Interleukin 9 induces expression of three cytokine signal inhibitors: cytokine-inducible SH2-containing protein, suppressor of cytokine signalling (SOCS)-2 and SOCS-3, but only SOCS-3 overexpression suppresses interleukin 9 signalling

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Interleukin 9 (IL-9) is a cytokine preferentially produced by T helper type 2 lymphocytes and active on various cell types such as T- and B-lymphocytes, mast cells and haemopoietic progenitors. The IL-9 receptor (IL-9R) belongs to the haemopoietic receptor superfamily and its signal transduction involves mainly the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway. Here we studied the implication of a novel family of suppressors of cytokine signalling (called CIS, for cytokine-inducible SH2-containing protein, and SOCS, for suppressor of cytokine signalling) in IL-9 signal attenuation. In BW5147 T-cell lymphoma, IL-9 induced the rapid expression of CIS, SOCS-2 and SOCS-3 with a peak after 2 h of stimulation. Using IL-9R mutants, we showed that STAT activation is required for CIS/SOCS induction: CIS and SOCS-2 expression was induced either via STAT1 and/or STAT3 or via STAT5 but only STAT1 and/or STAT3 were involved in SOCS-3 expression. The effect of these three proteins on IL-9 signal transduction was assessed by transient transfection in HEK-293 cells expressing the components of the IL-9 signalling pathway and a STAT-responsive reporter construct. These experiments showed that only SOCS-3 is able to inhibit IL-9-induced signal transduction; neither CIS nor SOCS-2 exerted any effect. Stable transfection of CIS and SOCS-3 in BW5147 lymphoma cells showed that only overexpression of SOCS-3 had an inhibitory activity on STAT activation, gene induction and the anti-apoptotic activity of IL-9. By contrast, CIS failed to affect the IL-9 response.

Key words: interleukin 9 receptor, signal regulator, signal transduction.

INTRODUCTION

Interleukin 9 (IL-9) is a T-helper type 2 cytokine originally characterized as a factor produced by activated T-cells and able to support the long-term growth of some T-helper clones [1]. IL-9 activities now extend to various cell types including mast cells, B-lymphocytes, haemopoietic progenitors, eosinophils, lung epithelial cells, neuronal precursors and T-lymphocytes [2]. Increased IL-9 production seems to be implicated in major pathologies such as lymphomagenesis and asthma. Indeed, IL-9 stimulates the growth of murine thymic lymphomas [3] and an autocrine loop has been suggested in Hodgkin lymphoma [4]. In addition, IL-9 protects T-cell lymphomas from dexamethasone-induced apoptosis [5]. The involvement of IL-9 in asthma has been suggested by genetic studies [6–8] and is supported by its effects on IgE production [9], mucus production [10], mast cell differentiation [11], eosinophil activation [12] and bronchial hyper-responsiveness [13,14]. Finally, IL-9 is required for an efficient immune response against intestinal parasites such as *Trichuris muris* [15].

This cytokine exerts its effects through a receptor that belongs to the haemopoietic receptor superfamily and consists of two chains, the IL-9-specific α chain of IL-9 receptor (IL-9Rα) associated with γ_c, also involved in IL-2, IL-4, IL-7, and IL-15 signalling [16–18]. As with all haemopoietic receptors, signal transduction through IL-9R involves Janus kinase (JAK) tyrosine kinases and signal transducer and activator of transcription (STAT) proteins. IL-9Rα and γ_c are associated respectively with JAK1 and JAK3, which become activated on binding of IL-9. This results in the phosphorylation of the IL-9R on a single tyrosine residue, Tyr-116, creating a docking site for STAT1, STAT3 and STAT5 [19]. These factors are essential for most IL-9 activities, such as cell differentiation and proliferation and the inhibition of corticoid-induced apoptosis [19,20]. In parallel, stimulation with IL-9 involves the recruitment of insulin receptor substrate 1 and phosphoinositide 3-kinase, which might also have a role in the biological activities of this factor [21].

Even though the signal transduction of IL-9 seems to be well understood, the mechanisms for signal attenuation and termination are still unknown. A previous comparison of STAT5 activation by IL-9 and erythropoietin has shown that erythropoietin but not IL-9 signalling was rapidly down-regulated by phosphatases, leading to a distinct gene expression pattern for these two STAT5-activating cytokines [22]. A family of signalling inhibitors has been identified [23–28]. Expression of these proteins is induced by several cytokines and negatively controls signal transduction [29]. So far, eight members of this family have been identified: CIS (for cytokine-inducible SH2-containing protein) and SOCS-1 to SOCS-7 (for suppressor of cytokine signalling [30,31]). Alternative names for SOCS include SSI (for STAT-induced STAT-inhibitor) [24] and JAB (for JAK-binding protein) [23]. All these proteins contain a central SH2 domain [23,26] and a conserved C-terminal domain named the SOCS box, which might be involved in protein stabilization [32].

Abbreviations used: CIS, cytokine-inducible SH2-containing protein; GH, growth hormone; IFN, interferon; IL, interleukin; IL-9R, IL-9 receptor; JAK, Janus kinase; LIF, leukaemia inhibitory factor; RT–PCR, reverse-transcriptase-mediated PCR; SOCS, suppressor of cytokine signalling; STAT, signal transducer and activator of transcription.

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Two distinct mechanisms by which these proteins might inhibit cytokine signalling have been proposed. CIS negatively regulates STAT5-activating cytokines through interaction with the phosphorylated tyrosine of the receptor that recruits STAT5, thereby competing with this transducer [33]. By contrast, SOCS-1 inhibits signalling by binding to JAK kinases through its SH2 domain, thus inhibiting their kinase activity [23]. SOCS-3 is the only member to be phosphorylated after stimulation by cytokines and might act by both mechanisms [34,35]. In IL-6 signalling, SOCS-3 exerts its negative effect through the SHP2 recruitment site of gp130 [36]. The function of the other members of the SOCS family, particularly SOCS-4 to SOCS-7, remains to be elucidated.

Here we show that IL-9 induces the expression of CIS, SOCS-2 and SOCS-3 through the JAK/STAT pathway. This induction is rapid and transient. Using transient transfection in HEK-293-EBNA cells, we demonstrated that SOCS-3 affects IL-9 signalling but neither CIS nor SOCS-2 has an effect. In stably transfected BW5147C2 cells, we observed a negative effect of SOCS-3 on several activities of IL-9, such as STAT activation, Bcl-3 induction and protection against corticoid-induced apoptosis. By contrast, CIS did not interfere with IL-9 activities, suggesting a role in cytokine signalling cross-talk rather than feedback inhibition.

EXPERIMENTAL

Cytokines, cells and cell cultures

Recombinant human IL-9 (2×10^7 units/mg) and mouse IL-9 (5×10^7 units/mg) were produced in the baculovirus system in our laboratory and purified as described previously [37]. Recombinant mouse interferon γ (IFNγ) was provided by Dr W. Fiers (University of Gent, Gent, Belgium). Cytokines were added to the cultures at the following concentration: 400 units/ml for human and mouse IL-9, and 250 units/ml for IFNγ.

BW5147.C2 cells, a dexamethasone-sensitive subclone of the murine T-lymphoma cell line BW5147 [5], were cultured in Iscove–Dulbecco's medium supplemented with 10 % (v/v) foetal calf serum, 0.24 mM L-asparagine, 1.5 mM L-glutamine, 0.55 mM L-arginine and 50 μM 2-mercaptoethanol. BW5147.C2 cells were transfected with wild-type human IL-9R cDNA and its Phe-116 mutant, as described previously [19], and cultured in the presence of 1.5 μg/ml puromycin. Two additional mutated IL-9R cDNA species, mut6 (activating STAT1 and STAT3) and mut7 (activating STAT5), were similarly transfected in BW5147.C2 [20]. HEK-293 human embryonic kidney cells were cultured in Iscove–Dulbecco's medium containing 10 % (v/v) foetal calf serum.

mRNA preparation and Northern blotting

Total RNA was extracted from 10^7 cells with 1 ml of Trizol solution, in accordance with the manufacturer's instructions (Gibco BRL, Gaithersburg, MD, U.S.A.). Total cellular RNA (10 μg) was fractionated by electrophoresis in a 1.2 % (w/v) agarose gel containing 2.2 M formaldehyde, before transfer to a Hybond-C Extra nitrocellulose membrane (Amersham, Arlington Heights, IL, U.S.A.). A cDNA insert coding for CIS was retrieved from pEFBos/puro plasmid, then labelled with ^{32}P using the Rediprime kit (Amersham). The SOCS-2 probe was a reverse-transcriptase-mediated PCR (RT–PCR) product purified by QIAEx (Qiagen, Chatsworth, CA, U.S.A.) and ^{32}P-labelled with the Rediprime kit. Hybridization and washes were performed as described [38]. After autoradiography, all blots were reprobed with a β-actin probe to control for uniform loading of RNA.

RT–PCR analysis and Southern blotting

Reverse transcription was performed on 10 μg of Trizol-purified total RNA with an oligo(dT) primer. cDNA corresponding to 100 ng of total RNA was amplified for 27 cycles by PCR with specific primers as follows: mouse CIS sense, 5′-TCCGACTCTCGAGCCGCC-3′; mouse CIS anti-sense, 5′-GGGACTGTGTTCCCTCCAGG-3′; mouse SOCS-1 sense, 5′-GCAGCCGACAATGCGATCTC-3′; mouse SOCS-1 anti-sense, 5′-GTAGTCACGGAGTACCGGGT-3′; mouse SOCS-2 sense, 5′-CATCTCCCATGACCCTGCGG-3′; mouse SOCS-2 anti-sense, 5′-CAGTTATCCAGAGGAGGGCCT-C-3′; mouse SOCS-3 sense, 5′-CGTGCGCCATGGTCACCC-3′; mouse SOCS-3 anti-sense, 5′-GCCTCGGAGGAGAGGCGA-3′; mouse Bcl-3 sense, 5′-CCTGCGCAGCGGCTGCGACGT-3′; mouse Bcl-3 anti-sense, 5′-CATCCGTCTCAGCTGCTTCCT-3′; β-actin sense, 5′-ATGGATGACGATATCGCTGC-3′; β-actin anti-sense, 5′-GCTGGAAGGTGGACAGTGAG-3′.

Each 50 μl PCR reaction was performed with 1 μl of cDNA, 0.25 μl (1.25 units) of *Taq* polymerase (Takara Shuzo Co., Shiga, Japan), 1 μl of each oligonucleotide (10 μM), 4 μl of dNTPs (2.5 mM), 5 μl of buffer [100 mM Tris/HCl (pH 8.8)/500 mM KCl/15 mM $MgCl_2$/1 % (v/v) Triton X-100].

The post-PCR products were analysed in ethidium-bromide-stained 1 % (w/v) agarose gel. Specific amplification was confirmed by blotting (Zeta-Probe membrane; Bio-Rad, Hercules, CA, U.S.A.) and hybridization with internal radioactive probes. The internal probes were 5′-GTCCACGACATCATGCTACTG-3′ for β-actin, 5′-CCAGTCCCTGGAGCTGCCCG-3′ for CIS and 5′-CAGCCTGCGCCTCAAGACCT-3′ for SOCS-3. Radioactive signals were quantified by PhosphorImager (Molecular Dynamics, Sunnyvale, CA, U.S.A.) and the ratios of CIS to actin or SOCS-3 to actin were calculated.

Plasmid constructions, DNA transfections and analysis of transfected cells

For stable transfection of CIS and SOCS-3 expression plasmids in BW5147.C2 cells, the CIS and SOCS-3 cDNA species were cloned into pEFBos/puro plasmid [19], which contains a resistance gene to puromycin. BW5147.C2 cells were transfected by electroporation (270 V, 1500 μF, 74 Ω) with 50 μg of DNA, then selected with puromycin (1.5 μg/ml) (Sigma, Bornem, Belgium). Puromycin-resistant bulk populations and clones of cells were used as indicated.

For stable transfection of IL-9R in HEK-293 cells, hIL-9Rα cDNA was cloned into pEFBos/puro plasmid under the control of the EF-1α promoter [19,39] and the γ_c human cDNA (gift from Dr K. Sugamura, Sendai, Japan) was cloned into pcDNA3 plasmid (Invitrogen, Leek, The Netherlands), under the control of the cytomegalovirus promoter. Cells were seeded in six-well plates (Nunc, Rochester, NY, U.S.A.) at 8×10^5 cells per well 1 day before transfection. Transfections were performed with the LIPOFECTAMINE method (Gibco BRL), in accordance with the manufacturer's recommendations with 1 μg of each plasmid DNA. After transfection, cells were incubated in 2 ml of normal medium for 24 h; 1 day after transfection, cells were cultured in normal medium with puromycin (2 μg/ml) and G418 (0.5 mg/ml) (Sigma) until confluent populations had been obtained. Human IL-9R expression was similar in transfected cells, as tested by

FACS analysis with biotinylated anti-IL-9R antibody AH9R1 [19] followed by phycoerythrin-conjugated streptavidin (Becton Dickinson, San Jose, CA, U.S.A.).

For transient transfection of HEK-293 cells, the CIS, SOCS-2 and SOCS-3 cDNA species were cloned, under the control of the CMV promoter, into pcDNA3 or pcDNAI/Amp plasmids (Invitrogen). The JAK3 cDNA (received from Dr T. Taniguchi, Tokyo, Japan) was cloned into pEFBos/puro plasmid, under the control of the EF-1α promoter [19,39]. The reporter plasmids used were pGRR5–luciferase (gift from Dr P. Brennan, Imperial Cancer Research Fund, London, U.K.) and pRL-TK (Promega, Madison, WI, U.S.A.). Cells were seeded in 12-well plates (Nunc) at 4×10^5 cells per well 1 day before transfection. Transfections were performed with the LIPOFECTAMINE method, in accordance with the manufacturer's recommendations, with a total of 2 μg of plasmid DNA; the mixture contained 500 ng of JAK3 cDNA, 100 ng of pGRR5, 100 ng of pRL-TK, various quantities of CIS and SOCS cDNA species and empty vector to 2 μg. At 5 h after transfection, cells were treated with or without stimulation by human IL-9 (400 units/ml) for 24 h. Luciferase assays were performed with the dual luciferase reporter assay kit (Promega).

Staining with propidium iodide was performed to measure cell viability, as described previously [5]. In brief, BW5147.C2 cells as a control, together with transfectant BW-CIS.0 and BW-SOCS, were cultured for 24 h under various conditions: in the presence or not of IL-9 (100 units/ml), dexamethasone (100 ng/ml), or a combination of cyclosporin (500 ng/ml) and dexamethasone. Cells were then incubated for 30 min with propidium iodide (125 μg/ml) at room temperature before FACS analysis with a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ, U.S.A.). Under these conditions, dead cells are brightly stained but live cells are not. A minimum of 5000 cells were counted per sample.

Western blotting

CIS and SOCS-2 expression were assayed by Western blotting after transient transfection of HEK-293 cells. Cells were seeded in 6-well plates at 8×10^5 cells per well 1 day before transfection. Transfection was performed the day afterwards, as described above. On the third day, cells were lysed in 100 μl of SDS sample buffer. Proteins were fractionated on precast Novex (Carlsbad, CA, U.S.A.) SDS/PAGE [12% (w/v) gel] and transferred electrophoretically to nitrocellulose (Hybond-C; Amersham). Membranes were blocked in 5% (w/v) non-fat dried milk, washed and probed with diluted (1:500) affinity-purified goat polyclonal antibody directed against mouse CIS (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) or with rabbit polyclonal antibody raised against a recombinant protein corresponding to residues 89–162 of human SOCS-2 (Santa Cruz Biotechnology). Membranes were then probed with a secondary antibody: horseradish-peroxidase-linked donkey anti-goat Ig antibody (diluted 1:2000; Santa Cruz Biotechnology) for CIS or horseradish-peroxidase-linked goat anti-rabbit Ig antibody (diluted 1:5000; Transduction Laboratories, Lexington, KY, U.S.A.) for SOCS-2. An ECL® kit (Amersham) was used for detection by enhanced chemiluminescence.

STAT activation in BW5147 and in stable transfectants for CIS and SOCS-3 was also tested by Western blotting with the use of the same protocol. The anti-(phospho-STAT1) (Y701), anti-(phospho-STAT3) (Y705) and anti-(phospho-STAT5) (Y694) affinity-purified rabbit antibodies (New England Biolabs, Beverly, MA, U.S.A.) were diluted 1:1000. The β-actin affinity-purified polyclonal mouse antibody (Sigma, St Louis, MO, U.S.A.) was used at 1:3000 dilution.

RESULTS

IL-9 induces CIS, SOCS-2 and SOCS-3 expression

To study the role of SOCS family members in IL-9 signalling, we first examined their induction by RT–PCR. For this experiment, BW5147 lymphoma cells were stimulated in the presence of murine IL-9 (400 units/ml) for 2 h and mRNA expression was analysed by RT–PCR. As shown in Figure 1, CIS, SOCS-2 and SOCS-3, but not SOCS-1, were expressed on stimulation with IL-9. As a control for SOCS-1 amplification, we stimulated BW5147 cells with IFNγ (250 units/ml) for 2 h because it has previously been shown that IFNγ induces SOCS-1 in mouse myeloid leukaemia cells [40].

To determine the kinetics of induction, BW5147 cells were stimulated with IL-9 for various periods. We examined CIS expression with Northern blot hybridization. As shown in Figure 2(A), IL-9 rapidly induced CIS expression, reaching a maximum after 2 h of stimulation. The expression subsequently declined but remained detectable after 24 h. For SOCS-2 and SOCS-3 we used a more sensitive RT–PCR strategy. The kinetics of induction was similar for SOCS-2 and SOCS-3: the expression was maximal after 2 h and was maintained to a lower level for up to 24 h after stimulation (Figure 2B).

CIS and SOCS are induced by IL-9 through the JAK/STAT pathway

To determine the pathway used by IL-9 to induce these genes, we took advantage of BW5147 cells stably transfected with various mutants of the human IL-9R. In addition to the wild-type receptor (IL-9R), mutated IL-9R cDNA species, called Phe116, mut6 and mut7, have been obtained by mutagenesis and allowed to discriminate between the three STAT factors activated in response to IL-9 [19]. The Phe116 receptor does not activate any STAT; mut6 receptor activates STAT1 and STAT3; and mut7 receptor activates only STAT5. The corresponding BW5147 stable transfectants were cultivated in control medium, with human IL-9 (400 units/ml) or with murine IL-9 (400 units/ml)

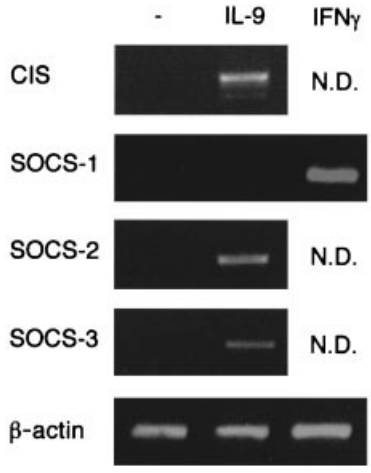

Figure 1 IL-9 induces CIS, SOCS-2 and SOCS-3 expression

BW5147 cells were cultured for 2 h in control medium or in the presence of murine IL-9 (400 units/ml) or murine IFNγ (250 units/ml) as a positive control for SOCS-1. RNA was isolated and a RT–PCR was performed with primers for CIS, SOCS-1, SOCS-2, SOCS-3 and β-actin. PCR products were analysed by ethidium bromide agarose-gel electrophoresis. Abbreviation: N.D.: not done.

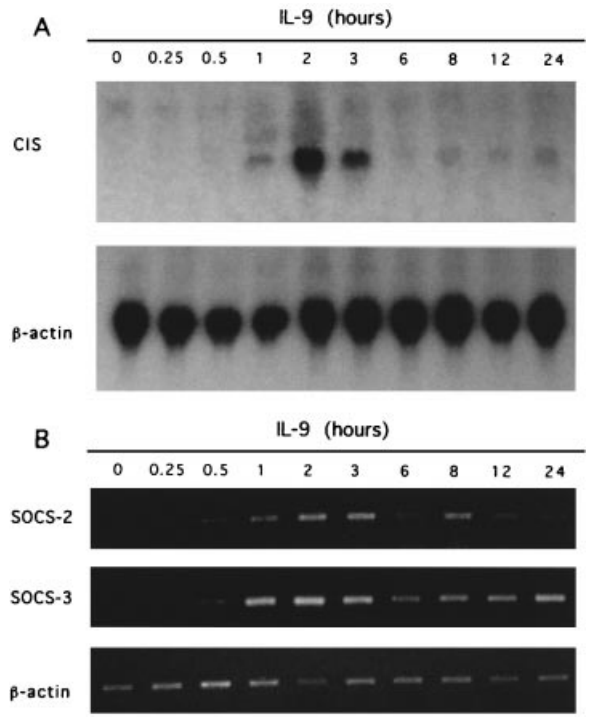

Figure 2 Induction of CIS, SOCS-2 and SOCS-3 by IL-9 is rapid and maintained for up to 24 h

BW5147 cells were cultured in control medium or with murine IL-9 (400 units/ml) for various periods between 15 min and 24 h before the isolation of RNA. (**A**) Northern blot analysis was performed with a cDNA probe for CIS or β-actin. (**B**) RT–PCR was performed with primers for SOCS-2, SOCS-3 or β-actin.

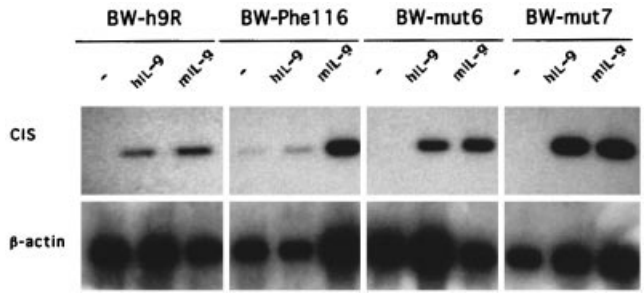

Figure 3 CIS expression is induced through IL-9R mutants that activate either STAT1 and STAT3 or STAT5

BW5147 transfectant cells were cultured for 2 h with control medium, human IL-9 (400 units/ml) or murine IL-9 (400 units/ml). RNA was isolated for RT–PCR, followed by Southern blotting and hybridization with an internal oligonucleotide for CIS or β-actin.

as a control. Parental BW5147 cells express the murine IL-9R and the stimulation of each transfectant with murine IL-9 should still lead to the expression of CIS and SOCS. As shown in Figure 3, CIS expression was induced by human IL-9 in BW-h9R but not in BW-Phe116 cells, in which IL-9 does not activate STAT factors. By contrast, both the mut6 and mut7 mutants allowed CIS induction, indicating that IL-9 redundantly regulated CIS expression via the activation of STAT1 and STAT3 (as in BW-mut6) or via STAT5 (as in BW-mut7). SOCS-2 followed the same pattern of induction (Figures 4A and 4B). By contrast,

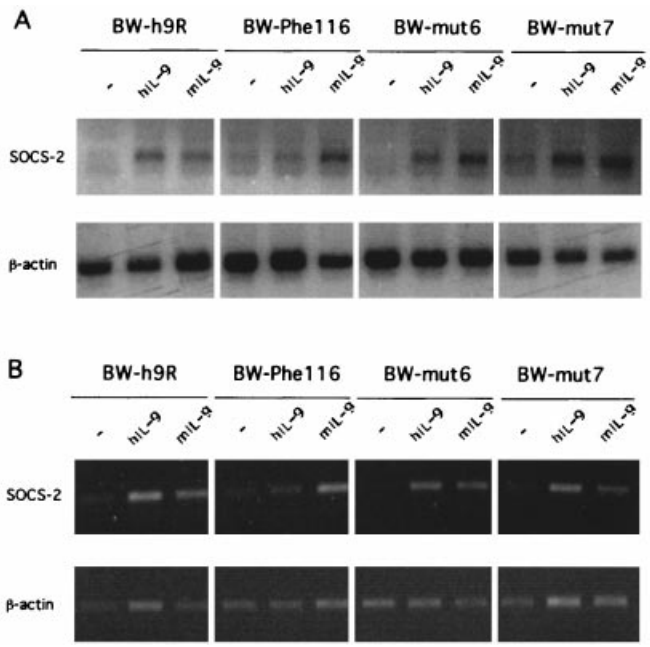

Figure 4 SOCS-2 expression is induced through IL-9R mutants that activate either STAT1 and STAT3 or STAT5

BW5147 transfectant cells were cultured for 2 h with control medium, human IL-9 (400 units/ml) or murine IL-9 (400 units/ml). RNA was isolated and a Northern blot with cDNA probes (**A**) and a RT–PCR with oligonucleotides for SOCS-2 or β-actin (**B**) were performed.

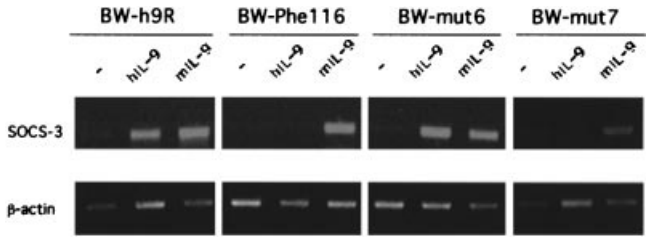

Figure 5 SOCS-3 expression is induced through STAT1 and STAT3 but not STAT5-activation

BW5147 transfectant cells were cultured for 2 h with control medium, human IL-9 (400 units/ml) or murine IL-9 (400 units/ml). RNA was isolated for RT–PCR with SOCS-3 and β-actin oligonucleotides. PCR products were analysed by ethidium bromide agarose-gel electrophoresis.

SOCS-3 is induced by human IL-9 in BW-mut6 (activating STAT1 and STAT3) and in BW-h9R (activating STAT1, STAT3 and STAT5) but is not induced in BW-mut7 (activating only STAT5) or in BW-Phe (deficient in STAT activation) (Figure 5). This suggested that SOCS-3 induction was mediated by STAT1 and STAT3 but not by STAT5.

Only SOCS-3 inhibits IL-9-induced signalling

Having demonstrated the induction of SOCS family members by IL-9, we investigated the potential role of these genes in IL-9 signal attenuation. We stably transfected HEK-293 cells with the IL-9Rα and γ_c cDNA. Then we transiently introduced JAK3, different amounts of CIS or SOCS cDNA and, as a reporter plasmid, the pGRR5–luciferase construct that is regulated by

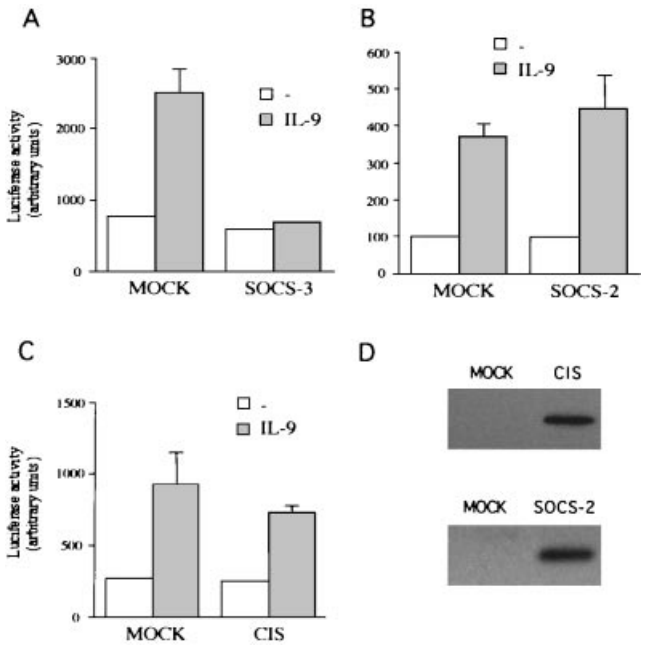

Figure 6 SOCS-3, but not CIS nor SOCS-2, inhibits IL-9 signal transduction

HEK-293 cells expressing IL-9R and γ_c were transfected with JAK3 and two reporter plasmids (pGRR5 and pRL-TK) in the presence or the absence of 500 ng of the pcDNA3 expression vector containing CIS (**A**), SOCS-2 (**B**) or SOCS-3 (**C**) cDNA. After transfection, cells were cultured for 24 h in control medium or with human IL-9 (400 units/ml) before measurement of luciferase activity. Similar results were obtained in five independent experiments. For CIS-transfected and SOCS-2-transfected cells, expression of the respective proteins was confirmed by Western blot analysis (**D**).

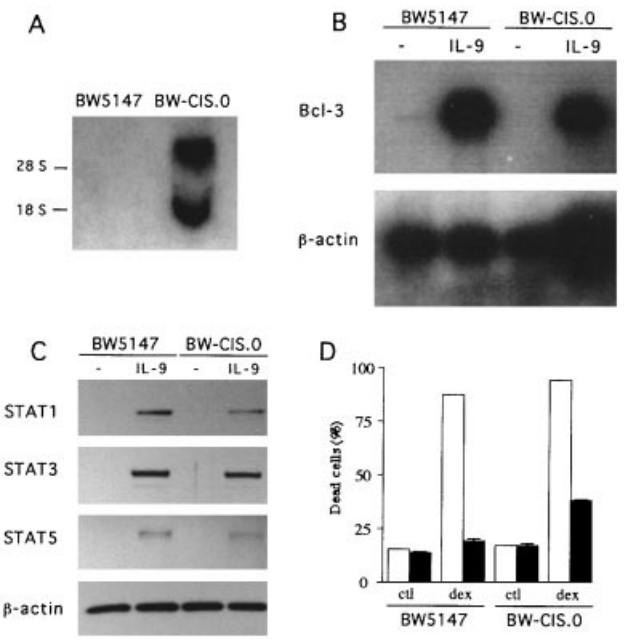

Figure 7 Stable CIS expression does not affect IL-9 activities on BW5147 cells

(**A**) CIS expression in BW5147 parental cells and BW-CIS.0 cells. RNA from BW5147 and BW-CIS.0 cells was isolated and Northern blotting with a CIS probe was performed. (**B**) Induction of Bcl-3 by IL-9 in BW5147 cells or cells stably transfected with CIS. Cells were cultured in control medium or with murine IL-9 (400 units/ml) for 12 h. RNA was isolated and RT–PCR was performed, followed by Southern blotting and hybridization with an internal probe for Bcl-3 or β-actin. (**C**) STATs activation in response to IL-9. BW5147 and BW-CIS.0 cells were stimulated for 15 min in the absence or the presence of IL-9 (400 units/ml) and analysed by Western blotting with anti-(phospho-STAT) antibodies or anti-(β-actin) antibody. (**D**) Effect of CIS expression on protection against dexamethasone-induced apoptosis. BW5147 and BW-CIS.0 cells were stimulated for 24 h in the absence (open bars) or the presence (filled bars) of murine IL-9 (100 units/ml), either in control medium (ctl) or with dexamethasone (100 ng/ml) and cyclosporin (500 ng/ml) (dex). Cyclosporin was added because it accelerates the effect of dexamethasone in this system [5]. Cells were analysed by flow cytometry after 30 min of incubation with propidium iodide. The graph shows percentages of dead cells. Similar results were obtained in three independent experiments.

five copies of a STAT-binding sequence recognizing at least STAT1, STAT3 and STAT5. Under these conditions, stimulation with IL-9 for 24 h induced a 3-fold increase in luciferase activity. As shown in Figure 6(A), SOCS-3 completely inhibited IL-9 signalling. By contrast, neither transfection with CIS nor transfection with SOCS-2 had any significant effect on IL-9 signal transduction (Figures 6B and 6C) although the proteins were produced, as shown by Western blotting after transfection with CIS and with SOCS-2 in HEK-293 cells (Figure 6D). These experiments indicated that SOCS-3, but not CIS or SOCS-2, might have a role in the attenuation of IL-9 signal transduction.

Only SOCS-3 inhibits IL-9-induced activities

To study the effect of the CIS and SOCS-3 proteins on the response to IL-9, we stably transfected into BW5147 expression plasmids encoding for CIS or SOCS-3 cDNA species. We first controlled the expression of these genes. For CIS, Northern blotting showed a high level of expression in bulk transfectant population (BW-CIS.0) (Figure 7A), higher than the induction by IL-9 in parental BW5147 cells (results not shown). The Northern blot showed two mRNA species for CIS, one transcript of 2 kb and another one of approx. 4 kb, as described previously [25]. We next examined the effect of this protein on several well-known effects of IL-9: the induction of Bcl-3, the activation of STATs, and protection against dexamethasone-induced apoptosis. To test for Bcl-3 induction by IL-9, cells were cultured with or without IL-9 for 12 h and RT–PCR reactions were used. As shown in Figure 7(B), IL-9 induced Bcl-3 in BW-CIS.0 as well as in BW5147. To study the activation of STATs in response to IL-9, we treated the cells without or with stimulation by IL-9 for 15 min; we then performed Western blotting with anti-(phospho-STAT) antibodies or with anti-(β-actin) antibodies. Figure 7(C) shows that IL-9 was still able to activate the STATs in BW-CIS.0. Finally we examined the effects of this protein on IL-9-induced protection against apoptosis. Dexamethasone induces the apoptosis of T lymphomas such as BW5147; IL-9 protects against this effect [5]. As shown in Figure 7(D), CIS did not inhibit the protective effect of IL-9 on dexamethasone-induced apoptosis.

For SOCS-3 we focused on two transfectant clones, BW-SOCS3.1 and BW-SOCS3.2, which showed different levels of expression (Figure 8A): although they both overexpressed SOCS-3, BW-SOCS3.2 cells expressed this gene at a higher level than BW-SOCS3.1 cells. This allowed us to investigate the potential relationship between SOCS-3 protein expression level and its inhibitory activity. As shown in Figure 8(B), IL-9 induced Bcl-3 in BW-SOCS3.1 as well as in parental BW5147 cells but Bcl-3 was no longer induced in BW-SOCS3.2 cells. STAT activation was also tested by Western blotting. Figure 8(C) shows that IL-9 induced a normal STAT activation in BW-SOCS3.1 cells but, in BW-SOCS3.2 cells, STAT3 activation was strongly

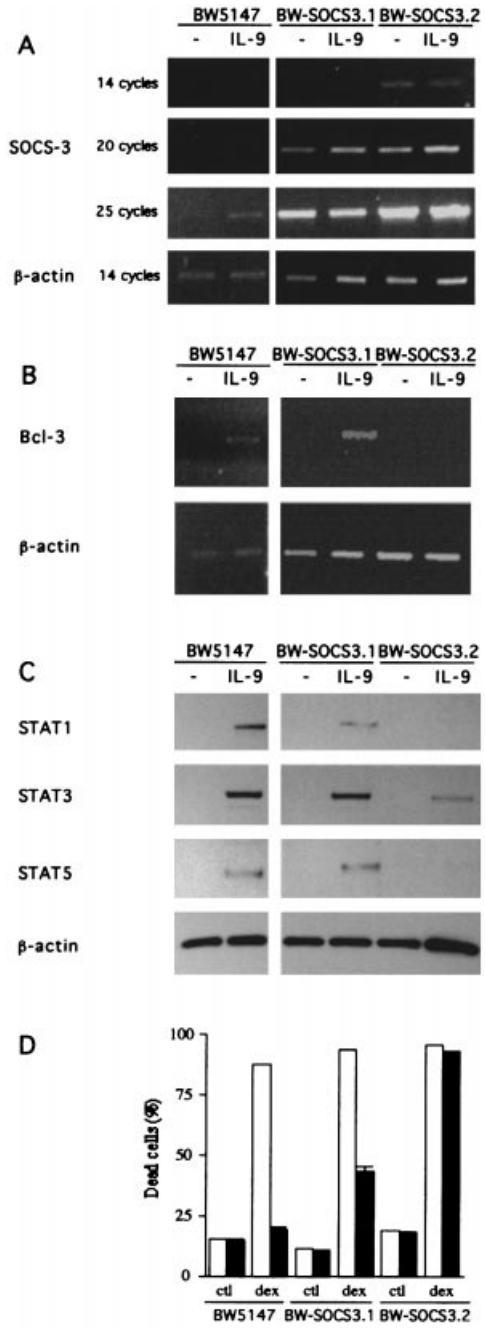

Figure 8 Effect of stable SOCS-3 expression on IL-9 activities in BW5147 cells

(**A**) SOCS-3 expression by BW5147 cells and stably transfected BW-SOCS3.1 and SOCS3.2 cells. Cells were cultured for 12 h in the absence or the presence of murine IL-9 (400 units/ml). RNA was isolated and RT–PCR was performed with primers for SOCS-3 or β-actin. (**B**) Induction of Bcl-3 by IL-9 in BW5147 cells or cells stably transfected with SOCS-3. Cells were cultured in medium or with IL-9 (400 units/ml) for 12 h; RNA was isolated and RT–PCR was performed, followed by Southern blotting and hybridization with an internal probe for Bcl-3 or β-actin. (**C**) STATs activation in response to IL-9. BW5147, BW-SOCS3.1 or BW-SOCS3.2 cells were stimulated for 15 min in the absence or the presence of murine IL-9 (400 units/ml) and analysed by Western blotting with anti-(phospho-STAT) antibodies or with anti-(β-actin) antibody. (**D**) Effect of SOCS-3 on protection against dexamethasone-induced apoptosis. BW5147.C2, BW-SOCS3.1 and BW-SOCS3.2 cells were stimulated for 24 h in the absence (open bars) or the presence (filled bars) of murine IL-9 (100 units/ml), either in control medium (ctl) or with dexamethasone (100 ng/ml) and cyclosporin (500 ng/ml) (dex). They were analysed by flow cytometry after 30 min of incubation with propidium iodide. The graph shows percentages of dead cells. Similar results were obtained in two independent experiments.

decreased and STAT1 and STAT5 activation was abolished. Finally we analysed the effect of SOCS-3 on IL-9-induced protection against apoptosis. With the same conditions as those described above, only the high level of SOCS-3 expressed in BW-SOCS3.2 cells was able to inhibit the protection exerted by IL-9 (Figure 8D).

DISCUSSION

Genes of the CIS/SOCS family are induced by a wide range of cytokines, including IL-1, IL-3, IL-4, IL-6, erythropoietin, IFNγ, leukaemia inhibitory factor (LIF), granulocyte colony-stimulating factor, granulocyte/macrophage colony-stimulating factor and growth hormone (GH) [26,29,41]. They are induced mainly by the JAK/STAT pathway and inhibit signal transduction either by binding to activated receptors and thus competing with STATs [33] or by inhibiting the kinase activity of JAKs [23]. In this paper we have shown that, in thymic lymphoma cells, IL-9 induces the expression of three genes of this family: CIS, SOCS-2 and SOCS-3. This rapid and transient induction is most probably mediated by the JAK/STAT pathway because a point mutation of the IL-9R that abolishes STAT activation also prevents CIS/SOCS induction. Using mutant IL-9Rs that selectively lost the ability to activate either STAT5 or STAT1 and STAT3, we found that CIS and SOCS-2 could be induced by IL-9 in both conditions. This contrasts with the initial paper suggesting that CIS might be specifically regulated by STAT5 [33] but is in line with the observation that GH induces CIS expression in STAT5b-deficient mice [42]. By contrast, SOCS-3 was found to be induced by IL-9 through an IL-9R mutant that activates STAT1 and STAT3, but not with a mutant receptor that activates only STAT5. This observation contrasts with a recent paper showing that STAT5 mediates the GH-induced expression of SOCS-3 in the liver [42]. This discrepancy might reflect tissue-specific differences as shown for GH, which induces CIS and SOCS-2 expression in liver and mammary gland but SOCS-3 only in the liver [42]. Another potential explanation would be a distinct regulation of STAT5 activity by IL-9 and GH through serine phosphorylation because, unlike many other cytokines, IL-9 does not induce serine phosphorylation of STAT factors [43–45].

Because of their ability to attenuate signal transduction from several cytokines, we focused on the possible role of these genes in a negative feedback loop for IL-9 signalling. We showed that only overexpression of SOCS-3 was able to inhibit signal transduction from IL-9, whereas CIS and SOCS-2 do not have such an effect. This might reflect the mode of action of CIS. Indeed, CIS has been shown to associate to tyrosine-phosphorylated erythropoietin receptors and phosphorylated β subunits of the IL-3 receptor [25]. This binding is thought to prevent association of STAT5, thereby inhibiting downstream signal transduction. As with IL-9R, one could hypothesize that its STAT5-binding motif has a weak affinity for the SH2 domain of CIS that might therefore be unable to interfere with IL-9 signalling. Little is known about the activity of SOCS-2 on cytokine signalling. Interestingly, it was shown previously that SOCS-2 is able to suppress the signal of LIF [30] but SOCS-2 was also shown to fail to inhibit prolactin signal transduction [46]. SOCS-3 activity might be mediated by two distinct mechanisms: on the one hand, it could bind to phosphorylated receptors such as gp130 [36]; on the other hand, SOCS-3 is thought to inhibit signalling by binding to JAK kinases through its SH2 domain, thereby inhibiting their kinase activity [34,35]. The experiments reported here do not permit discrimination between these modes of action for the IL-9R.

The observation that CIS, SOCS-2 and even lower levels of SOCS-3 failed to have a negative feedback activity on IL-9 signalling raises the question of the actual role of these proteins in the IL-9 response. Because a negative activity has been demonstrated for other cytokines such as IL-3 [25], erythropoietin [33] for CIS, and LIF for SOCS-2 [30], the induction of these two genes could inhibit the cell response to other cytokines and confer on IL-9-stimulated cells a resistant state to various signals. Alternatively, these proteins might have other roles than that of inhibitors of signalling. In this regard, it should be stressed that most reports of inhibitory activity, including the present one, involved the overexpression of these genes. For instance, the loss of the IL-9 response in stable SOCS-3 transfectants was seen only with cells expressing SOCS-3 mRNA levels that were much higher than those induced by IL-9. However, studies of mice deficient in SOCS-1 and in SOCS-3 point to a negative regulatory role for these genes in the development of T-cells and in haemopoiesis in foetal liver respectively, supporting the inhibitory hypothesis [47,48]. In IL-6 signal transduction, it has been recently shown that the recruitment site for SOCS-3 on gp130 corresponds to the consensus recruitment site for SHP2 [36]. The fact that the IL-9R sequence does not seem to include any related motif could explain why IL-9 signalling is relatively resistant to SOCS-3 inhibition. Further studies examining the IL-9 response of SOCS-3-deficient cells might shed some light on these questions.

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