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**SUPPRESSOR OF CYTOKINE SIGNALLING-3 INHIBITS TUMOR NECROSIS  
FACTOR-ALPHA INDUCED APOPTOSIS AND SIGNALLING IN BETA CELLS**

Christine Bruun<sup>1</sup>, Peter E. Heding<sup>1</sup>, Sif G. Rønn<sup>1</sup>, Helle Frobøse<sup>1</sup>, Christopher J. Rhodes<sup>2</sup>,  
Thomas Mandrup-Poulsen<sup>1,3</sup> and Nils Billestrup<sup>1</sup>

<sup>1</sup>Hagedorn Research Institute, Niels Steensens Vej 6, DK-2820 Gentofte, Denmark.

<sup>2</sup>Section of Endocrinology, Diabetes, and Metabolism, University of Chicago, IL, U.S.A.

<sup>3</sup>Dept. of Biomedical Sciences, University of Copenhagen, Blegdamsvej 3, DK-2200  
Copenhagen, Denmark

Corresponding author: Nils Billestrup, Hagedorn Research Institute, Niels Steensens Vej 6  
NSK2.02, DK-2820 Gentofte, Denmark, Tlf. +45 44439182; Fax. +45 44438000; E-mail:  
[nbil@hagedorn.dk](mailto:nbil@hagedorn.dk)

**Key words:**

SOCS-3, TNF $\alpha$ , MAP kinase, NF $\kappa$ B, I $\kappa$ B, T1DM, (Rat islets), (INS-1 beta cells).

**ABSTRACT**

Tumor necrosis factor-alpha (TNF $\alpha$ ) is a pro-inflammatory cytokine involved in the pathogenesis of several diseases including type 1 diabetes mellitus (T1DM). TNF $\alpha$  in combination with interleukin-1-beta (IL-1 $\beta$ ) and/or interferon-gamma (IFN $\gamma$ ) induce specific destruction of the pancreatic insulin-producing beta cells. Suppressor of cytokine signalling-3 (SOCS-3) proteins regulate signalling induced by a number of cytokines including growth hormone, IFN $\gamma$  and IL-1 $\beta$  which signals via very distinctive pathways. The objective of this study was to investigate the effect of SOCS-3 on TNF $\alpha$ -induced signalling in beta cells. We found that apoptosis induced by TNF $\alpha$  alone or in combination with IL-1 $\beta$  was suppressed by expression of SOCS-3 in the beta cell line INSr3#2. SOCS-3 inhibited TNF $\alpha$ -induced phosphorylation of the mitogen activated protein kinases ERK1/2, p38 and JNK in INSr3#2 cells and in primary rat islets. Furthermore, SOCS-3 repressed TNF $\alpha$ -induced degradation of I $\kappa$ B, NF $\kappa$ B DNA binding and transcription of the NF $\kappa$ B dependent *MnSOD* promoter. Finally, expression of *Socs-3* mRNA was induced by TNF $\alpha$  in rat islets in a transient manner with maximum expression after 1-2 hours. The ability of SOCS-3 to regulate signalling induced by the three major pro-inflammatory cytokines involved in the pathogenesis of T1DM makes SOCS-3 an interesting therapeutic candidate for protection of the beta cell mass.

## INTRODUCTION

Tumor necrosis factor-alpha (TNF $\alpha$ ) is an important mediator of inflammatory and immunological reactions. Apart from its beneficial effects under normal physiological conditions TNF $\alpha$  is implicated in the pathogenesis of a wide spectrum of human diseases, including rheumatoid arthritis, multiple sclerosis, inflammatory bowel disease and type 1 diabetes mellitus (T1DM) (Chen & Goeddel 2002) (Eizirik & Mandrup-Poulsen 2001). T1DM is an immune-mediated disease caused by inflammation in the islets of Langerhans. Infiltrating activated macrophages and T-cells secrete pro-inflammatory cytokines including TNF $\alpha$ , interleukin-1-beta (IL-1 $\beta$ ) and interferon-gamma (IFN $\gamma$ ) which are potent inducers of beta cell death (Eizirik & Mandrup-Poulsen 2001; Thomas & Kay 2000).

Binding of TNF $\alpha$  to its receptors leads to recruitment and activation of several proteins including TNF receptor-associated death domain (TRADD) and TNF receptor-associated factor (TRAF)-2 ultimately leading to phosphorylation and activation of mitogen activated protein (MAP) kinases and translocation of the transcription factor nuclear factor kappa B (NF $\kappa$ B) from the cytoplasm to the nucleus (Devin *et al.* 2000; Hsu *et al.* 1995; Hsu *et al.* 1996; Kanayama *et al.* 2004; Stanger *et al.* 1995). In the nucleus NF $\kappa$ B regulates transcription of a wide range of both anti- and pro-apoptotic genes. The role of NF $\kappa$ B in beta cells is atypical as NF $\kappa$ B in most cell types mediate anti-apoptotic signalling whereas activation of NF $\kappa$ B in beta cells seems to have a pro-apoptotic outcome (Eldor *et al.* 2006; Giannoukakis *et al.* 2000; Heimberg *et al.* 2001).

Exposure of beta cells to TNF $\alpha$  alone has been found to cause only modest or no cell death depending on the beta cell model system investigated. However, TNF $\alpha$  in combination with IFN $\gamma$  and/or IL-1 $\beta$  potently induces cell death in rodent and human beta cells through both

NO-dependent and independent pathways (Chong *et al.* 2002; Eizirik & Mandrup-Poulsen 2001; Mandrup-Poulsen *et al.* 1987; Saldeen 2000). Upregulation and activation of iNOS which leads to production of NO is dependent on activation of both NF $\kappa$ B and the MAP kinases p38 and ERK. Activation of JNK has been shown to induce beta cell death independently of NO production (Darville & Eizirik 1998; Flodstrom *et al.* 1996; Larsen *et al.* 2005). Activation of both NF $\kappa$ B and phosphorylation of JNK is necessary for induction of beta cell death since inhibition of either of these signalling molecules inhibits apoptosis (Ammendrup *et al.* 2000; Giannoukakis *et al.* 2000; Bonny *et al.* 2000; Bonny *et al.* 2001).

A tight regulation of the signal transduction pathways activated by TNF $\alpha$  is required in order to prevent potentially harmful effects. Suppressor of cytokine signalling (SOCS) proteins are a class of inhibitory molecules shown to inhibit signalling induced by cytokines that signal through the janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway as well as pathways of other cytokines and hormones including IL-1 $\beta$  and insulin (Adams *et al.* 1998; Rui *et al.* 2002; Alexander *et al.* 1999; Karlsen *et al.* 2001). SOCS-1 and SOCS-3 are up-regulated in response to various factors such as IL-2, IL-3, IL-4, IL-6, IFN $\gamma$ , erythropoietin and GH in a JAK/STAT dependent manner (Krebs & Hilton 2001). However, SOCS-1 and SOCS-3 are also induced by stimuli such as TNF $\alpha$ , IL-1 $\beta$ , and lipopolysaccharide (LPS) that do not utilize JAK/STAT proteins in signalling but rather NF $\kappa$ B and MAP kinases (Karlsen *et al.* 2001; Emanuelli *et al.* 2001; Stoiber *et al.* 1999).

SOCS-3 is able to inhibit IL-1 $\beta$  and IL-1 $\beta$ +IFN $\gamma$  induced apoptosis and NO production in INS-1 cells and primary mouse and rat beta cells (Karlsen *et al.* 2001; Rønn *et al.* 2008). Furthermore, *Socs-3* transgenic islets transplanted into BALB/c mice shows prolonged graft survival indicating a better protection of these islets from immune-mediated cytokine damage

(Rønn *et al.* 2008). Gene array analysis conducted in INS-1 cells has shown that SOCS-3 suppresses the expression of several IL-1 $\beta$ -induced pro-apoptotic genes, many of these known to be NF $\kappa$ B-dependent (Karlsen *et al.* 2004).

TNF $\alpha$  and IL-1 $\beta$  both induce MAP kinase and NF $\kappa$ B activation but they do so by quite different mechanisms. Following activation by TNF $\alpha$ , the TNF receptor 1 recruits the adaptor protein TRADD which subsequently binds the receptor-interacting protein RIP (Chen & Goeddel 2002). This results in the activation of TRAF2 which is responsible for activation of NF $\kappa$ B and MAP kinases. In contrast, IL-1 stimulation of the IL-1 receptor initiates recruitment of IL-1R activated kinase (IRAK)-1 and -4 which activates TRAF6 and subsequently activates the TGF- $\beta$ -activated kinase 1 (TAK1)/TAK1 binding protein (TAB) complex which is required for NF $\kappa$ B and MAP kinase activation. Based on experiments with INS-1 cells it seems likely that SOCS-3 inhibit IL-1 $\beta$  signalling in beta cells through binding to the TAK/TAB complex but the exact mechanism of SOCS-3 inhibition of IL-1 $\beta$ -induced signalling is not yet fully understood (Frobøse *et al.* 2006).

The aim of this study was to investigate the effect of SOCS-3 on TNF $\alpha$  induced cell death, MAP kinase and NF $\kappa$ B activity in beta cells and to evaluate TNF $\alpha$  induced *Socs-3* expression in beta cells. We found that INS-1 cell death induced by TNF $\alpha$  alone or in combination with IL-1 $\beta$  was suppressed by SOCS-3 expression. In INS-1 cells TNF $\alpha$ -induced p38 phosphorylation was reduced by SOCS-3. In primary rat beta cells TNF $\alpha$  induced phosphorylation of p38, JNK and ERK and SOCS-3 inhibited TNF $\alpha$ -induced phosphorylation of all three MAP kinases. I $\kappa$ B degradation and NF $\kappa$ B DNA binding and activity induced by TNF $\alpha$  was repressed by SOCS-3 in INS-1 and finally, exposure of primary rat islet cells to TNF $\alpha$  led to up-regulation of *Socs-3* mRNA expression.

## RESEARCH DESIGN AND METHODS

*Cell culture and cytokines* - INS-1 cells with doxycycline-inducible *Socs-3* expression (INS-r3#2) were generated and cultured as previously described (Karlsen *et al.* 2001). For analysis of TNF $\alpha$  and IL-1 $\beta$ -induced effects the INS-1 and islet cells were stimulated for 15 min with 8 ng/ml recombinant human TNF $\alpha$  ( $\geq 2 \times 10^4$  U/ $\mu$ g) (Endogen, Cambridge, MA, USA) or 150 pg/ml recombinant mouse IL-1 $\beta$  ( $5-15 \times 10^5$  U/ $\mu$ g) (BD Pharmingen, San Diego, CA, USA).

*Socs-3 expression systems* – In order to induce *Socs-3* expression and SOCS-3 protein production in INSR3#2 cells the cells were cultured in the presence of 1  $\mu$ g/ml doxycycline (Sigma-Aldrich, Saint Louis, MO, USA) for 24 hrs. *Socs-3* expression was induced in rat islet cells by transduction with a *Socs-3*-encoding adenoviral construct at a final concentration of  $5 \times 10^8$  pfu/ml. A *Luciferase*-encoding adenoviral construct was used as control. After 2 days of incubation with Adenovirus the cells were stimulated with TNF $\alpha$ . (Jacobsen 2009). The level of SOCS-3 expression following doxycycline induction was comparable to that obtained after cytokine stimulation of INS-1 cells and can thus be considered to be physiological relevant. (Rønn *et al.*). By adenoviral transduction of primary islet cells SOCS-3 expression was found in more than 97 % of beta cells and the level of SOCS-3 mRNA expression was approximately two times higher compared to that obtained using doxycycline induced expression (Jacobsen *et al.*)

*Rat islet isolation and culture* - Islets were isolated from neonatal (5- to 7-day-old) Wistar Furth rats (Taconic, Rye, Denmark) as described (Brunstedt 1980). Following isolation, islets



were precultured for 5-7 days in RPMI 1640 glutamax-1 (Gibco BRL, Paisley, Scotland) supplemented with 10% foetal calf serum (FCS) (Gibco BRL), 100 U/ml Penicillin and 100 µg/ml Streptomycin (Gibco BRL), in an atmosphere of air with 5% CO<sub>2</sub> at 37°C. The islets were dispersed into single cells using 0.2% trypsin, 10 mM EDTA (Gibco BRL) in 1 x HANKS. Immediately hereafter, the islet cells were exposed to adenovirus.

*Human islet culture* - Human islets were obtained from Olle Korsgren, the Department of Clinical Immunology, Rudbeck Laboratory, Uppsala University Hospital, Sweden through the Juvenile Diabetes Research Foundation (JDRF) Islet Distribution Program. Islets used in this study were obtained from a 59 year old female cadaveric donor and had a purity of 45%. Islets were cultured in RPMI 1640 without glucose (Gibco BRL) supplemented with 5.6 mM D-glucose (Sigma-Aldrich), 10% FCS and 100 U/ml Penicillin and 100 µg/ml Streptomycin, in an atmosphere of air with 5% CO<sub>2</sub> at 37°C. Before stimulation islets were transferred to 100 mm petri-dishes (1200 islets per dish) in RPMI 1640 supplemented with 2% human serum (Lonza (BioWhittaker), Basel, Switzerland).

*Apoptosis assay*: Apoptosis was measured by examination of the presence of cytoplasmic histone-associated DNA fragments according to the manufacturer's instructions (Cell Death Detection ELISA<sub>PLUS</sub>, Roche, Indianapolis, IN, USA). In short, INS-r3#2 cells, seeded at a density of  $0.5 \times 10^6$  in 48-well culture plates (Nalge Nunc International, Rochester, NY, USA), were cultured for 48 hrs prior addition of doxycycline. After 24 hrs incubation the cells were stimulated with 8 ng/ml TNF $\alpha$ , 150 pg/ml IL-1 $\beta$  or both for 24 hrs. Cell lysates were incubated with anti-DNAperoxidase and anti-histone-biotin and transferred to streptavidin-coated wells. Absorbance was measured after addition of peroxidase substrate ABTS.

*Western Blotting* - INS-r3#2: Cells were seeded at a density of  $10^6$  cells/well in 6-well culture plates (Nalge Nunc International). Rat islets: Approximately 400 islets were used per condition. Lysis and Western Blotting was performed as described (Frobøse *et al.* 2006). Protein concentrations were measured and equal amounts were loaded in each condition. To detect expression levels of SOCS-3, JNK, p38, ERK1/2, I $\kappa$ B, and  $\beta$ -actin and phosphorylation of JNK, p38, and ERK1/2 we used following specific primary antibodies: (rabbit-anti-p-JNK/JNK IgG, rabbit-anti-p-p38/p38 IgG, rabbit-anti-p-ERK1/2/ERK1/2 IgG (Cell Signalling Technologies, Cambridge, MA, USA), mouse-anti-I $\kappa$ B IgG (Active Motif, San Francisco, CA, USA), mouse-anti-Flag IgG (Sigma Aldrich), and mouse-anti- $\beta$ -actin (Abcam, Cambridge, UK). Secondary antibodies were either goat anti-rabbit IgG or horse anti-mouse IgG conjugated to horseradish peroxidase (HRP) (Cell Signalling Technologies).  $\beta$ -actin was used as loading control. The protein of interest was detected by a chemiluminescence detection system (Lumi-GLO; Cell Signalling Technologies) and visualized using an imaging system (Las3000; Fuji Film, Tokyo, Japan).

*NF $\kappa$ B promoter activity* - INS-r3#2 cells were seeded at a density of  $1.5 \times 10^5$  cells/well in 24-well culture dishes and cultured for two days in RPMI 1640 containing 10% FCS, transfected and exposed to doxycycline. Transient transfection was performed for 4 hrs with a total of 2  $\mu$ g of plasmid DNA (0.2  $\mu$ g of an internal control (pRL-TK), 0.4  $\mu$ g of *MnSOD* promoter construct (kindly provided by M. Darville and D. Eizirik, Brussels, Belgium (Darville *et al.* 2000)) and 1.4  $\mu$ g of empty vector (pcDNA3, Invitrogen)) using SuperFect (Qiagen, Hilden, Germany) according to the manufacturer's manual. Culture was continued overnight in medium containing 0.5% FCS in the presence or absence of doxycycline. The cells were cultured for an additionally 6 hrs in the absence or presence of TNF $\alpha$  and doxycycline. Luciferase activities were assayed with the Dual-Luciferase Reporter Assay System

(Promega, Madison) according to the manufacturer's description and measured using a Luminometer (Berthold Technologies, Bad Wildbad, Germany). *MnSOD* promoter activity was normalised to the activity of co-expressed *Renilla luciferase*.

*Nuclear extraction and electrophoretic mobility-shift assay (EMSA) of NFκB binding* - INS-1#2 cells were seeded at a density of  $4 \times 10^6$  cells in 100-mm dishes and cultured for 2 days. Culture was continued with 0.5% FCS with or without doxycycline for 24 hrs prior exposure to TNFα. Nuclear extracts were isolated and EMSA was performed as described (Ronn *et al.* 2002). A double-stranded oligonucleotide (5'-agctAGCTTCAGAGGGGACTTTCCGAGAGG-3' (+ strand), DNA Technology A/S, Aarhus, Denmark) was used to detect NFκB DNA binding. In super-shift analysis, nuclear extracts were pre-incubated for 30 min at 4°C with a NFκB(p65) antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). In competition experiments a 10 time excess of unlabeled NFκB oligonucleotide was added to the binding reaction.

*Real-Time PCR* – Neonatal rat or human islets were exposed to TNFα for 1, 2, 4, or 24 hrs and total RNA was extracted using the TRIZOL method (Invitrogen). cDNA synthesis was performed by the TaqMan Gold RT-PCR Kit (Perkin-Elmer, Boston, MA, USA). Primers used for the real-time PCR reaction were: rat *Socs-3*, forward 5' TGAGCGTCAAGACCCAGTCG-3', and reverse 5'- CACAGTCGAAGCGGGGAACT-3', 114 bp; human *SOCS-3*, forward 5'- ACACGCACTTCCGCACATTC-3' and reverse 5'- CGAGGCCATCTTCACGCTAA-3', 110 bp, rat *Sp1*, forward 5'- GGCTACCCCTACCTCAAAGG-3', and reverse 5'-CACAACATACTGCCACCAG-3', 103 bp, human *GAPDH*, forward 5'- GGTGAAGGTCGGAGTCAAC-3', and reverse 5'- CCATGGGTGGAATCATATTG-3', 154 bp.

The experiments were performed as described in the manual for ABI Prism 7700 Sequence Detector (AB Applied Biosystems) with minor modifications. Each cDNA sample in duplicate was subjected to two individual PCR analyses using either the *Socs-3/SOCS-3* or the *Sp1/GAPDH* primer pair. For the Real-Time analysis, every PCR reaction was amplified in a PCR Mastermix supplemented with the DNA binding dye SYBR Green (Applied Biosystems, Forster City, CA, USA).

*Statistical analysis* - Results are given as means + SEM. Comparisons versus the respective control groups were made by Student's paired *t*-test. A *p* value of less than 0.05 was considered statistically significant.

## RESULTS

*SOCS-3 suppresses beta cell death induced by TNF $\alpha$  and IL-1 $\beta$  alone or in combination.* In order to study the effect TNF $\alpha$  on beta cell apoptosis and the ability of SOCS-3 to inhibit a potential effect of TNF $\alpha$  we used the INS-1 beta cell line with inducible *Socs-3* expression (INS-r3#2). Exposure of INS-1 cells to 8 ng/ml TNF $\alpha$  for 24 hrs. induced a 1.6-fold increase in beta cell apoptosis. Expression of SOCS-3 significantly suppressed TNF $\alpha$ -induced apoptosis by 62% (Fig. 1). Similarly, 150 pg/ml IL-1 $\beta$  induced a 2.1-fold increase in beta cell death that were reduced by 75% by SOCS-3. When TNF $\alpha$  and IL-1 $\beta$  were applied in combination we observed a 2.7-fold increase in cell death and SOCS-3 expression reduced this by 59%.

*SOCS-3 expression suppresses TNF $\alpha$ -induced MAP kinase activation in beta cells.* Activation of the MAP kinases (ERK1/2, JNK, and p38) after stimulation with TNF $\alpha$  was analyzed by measuring their phosphorylation by Western blotting using phospho-specific antibodies. TNF $\alpha$  significantly induced ( $p=0.04$ ) phosphorylation of p38 but did not affect JNK and ERK1/2 phosphorylation in the beta cell line (Fig. 2 panels A and B). Doxycycline-induced SOCS-3 suppressed TNF $\alpha$ -induced phosphorylation of p38 without affecting the total amount of p38 protein. A similar induction of p38 phosphorylation was observed in IL-1 stimulated cells and this phosphorylation was also reduced by SOCS-3. In INS-r3#2 cells the basal phosphorylation level of ERK was high compared to primary rat beta cells, even when cultured in 0.5% serum. Higher concentrations of TNF $\alpha$  induced a more marked phosphorylation of p38 but did not induce phosphorylation of JNK and ERK (data not shown). In order to confirm and extend the results obtained in the beta cell line, we analyzed the effect of SOCS-3 on MAP kinase activation in response to TNF $\alpha$  in primary rat islets exposed to *Luciferase*- or *Socs-3*-encoding Adenovirus (Fig. 3). TNF $\alpha$  significantly induced

phosphorylation of not only p38 ( $p=0.01$ ) but also JNK ( $p=0.003$ ) and ERK1/2 ( $p=0.02$ ) in islet cells. SOCS-3 induced by *Socs-3*-encoding Adenovirus completely prevented the TNF $\alpha$ -induced phosphorylation of all three MAP kinases without affecting the total amount of MAP kinase protein. In contrast, islets transduced with a control *Luciferase*-encoding Adenovirus exhibited TNF $\alpha$ -induced MAP kinase phosphorylation similar to non-transduced islet cells (Fig. 3).

*SOCS-3 expression suppresses TNF $\alpha$ -induced I $\kappa$ B degradation in beta cells.*

In order to investigate if SOCS-3 also regulates TNF $\alpha$ -induced activation of the NF $\kappa$ B pathway we analyzed I $\kappa$ B degradation in response to TNF $\alpha$  in INSr3#2 and rat islet cells. After a 15 min exposure to TNF $\alpha$  a significant decrease in the level of I $\kappa$ B was observed in both INSr3#2 cells ( $p=0.006$ ) (Fig. 4 panel A and C) as well as in primary rat islet cells ( $p=0.006$ ) (Fig. 4 panel B and D). This degradation was significantly inhibited by SOCS-3 in both cell types. IL-1 $\beta$  was included as a control in the INSr3#2 experiments and was as expected able to induce I $\kappa$ B degradation in INS-1 cells in a SOCS-3 sensitive manner (Fig. 4A).

*SOCS-3 expression inhibits TNF $\alpha$ -induced NF $\kappa$ B DNA-binding activity in INS-1 cells.*

We next analyzed the possible role of SOCS-3 on TNF $\alpha$ -induced NF $\kappa$ B DNA binding activity. Nuclear extracts from INSr3#2 cells stimulated with TNF $\alpha$  in the absence or presence of SOCS-3 were isolated and analyzed by EMSA using a radio-labelled NF $\kappa$ B binding probe. As shown in Fig. 5A, TNF $\alpha$  induced an increase in NF $\kappa$ B DNA binding in the absence of induced SOCS-3 production whereas in the presence of SOCS-3 no increase in NF $\kappa$ B binding could be observed. Incubation with non-labelled oligonucleotide showed

specific competition of binding between radio labelled probe and protein and super-shift using a p65 antibody confirmed the presence of NF $\kappa$ B in the complex.

*SOCS-3 expression reduces TNF $\alpha$ -induced MnSOD promoter activity.*

NF $\kappa$ B regulate transcription of a wide range of genes by binding to specific sequences in the promoter regions. A classical example of a NF $\kappa$ B responsive gene is *MnSOD* (Wong 1988). We used a reporter construct containing the *MnSOD* promoter to investigate whether SOCS-3 could inhibit TNF $\alpha$ -induced gene transcription. As seen in Fig. 5B, TNF $\alpha$  induced *MnSOD* promoter activity 2.2 fold and this stimulation was reduced by 40% in the presence of SOCS-3.

*TNF $\alpha$  induces SOCS-3 expression in rat islets.*

In order to investigate if SOCS-3 not only regulates TNF $\alpha$  signalling but also itself is regulated by TNF $\alpha$  we analysed the level of *Socs-3* mRNA expression in primary rat islets after exposure to TNF $\alpha$  for up to 24 hours. Based on three independent experiments we observed that TNF $\alpha$  induced a rapid and transient increase in expression of *Socs-3* mRNA with a maximal (5-21-fold) induction observed after 1-2 hrs and *Socs-3* mRNA levels returning to near basal levels after 2-4 hrs (Fig. 6). Finally, we investigated TNF $\alpha$ -induced *SOCS-3* mRNA expression in human islets from one donor with an islet cell purity of 45% and found the same *SOCS-3* mRNA expression pattern as observed in rat islets with a maximal expression (2-fold compared to control) after 2-3 hours of exposure to TNF $\alpha$  (data not shown).

## DISCUSSION

The results from this study show that SOCS-3 is able to suppress TNF $\alpha$ -induced signalling mediated through the NF $\kappa$ B and MAP kinase pathways in primary islets and in the INSr3#2 beta cell line. We observed that expression of SOCS-3 in INS-1 cells inhibited beta cell death induced by TNF $\alpha$ . Since it is well established that TNF $\alpha$  stimulation activates signalling pathways leading to activation of MAP kinases and NF $\kappa$ B we next sought to investigate the effect of SOCS-3 expression on these central signalling pathways. SOCS-3 inhibited TNF $\alpha$ -induced MAP kinase phosphorylation, prevented TNF $\alpha$ -induced I $\kappa$ B degradation and reduced NF $\kappa$ B DNA binding and *MnSOD* promoter activity. These data indicate that SOCS-3 inhibits TNF $\alpha$  signalling in beta cells at a level upstream to the branching point between MAP kinases and NF $\kappa$ B and suggest that SOCS-3 is able to protect cells from the potential harmful effects of TNF $\alpha$  signalling.

In un-stimulated INS-1 and primary rat beta cells the basal expression level of *Socs-3* is low (Karlsen *et al.* 2001), but upon exposure to cytokines *Socs-3* expression is up-regulated in a cytokine-, cell type-, and time-dependent manner (Krebs & Hilton 2001; Rønn *et al.* 2008). We have previously compared the level of endogenous SOCS-3 protein in cytokine-stimulated beta cells with production of exogenous SOCS-3 in beta cells transfected with a *Socs-3*-encoding plasmid or transduced with a *Socs-3*-encoding Adenovirus. Compared to cytokine-induced endogenous levels the SOCS-3 protein level was slightly higher in cells transduced with *Socs-3*-encoding Adenovirus (N. Billestrup, unpublished observations) but comparable in cells transfected with *Socs-3*-encoding plasmids (Karlsen *et al.* 2001). Based on these studies we believe that the induced levels of SOCS-3 used in these studies lie within physiologically relevant levels. However, the slightly higher SOCS-3 levels in the Adv- *Socs-3* transduced rat islets could in part explain the more profound SOCS-3 effects on MAP



kinase phosphorylation observed in rat islet cells compared to INSr3#2 cells. Increasing the concentration of doxycycline above 1  $\mu\text{g/ml}$  does not induce the SOCS-3 protein production in INSr3#2 cells further. However, the differences in the ability of SOCS-3 to inhibit MAP kinase activation in INS-1 cell compared to primary rat islets might also be explained by fundamental differences between these cells. As INS-1 are transformed insulinoma cells, it is possible that deregulation of the MAP kinases is present in transformed tumor cells. Indeed ERK is known to be an important regulator of cell proliferation in general and is often found to be up-regulated in tumor cells. In INS-1 cells we observe an increased phosphorylation of ERK under basal conditions when compared to primary islets.

It has previously been shown that exposure of primary rat beta-cells to IL-1 $\beta$  markedly induced *Socs-3* mRNA with a maximum after 4 hrs of stimulation, whereas IFN $\gamma$  induced a transient up-regulation of *Socs-3* mRNA peaking at 1 hr (Rønn *et al.* 2008). In the present study we observed that TNF $\alpha$  like IFN $\gamma$  induced a rather rapid and transient expression of *Socs-3* peaking after 1-2 hours in primary rat beta cells. We found that TNF $\alpha$ -induced *SOCS-3* expression in human islets reached its maximum after two hours. Although the level of expression was lower than observed in rat islets the kinetics was in agreement between the two species. The lower level of *SOCS-3* RNA expression in human islets versus rat islets might be explained by a generally lower content of beta cells in human islets compared to rat islets and by the less pure fraction of islets in the preparation. In addition, differences in species and in age of the islet donors (rat islets were from 4-5 days old rats and human islets were from a 59 year old donor) might also affect *SOCS-3* expression. The differences in the kinetics of cytokine-induced *Socs-3* expression may account for the fact that stimulation with IL-1 $\beta$  alone in general is more cytotoxic to primary rat beta cells than IFN $\gamma$  and TNF $\alpha$ . In rat islet non-beta cells *Socs-3* expression induced by IL-1 $\beta$  reaches maximum after 1-2 hrs (Rønn

*et al.* 2008) which in part could explain why only the beta cells in the islets die when exposed to pro-apoptotic cytokines. Thus, insufficient or delayed *Socs-3* up-regulation could make beta cells more susceptible to cytokine-induced cell death.

The mechanisms by which SOCS-3 inhibits cytokine signalling are still being investigated and there are clearly several different ways depending on the target pathway. SOCS-3 inhibits JAK/STAT signalling by direct interaction with JAK after binding to phospho-tyrosine residues at receptors such as GH, Epo, LIF/IL-6, and leptin receptors (Krebs & Hilton 2001). Further, SOCS-3 has been shown to inhibit insulin signalling by binding to insulin receptor substrate-1 and -2 thereby facilitating their proteasomal degradation (Rui *et al.* 2002). However, the mechanism by which SOCS-3 inhibits TNF $\alpha$  signalling is still not clear.

In summary, we have shown that SOCS-3 inhibited TNF $\alpha$ -induced INS-1 cell death and that SOCS-3 inhibited TNF $\alpha$ -induced MAP kinase phosphorylation and NF $\kappa$ B activation, suggesting a novel inhibitory pathway by which the magnitude and duration of TNF $\alpha$  action in beta cells is regulated. Since SOCS-3 is able to inhibit signalling induced by the three major pro-inflammatory cytokines (IL-1 $\beta$ , TNF $\alpha$  and IFN- $\gamma$ ) known to mediate beta cell destruction in type 1 diabetes, SOCS-3 could serve as a possible therapeutic target in the prevention and treatment of diabetes.

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## Reference List

1. Adams TE, Hansen JA, Starr R, Nicola NA, Hilton DJ & Billestrup N 1998 Growth hormone preferentially induces the rapid, transient expression of SOCS-3, a novel inhibitor of cytokine receptor signaling. *Journal of biological chemistry* **273** 1285-1287.
2. Alexander WS, Starr R, Fenner JE, Scott CL, Handman E, Sprigg NS, Corbin JE, Cornish AL, Darwiche R, Owczarek CM, Kay TW, Nicola NA, Hertzog PJ, Metcalf D & Hilton DJ 1999 SOCS1 is a critical inhibitor of interferon gamma signaling and prevents the potentially fatal neonatal actions of this cytokine. *Cell* **98** 597-608.
3. Ammendrup A, Maillard A, Nielsen K, Aabenhus Andersen N, Serup P, Dragsbæk Madsen O, Mandrup-Poulsen T & Bonny C 2000 The c-Jun amino-terminal kinase pathway is preferentially activated by interleukin-1 and controls apoptosis in differentiating pancreatic beta-cells. *Diabetes* **49** 1468-1476.
4. Bonny C, Oberson A, Negri S, Sauser C & Schorderet DF 2001 Cell-permeable peptide inhibitors of JNK: novel blockers of beta-cell death. *Diabetes* **50** 77-82.
5. Bonny C, Oberson A, Steinmann M, Schorderet DF, Nicod P & Waeber G 2000 IB1 reduces cytokine-induced apoptosis of insulin-secreting cells. *Journal of biological chemistry* **275** 16466-16472.
6. Brunstedt J 1980 Rapid isolation of functionally intact pancreatic islets from mice and rats by percoll<sup>TM</sup> gradient centrifugation. *Diabète & métabolisme* **6** 87-89.

7. Chen GQ & Goeddel DV 2002 TNF-R1 signaling: A beautiful pathway. *Science (New York, N.Y.)* **296** 1634-1635.
8. Chong MMW, Thomas HE & Kay TW 2002 Suppressor of cytokine signaling-1 regulates the sensitivity of pancreatic beta cells to tumor necrosis factor. *The Journal of biological chemistry* **277** 27945-27952.
9. Darville MI & Eizirik DL 1998 Regulation by cytokines of the inducible nitric oxide synthase promoter in insulin-producing cells. *Diabetologia* **41** 1101-1108.
10. Darville MI, Ho YS & Eizirik DL 2000 NF-kappaB is required for cytokine-induced manganese superoxide dismutase expression in insulin-producing cells. *Endocrinology* **141** 153-162.
11. Devin A, Cook A, Lin Y, Rodriguez Y, Kelliher M & Liu Z 2000 The distinct roles of TRAF2 and RIP in IKK activation by TNF-R1: TRAF2 recruits IKK to TNF-R1 while RIP mediates IKK activation. *Immunity* **12** 419-429.
12. Eizirik DL & Mandrup-Poulsen T 2001 A choice of death--the signal-transduction of immune-mediated beta-cell apoptosis. *Diabetologia* **44** 2115-2133.
13. Eldor R, Yeffet A, Baum K, Doviner V, Amar D, Ben-Neriah Y, Christofori G, Peled A, Carel JC, Boitard C, Klein T, Serup P, Eizirik DL & Melloul D 2006 Conditional and specific NF-kappa B blockade protects pancreatic beta cells from diabetogenic agents. *Proceedings of the National Academy of Sciences of the United States of America* **103** 5072-5077.
14. Emanuelli B, Peraldi P, Filloux C, Chavey C, Freidinger K, Hilton DJ, Hotamisligil GS & Van Obberghen E 2001 SOCS-3 inhibits insulin signaling and is up-regulated in response to tumor necrosis factor-alpha in the adipose tissue of obese mice. *Journal of biological chemistry* **276** 47944-47949.
15. Flodstrom M, Welsh N & Eizirik DL 1996 Cytokines activate the nuclear factor kappa-B (NF-kappa-B) and induce nitric oxide production in human pancreatic islets. *FEBS letters* **385** 4-6.

16. Frobøse H, Rønn SG, Heding PE, Mendoza H, Cohen P, Mandrup-Poulsen T & Billestrup N 2006 Suppressor of cytokine Signaling-3 inhibits interleukin-1 signaling by targeting the TRAF-6/TAK1 complex. *Molecular endocrinology (Baltimore, Md.)* **20** 1587-1596.
17. Giannoukakis N, Rudert WA, Trucco M & Robbins PD 2000 Protection of human islets from the effects of interleukin-1beta by adenoviral gene transfer of an Ikappa B repressor. *The Journal of biological chemistry* **275** 36509-36513.
18. Heimberg H, Heremans Y, Jobin C, Leemans R, Cardozo AK, Darville M & Eizirik DL 2001 Inhibition of cytokine-induced NF-kappa B activation by adenovirus-mediated expression of a NF-kappa B super-repressor prevents beta-cell apoptosis. *Diabetes* **50** 2219-2224.
19. Hsu H, Huang J, Shu HB, Baichwal V & Goeddel DV 1996 TNF-dependent recruitment of the protein kinase RIP to the TNF receptor-1 signaling complex. *Immunity* **4** 387-396.
20. Hsu H, Xiong J & Goeddel DV 1995 The TNF receptor 1-associated protein TRADD signals cell death and NF-kappa B activation. *Cell* **81** 495-504.
21. Jacobsen MLB 2009 IL-1 $\beta$ -induced chemokine and Fas expression are inhibited by suppressor of cytokine signalling-3 in insulin-producing cells. *Diabetologia* **52** 281-288.
22. Kanayama A, Seth RB, Sun L, Ea CK, Hong M, Shaito A, Chiu YH, Deng L & Chen ZJ 2004 TAB2 and TAB3 activate the NF-kappaB pathway through binding to polyubiquitin chains. *Molecular cell* **15** 535-548.
23. Karlsen AE, Heding PE, Frobøse H, Rønn SG, Kruhoffer M, Orntoft TF, Darville M, Eizirik DL, Pociot F, Nerup J, Mandrup-Poulsen T & Billestrup N 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.
24. Karlsen AE, Rønn SG, Lindberg K, Johannesen J, Galsgaard ED, Pociot F, Nielsen JH, Mandrup-Poulsen T, Nerup J & Billestrup N 2001 Suppressor of cytokine signaling 3 (SOCS-3) protects beta-cells against interleukin-1 beta- and interferon-gamma-

- mediated toxicity. *Proceedings of the National Academy of Sciences of the United States of America* **98** 12191-12196.
25. Krebs DL & Hilton DJ 2001 SOCS proteins: Negative regulators of cytokine signaling. *Stem cells (Dayton, Ohio)* **19** 378-387.
  26. Larsen L, Storling J, Darville M, Eizirik DL, Bonny C, Billestrup N & Mandrup-Poulsen T 2005 Extracellular signal-regulated kinase is essential for interleukin-1-induced and nuclear factor kappa B-mediated gene expression in insulin-producing INS-1E cells. *Diabetologia* **48** 2582-2590.
  27. Mandrup-Poulsen T, Bendtzen K, Dinarello CA & Nerup J 1987 Human tumor necrosis factor potentiates human interleukin 1-mediated rat pancreatic beta-cell cytotoxicity. *Journal of immunology (Baltimore, MD)* **139** 4077-4082.
  28. Rønn SG, Börjesson A, Bruun C, Heding PE, Frobøse H, Mandrup-Poulsen T, Karlens AE, Rasschaert J, Sandler S & Billestrup N 2008 Suppressor of cytokine signalling-3 expression inhibits cytokine-mediated destruction of primary mouse and rat pancreatic islets and delays allograft rejection. *Diabetologia* **51** 1873-1882.
  29. Rønn SG, Hansen JA, Lindberg K, Karlens AE & Billestrup N 2002 The effect of suppressor of cytokine signaling 3 on GH signaling in beta-cells. *Molecular endocrinology* **16** 2124-2134.
  30. Rui L, Yuan M, Frantz D, Shoelson S & White MF 2002 SOCS-1 and SOCS-3 block insulin signaling by ubiquitin-mediated degradation of IRS1 and IRS2. *The Journal of biological chemistry* **277** 42394-42398.
  31. Saldeen J 2000 Cytokines induce both necrosis and apoptosis via a common Bcl-2-inhibitable pathway in rat insulin-producing cells. *Endocrinology* **141** 2003-2010.
  32. Stanger BZ, Leder P, Lee TH, Kim E & Seed B 1995 RIP: a novel protein containing a death domain that interacts with Fas/APO-1 (CD95) in yeast and causes cell death. *Cell* **81** 513-523.
  33. Stoiber D, Kovarik P, Cohn S, Johnston JA, Steinlein P & Decker T 1999 Lipopolysaccharide induces in macrophages the synthesis of the suppressor of cytokine

signaling 3 and suppresses signal transduction in response to the activating factor IFN-gamma. *The journal of immunology* **163** 2640-2647.

34. Thomas HE & Kay TWH 2000 Beta cell destruction in the development of autoimmune diabetes in the non-obese diabetic (NOD) mouse. *Diabetes-Metabolism Research and Reviews* **16** 251-261.
35. Wong GH 1988 Induction of manganous superoxide dismutase by tumor necrosis factor: possible protective mechanism. *Science (New York, N.Y.)* **242** 941-944.

## FIGURE LEGENDS

Fig. 1. SOCS-3 inhibition of TNF $\alpha$ - and IL-1-induced apoptosis in INSr3#2 cells. INSr3#2 cells were pre-incubated with (black bars) or without doxycycline (Dox) (open bars) and stimulated for 24 hrs with 8 ng/ml TNF $\alpha$ , 150 pg/ml IL-1 $\beta$  or the combination. Apoptosis was measured as cytoplasmic appearance of histone-associated DNA fragments. Data are shown as fold induction compared to the control without SOCS-3 expression and are presented as means + SEM of four independent experiments. \*:  $p < 0.05$ , \*\*:  $p < 0.01$ .

Fig. 2. Effect of SOCS-3 on TNF $\alpha$ - and IL-1 $\beta$ -induced MAP kinase phosphorylation in INSr3#2 cells. INSr3#2 cells were pre-incubated with (black bars) or without (open bars) doxycycline (Dox) prior to stimulation for 15 min with either TNF $\alpha$  (8 ng/ml) or IL-1 $\beta$  (150 pg/ml). Cell lysates were subjected to Western blot analysis using specific antibodies directed against phospho- or total MAP kinases (p38, JNK, ERK1/2) in order to determine MAP kinase activation. Total MAP kinase protein served as loading controls. SOCS-3 production was confirmed using an antibody towards flag-tagged SOCS-3. A: The figures are representative immunoblots of three to four experiments. B: Phospho-p38 bands quantified by densitometry and expressed as fold-change compared to -TNF $\alpha$ /-Dox cells. Values are means

+ SEM determined from three to four experiments. \* $p < 0.05$ , vs. TNF $\alpha$ -stimulated cells not expressing *Socs-3*.

Fig. 3. Effect of SOCS-3 on TNF $\alpha$ -induced MAP kinase phosphorylation in rat islets. Dispersed rat islet cells were transduced with either; No virus (open bars), *Luciferase*-encoding Adenovirus (Adv-Luc) (grey bars), or *Socs-3*-encoding Adenovirus (Adv-SOCS-3) (black bars). The cells were either stimulated or not with 8 ng/ml TNF $\alpha$  for 15 min. Cell lysates were subjected to Western blot analysis using specific antibodies directed against phospho- or total MAP kinases (p38, JNK, ERK1/2) in order to determine MAP kinase activation. Total MAP kinase protein served as loading controls. A: Representative immunoblots of three independent experiments. B-D: Quantification by densitometry of phosphorylation of p38 (B), JNK (C) and ERK (D). Data are presented as means + SEM and shown as fold change compared to cells transduced with the control-virus and exposed to TNF $\alpha$ . \* $p < 0.05$ , vs. TNF $\alpha$ -stimulated cells not expressing *Socs-3*.

Fig. 4. Effect of SOCS-3 on TNF $\alpha$ - and IL-1 $\beta$ -induced I $\kappa$ B degradation in INSR3#2 and rat islet cells. A: INSR3#2 cells were pre-incubated with or without doxycycline (Dox) prior to stimulation for 15 min with either TNF $\alpha$  (8 ng/ml) or IL-1 $\beta$  (150 pg/ml). B: Dispersed rat islet cells were transduced with either; No virus, *Luciferase*-encoding Adenovirus (Adv-Luc), or *Socs-3*-encoding Adenovirus (Adv-SOCS-3). The cells were stimulated with or without 8 ng/ml TNF $\alpha$  for 15 min. Cell lysates were subjected to Western blot analyses using specific antibodies directed against I $\kappa$ B in order to determine I $\kappa$ B degradation. Equal loading was verified by blotting against  $\beta$ -actin. A-B: Representative blots of three independent experiments. C-D: Quantification by densitometry of I $\kappa$ B in: (C) INSR3#2 cells with data



presented as means + SEM and shown as fold-change compared to  $-TNF\alpha$ -Dox cells. Cells not treated with dox (open bars) and dox-treated cells (black bars), (D) rat islet cells with data presented as means + SEM and shown as fold-change compared to cells transduced with the control-virus and exposed to  $TNF\alpha$ . Non-transduced cells (open bars), cells transduced with Adv-Luc (grey bars) and Adv-SOCS-3 (black bars). \* $p < 0.05$ , vs.  $TNF\alpha$ -stimulated cells not expressing *Socs-3*.

Fig. 5. Effect of SOCS-3 on  $TNF\alpha$ -induced NF $\kappa$ B-binding and *MnSOD* promoter activity. A: INSr3#2 cells were pre-incubated with or without doxycycline (Dox) and stimulated for 15 min with 8 ng/ml  $TNF\alpha$ . Nuclear extracts were prepared and EMSA was performed using an NF $\kappa$ B binding probe. The figure is representative of four independent experiments. For competition a 10-fold excess of specific competitor (10 x sc) was used. Supershift was performed by use of a p65 subunit specific NF $\kappa$ B antibody ( $\alpha$ -p65). B: INSr3#2 cells were transfected with a *MnSOD* promoter reporter construct and a *Renilla* control plasmid and cultured in the presence or absence of doxycycline. The cells were stimulated for 6 hrs with or without 8 ng/ml  $TNF\alpha$  and luciferase activity was measured. Data are shown as the ratio of Luciferase/*Renilla* with both controls set to 1 and presented as means + SEM of four independent experiments. \*:  $p < 0.05$ , vs.  $TNF\alpha$ -stimulated cells not expressing *Socs-3*.

Fig. 6.  $TNF\alpha$ -induced *Socs-3* expression in primary rat islet-cells. Rat islet-cells were stimulated with 8 ng/ml  $TNF\alpha$  for up to 24 hrs. Total RNA was isolated, reverse-transcribed and *Socs-3* expression was quantified by Real-Time PCR. To correct for variation in input RNA, data were normalized to the expression level of *Sp-1*. Data from each of three

independent experiments are presented as fold-change compared to controls. Experiment (Exp.) 1 (diamonds), Exp.2 (squares), Exp.3 (triangles).

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Figure 1

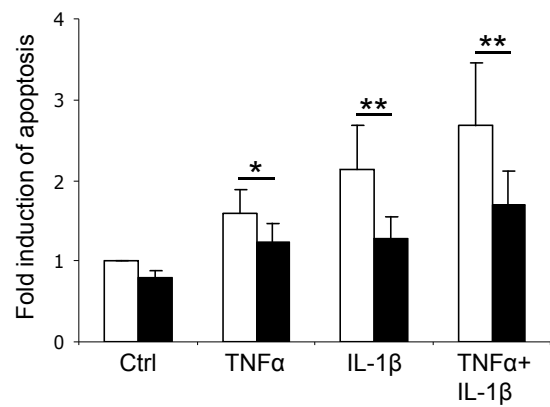


Figure 2

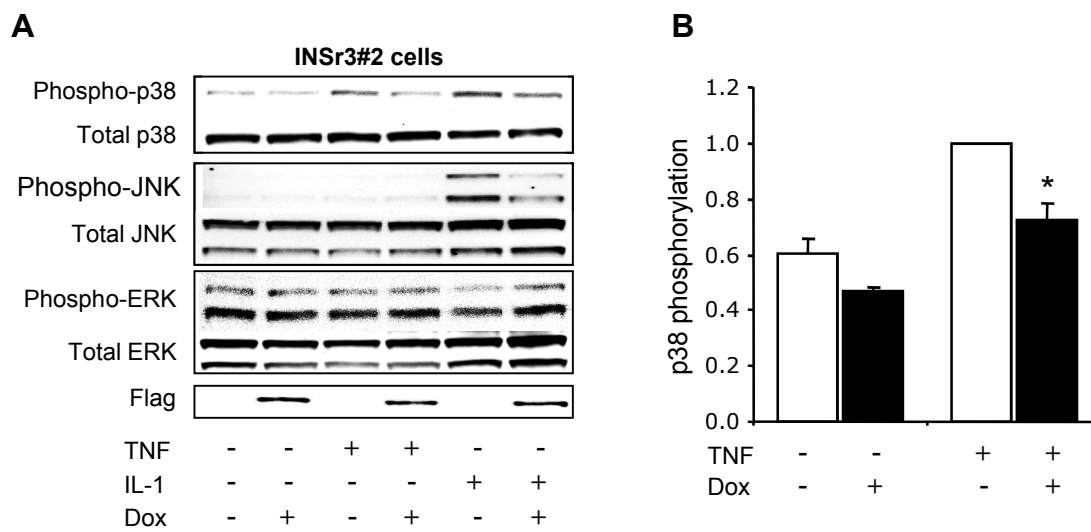


Figure 3

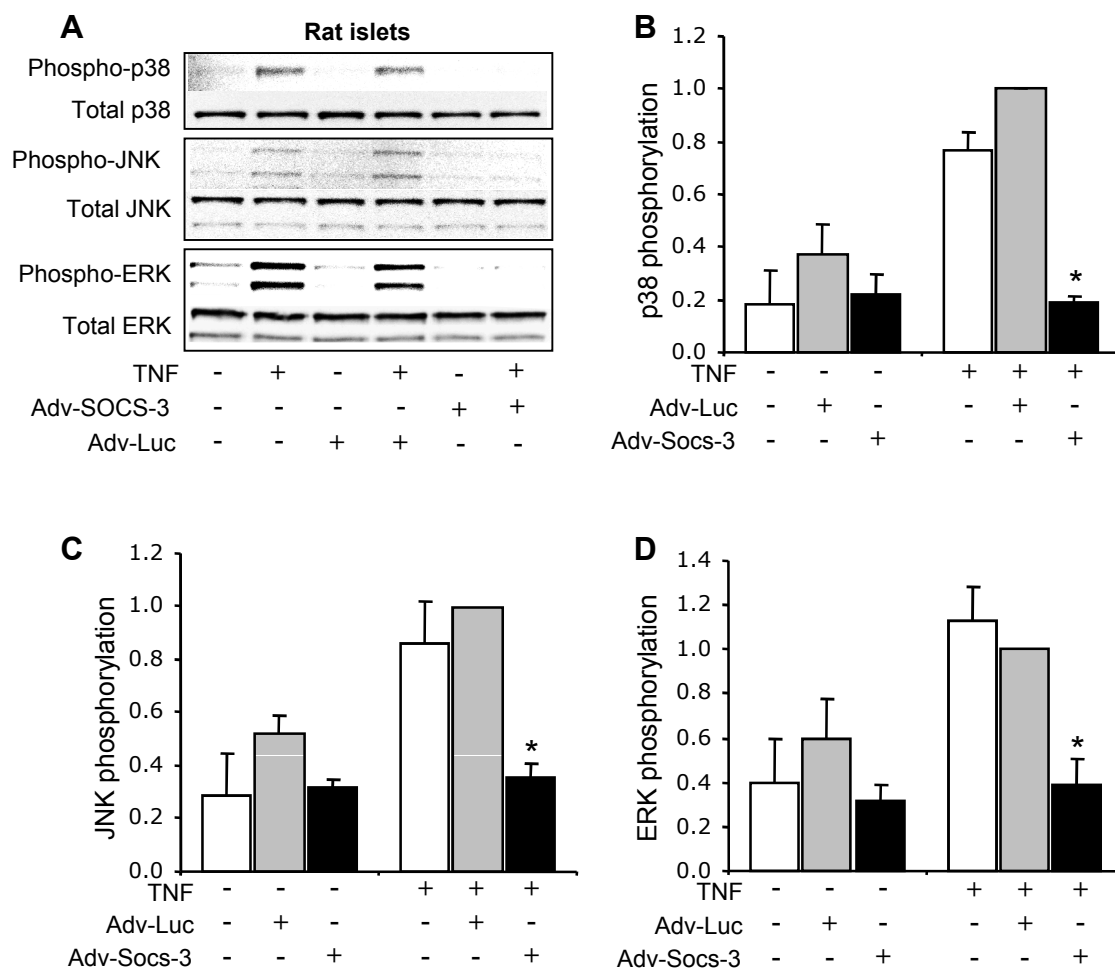


Figure 4

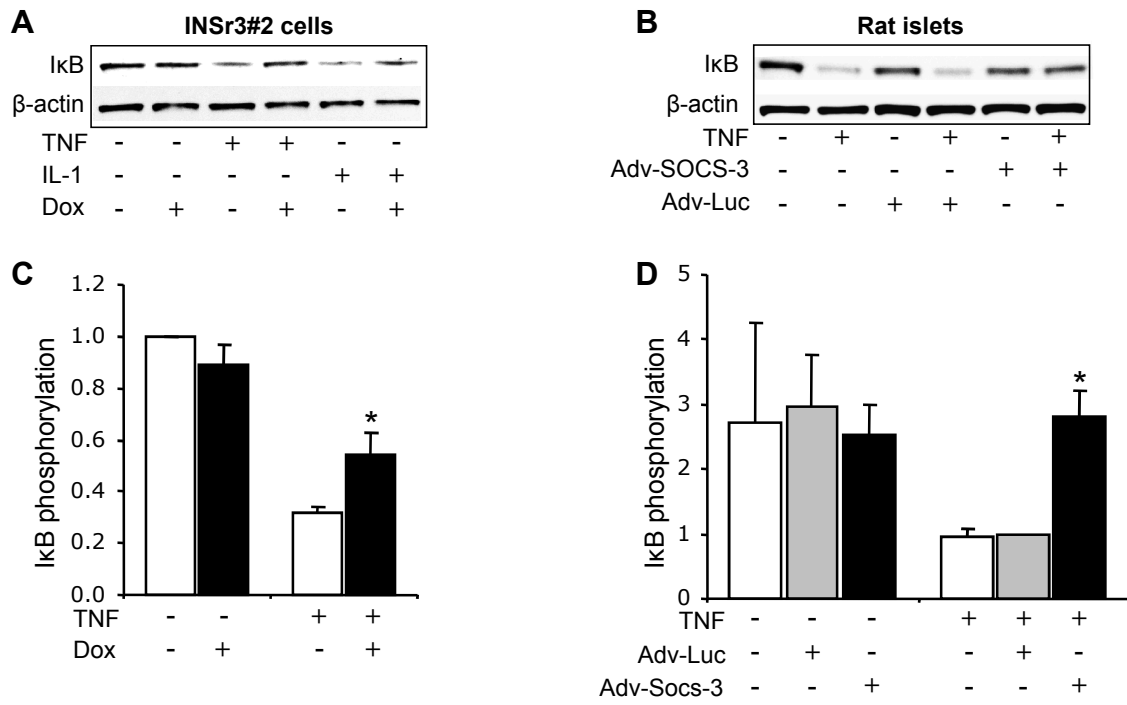


Figure 5

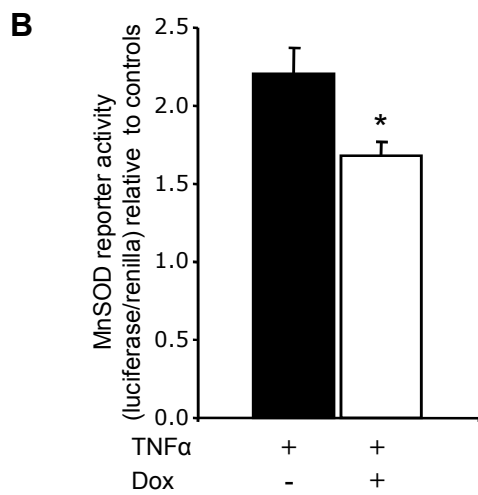
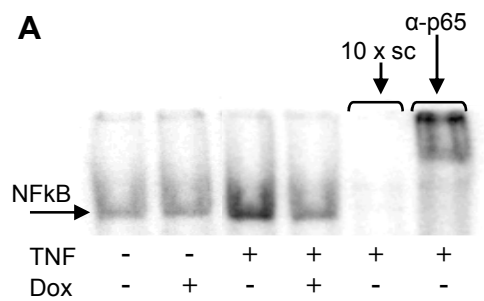


Figure 6

