ml or 1 ng/ml IL-1β, a 1.6- and 2.6-fold increase,

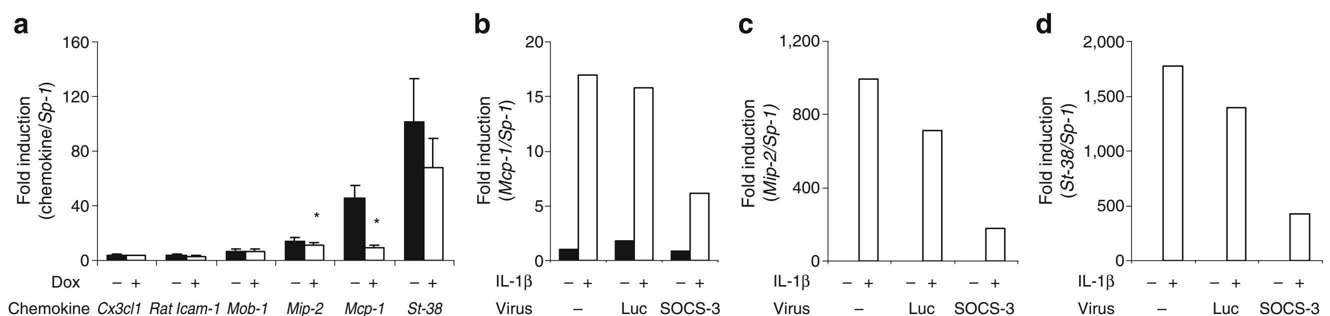

Fig. 1 SOCS-3 inhibits IL-1β-induced *Mip-2*, *Mcp-1* and *St-38* expression. **a** INS-r3#2 cells were cultured for 24 h in the presence (white bars) or absence (black bars) of doxycycline (Dox; 1 μg/ml) to induce SOCS-3 expression. Cells were subsequently exposed to 150 pg/ml IL-1β for 6 h and total RNA was isolated. **b**–**d** Monolayers of rat islet cells were exposed for 48 h to adenovirus encoding Luciferase (Luc) or SOCS-3 and subsequently stimulated with (white bars) or without (black bars) 150 pg/ml IL-1β for 6 h, after which total RNA for *Mcp-1* (**b**), *Mip-2* (**c**) and *St-38* (**d**) was isolated. Chemokine expression was quantified using real-time PCR and results are presented as mean fold induction by IL-1β. *$p<0.05$; $n=7$ for *Mcp-1* and *Cx3cl1*, $n=5$ for rat *Icam1*, $n=6$ *Mob-1*, $n=9$ for *Mip-2* and *St-38* (a), $n=2$ (b–d)

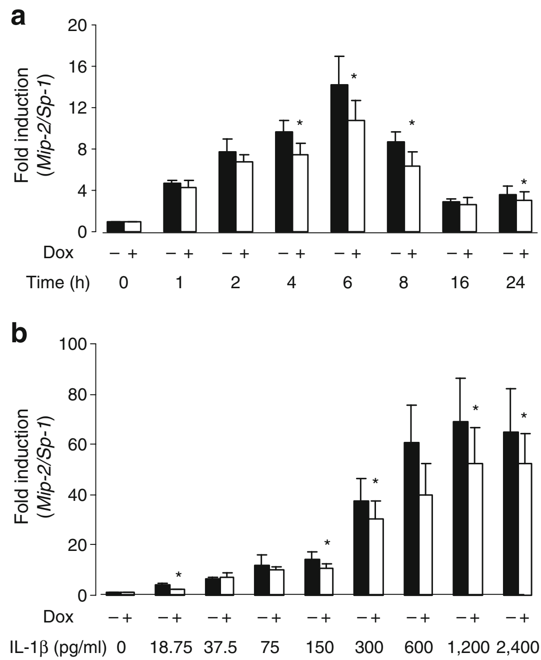

Fig. 2 SOCS-3 inhibits IL-1β-induced *Mip-2* expression in a time- and dose-dependent manner. INS-r3#2 cells were cultured for 24 h in the presence (white bars) or absence (black bars) of doxycycline (Dox; 1 μg/ml) to induce SOCS-3-expression. Cells were subsequently exposed to 150 pg/ml IL-1β for the time indicated (**a**) or to the indicated concentrations of IL-1β for 6 h (**b**) and total RNA was isolated. *Mip-2*-expression was quantified using real-time PCR and results are shown as fold stimulation by IL-1β. $^*p<0.05$, $n=4$–9

respectively was seen in promoter activity. SOCS-3 expression significantly inhibited the *Mcp-1* promoter activity in response to 150 pg/ml or 1 ng/ml IL-1β (Fig. 4).

SOCS-3 inhibits cytokine-induced Fas expression in beta cells IL-1β at 150 pg/ml or 1 ng/ml caused significant induction of *Fas* mRNA expression at 6 or 24 h, but SOCS-3 did not affect this induction (Fig. 5). The combination of IL-1β and IFN-γ enhanced stimulation of *Fas* mRNA expression, which in turn was significantly reduced by SOCS-3.

SOCS-3 inhibits cytokine-induced Fas promoter activity in beta cells To study whether the regulation of *Fas* expression by SOCS-3 was caused by a change at the transcriptional level, a *Fas* promoter–reporter analysis was performed (Fig. 6). Cells were exposed to either 1 ng/ml of IL-1β or a mixture of IL-1β and IFN-γ (1 ng/ml and 10 ng/ml, respectively) for 2, 4 or 6 h in the presence or absence of SOCS-3. IL-1β and IL-1β in combination with IFN-γ induced *Fas* promoter activity in a time-dependent manner. There was no difference in induction between IL-1β alone and the combination of IL-1β with IFN-γ. At all time points the promoter activity induced by IL-1β plus IFN-γ was reduced by SOCS-3. At two of the three time points (2 and 6 h) SOCS-3 also inhibited transcription induced by IL-1β alone.

Discussion

In this study, we investigated the expression patterns of six chemokines upon cytokine exposure and the ability of SOCS-3 to regulate this expression. First, we were able to show an increase in mRNA expression in response to cytokine exposure for all six chemokines in INS-1 cells. Regulation by IL-1β was verified in primary rat islet cells for *Mcp-1*, *Mip-2* and *St-38*. Second, we showed that SOCS-3 inhibited the expression of two of these, *Mcp-1* and *Mip-2* in INS-1 cells, and in addition inhibited IL-1β-induced production of ST-38 in primary neonatal rat cells, with the reduction in mRNA expression of *Mcp-1* being associated with reduced promoter activity. However, we were not able to show that SOCS-3 inhibited the cytokine-induced expression of chemokines *Cx3cl1*, *Mob-1* and rat *Icam1* in INS-1 cells.

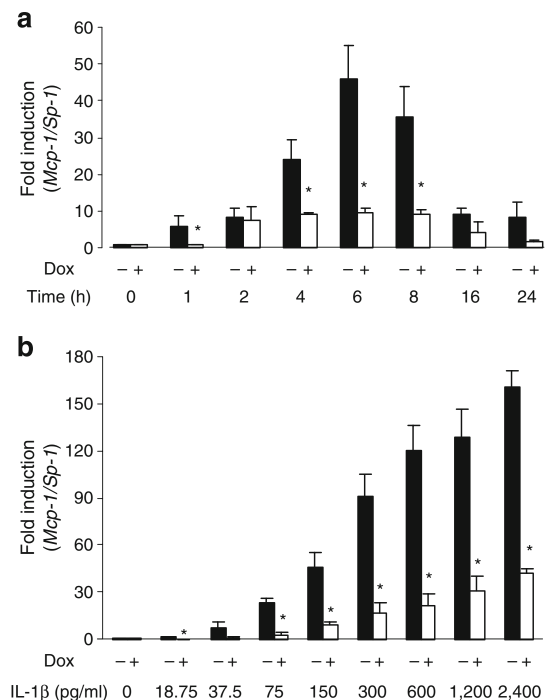

Fig. 3 SOCS-3 inhibits IL-1β-induced *Mcp-1* expression in a time- and dose-dependent manner. INS-r3#2 cells were cultured for 24 h in the presence (white bars) or absence (black bars) of doxycycline (Dox; 1 μg/ml) to induce SOCS-3-expression. Cells were subsequently exposed to 150 pg/ml IL-1β for the time indicated (**a**) or to the indicated concentrations of IL-1β for 6 h (**b**) and total RNA was isolated. *Mcp-1*-expression was quantified using real-time PCR and results are shown as fold stimulation by IL-1β. $^*p<0.05$, $n=4$–9

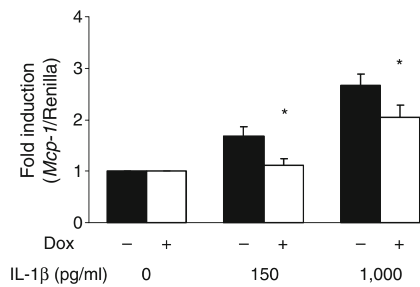

Fig. 4 SOCS-3 inhibits cytokine-induced *Mcp-1* promotor activity in INS-1 cells. INS-r3#2 cells were transfected with a *Mcp-1* promoter–Luciferase plasmid and a Renilla control plasmid for 4 h and subsequently incubated overnight in the presence (white bars) or absence (black bars) of doxycycline (Dox; 1 μg/ml). The transfected cells were exposed to IL-1β for 6 h and lysed. The lysates were subjected to Dual-Luciferase assay to measure the activity of the promoter. Results are presented as promoter activity normalised to the Renilla internal control. *$p<0.05$, $n=7$

Our study also shows induction of *Fas* mRNA expression upon cytokine exposure, both after 6 and 24 h, and that this expression was inhibited by SOCS-3.

Fas promoter activity was significantly inhibited when expression of SOCS-3 was induced in the cells. SOCS-3 inhibited the activity of the promoter when induced by IL-1β alone or by IL-1β + IFN-γ.

In an mRNA array study, it has previously been shown that the chemokines *Cx3cl1*, *St-38*, *Mob-1*, *Mip-2* and the intracellular adhesion molecule rat *Icam-1* are upregulated in response to cytokine exposure [13]. The array study also showed that this upregulation was significantly inhibited by SOCS-3. In the present study, using real-time PCR, we were able to confirm this inhibitory effect of SOCS-3 for the chemokine *Mip-2*.

The chemokine MCP-1 has previously also been shown to be upregulated by IL-1β [38]. The transcriptional regulation of *Mcp-1* by cytokines has been characterised

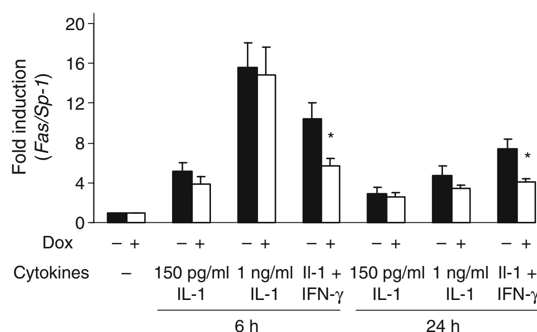

Fig. 5 SOCS-3 inhibits cytokine-induced *Fas* expression. INS-r3#2 cells were cultured in the presence (white bars) or absence (black bars) of doxycycline Dox; 1 μg/ml) to induce SOCS-3-expression. Cells were subsequently exposed to the cytokines indicated for 6 or 24 h and total RNA was isolated. *Fas* mRNA expression was quantified using real-time PCR. Results are shown as fold stimulation by IL-1β. *$p<0.05$, $n=6$

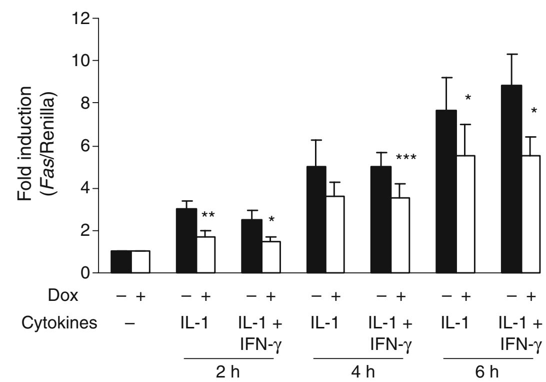

Fig. 6 SOCS-3 inhibits cytokine-induced *Fas* promotor activity. INS-r3#2 cells were transfected with a *Fas* promoter–Luciferase plasmid and a Renilla control plasmid for 4 h and subsequently incubated overnight in the presence (white bars) or absence (black bars) of doxycycline (Dox; 1 μg/ml). The transfected cells were exposed to IL-1β 150 pg/ml alone or IL-1β in combination with IFN-γ (1 ng/ml) as indicated for 2, 4 and 6 h and were then lysed. The lysates were subjected to Dual-Luciferase assay to measure the activity of the promoter. Results are shown as promoter activity normalised to Renilla internal control. *$p<0.05$, $n=5$

[39] showing an IL-1β-responsive enhancer region in rat *Mcp-1* gene between −2,180 and −2,478. This region contains two NFκB sites, and mutation in either of these abrogated IL-1β-induced *Mcp-1* promoter activity [40]. In the present study we show that SOCS-3 did indeed suppress IL-1β-induced *Mcp-1* promoter-activity. All chemokines investigated in the present study are dependent on the transcription factor NFκB, and since SOCS-3 blocks this transcription factor, this might be the mechanism by which SOCS-3 inhibits chemokine expression.

Fas has been shown to be upregulated in rat beta cells following IL-1β stimulation, but not by IFN-γ [40]. Also in human [23] and in mouse islets [19] IL-1β stimulation resulted in increased production of FAS and associated beta cell apoptosis. In line with this, we observed here that *Fas* mRNA expression was increased upon cytokine exposure after both 6 and 24 h. Because it is known that NFκB is necessary for the transcription of *Fas*, it was not surprising that in the present study SOCS-3 inhibited expression of *Fas* as well as the activity of the *Fas* promoter.

Since all six chemokines examined in this study are dependent on the transcription factor NFκB for their expression, we would expect them all to be similarly inhibited. The differential effect of SOCS-3 on expression of the six chemokines could have been caused by different mechanisms. First, the number of experiments carried out for each chemokine might not have been large enough to show a small but statistically significant inhibition by SOCS-3. Thus, increasing the number of experiments might increase the likelihood of identifying a slight inhibition. Another possible explanation could be that the level of

SOCS-3 produced by the cells upon doxycycline exposure is insufficient to inhibit the cytokine-induced chemokine expression of the four chemokines that did not show a statistically significant inhibition by SOCS-3 in INS-1 cells. In support of this hypothesis, we found that in primary islet cells *Socs* expression induced by adenoviral transduction was able to inhibit IL-1β-stimulated expression of *Mip-2*, *Mcp-2* and *St-38*. With the concentration of adenovirus used in these studies, a two- to threefold higher expression of *Socs* is achieved compared with the levels observed using the doxycycline-inducible system in INS-1 cells. Finally, other pathways are involved in regulating expression of these chemokines in addition to the NFκB pathway. IL-1β also signals via protein kinase C and small G-proteins, and it is possible that some of these pathways influence expression of the chemokines even in the presence of SOCS-3.

All of the experiments in the present study only addressed expression of chemokines at the mRNA level. It could be interesting to investigate whether this inhibition seen at the mRNA level translates to the protein level. Additionally, using motility assays, it could be elucidated whether the number of attracted immune cells in a chamber can be reduced by inducing SOCS-3 expression in beta cells.

The presented data show that SOCS-3 is able to inhibit IL-1β-induced expression of the chemokines *Mcp-1*, *St-38* and *Mip-2* as well as of the death receptor *Fas*. The perspectives of such an inhibition are interesting, as the inflammatory response in beta cells and the susceptibility to T cell-mediated killing could potentially be inhibited, thus preventing the development of type 1 diabetes.

Acknowledgements M. L. B. Jacobsen was supported by a PhD fellowship from the Danish Agency for Science, Technology and Innovation. S. G. Rønn and C. Bruun were supported by the Juvenile Diabetes Research Foundation International (no. 1-2004-736). D. L. Eizirik was supported by grants from the Fonds National de la Recherche Scientifique (FNRS) Belgium and the European Union (STREP Savebeta, contract no. 036903; in the Framework programme 6 of the European Community). We thank A. Hellgren and H. Fjordvang for excellent technical assistance.

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

References

1. Mandrup-Poulsen T (2001) beta-cell apoptosis: stimuli and signaling. Diabetes 50(Suppl 1):S58–S63
2. Donath MY, Størling J, Berchtold LA, Billestrup N, Mandrup-Poulsen T (2008) Cytokine and β-cell biology: from concept to clinical translation. Endo Rev 29:334–350
3. Mandrup-Poulsen T (2003) Apoptotic signal transduction pathways in diabetes. Biochem Pharmacol 66:1433–1440
4. Shuai K, Stark GR, Kerr IM, Darnell JE (1993) A single phosphotyrosine residue in Stat91 required for gene activation by interferon-gamma. Science 261:1744–1746
5. Baggiolini M (1998) Chemokines and leukocyte traffic. Nature 392:565–568
6. Moser B, Wolf M, Walz A, Loetscher P (2004) Chemokines: multiple levels of leukocyte migration control. Trends Immunol 25:75–84
7. Moser B, Willimann K (2004) Chemokines: role in inflammation and immune surveillance. Ann Rheum Dis 63(Suppl 2):ii84–ii89
8. Baggiolini M, Dewald B, Moser B (1997) Human chemokines: an update. Annu Rev Immunol 15:675–705
9. Olson TS, Ley K (2002) Chemokines and chemokine receptors in leukocyte trafficking. Am J Physiol Regul Integr Comp Physiol 283:R7–R28
10. Baggiolini M (2001) Chemokines in pathology and medicine. J Intern Med 250:91–9104
11. Chen MC, Proost P, Gysemans C, Mathieu C, Eizirik DL (2001) Monocyte chemoattractant protein-1 is expressed in pancreatic islets from prediabetic NOD mice and in interleukin-1 beta-exposed human and rat islet cells. Diabetologia 44:325–332
12. Cardozo AK, Kruhoffer M, Leeman R, Orntoft T, Eizirik DL (2001) Identification of novel cytokine-induced genes in pancreatic beta-cells by high-density oligonucleotide arrays. Diabetes 50:909–920
13. Karlsen AE, Heding PE, Frobose H et al (2004) Suppressor of cytokine signalling (SOCS)-3 protects beta cells against IL-1beta-mediated toxicity through inhibition of multiple nuclear factor-kappaB-regulated proapoptotic pathways. Diabetologia 47:1998–2011
14. Cardozo AK, Heimberg H, Heremans Y et al (2001) A comprehensive analysis of cytokine-induced and nuclear factor-kappa B-dependent genes in primary rat pancreatic beta-cells. J Biol Chem 276:48879–48886
15. Garcia GE, Xia YY, Chen SZ et al (2000) NF-kappa B-dependent fractalkine induction in rat aortic endothelial cells stimulated by IL-1 beta, TNF-alpha, and LPS. J Leukoc Biol 67:577–584
16. Hellerbrand C, Jobin C, Licato LL, Sartor RB, Brenner DA (1998) Cytokines induce NF-kappaB in activated but not in quiescent rat hepatic stellate cells. Am J Physiol 275:G269–G278
17. Imaizumi Y, Sugita S, Yamamoto K et al (2002) Human T cell leukemia virus type-I Tax activates human macrophage inflammatory protein-3 alpha/CCL20 gene transcription via the NF-kappa B pathway. Int Immunol 14:147–155
18. Stassi G, De-Maria R, Trucco G et al (1997) Nitric oxide primes pancreatic beta cells for Fas-mediated destruction in insulin-dependent diabetes mellitus. J Exp Med 186:1193–1200
19. Yamada K, Takane-Gyotoku N, Yuan X, Ichikawa F, Inada C, Nonaka K (1996) Mouse islet cell lysis mediated by interleukin-1-induced Fas. Diabetologia 39:1306–1312
20. Nagata S, Golstein P (1995) The Fas death factor. Science 267:1449–1456
21. Nagata S (1997) Apoptosis by death factor. Cell 88:355–365
22. Krammer PH (2000) CD95's deadly mission in the immune system. Nature 407:789–795
23. Stassi G, Todaro M, Richiusa P et al (1995) Expression of apoptosis-inducing CD95 (Fas/Apo-1) on human beta-cells sorted by flow-cytometry and cultured in vitro. Transplant Proc 27:3271–3275
24. Su X, Hu Q, Kristan JM et al (2000) Significant role for Fas in the pathogenesis of autoimmune diabetes. J Immunol 164:2523–2532
25. Dudek NL, Thomas HE, Mariana L et al (2006) Cytotoxic T-cells from T-cell receptor transgenic NOD8.3 mice destroy beta-cells via the perforin and Fas pathways. Diabetes 55:2412–2418
26. Allison J, Thomas HE, Catterall T, Kay TW, Strasser A (2005) Transgenic expression of dominant-negative fas-associated death domain protein in β cells protects against Fas ligand-induced apoptosis and reduces spontaneous diabetes in nonobese diabetic mice. J Immunol 175:293–301

27. Kim S, Kim KA, Hwang DY et al (2000) Inhibition of autoimmune diabetes by Fas ligand: the paradox is solved. J Immunol 164:2931–2936
28. Kim YH, Kim S, Kim KA et al (1999) Apoptosis of pancreatic B cells detected in accelerated diabetes of NOD mice: no role of Fas-Fas ligand interaction in autoimmune diabetes. Eur J Immunol 29:455–465
29. Wormald S, Hilton DJ (2004) Inhibitors of cytokine signal transduction. J Biol Chem 279:821–824
30. Krebs DL, Hilton DJ (2001) SOCS proteins: negative regulators of cytokine signaling. Stem Cells 19:378–387
31. Lavens D, Ulrichts P, Catteeuw D et al (2007) The C-terminus of CIS defines its interaction pattern. Biochem J 401:257–267
32. Karlsen AE, Rønn SG, Lindberg K et al (2001) Suppressor of cytokine signaling 3 (SOCS-3) protects beta-cells against interleukin-1beta- and interferon-gamma-mediated toxicity. Proc Natl Acad Sci U S A 98:12191–12196
33. Frobose H, Groth Rønn S, Heding PE et al (2006) Suppressor of cytokine signaling-3 inhibits interleukin-1 signaling by targeting the TRAF-6/TAK1 complex. Mol Endocrinol 20:1587–1596
34. Ronn SG, Hansen JA, Lindberg K, Karlsen AE, Billestrup N (2002) The effect of suppressor of cytokine signaling 3 on GH signaling in beta-cells. Mol Endocrinol 16:2124–2134
35. Parnaud G, Bosco D, Berrey T et al (2008) Proliferation of sorted human and rat beta cells. Diabetologia 51:91–100
36. Friedrichsen BN, Richter HE, Hansen JA et al (2003) Signal transducer and activator of transcription 5 activation is sufficient to drive transcriptional induction of cyclin D2 gene and proliferation of rat pancreatic β-cells. Mol Endocrinol 17:945–958
37. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25:402–408
38. Chen MC, Schuit F, Eizirik DL (1999) Identification of IL-1beta-induced messenger RNAs in rat pancreatic beta cells by differential display of messenger RNA. Diabetologia 42:1199–1203
39. Kutlu B, Darville MI, Cardozo AK, Eizirik DL (2003) Molecular regulation of monocyte chemoattractant protein-1 (MCP-1) expression in pancreatic β-cells. Diabetes 52:348–355
40. Darville MI, Eizirik DL (2001) Cytokine induction of Fas gene expression in insulin-producing cells requires the transcription factors NF-kappaB and C/EBP. Diabetes 50:1741–1748