

# $\gamma$ -Interferon Signaling in Pancreatic $\beta$ -Cells Is Persistent but Can Be Terminated by Overexpression of Suppressor of Cytokine Signaling-1

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**Proinflammatory cytokines, including  $\gamma$ -interferon (IFN- $\gamma$ ), have been implicated in the destruction of  $\beta$ -cells in autoimmune diabetes. IFN- $\gamma$  signaling is transient in some cell types, but there is indirect evidence that it may be prolonged in  $\beta$ -cells. In this study, we have shown that IFN- $\gamma$  signaling, measured by signal transducer and activator of transcription-1 (STAT1) activation and the expression of IFN- $\gamma$ -responsive genes, is persistent in  $\beta$ -cells for as long as the cytokine is present. Because members of the suppressor of cytokine signaling (SOCS) family may regulate the duration of IFN- $\gamma$  signaling, their expression was investigated in  $\beta$ -cells. We found that cytokine-inducible SH2-containing protein, SOCS-1, and SOCS-2 are expressed in primary islets and NIT-1 insulinoma cells, both at the mRNA and protein levels, after treatment with IFN- $\gamma$  and other proinflammatory cytokines. Transfected SOCS-1 was found to inhibit responses to IFN- $\gamma$  in NIT-1 insulinoma cells, including STAT1 activation, class I major histocompatibility complex upregulation, and IFN- $\gamma$ -induced cell death, but only when expressed at levels higher than those found in untransfected cells. Consistent with this, IFN- $\gamma$  signaling was not affected in SOCS-1-deficient  $\beta$ -cells. Therefore, persistent IFN- $\gamma$  signaling in  $\beta$ -cells is associated with SOCS-1 expression that is not sufficient to terminate signaling. Because overexpression of SOCS-1 can suppress responses to IFN- $\gamma$ , this may be a useful strategy for protecting  $\beta$ -cells from cytotoxicity mediated by IFN- $\gamma$  and possibly other proinflammatory cytokines. *Diabetes* 50:2744–2751, 2001**

**D**uring the pathogenesis of autoimmune diabetes, proinflammatory cytokines, such as  $\gamma$ -interferon (IFN- $\gamma$ ), interleukin-1 (IL-1), and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), are secreted by T-cells and macrophages within the inflammatory infiltrate of pancreatic islets, and they act upon  $\beta$ -cells. The inter-

action between cytokines and  $\beta$ -cells results in the expression of cytokine-regulated genes, including class I major histocompatibility complex (MHC) (1), inducible nitric oxide synthase (iNOS) (2), and Fas (3–5). In vitro, combinations of these cytokines result in  $\beta$ -cell damage (6–11). In vivo, we have shown that  $\beta$ -cells unresponsive to IFN- $\gamma$  are protected from destruction in the lymphocytic choriomeningitis virus-induced transgenic mouse model of diabetes (12). On the other hand, we have described that IFN- $\gamma$  is a key regulator of class I MHC expression but is not required for  $\beta$ -cell destruction in the nonobese diabetic (NOD) mouse model (13,14). TNF- $\alpha$  and IL-1 may also be important in  $\beta$ -cell damage, although their precise roles remain inconclusive.

The binding of a cytokine to its receptor on a target cell results in the activation of a variety of signal-transduction pathways. IFN- $\gamma$  signaling involves the activation of the Jak-signal transducer and activator of transcription (STAT) pathway (15). Ligation of the receptor results in the dimerization of receptor subunits and the activation of associated Jak kinases. Activated Jak kinases then phosphorylate tyrosine residues within the intracellular domain of the receptor, which act as docking sites for the transcription factor STAT1. Recruited STAT1 molecules are activated by phosphorylation and then dimerize and translocate to the nucleus, where they bind to activated sequence (GAS) elements within the promoters of IFN- $\gamma$ -responsive genes in order to drive transcription.

Previous studies have demonstrated that IFN- $\gamma$ -induced STAT1 activation is transient in many cell types, such as Bud-8 human fibroblasts, despite the continuous presence of the cytokine (16,17). STAT1 is rapidly activated by IFN- $\gamma$ , reaches a peak after 30 min, but then wanes by 1–4 h, suggesting that mechanisms exist that negatively regulate IFN- $\gamma$  signaling in these cells. Termination of IFN- $\gamma$  signaling requires at least two events: inactivation of the activated receptor-Jak kinase complex and inactivation of phosphorylated STAT1. Termination of receptor signaling may involve internalization of the receptor complex (18) as well as inhibition by members of the suppressor of cytokine signaling (SOCS) family (19). In particular, SOCS-1 has been shown to inhibit IFN- $\gamma$  signaling in a variety of cell lines, such as M1 monocytic cells and NIH3T3 fibroblasts, when it is overexpressed (20–22). SOCS-1 is thought to function by binding to and inhibiting activated Jak kinases (23–26). Additionally, it may act to target the activated complex for proteosomal degradation (27,28). Inactivation of phosphorylated STAT1 involves a

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Received for publication 5 July 2001 and accepted in revised form 14 September 2001.

CIS, cytokine-inducible SH2-containing protein; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; FCS, fetal calf serum; GAS,  $\gamma$ -interferon-activated sequence; IFN- $\gamma$ ,  $\gamma$ -interferon; IL-1, interleukin-1; iNOS, inducible nitric oxide synthase; MHC, major histocompatibility complex; NEAA, nonessential amino acids; PCR, polymerase chain reaction; RT, reverse transcriptase; SOCS, suppressor of cytokine signaling; STAT, signal transducer and activator of transcription; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ .

nuclear protein tyrosine phosphatase, because in the absence of continued IFN- $\gamma$ -receptor signaling, previously phosphorylated STAT1 is rapidly dephosphorylated (17).

It was recently shown that in insulinoma cells, resistance to IFN- $\gamma$ -mediated cytotoxicity could be selected for by continuous exposure to IFN- $\gamma$  (in combination with IL-1 $\beta$ ) over an 8-week period (29). Resistance was found to be associated with increased expression of STAT1 $\alpha$  and could be reproduced by overexpression of STAT1 $\alpha$  in nonresistant cells, although the biochemical mechanism remains unclear (30). The continual expression of STAT1 in response to IFN- $\gamma$  appears to be at odds with the idea of transient IFN- $\gamma$ -receptor signaling. It was also found that the ability of IFN- $\gamma$  to prime IL-1 $\beta$ -induced iNOS expression in primary  $\beta$ -cells was associated with prolonged STAT1 activation (31). Residual activated STAT1 molecules were reported to be detectable up to a week after a transient pulse of IFN- $\gamma$ . As possible explanations for this phenomenon, the authors postulated that either  $\beta$ -cells lacked the protein tyrosine phosphatase, which was responsible for nuclear inactivation of STAT1, or Jak kinase activity was prolonged.

Both of these observations suggest that IFN- $\gamma$  signaling in  $\beta$ -cells may not be transient, as described for other cell types. Prolonged IFN- $\gamma$  signaling may contribute to the cytotoxicity of IFN- $\gamma$  for  $\beta$ -cells. Therefore, the present study was undertaken to clarify the kinetics of IFN- $\gamma$  signaling in  $\beta$ -cells. The kinetics of IFN- $\gamma$  signaling were directly analyzed in primary islets and in an insulinoma cell line. In particular, the potential role of SOCS-1 in the negative regulation of IFN- $\gamma$  signaling in  $\beta$ -cells was explored using gene deletion and overexpression approaches.

## RESEARCH DESIGN AND METHODS

**Mice, cell lines, transfections, and cytokines.** NOD $^{scid}$  mice were bred in the animal facilities of the Walter and Eliza Hall Institute of Medical Research. IFN- $\gamma^{-/-}$  and SOCS-1 $^{-/-}$  IFN- $\gamma^{-/-}$  mice, on mixed C57BL/6-129Sv backgrounds, were obtained from Dr. W. Alexander of the Walter and Eliza Hall Institute of Medical Research.

NIT-1 insulinoma cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, Gaithersburg, MD) supplemented with nonessential amino acids (NEAA), 100 units/ml penicillin, 100  $\mu$ g/ml streptomycin, and 10% fetal calf serum (FCS). NIT-1 cells were transfected with the SOCS-1 expression plasmid REP9SOCS-1 (see below) by electroporation as previously described (11), and stable transfectants were selected with 500  $\mu$ g/ml Geneticin (Life Technologies).

Unless otherwise stated, recombinant murine IFN- $\gamma$  (Genentech, South San Francisco, CA) was used at 100 units/ml; recombinant human IL-1 $\beta$  (Genzyme, Cambridge MA) was used at 10 units/ml; recombinant murine TNF- $\alpha$  (Genentech) was used at 250 units/ml; and recombinant murine IFN $\alpha$  (provided by Dr. S. Gerondakis, the Walter and Eliza Hall Institute of Medical Research) was used at 100 units/ml.

**Plasmids.** pGLB928LUC (provided by Dr. D. Paulnock, University of Wisconsin, Madison, WI) contains 928 bp of the murine guanylate-binding protein-1 promoter (containing GAS elements) (32) ligated to the luciferase gene. The SOCS-1 expression plasmid, REP9SOCS-1, was constructed by subcloning the SOCS-1 coding region from the genomic murine SOCS-1 clone 95-11-10 (obtained from Dr. T. Willson, the Walter and Eliza Hall Institute of Medical Research) into the mammalian expression vector REP9 (RSV promoter).

**Luciferase reporter assays.** NIT-1 cells were transiently transfected with the STAT1-responsive reporter pGLB928LUC ( $\pm$ REP9SOCS-1) by electroporation. The total number of cells required for each experiment was electroporated in the same tube. They were then plated out into six-well plates at  $8 \times 10^5$  cells per well. After 1 day, transfected cells were stimulated with IFN- $\gamma$ , and then a luciferase assay was performed. Luciferase assays were performed by harvesting the cells in 300  $\mu$ l lysis buffer (1% Triton X-100 in 25 mmol/l glycylglycine, 15 mmol/l MgSO $_4$ , 4 mmol/l EGTA, and 1 mmol/l dithiothreitol

[DTT], pH 7.8). The samples were centrifuged, and 100  $\mu$ l supernatant was added to 365  $\mu$ l luciferase assay buffer (25 mmol/l glycylglycine, 15 mmol/l MgSO $_4$ , 4 mmol/l EGTA, 16 mmol/l potassium phosphate, 1 mmol/l DTT, and 2 mmol/l ATP, pH 7.8). The reactions were performed at room temperature using a Lumat LB950 luminometer (Berthold, Wildbald, Germany), which injects 100  $\mu$ l of 25 mmol/l luciferin in glycylglycine buffer (25 mmol/l glycylglycine, 15 mmol/l MgSO $_4$ , 4 mmol/l EGTA, and 10 mmol/l DTT, pH 7.8) into the extract and assay buffer. Luciferase activity was measured by calculating the light emitted during the initial 10 s of the reaction, and the values are expressed in arbitrary light units. In some experiments, SV40 early promoter- $\beta$ -gal was cotransfected to determine whether transfection efficiency could confound the results. However, correction for  $\beta$ -gal expression made little difference to the results (data not shown).

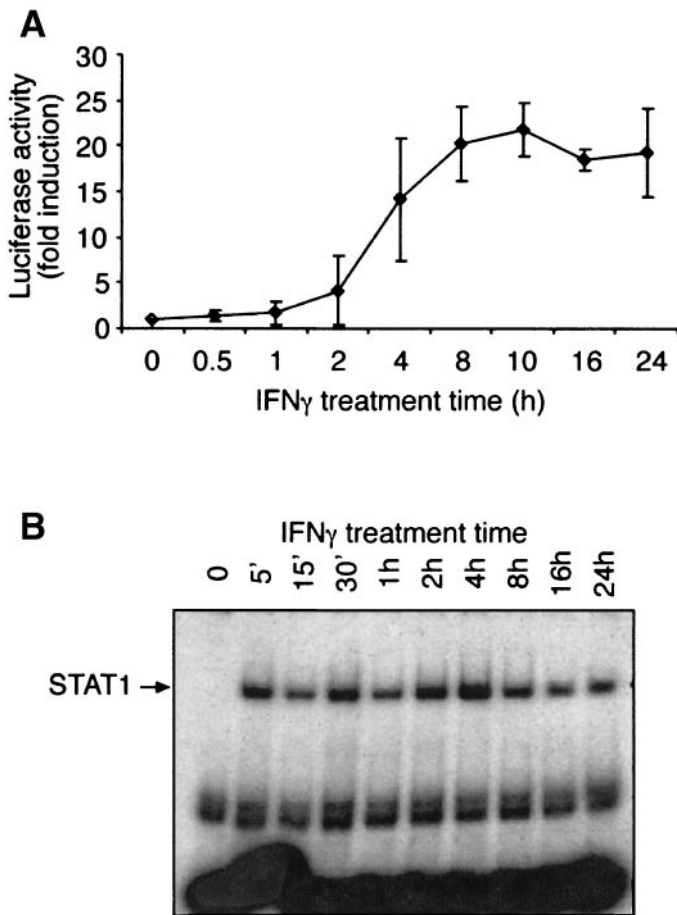
**Gel shift.** Nuclear extracts were prepared according to the method of Schreiber et al. (33), and gel shifts were performed as described elsewhere (13). STAT1 DNA binding activity was measured using the GAS from the Fc $\gamma$ RI promoter: 5'-GTACGAGATGTTATTTCCAGAAAA-3'.

**Isolation of pancreatic islets.** Islets were isolated from mice by intraductal digestion of the pancreas with collagenase P (Roche Diagnostics, Basel) as previously described (13) and separated on a BSA density gradient. Islets were handpicked to purity and cultured in CMRL-1066 (Life Technologies) supplemented with 100 units/ml penicillin, 100  $\mu$ g/ml streptomycin, and 10% FCS.

**Analysis of mRNA expression.** Total RNA was purified from primary islets and NIT-1 cells using RNazol B (Tel-Test, Friendswood, TX). mRNA analysis was performed by reverse transcriptase (RT)-polymerase chain reaction (PCR). First-strand cDNA synthesis from 1  $\mu$ g total RNA was performed in a 20  $\mu$ l reaction using MLV reverse transcriptase (Promega, Madison, WI). cDNAs of interest were amplified from 1  $\mu$ l RT product per PCR using REDTaq DNA polymerase (Sigma, St. Louis, MO). The primers 5'-GTGGGC CGCCTAGGACCA-3' and 5'-CTCTTTGATGTACGCACGATTTC-3' were used to amplify a 530-bp fragment of the murine  $\beta$ -actin sequence; 5'-ATGGTCTCTGCGTACAG-3' and 5'-TGACAAGCAGTTAGAGTC-3' amplified a 440-bp fragment of the murine CIS sequence; 5'-ATGGTACGACGCAACCAG GTG-3' and 5'-CTCCAGCAGCTCGAAAAGGCA-3' amplified a 460-bp fragment of the murine SOCS-1 sequence; and 5'-CCATGACCCTGCGGTGCGTGG-3' and 5'-GGAATTTATATTCTTCCAAGT-3' amplified a 593-bp fragment of the murine SOCS-2 sequence. PCRs were optimized such that the number of cycles that were used allowed for the analysis of products within the linear part of the amplification reaction. Amplification of  $\beta$ -actin (22 cycles), CIS (28 cycles), SOCS-1 (28 cycles), and SOCS-2 (30 cycles) was performed for 20 s at 96°C, 30 s at 55°C, and 40 s at 72°C. PCR products were resolved on an agarose gel, transferred onto a nylon membrane, and Southern hybridized with [ $\alpha$ - $^{32}$ P]dATP-labeled cDNA probes (Megaprime DNA Labeling; Amersham Pharmacia Biotech, Uppsala, Sweden) for CIS, SOCS-1, SOCS-2, or  $\beta$ -actin (SOCS cDNA probes were provided by Dr. W. Alexander). Radiation counts for SOCS RT-PCR products were quantitated by Phosphorimager (Molecular Dynamics, Sunnyvale, CA) analysis using ImageQuant version 5.0 and were corrected against  $\beta$ -actin.

**Immunoprecipitation and Western blot analysis.** NIT-1 cells ( $2 \times 10^7$ ) were lysed on ice for 30 min in 1 ml KALB lysis buffer (34) supplemented with 1 mmol/l phenylmethylsulfonyl fluoride, 1  $\mu$ g/ml pepstatin A, and 2  $\mu$ g/ml aprotinin, and then they were centrifuged for 10 min at 4°C. The clarified supernatant was collected and incubated with 3  $\mu$ g anti-SOCS-1 antibody (2E1; anti-SOCS-1 antibodies were provided by Dr. J.-G. Zhang, the Walter and Eliza Hall Institute of Medical Research) on ice for 1 h. Forty  $\mu$ l of 50% protein G-Sepharose gel slurry was added, with a further incubation on a rotating wheel for 2 h at 4°C. The protein G beads were washed three times with KALB lysis buffer, and then the immunoprecipitate was eluted with 2 $\times$  protein sample buffer (8% SDS, 40% 2-mecaptoethanol, and 0.5 mol/l Tris-HCl, pH 6.6, bromophenol blue). The immunoprecipitates were resolved on a 14% polyacrylamide gel, transferred onto a nitrocellulose membrane, and probed with a biotinylated anti-SOCS-1 antibody (4H1), followed by a secondary horse radish peroxidase-conjugated streptavidin (Amersham Pharmacia Biotech). SOCS-1 protein was visualized with Lumi-Light (Roche Diagnostics).

**Flow cytometry.** NIT-1 insulinoma cells were liberated from tissue culture plates with 0.2% trypsin and 10 mmol/l EDTA in phosphate-buffered saline (37°C for 4 min), washed twice with DMEM NEAA + 10% FCS, and then allowed to recover for 30 min at 37°C. A biotinylated anti-H-2D $^b$  monoclonal antibody (28-14-8; Pharmingen, San Diego, CA) was used to stain for class I MHC, followed by phycoerythrin-conjugated streptavidin (Caltag, Buringame, CA). Analysis was performed on a FACScan (Becton Dickinson, Franklin Lakes, NJ). The method for dispersing islets into single cells for flow cytometric analysis is described elsewhere (4). Islet cells were stained for class I MHC expression as described for NIT-1 cells. The  $\beta$ -cell population was identified by their high flavin adenine dinucleotide autofluorescence (4).



**FIG. 1.** IFN- $\gamma$  signaling is persistent in  $\beta$ -cells. **A:** The kinetics of IFN- $\gamma$  signaling in NIT-1 cells were measured in a luciferase reporter assay for STAT1 activation. NIT-1 cells were transfected with the IFN- $\gamma$ -responsive reporter pGLB928LUC (containing GAS elements) and stimulated with IFN- $\gamma$  for 0–24 h. Cellular extracts were then assayed for luciferase activity. Values are expressed as a fold induction from untreated cells. Each time point is the mean  $\pm$  SD of four independent experiments. **B:** Gel shift analysis for STAT1 activation in NIT-1 cells treated with IFN- $\gamma$  for 0–24 h.

**Apoptosis assays.** NIT-1 cells were seeded into 12-well plates at a density of  $10^5$  cells per well. After cytokine treatment, apoptosis was quantitated according to the method described by Nicoletti et al. (35), which measures the fragmentation of nuclei. Briefly, the cells were harvested and then incubated overnight at 4°C in 50  $\mu$ g/ml propidium iodide in 0.1% sodium citrate with 0.1% Triton X-100, which stains nuclear DNA. The cells were then analyzed on a FACScan. Apoptotic bodies were identified by their reduced DNA content.

**Statistical analysis.** Statistical comparisons were made using two-way analyses of variance. Calculations were performed with the aid of Prism version 2.0a (GraphPad Software, San Diego, CA).

## RESULTS

**IFN- $\gamma$  signaling is persistent in pancreatic  $\beta$ -cells.** The kinetics of IFN- $\gamma$  signaling in NIT-1 insulinoma cells were examined in a luciferase reporter assay for STAT1 activity (Fig. 1A). NIT-1 cells were transiently transfected with the IFN- $\gamma$ -responsive luciferase reporter pGLB928LUC (containing GAS elements) and then treated with IFN- $\gamma$  over a 24-h time period. IFN- $\gamma$  treatment of NIT-1 cells resulted in maximal luciferase expression ( $\sim$ 20-fold over untreated) after 8 h, which remained unchanged after 24 h, suggesting the persistence of activated STAT1. This was confirmed when STAT1 activity was directly analyzed by gel shift (Fig. 1B). Correlating with luciferase expression, STAT1

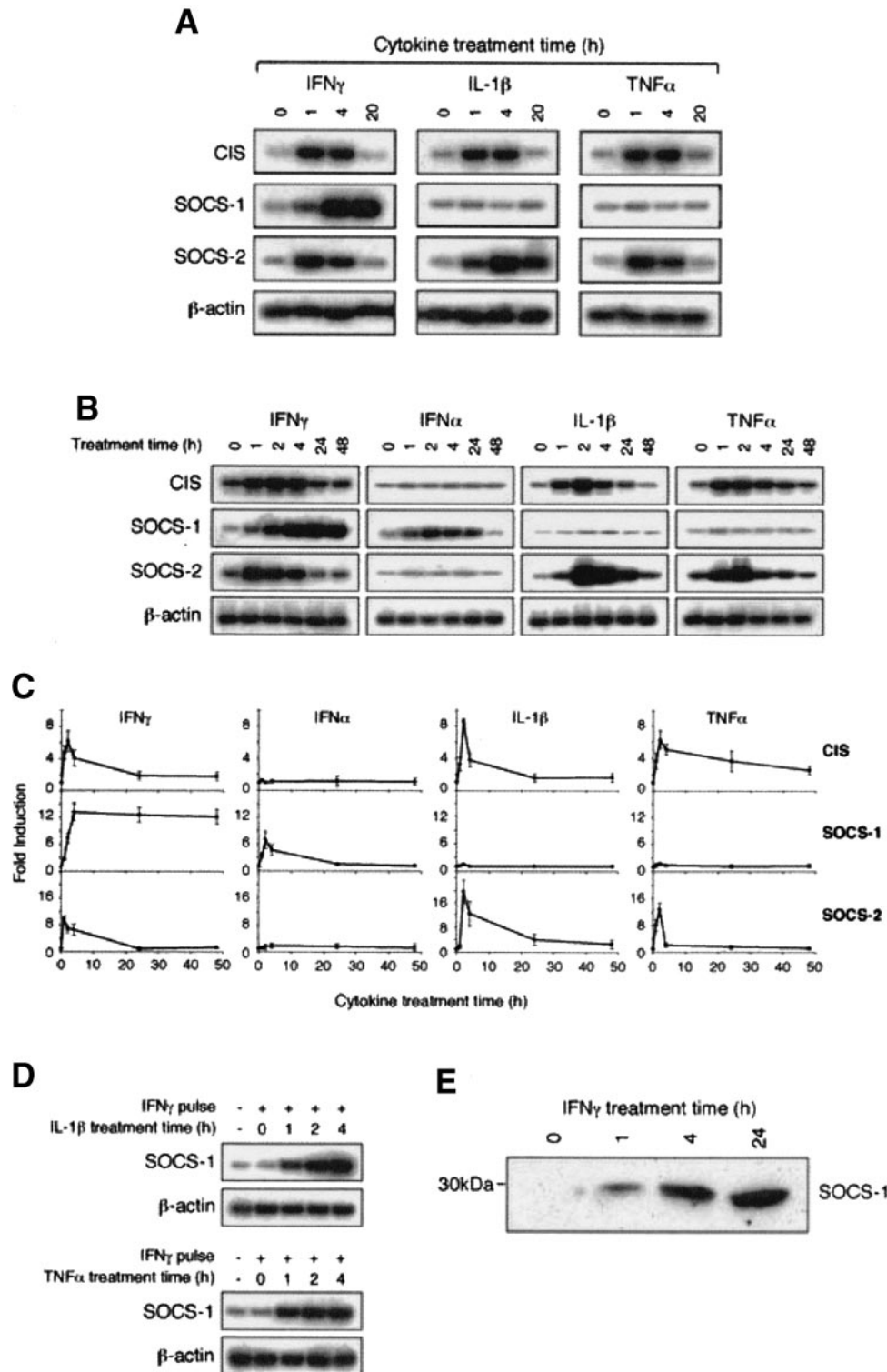
homodimers were detected within minutes but then persisted as long as the cytokine was present, even after 24 h. Expression of IFN- $\gamma$ -responsive genes, such as class I MHC, also remained maximal in NIT-1 cells for as long as the cytokine was present (not shown). The same kinetics of signaling were also evident in primary pancreatic islets after IFN- $\gamma$  stimulation. The induction of both STAT1 activation, measured by gel shift, and class I MHC expression, measured by flow cytometry, persisted throughout the time that IFN- $\gamma$  was present. These findings demonstrate that IFN- $\gamma$  signaling is prolonged in pancreatic  $\beta$ -cells and is not terminated in the manner seen in Bud-8 human fibroblasts (17).

**SOCS family members are expressed in primary islets and NIT-1 insulinoma cells after treatment with proinflammatory cytokines.** The discovery of the SOCS family of proteins has provided a better understanding of how cytokine signaling is negatively regulated. It has been shown in cell lines such as M1 monocytic and NIH3T3 fibroblast cells that SOCS-1 expression is induced by IFN- $\gamma$ , whereas overexpression of SOCS-1 blocks IFN- $\gamma$  signaling (21,22). Induction of SOCS-1 is thought to function in an intracellular negative-feedback loop to switch off IFN- $\gamma$ -receptor signaling. The transient nature of IFN- $\gamma$  signaling in some cell types may be a result of negative regulation by SOCS-1. The persistence of IFN- $\gamma$  signaling in pancreatic  $\beta$ -cells could therefore be due to lack of SOCS-1 expression. Thus, we analyzed  $\beta$ -cells for the expression of SOCS genes.

The expression of SOCS genes in primary murine islets was analyzed by RT-PCR after treatment with IFN- $\gamma$ , IL-1 $\beta$ , or TNF- $\alpha$  (Fig. 2A). Two members of the family, CIS and SOCS-2, were transiently expressed in primary islets after treatment with all three cytokines. SOCS-1 was also expressed after treatment with IFN- $\gamma$  but not IL-1 $\beta$  or TNF- $\alpha$ . Islets from NOD mice were also analyzed for SOCS transcripts because the presence of proinflammatory cytokines during the progression of diabetes may also induce SOCS gene expression. Both CIS and SOCS-2 message were present in the islets of mice at an early stage of disease (50 days), whereas SOCS-1 was not detected until 70 days (data not shown).

Although  $\beta$ -cells constitute the majority of cells within an islet, other cell types are also present. To confirm that  $\beta$ -cells express SOCS genes, the NIT-1 insulinoma cell line was used as a pure source of  $\beta$ -cells. SOCS gene expression was analyzed in NIT-1 cells after treatment with IFN- $\gamma$ , IFN $\alpha$ , IL-1 $\beta$ , or TNF- $\alpha$  (Fig. 2B and C). As in primary islets, CIS and SOCS-2 were transiently induced by IFN- $\gamma$ , IL-1 $\beta$ , and TNF- $\alpha$  but not IFN $\alpha$ . SOCS-1 was transiently expressed after IFN $\alpha$  treatment, peaking at 2 h and then decaying. SOCS-1 was also induced by IFN- $\gamma$  and reached maximal expression by 4 h but was not downregulated within the time course. This prolonged SOCS-1 expression after IFN- $\gamma$  treatment was also observed in primary islets. Although IL-1 $\beta$  or TNF- $\alpha$  alone did not induce SOCS-1 expression in NIT-1 cells, both were able to do so in combination with a low concentration of IFN- $\gamma$ , suggesting synergistic interactions between these cytokines (Fig. 2D).

IFN- $\gamma$  signaling was found to be persistent in  $\beta$ -cells, despite the induction of SOCS-1 mRNA expression. The presence of message, however, does not necessarily indi-



**FIG. 2.** Expression of SOCS genes in pancreatic  $\beta$ -cells is induced by proinflammatory cytokines. **A:** Primary islets were isolated from 8-week-old NODSCID mice and treated with IFN- $\gamma$ , IL-1 $\beta$ , or TNF- $\alpha$  for 0–20 h. SOCS mRNA expression was analyzed by RT-PCR. A representative of three independent experiments is shown. **B:** NIT-1 insulinoma cells were stimulated with IFN- $\gamma$ , IFN- $\alpha$ , IL-1 $\beta$ , or TNF- $\alpha$  for 0–48 h, and SOCS mRNA expression was analyzed by RT-PCR. A representative of four independent experiments is shown. **C:** Phosphorimager quantification of SOCS gene expression levels in NIT-1 cells (corrected against  $\beta$ -actin). Values are expressed as the fold induction from basal levels in untreated cells. The means  $\pm$  SD of the four experiments are shown. **D:** IL-1 $\beta$  and TNF- $\alpha$  can induce SOCS-1 expression in synergy with IFN- $\gamma$ . NIT-1 cells were primed with a low concentration of IFN- $\gamma$  (1 unit/ml) for 48 h and then stimulated with IL-1 $\beta$  or TNF- $\alpha$  for 0–4 h. SOCS-1 mRNA expression was analyzed by RT-PCR. One of two experiments is shown (**E**); SOCS-1 protein levels correlate with mRNA expression in NIT-1 cells after IFN- $\gamma$  treatment. NIT-1 cells were treated with IFN- $\gamma$  for 0–24 h, and then SOCS-1 protein expression was analyzed by immunoprecipitation/Western blotting. A representative of three experiments is shown.

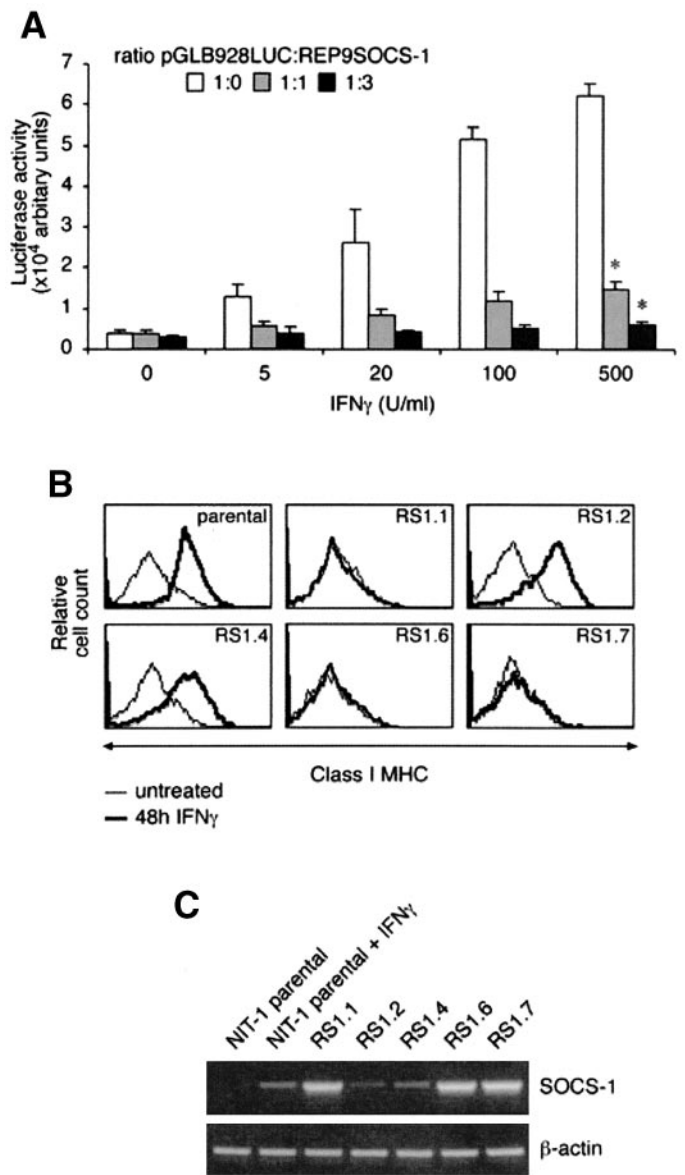
cate translation into protein. Therefore, we analyzed NIT-1 cells for SOCS-1 protein expression by immunoprecipitation/Western blotting (Fig. 2E). SOCS-1 protein was first detected 1 h after IFN- $\gamma$  treatment, which increased after 4 and 24 h, thus correlating with SOCS-1 mRNA expression.

**Overexpression of SOCS-1 blocks IFN- $\gamma$  responsiveness in NIT-1 insulinoma cells.** SOCS-1 is expressed in  $\beta$ -cells in response to IFN- $\gamma$ , but IFN- $\gamma$  signaling remains persistent. To examine whether SOCS-1 is functional in  $\beta$ -cells, the ability of overexpressed SOCS-1 to suppress IFN- $\gamma$  signaling was studied in NIT-1 insulinoma cells. The effect of transfected SOCS-1 was analyzed in a luciferase reporter assay for STAT1 activation. NIT-1 cells were transiently transfected with the IFN- $\gamma$ -responsive luciferase reporter pGLB928LUC, with or without the cotransfection of a SOCS-1 expression vector. Cotransfection of SOCS-1 into NIT-1 cells effectively blocked the induction of luciferase by IFN- $\gamma$ , even when high concentrations of IFN- $\gamma$  (500 units/ml) were used (Fig. 3A). Furthermore, NIT-1 cells that were stably transfected with SOCS-1 did not upregulate class I MHC expression in response to IFN- $\gamma$  (RS1.1, -1.6, and -1.7) (Fig. 3B).

**Termination of IFN- $\gamma$  signaling in  $\beta$ -cells requires high levels of SOCS-1.** When different SOCS-1-transfected NIT-1 clones were analyzed for IFN- $\gamma$  responsiveness, we found that IFN- $\gamma$  signaling was unaffected in some clones (RS1.2 and -1.4), as measured by the upregulation of class I MHC expression (Fig. 4). These clones expressed transfected SOCS-1 at levels similar to endogenous SOCS-1 in untransfected NIT-1 cells after IFN- $\gamma$  treatment (Fig. 3C). In contrast, the level of SOCS-1 overexpressed in the IFN- $\gamma$ -unresponsive clones RS1.1, -1.6, and -1.7 was much greater than endogenous SOCS-1. The ability of SOCS-1 to suppress IFN- $\gamma$  signaling in  $\beta$ -cells thus depends on constitutive overexpression of high levels of the protein. These results suggest that in  $\beta$ -cells, endogenous SOCS-1 is expressed at levels (even after IFN- $\gamma$  treatment) insufficient to terminate IFN- $\gamma$  signaling.

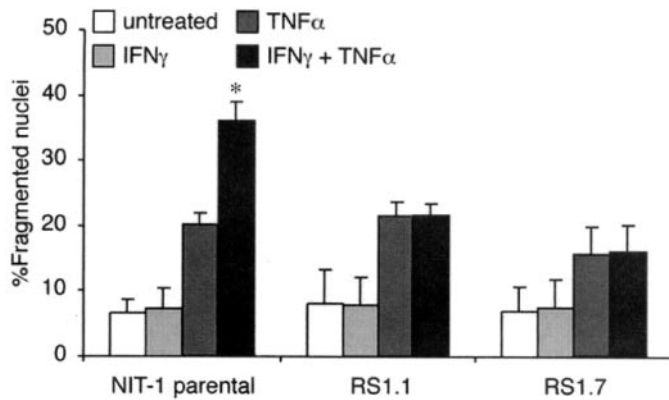
**Overexpression of SOCS-1 protects NIT-1 insulinoma cells from IFN- $\gamma$ -mediated cytotoxicity.** IFN- $\gamma$ , in combination with other proinflammatory cytokines such as TNF- $\alpha$ , is cytotoxic to  $\beta$ -cells (11,31,36). Therefore, we investigated whether SOCS-1 overexpression could inhibit IFN- $\gamma$ -induced cell death in NIT-1 insulinoma cells. The SOCS-1-overexpressing NIT-1 clones RS1.1 and -1.7 and parental NIT-1 cells were treated with either IFN- $\gamma$  or TNF- $\alpha$  or both. Cell death was then measured in a flow cytometric analysis for nuclear fragmentation (Fig. 4). Consistent with our previous findings (11), IFN- $\gamma$  alone did not induce cell death in parental NIT-1 cells but significantly enhanced the cytotoxicity of TNF- $\alpha$  for these cells. However, this enhancement was not observed in NIT-1 cells that constitutively overexpress SOCS-1 (RS1.1 and -1.7). SOCS-1 overexpression therefore protects NIT-1 cells from IFN- $\gamma$ -enhanced death from TNF- $\alpha$ .

**SOCS-1 neither modulates the kinetics or intensity of IFN- $\gamma$  signaling in  $\beta$ -cells nor the sensitivity of  $\beta$ -cells to the cytokine.** To confirm that physiological levels of SOCS-1 do not terminate IFN- $\gamma$  signaling in  $\beta$ -cells, the effect of SOCS-1 deficiency on IFN- $\gamma$  signaling was examined. SOCS-1<sup>-/-</sup> mice die by 3 weeks of age



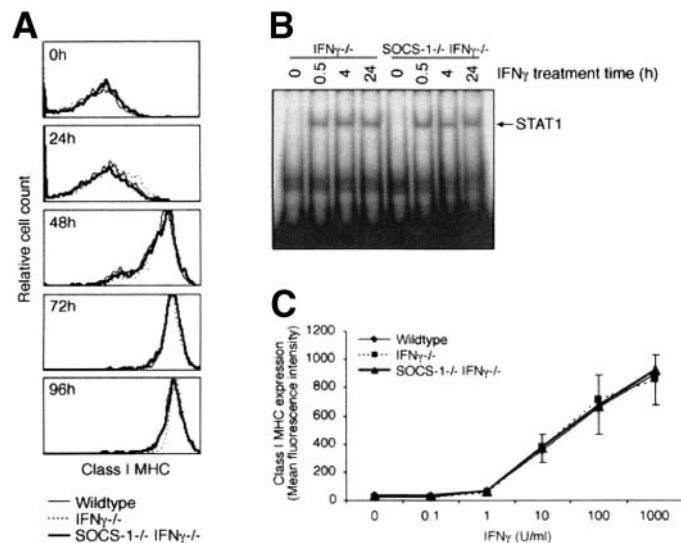
**FIG. 3.** IFN- $\gamma$  signaling in NIT-1 insulinoma cells is suppressed by overexpression of SOCS-1 at high levels. **A:** Overexpression of SOCS-1 blocks IFN- $\gamma$ -induced luciferase expression in a luciferase reporter assay for activated STAT1. NIT-1 cells were transfected with pGLB928LUC in a 1:0, 1:1, or 1:3 ratio with the SOCS-1 expression plasmid REP9SOCS-1. Total transfected DNA was kept constant by the addition of REP9 empty vector. The cells were then treated with 0–500 units/ml IFN- $\gamma$  for 12 h, and cellular extracts were assayed for luciferase activity. Values are expressed as the means  $\pm$  SD (arbitrary light units) of three independent experiments. \* $P$  < 0.05 compared with pGLB928LUC alone. **B:** Constitutive SOCS-1 overexpression blocks IFN- $\gamma$ -induced class I MHC expression in NIT-1 cells. NIT-1 stable transfectants (clones RS1.1, -1.2, -1.4, -1.6, and -1.7) and parental cells were treated with 1,000 units/ml IFN- $\gamma$  for 48 h, and class I MHC expression was analyzed by flow cytometry. Thirty individual SOCS-1-transfected clones were analyzed for IFN- $\gamma$ -induced class I MHC expression. Five representative clones are shown. **C:** Levels of SOCS-1 expressed in the NIT-1 transfectants (untreated) compared with endogenous SOCS-1 expression in parental cells treated with IFN- $\gamma$  for 24 h, as measured by RT-PCR. SOCS-1 protein levels were also confirmed by immunoprecipitation/Western blot analysis (not shown).

(37,38), but they can be rescued by removing the IFN- $\gamma$  gene (39,40). Islets from SOCS-1<sup>-/-</sup> IFN- $\gamma$ <sup>-/-</sup>, IFN- $\gamma$ <sup>-/-</sup>, and wild-type mice were stimulated with IFN- $\gamma$  over a 96-h time course. The  $\beta$ -cell population was analyzed by flow cytometry for class I MHC expression as a marker of IFN- $\gamma$

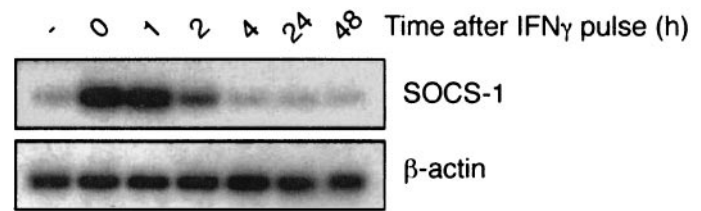


**FIG. 4.** NIT-1 insulinoma cells are protected from IFN- $\gamma$ -induced cell death by overexpression of SOCS-1. Parental NIT-1 insulinoma cells and the SOCS-1-overexpressing NIT-1 clones RS1.1 and -1.7 were treated with 100 units/ml IFN- $\gamma$ , 1,000 units/ml TNF- $\alpha$ , or both. After 3 days, cell death was assessed by measuring nuclear fragmentation in a flow cytometric analysis for DNA content. Apoptotic cells were identified by their reduced DNA content after staining with propidium iodide. The results represent the mean percentage fragmented nuclei  $\pm$  SD of three independent experiments. \* $P < 0.005$  vs. TNF- $\alpha$  alone.

signaling (Fig. 5A). Maximal class I MHC expression on the  $\beta$ -cells of all three genotypes was observed 72 h after the addition of IFN- $\gamma$ , which remained at the same level after 96 h. Similarly, we found that IFN- $\gamma$ -induced STAT1 activation was also not altered by SOCS-1 deficiency (Fig. 5B). The levels of activated STAT1 at 0.5, 4, and 24 h after IFN- $\gamma$  treatment were the same in SOCS-1 $^{-/-}$  islets as in con-



**FIG. 5.** The kinetics and intensity of IFN- $\gamma$  signaling in  $\beta$ -cells and the sensitivity of  $\beta$ -cells to IFN- $\gamma$  are not affected by loss of SOCS-1. **A:** Primary islets from SOCS-1 $^{-/-}$  IFN- $\gamma$  $^{-/-}$ , IFN- $\gamma$  $^{-/-}$ , and wild-type mice were stimulated with IFN- $\gamma$  for 0–96 h. The  $\beta$ -cell population (electronically gated as described in RESEARCH DESIGN AND METHODS) was analyzed for class I MHC expression by flow cytometry. A representative of four experiments is shown. **B:** Islets from SOCS-1 $^{-/-}$  IFN- $\gamma$  $^{-/-}$  and IFN- $\gamma$  $^{-/-}$  mice were treated with IFN- $\gamma$  for 0–24 h and then analyzed for STAT1 activation by gel shift. **C:** Islets from SOCS-1 $^{-/-}$  IFN- $\gamma$  $^{-/-}$ , IFN- $\gamma$  $^{-/-}$ , and wild-type mice were treated with 0–1,000 units/ml IFN- $\gamma$  for 48 h, and the  $\beta$ -cell population was analyzed for class I MHC expression. Class I MHC staining is expressed as the mean fluorescence intensity. The means  $\pm$  SD of three independent experiments are shown. Smaller increments in IFN- $\gamma$  concentration (i.e., 0, 0.5, 1 unit/ml, etc.) were also used, but no difference was found (not shown).



**FIG. 6.** IFN- $\gamma$ -induced SOCS-1 expression in NIT-1 insulinoma cells rapidly decays on removal of IFN- $\gamma$ . NIT-1 insulinoma cells were pulsed with IFN- $\gamma$  for 4 h. The cells were then washed, fresh medium was added, and SOCS-1 expression was allowed to decay for 0–24 h after the IFN- $\gamma$  pulse. SOCS-1 message levels were measured by RT-PCR. A representative of three experiments is shown.

trols. Therefore, SOCS-1 does not modulate the kinetics or intensity of IFN- $\gamma$  signaling in  $\beta$ -cells.

Although SOCS-1 does not modulate the kinetics of IFN- $\gamma$  signaling in  $\beta$ -cells, it may regulate the sensitivity of  $\beta$ -cells to IFN- $\gamma$ . The sensitivity of SOCS-1 $^{-/-}$   $\beta$ -cells to IFN- $\gamma$  was assessed by measuring the upregulation of class I MHC as a readout for responsiveness to the cytokine. Pancreatic islets were treated with 0–1,000 units/ml IFN- $\gamma$ , and then the  $\beta$ -cell population was analyzed for class I MHC expression by flow cytometry (Fig. 5C). Class I MHC expression on both SOCS-1 $^{-/-}$  and wild-type  $\beta$ -cells was upregulated by IFN- $\gamma$  in a dose-dependent manner. However, SOCS-1 deficiency did not augment the sensitivity of  $\beta$ -cells to IFN- $\gamma$ . At all concentrations (1–1,000 units/ml), IFN- $\gamma$  induced the same level of class I MHC on SOCS-1 $^{-/-}$   $\beta$ -cells as on wild-type cells. These results confirm that physiological levels of SOCS-1 do not negatively regulate IFN- $\gamma$  signaling in  $\beta$ -cells.

We have also begun to analyze death of SOCS-1 $^{-/-}$   $\beta$ -cells induced by cytokine combinations. These data also indicate that the contribution of IFN- $\gamma$  to cytokine-induced  $\beta$ -cell death and iNOS induction is not increased with SOCS-1 deficiency (M.M.W. Chong and T.W.H. Kay, unpublished data).

**IFN- $\gamma$ -induced gene transcription in  $\beta$ -cells decays on removal of the cytokine.** The decay of phosphorylated STAT1 is also required for the termination of IFN- $\gamma$  signaling. This has been shown to be a result of a nuclear protein tyrosine phosphatase (17). We investigated whether this mechanism was intact in  $\beta$ -cells. The expression of SOCS-1 is directly regulated by STAT1 (41) and could be used as a readout for STAT1 activity. Consistent with persistent STAT1 activation in the presence of continuous exposure to IFN- $\gamma$  (Fig. 1), maximal SOCS-1 expression was sustained in NIT-1 insulinoma cells for as long as IFN- $\gamma$  was present (Fig. 5). SOCS-1 expression was not downregulated even after 1 week (not shown). Removal of the cytokine, however, resulted in rapid decay of SOCS-1 transcription back to basal levels within 2–4 h (Fig. 6). This suggests that phosphorylated STAT1 is rapidly inactivated on removal of IFN- $\gamma$ , at least to levels insufficient to stimulate gene transcription. A mechanism to inactivate phosphorylated STAT1 therefore exists in  $\beta$ -cells.

## DISCUSSION

IFN- $\gamma$  signaling in some cell types is transient (16,17), suggesting the existence of mechanisms that negatively regulate signaling in these cells. In pancreatic  $\beta$ -cells,

however, we have found that IFN- $\gamma$  signaling is persistent. Signaling is only terminated on removal of the cytokine. Persistence of signaling may be important in the cytotoxicity of IFN- $\gamma$  for  $\beta$ -cells because the induction of many mediators of  $\beta$ -cell damage is dependent on prolonged treatment with the cytokine. For example, treatment with IFN- $\gamma$  (plus IL-1 $\beta$ ) for 24–48 h is required to induce Fas and iNOS expression in  $\beta$ -cells (3,4,11,31,36,42).

Members of the SOCS family of proteins have been shown to act as negative regulators of the Jak-STAT pathway by acting on the cytokine receptor or associated Jak kinase (19). In particular, SOCS-1 has been implicated in the negative regulation of IFN- $\gamma$  signaling, both in vitro and in vivo. Overexpression of SOCS-1 has been shown to inhibit IFN- $\gamma$  responsiveness in various cell lines (20–22). Analysis of SOCS-1-deficient mice has also suggested a role for SOCS-1 in regulating the IFN- $\gamma$ -signaling pathway. These mice succumb to complex inflammatory pathology but can be rescued by IFN- $\gamma$  deficiency (39,40), implicating IFN- $\gamma$  overproduction, IFN- $\gamma$  hypersensitivity, or both in the SOCS-1<sup>-/-</sup> phenotype. In SOCS-1<sup>-/-</sup> IFN- $\gamma$ <sup>-/-</sup> mice injected with IFN- $\gamma$ , it was recently reported that STAT1 activation was prolonged in liver (43). STAT1 activation was also prolonged in SOCS-1<sup>-/-</sup> hepatocytes treated with IFN- $\gamma$  in vitro. Furthermore, in some cells of the hematopoietic lineage, SOCS-1 deficiency has been shown to result in hypersensitivity to IFN- $\gamma$  (39,44). Consistent with studies in other cell types, we found that overexpression of SOCS-1 suppressed responses to IFN- $\gamma$  in NIT-1 insulinoma cells, such as IFN- $\gamma$ -induced STAT1 activation, class I MHC upregulation, and cell death. By analyzing the levels of transfected SOCS-1 required to suppress IFN- $\gamma$  signaling, we found that physiological levels of SOCS-1, even after induction by IFN- $\gamma$ , are insufficient to terminate IFN- $\gamma$  signaling in  $\beta$ -cells. Although IFN- $\gamma$  signaling was found to be persistent, we have indirectly shown that STAT1 activity is rapidly downregulated in  $\beta$ -cells on removal of IFN- $\gamma$ , potentially by the nuclear protein tyrosine phosphatase, as previously described (17).

As IFN- $\gamma$  has been shown to be an important mediator of  $\beta$ -cell death both in vitro and in vivo, the blockade of IFN- $\gamma$  signaling remains important in designing therapies for the prevention of autoimmune  $\beta$ -cell destruction. We have demonstrated that overexpression of SOCS-1 (at high levels) terminates the otherwise persistent IFN- $\gamma$  signaling in  $\beta$ -cells and may therefore be a useful strategy for protecting  $\beta$ -cells from the cytotoxic effects of the cytokine. Cottet et al. (45) recently demonstrated that IFN- $\gamma$  activation of the Jak-STAT pathway is also involved in the suppression of insulin expression and glucose-stimulated insulin secretion by CDM3D insulinoma cells. This effect of IFN- $\gamma$  on insulin-producing cells may also be dependent on persistent signaling because maximal suppression of insulin expression only occurred after prolonged exposure (48 h) to IFN- $\gamma$ . However, it was found that IFN- $\gamma$ -induced inhibition of both insulin expression and secretion could be prevented by overexpression of SOCS-1. SOCS-1 overexpression therefore protects  $\beta$ -cells from many effects of IFN- $\gamma$ . Another potential benefit of manipulating SOCS-1 expression is that responses to multiple cytokines may be suppressed. Overexpression of SOCS-1 in other cell types has been shown to block signaling by a diverse range of

cytokines, including members of the IL-6 cytokine family (20), type 1 interferons (22), IL-4 (46), and TNF- $\alpha$  (47). Thus, SOCS-1 overexpression could protect  $\beta$ -cells from multiple cytokines thought to be important in the progression of autoimmune diabetes.

#### ACKNOWLEDGMENTS

This work was supported by the National Health and Medical Research Council, Australia, (Regkey 973002) and the Juvenile Diabetes Research Foundation's Center grant for research into "Immune mechanisms of  $\beta$ -cell life and death." We are grateful to Dr. Warren Alexander for mice and other reagents, Dr. Donna Paulnock for the pGLB928LUC plasmid, Dr. Tracy Willson for the genomic SOCS-1 clone, and Dr. Jian-guo Zhang for assistance with the detection of SOCS-1 protein. We also thank Rima Darwiche for her excellent technical assistance.

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