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Suppressor of Cytokine Signaling 1 Inhibits Cytokine Induction of CD40 Expression in Macrophages

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CD40 is a type 1 membrane-bound molecule belonging to the TNFR superfamily that is expressed on various immune cells including macrophages and microglia. The aberrant expression of CD40 is involved in the initiation and maintenance of various human diseases including multiple sclerosis, arthritis, atherosclerosis, and Alzheimer’s disease. Inhibition of CD40 signaling has been shown to provide a significant beneficial effect in a number of animal models of human diseases including the aforementioned examples. We have previously shown that IFN-γ induces CD40 expression in macrophages and microglia. IFN-γ leads to STAT-1α activation directly and up-regulation of NF-κB activity due to the secretion and subsequent autocrine signaling of TNF-α. However, TNF-α alone is not capable of inducing CD40 expression in these cells.Suppressor of cytokine signaling 1 protein (SOCS-1) is a cytokine-inducible Src homology 2-containing protein that regulates cytokine receptor signaling by inhibiting STAT-1α activation via a specific interaction with activated Janus kinase 2. Given the important role of CD40 in inflammatory events in the CNS as well as other organ systems, it is imperative to understand the molecular mechanisms contributing to both CD40 induction and repression. We show that ectopic expression of SOCS-1 abrogates IFN-γ-induced CD40 protein expression, mRNA levels, and promoter activity. Additionally, IFN-γ-induced TNF-α secretion, as well as STAT-1α and NF-κB activation, are inhibited in the presence of SOCS-1. We conclude that SOCS-1 inhibits cytokine-induced CD40 expression by blocking IFN-γ-mediated STAT-1α activation, which also then results in suppression of IFN-γ-induced TNF-α secretion and subsequent NF-κB activation. The Journal of Immunology, 2002, 169: 2354–2360.

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TNF-α treatment in and of itself does not induce CD40 expression in macrophages/microglia (24).

The suppressor of cytokine signaling 1 protein (SOCS-1) is one of seven recently discovered cytokine-inducible down-regulators of cytokine signaling (27–29). SOCS-1 interacts with phosphorylated Janus kinase (JAK) 2 via an Src homology 2 domain and has been shown to specifically inhibit signaling by IFN-γ (30). Additionally, a conserved C-terminal motif among SOCS members, known as the SOCS box, has been implicated in coupling proteins to proteasomal degradation (31, 32). A multitude of studies have implicated SOCS-1 involvement in the inhibition of a number of signaling pathways (27, 28, 33–36). However, it appears that the most important in vivo target of SOCS-1 is the IFN-γ-activated JAK-STAT pathway. This has been demonstrated by the fact that the lethal perinatal syndrome observed in SOCS-1-deficient mice, which includes fatty degeneration, necrosis of the liver, and damage to the pancreas, heart, and skin due to infiltrating T lymphocytes, macrophages, and eosinophils (37–39), is eliminated with Abs to IFN-γ or in mice deficient in IFN-γ in addition to SOCS-1 (40). This clearly implicates SOCS-1 as a specific inhibitor of the IFN-γ-mediated JAK-STAT pathway. It is worthy of comment, however, that mice lacking both SOCS-1 and IFN-γ, though saved from the lethal perinatal syndrome observed in SOCS-1-deficient mice, develop a variety of chronic infections or inflammatory lesions as adults (41).

To further define the role of the JAK-STAT pathway in the induction of CD40 expression in macrophages and to identify a potential means to inhibit pathological inflammation by down-regulating CD40 expression, we constructed a murine macrophage cell line stably expressing murine SOCS-1 to determine its effect on IFN-γ-mediated CD40 induction. We have previously shown that IFN-γ-induced STAT-1α phosphorylation is blunted by ectopic SOCS-1 expression in these cells (42). In this study, we demonstrate that IFN-γ-mediated CD40 up-regulation in macrophages is abrogated by ectopic SOCS-1 expression. Clues to the potential mechanisms of this inhibition include the observations that SOCS-1 inhibits IFN-γ-mediated STAT-1α phosphorylation and binding to the medial GAS element of the CD40 promoter, as well as IFN-γ-mediated NF-κB activation and binding to the distal κB binding site of the CD40 promoter. We also show that the latter effect is likely due to the inhibition by SOCS-1 of IFN-γ-mediated induction of TNF-α and its receptor.

Materials and Methods

Recombinant proteins and reagents

Recombinant murine IFN-γ was purchased from Genzyme (Boston, MA) and murine TNF-α and neutralizing anti-murine TNF-α Ab were purchased from Endogen (Woburn, MA). Rat IgG2α-κ anti-mouse CD4 Ab (clone 3/23), biotinylated mouse anti-rat IgG2a, and PE-conjugated streptavidin were purchased from BD PharMingen (San Diego, CA). Mouse anti-human c-myc Ab was purchased from Calbiochem (La Jolla, CA). Goat anti-human NF-κB p50 and p65 Abs, rabbit anti-SOCS-1 Ab, and consensus NF-κB binding oligonucleotide were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Abs to STAT-1α and phosphotyrosine-701-STAT-1α were purchased from Upstate Biotechnology (Lake Placid, NY).

Cells

The murine macrophage cell line RAW264.7 was maintained in DMEM supplemented with 10% FBS as previously described (25).

Stable transfection of SOCS-1

SOCS-1 stable transfectants were created by transfecting RAW cells with the pcDNA3 expression vector containing N-terminal myc-tagged cDNA of mouse SOCS-1 (a generous gift from Prof. A. Yoshimura, Kurume University, Kurume, Japan) using the Lipofectamine Plus method according to the manufacturer (Life Technologies, Rockville, MD) (42). RAW264.7 cells stably transfected with the pcDNA3 plasmid only were used as a negative control. Cells were selected in G418 sulfate (100 μg/ml) and screened for SOCS-1 expression by immunoblotting for c-myc expression as well as fluorescence microscopy for SOCS-1.

CD40 promoter constructs

The characterization of the human CD40 promoter construct (hCD40p0.7) was described previously (25).

RNA isolation, riboprobes, and ribonuclease protection assay (RPA)

Total cellular RNA was isolated from confluent monolayers of RAW264.7 cells. The riboprobes for murine CD40, IFN regulatory factor 1 (IRF-1), TNF-α, and GAPDH prepared from in vitro transcription with T7 polymerase are 576, 367, 312, and 270 nt, respectively. The riboprobes for murine receptor-interacting protein (RIP), L32, and TNFR1 were purchased from BD PharMingen and prepared similarly following the manufacturer’s guidelines. Twenty micrograms of total RNA from RAW264.7 cells was hybridized with CD40, IRF-1, TNF-α, and GAPDH riboprobes or with RIP, TNFR1, L32, and GAPDH (25 × 10^3 cpm) at 42°C overnight in 20 μl of 40 mM PIPES (pH 6.4), 80% deionized formamide, 400 mM NaOAc, and 1 mM EDTA. The hybridized mixture was then treated with RNase A/T1 (1/200 dilution in 200 μl of the RNase digestion buffer) at 37°C for 30 min, analyzed by 5% denaturing (8 M urea) PAGE, and the gels were exposed to phosphorimaging cassettes. The protected fragments of the CD40, IRF-1, TNF-α, and GAPDH riboprobes are 419, 314, 270, and 212 nt in length, respectively. Quantification of the protected RNA fragments was performed by scanning with the PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Values for CD40, IRF-1, TNF-α, RIP, and TNFR1 mRNA expression were normalized to GAPDH mRNA levels for each experimental condition. GAPDH mRNA was used as a housekeeping gene because its levels are not affected by cytokine treatment.

Nuclear extracts and EMSA

Cells were incubated with medium or IFN-γ (10 ng/ml) for various time periods (0–24 h), and nuclear extracts were prepared. EMSA was performed with 5–10 μg of nuclear extract in a final volume of 15 μl of binding buffer (50 mM NaCl, 1 mM MgCl2, 0.1 mM EDTA, 4% glycerol, 0.5 mM DTT, 4 mM Tris-HCl (pH 7.5), 1 μg polydeoxyinosinoc-deoxy- cytidyl acid, and 20,000 cpm 32P-labeled oligonucleotide probe), and incubated on ice for 15 min. Bound and free DNA were then resolved by electrophoresis through a 6% polyacrylamide gel in 0.5× Tris-borate-EDTA buffer at 250 V for 1 h. For supershift analysis, 1 μl of nuclear extract in a total volume of 40 mM PIPES (pH 6.4), 80% deionized formamide, 400 mM NaOAc, and 1 mM EDTA. The hybridized mixture was then treated with RNase A/T1 (1/200 dilution in 200 μl of the RNase digestion buffer) at 37°C for 30 min, analyzed by 5% denaturing (8 M urea) PAGE, and the gels were exposed to phosphorimaging cassettes. The protected fragments of the CD40, IRF-1, TNF-α, and GAPDH riboprobes are 419, 314, 270, and 212 nt in length, respectively. Quantification of the protected RNA fragments was performed by scanning with the PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Values for CD40, IRF-1, TNF-α, RIP, and TNFR1 mRNA expression were normalized to GAPDH mRNA levels for each experimental condition. GAPDH mRNA was used as a housekeeping gene because its levels are not affected by cytokine treatment.

Transient transfection and analysis

A total of 0.7 μg of the human CD40 (hCD40) promoter construct was transiently transfected into 2 × 10^5 SOCS-1 stable transfectants or pcDNA3-transfected RAW264.7 cells in 12-well plates using the Lipo- fectAMINE Plus method as previously described (25). As a comparison, wild-type RAW264.7 cells were also transiently transfected with 0.35 μg of the hCD40 promoter construct and 0.35 μg of either the SOCS-1 plasmid or the empty pcDNA3 vector. Differences in the amount of DNA were adjusted with the appropriate empty vector. After 3 h of transfection, cells were allowed to recover for 4 h before treatment with IFN-γ (10 ng/ml) for 12 h, which we have previously determined to be optimal for IFN-γ-induced activation of the hCD40p0.7 construct (25). Cells were washed with PBS and lysed with 200 μl of lysis buffer (25 mM trisphosphate (pH 7.8), 2 mM DTT, 2 mM dianimocyclohexane tetracetic acid, 10% glycerol, and 1% Triton X-100). Extracts were assayed in triplicate for luciferase activity in a total volume of 20 μl (10 μl cell extract, 20 μl Tricine, 0.1 mM EDTA, 1 mM MgCl2, 2.67 mM MgSO4, 33.3 mM DTT, 0.27 mM CoA, 0.47 mM luciferin, and 0.53 mM ATP), and light intensity was measured using a luminometer (Promega, Madison, WI). Luciferase activity was integrated over a 10-s time period. Extracts were also assayed in triplicate for protein concentration. The luciferase activity of each sample was normalized to total protein concentration to yield relative luciferase activity. Fold induction was calculated as the ratio of relative luciferase activity between IFN-γ and medium-treated samples that were transfected with the same construct.
Measurement of TNF-α secretion

Cells were incubated in the absence or presence of IFN-γ (10 ng/ml) for various time points after which supernatants were collected and analyzed with a murine TNF-α ELISA kit purchased from R&D Systems (Minneapolis, MN) following the manufacturer’s instructions. Samples were read in triplicate and normalized to total protein levels which were also determined in triplicate.

Western blotting

For detection of tyrosine-phosphorylated STAT-1α and total STAT-1α, 100 μg of cell lysates were boiled in sample buffer, separated on 10% SDS-PAGE, transferred to a nitrocellulose membrane, and probed with anti-phospho-701-STAT-1α. Membranes were stripped at 50°C in buffer containing 100 mM 2-ME, 2% SDS, 62.5 mM Tris-HCl (pH 6.7) with occasional shaking, and reprobed for total STAT-1α protein. The ECL method was used for protein detection.

Results

TNF-α is required for IFN-γ induction of CD40, but alone does not induce CD40 expression

We have recently shown that both IFN-γ and TNF-α are required for CD40 gene expression in macrophages and microglia (26). FACS analysis demonstrated that IFN-γ increases CD40 protein expression on the cell surface, as measured by mean fluorescence intensity (Fig. 1A). TNF-α is required for IFN-γ induction of CD40 expression in RAW264.7 cells. This is demonstrated by the attenuation of IFN-γ-induced CD40 protein expression by a neutralizing anti-TNF-α mAb (Fig. 1A). However, TNF-α treatment alone is unable to induce CD40 expression (Fig. 1A). We have previously demonstrated that IFN-γ induction of CD40 in these cells requires both STAT-1α and NF-κB activation (25, 26). TNF-α is a well-known activator of NF-κB and does so in the RAW264.7 cells (data not shown). Additionally, recent studies implicate TNF-α as an activator of STAT-1α in certain cell lines (43–45). To determine the reason for the failure of TNF-α to induce CD40, we tested whether or not TNF-α is able to induce STAT-1α tyrosine phosphorylation. As shown in Fig. 1B, TNF-α was unable to induce tyrosine phosphorylation of STAT-1α in RAW264.7 cells, in contrast to that seen by IFN-γ. The inability of TNF-α to induce tyrosine phosphorylation of STAT-1α may preclude it from being sufficient to induce CD40 expression since activated STAT-1α is critical for CD40 induction in these cells (25). Thus, TNF-α alone does not induce CD40 expression in RAW264.7 cells, but is an important component of IFN-γ-induced CD40 expression.

Ectopic SOCS-1 abrogates IFN-γ induction of CD40 mRNA and protein expression

As shown above, IFN-γ is a potent inducer of CD40 expression on macrophages. FACS analysis confirmed a robust induction of CD40 surface expression in RAW264.7 cells stably transfected with the pcDNA3 control vector (RAW-pcDNA3) after a 48-h treatment with 10 ng/ml murine IFN-γ (Fig. 2A), comparable to that seen in untransfected cells (Fig. 1A). However, in the RAW264.7 cells stably transfected with SOCS-1, IFN-γ-induced CD40 expression is abrogated (Fig. 2B). mRNA levels of CD40 were also monitored using ribonuclease protection assay. An 8-h IFN-γ treatment (10 ng/ml) led to a 20-fold induction of CD40 mRNA levels in the RAW-pcDNA3 cells (Fig. 3, lane 2) and this induction was inhibited by ~80% in the presence of SOCS-1 (lane 6). IRF-1 mRNA induction by IFN-γ was also examined since it is an IFN-γ-inducible gene (46). A 24-fold induction of IRF-1 was observed upon IFN-γ treatment (Fig. 3, lane 3) and ~75% inhibition was noted in the presence of SOCS-1 (Fig. 3, lane 6). LPS induction of TNF-α was also monitored in this experiment. This signaling pathway was not affected by SOCS-1 overexpression (Fig. 3, lanes 2 and 5). These results demonstrate that IFN-γ induction of both CD40 and IRF-1 mRNA expression is inhibited by ectopic SOCS-1 expression in RAW264.7 cells, whereas LPS induction of TNF-α mRNA is not affected, indicating specificity of SOCS-1 for IFN-γ-induced signaling.

SOCS-1 inhibits IFN-γ-induced CD40 promoter activity

The effect of SOCS-1 overexpression on CD40 promoter activity was analyzed next to determine whether the inhibitory effect was
mediated at the transcriptional level. A 12-h treatment with IFN-γ (10 ng/ml) led to a 3-fold induction of CD40 promoter activity in the RAW-pcDNA3 cells, whereas no induction of CD40 promoter activity was detected in the RAW-SOCS-1 cells (Fig. 4A). To determine whether or not this inhibitory effect was an artifact of chronic SOCS-1 overexpression, wild-type RAW264.7 cells were transiently cotransfected with the human CD40 promoter construct and either SOCS-1 or the pcDNA3 vector. IFN-γ treatment enhanced CD40 promoter activity 4.5-fold, which was inhibited by transient expression of SOCS-1 (Fig. 4B).

**FIGURE 3.** SOCS-1 inhibits IFN-γ-induced CD40 promoter activity. RAW264.7 cells stably transfected with either SOCS-1 (St-SOCS-1) or the pcDNA3 vector (St-pcDNA3) were transiently transfected with either pcDNA3 vector (T-pcDNA3, 0.35 μg) or SOCS-1 (T-SOCS-1, 0.35 μg) with the hCD40p0.7 (0.35 μg) construct and then treated as described in A. Mean ± SD of three experiments.

**FIGURE 4.** SOCS-1 inhibition of CD40 promoter activity. A. RAW264.7 cells stably transfected with either SOCS-1 (St-SOCS-1) or the pcDNA3 vector (St-pcDNA3) were transiently transfected with 0.7 μg of the hCD40p0.7 construct, allowed to recover for 4 h, then were treated with medium or IFN-γ (10 ng/ml) for 12 h and analyzed for luciferase activity. Values were normalized to total protein and fold induction was calculated by dividing treatment values by untreated levels. B. Wild-type RAW264.7 cells were cotransfected with either pcDNA3 vector (T-pcDNA3, 0.35 μg) or SOCS-1 (T-SOCS-1, 0.35 μg) with the hCD40p0.7 (0.35 μg) construct and then treated as described in A. Mean ± SD of three experiments.

STAT-1α DNA-binding activity in the RAW264.7-pcDNA3 cells, whereas no induction of CD40 promoter activity was detected in the RAW-SOCS-1 cells (Fig. 4A). To determine whether or not this inhibitory effect was an artifact of chronic SOCS-1 overexpression, wild-type RAW264.7 cells were transiently cotransfected with the human CD40 promoter construct and either SOCS-1 or the pcDNA3 vector. IFN-γ treatment enhanced CD40 promoter activity 4.5-fold, which was inhibited by transient expression of SOCS-1 (Fig. 4B).

**FIGURE 5.** SOCS-1 inhibits IFN-γ-mediated NF-κB DNA-binding activity and TNF-α secretion

We have previously shown that IFN-γ induction of CD40 promoter activity is dependent on the STAT-1α transcription factor that activated STAT-1α binds to two GAS elements within the CD40 promoter (25). Given the strong inhibitory effect of SOCS-1 on IFN-γ-induced CD40 expression, we reasoned that IFN-γ-activated STAT-1α may be inhibited by ectopic SOCS-1 expression. To determine the effect of SOCS-1 on IFN-γ activation of STAT-1α, we performed EMSA using the medial GAS sequence of the CD40 promoter as a probe. As expected, IFN-γ treatment induced STAT-1α DNA-binding activity in the RAW264.7-pcDNA3 cells in a time-dependent manner, and this was inhibited in the presence of SOCS-1 (Fig. 5). This result suggests that the inhibitory effect of SOCS-1 may be due in part to inhibition of STAT-1α binding to the medial GAS element of the CD40 promoter.

**Ectopic SOCS-1 inhibits IFN-γ-mediated NF-κB DNA-binding activity and TNF-α secretion**

We have recently shown that another important component of IFN-γ-induced CD40 expression is contributed by TNF-α. IFN-γ induces TNF-α secretion in macrophages and microglia, and the subsequent autocrine or paracrine signaling by TNF-α is necessary for maximal IFN-γ induction of CD40 expression in these cells. This is in part due to NF-κB activation and binding to the CD40 promoter (26). To determine the effect of SOCS-1 on IFN-γ-induced NF-κB-binding activity, EMSA experiments were performed using the distal κB CD40-binding sequence as a probe. In the presence of SOCS-1, IFN-γ was unable to induce NF-κB-binding activity compared with that observed in pcDNA3 stable transfectants (Fig. 6A). We confirmed that IFN-γ-induced NF-κB activation is due to induction and subsequent autocrine/paracrine action of TNF-α by showing that IFN-γ-induced NF-κB activity is attenuated in the presence of a neutralizing anti-TNF-α Ab (Fig. 6B). Supershift analysis suggests that both p65 and p50 subunits of NF-κB are involved in this response (Fig. 6C).

Given that IFN-γ-mediated TNF-α secretion is necessary for subsequent NF-κB activation, we next assessed the effect of ectopic SOCS-1 expression on TNF-α production. RAW cells (pcDNA3 and SOCS-1) were incubated with IFN-γ (10 ng/ml) for various times (0–24 h), then supernatants were harvested and analyzed for TNF-α production by ELISA. IFN-γ treatment of pcDNA3-RAW cells led to a time-dependent induction of TNF-α, while this same response was abrogated in SOCS-1 overexpressors (Fig. 7). These results indicated that ectopic expression of SOCS-1 inhibits IFN-γ-induced TNF-α production and subsequent activation of NF-κB.

**SOCS-1 inhibits IFN-γ-induced TNFR1 and RIP mRNA levels**

In addition to the enhanced secretion of TNF-α that leads to activation of the TNF-α signaling cascade and activation of NF-κB, we considered the potential role of IFN-γ to enhance the sensitivity of the cell to TNF-α signaling. We examined whether IFN-γ may enhance the expression of TNFR1, as well as RIP, an adaptor protein important for TNF-α-mediated NF-κB activation (47). Moreover, we reasoned that since SOCS-1 appears to be a specific inhibitor of the IFN-γ pathway in our system, any IFN-γ-mediated sensitization to TNF-α signaling may be abrogated by SOCS-1 overexpression. As shown in Fig. 8, RAW-pcDNA3 cells constitutively express TNFR1 mRNA (lane 1) which is enhanced by an 8-h treatment with IFN-γ (3.1-fold induction; lane 2). In addition, a 2.6-fold induction of RIP mRNA is observed in the RAW-pcDNA3 cells (Fig. 8, lane 2). Ectopic expression of SOCS-1 abrogates the IFN-γ induction of both TNFR1 and RIP (Fig. 8, lane 4). This result suggests that IFN-γ may be involved in priming
macrophages for TNF-α action by enhancing the expression of TNFR1 and RIP. As well, attenuation of IFN-γ-mediated TNF1 and RIP up-regulation by ectopic SOCS-1 may contribute to the mechanism of its inhibitory effect on IFN-γ-mediated CD40 up-regulation.

Exogenous addition of TNF-α does not restore IFN-γ-induced CD40 expression in the SOCS-1 transfectants

IFN-γ-induced TNF-α production is attenuated in the SOCS-1 transfectants, which is in part responsible for SOCS-1 inhibition of IFN-γ-induced CD40 expression. We next examined whether exogenously provided TNF-α compensates for the loss of TNF-α production in the SOCS-1 transfectants for inducing CD40 expression. As shown in Fig. 9, TNF-α alone does not induce CD40 mRNA expression in RAW-pcDNA3 cells (lane 2), nor does it enhance IFN-γ-induced CD40 mRNA expression (compare lanes 3 and 4). In the SOCS-1 transfectants, IFN-γ alone does not induce CD40 expression (Fig. 9, lane 7), and the addition of TNF-α with IFN-γ treatment was unable to restore CD40 expression (Fig. 9, lane 8). Thus, exogenously provided TNF-α does not compensate for the loss of IFN-γ-induced TNF-α production in SOCS-1 transfectants. Although exogenous TNF-α does activate NF-κB in the SOCS-1 transfectants (data not shown), this signal, in the absence of IFN-γ activation of STAT-1α, is not sufficient for CD40 expression.

Discussion

CD40 has been implicated as a proinflammatory molecule that is involved in a variety of critical immunologic functions. The signaling pathway initiated by CD40 ligation with its cognate ligand, CD154, is a necessary and nonredundant step in a number of pathological inflammatory conditions (8, 9, 48–52). Despite the importance of the immune regulatory events initiated by CD40 signaling, surprisingly little is known about regulation of this gene. IFN-γ leads to the up-regulation of CD40 on macrophages and microglia. Necessary for such induction is STAT-1α activation as well as TNF-α secretion and subsequent autocrine/paracrine induction of TNF-α-dependent NF-κB activation (25, 26). Although TNF-α signaling is necessary for CD40 induction, TNF-α treatment alone does not induce CD40 expression (Figs. 1 and 9). IFN-γ-activated STAT-1α and NF-κB, along with constitutively expressed PU.1/Spi-B, bind to GAS, κB, and etsA and etsB elements within the CD40 promoter (25, 26). SOCS-1 attenuates IFN-γ signaling in vivo (40) and may have additional intracellular actions whose importance emerge later in life (41). It was of interest to us to identify a means to inhibit CD40 expression in macrophages as well as to further define the role of IFN-γ in CD40 gene induction.

In this current study, we show that IFN-γ is unable to induce CD40 protein expression in RAW264.7 cells ectopically expressing SOCS-1 (Fig. 2). SOCS-1 attenuates IFN-γ-induced CD40 mRNA expression (Fig. 3) as well as promoter activity (Fig. 4). Additionally, IFN-γ-induced STAT-1α phosphorylation and binding to the medial GAS element of the CD40 promoter is abrogated (Fig. 5), and IFN-γ induction of NF-κB binding to the distal κB element of the CD40 promoter is significantly decreased in RAW264.7 cells ectopically expressing SOCS-1 (Fig. 6A). The later effect is likely due to the abrogation of IFN-γ mediated TNF-α secretion (Fig. 7) as IFN-γ is unable to activate NF-κB in

FIGURE 6. SOCS-1 inhibits IFN-γ-induced NF-κB DNA-binding activity. A, EMSA experiments were performed using nuclear extracts prepared from RAW-pcDNA3 and RAW-SOCS-1 stable cells treated with IFN-γ (10 ng/ml) for 0, 1, 3, and 6 h and assayed using a radiolabeled oligonucleotide containing the distal NF-κB DNA sequence of the hCD40 promoter. B, EMSA was performed using nuclear extracts (7 μg) prepared from RAW cells treated with IFN-γ (10 ng/ml) alone or in the presence of anti-TNF-α-neutralizing Ab for 3 h and assayed as described above. C, Super-shift analysis using Abs to the p50 and p65 subunits (1 μg) of NF-κB alone or in combination. Normal rabbit serum (NRS) was included as a negative control. Representative of three independent experiments.

FIGURE 7. SOCS-1 inhibits IFN-γ-induced TNF-α secretion. ELISA were performed on supernatants from RAW-pcDNA3 or RAW-SOCS-1 in the absence or presence of 10 ng/ml IFN-γ for 0, 3, 6, 12, and 24 h. Average triplicate TNF-α values were normalized with total protein and “relative TNF-α secretion” was derived by dividing the total normalized amount of TNF-α induced by IFN-γ by a given time point by the normalized value of TNF-α secretion from untreated cells. Mean ± SD of triplicate samples of a representative experiment.

FIGURE 8. SOCS-1 inhibits IFN-γ-induced TNFR1 and RIP mRNA levels. Raw-pcDNA3 and RAW-SOCS-1 cells were treated with medium or IFN-γ (10 ng/ml) for 8 h. RNA was isolated and analyzed by RPA for TNFR1, RIP, L32, and GAPDH mRNA.
the presence of a neutralizing TNF-α Ab (Fig. 6B). Also in this study is the observation that IFN-γ leads to an up-regulation of TNFR1 and RIP (Fig. 8), both important for TNF-α-mediated NF-κB activation (53, 54). This finding provides an additional clue as to how IFN-γ is involved in sensitizing macrophages to TNF-α signaling. Ectopic SOCS-1 expression inhibits IFN-γ-induced TNFR1 and RIP mRNA expression (Fig. 8). Further studies need to be done to determine whether TNFR1 and/or RIP up-regulation is necessary for IFN-γ-induced CD40 expression in macrophages and microglia. Since SOCS-1 is a potent inhibitor of IFN-γ-mediated STAT-1α activation, these results imply that IFN-γ induction of TNFR1 and RIP mRNA levels as well as TNF-α secretion are STAT-1α dependent.

There is one study in the literature implicating SOCS-1 as an inhibitor of TNF-α signaling (35) and an accumulating amount of evidence stands to implicate STAT-1α and JAK2 in the TNF-α signaling cascade (43–45). However, in the RAW264.7 cells, TNF-α does not activate STAT-1α, as assessed by STAT-1α tyrosine phosphorylation (Fig. 1B). As mentioned previously, in macrophages, IFN-γ-mediated TNF-α secretion (which is inhibited by SOCS-1) is necessary for CD40 up-regulation (26). We tested the possibility of restoring IFN-γ-mediated CD40 up-regulation in SOCS-1 overexpressing cells by adding exogenous TNF-α in addition to IFN-γ. Under these conditions, we observed no restoration of CD40 mRNA expression (Fig. 9) or CD40 promoter activity and protein levels (data not shown). This result suggests that IFN-γ activated STAT-1α (which is effectively blocked by SOCS-1), in addition to IFN-γ-induced TNF-α secretion and subsequent NF-κB activation, are necessary for optimal CD40 up-regulation. Alternatively, it is also possible that IFN-γ mediates a JAK/STAT1-dependent sensitization of the cells to TNF-α signaling that is necessary for CD40 induction, such as TNFR1 and RIP up-regulation, and/or that SOCS-1 blocks some aspect of TNF-α signaling independent of the JAK-STAT pathway. The mechanism of these effects and the consequences this may have on CD40 expression are currently being investigated.

Recently, it has been shown that an important form of regulation of CD40 function is through alternative splicing. Tone et al. (55) have identified a number of CD40 isoforms generated by alternative splicing. Type I CD40 is the functional form, which contains the signal-transducing domain. Type II CD40 lacks the membrane-associated endodomain and seems to inhibit the expression of signal-transducing CD40 on the cell surface. Type III and IV are membrane-bound CD40 isoforms with cytoplasmic domains not capable of signal transduction. Using RT-PCR analysis, SOCS-1 was previously shown to inhibit IFN-γ-mediated CD40 mRNA levels (55); however, the mechanism of this effect was not explored. Furthermore, in this same study, SOCS-1 was shown to preferentially inhibit LPS-mediated type II CD40, but not type I. How SOCS-1 is involved in the differential splicing is not clear. In light of these recent findings, it is noteworthy that our riboprobe corresponds to the first 452 bp common to all CD40 isoforms.

CD40 expression by resident cells of the CNS, most likely microglia, is critical for the infiltration/retention of inflammatory cells in the CNS, leading to the disease of experimental autoimmune encephalomyelitis (50). As well, chronic neuroinflammatory processes including glial activation may play a role in the pathogenesis of AD. Interestingly, blockade of CD40-CD154 interaction opposes reactive microglial-mediated neurotoxicity in vitro and is a candidate for mitigating hyperphosphorylation of tau in a mouse model for AD (for review, see Ref. 8). Given the important role of CD40 in inflammatory events in the CNS as well as other organ systems (for review, see Refs. 6 and 56), it is imperative to understand the molecular mechanisms contributing to both CD40 induction and repression in various cell types. We have previously shown that IL-4 inhibits IFN-γ-induced CD40 expression in microglia in a STAT-6-dependent and SOCS-1-independent manner (57). Interestingly, unlike SOCS-1, IL-4 does not inhibit IFN-γ activation of STAT-1α and subsequent IRF-1 up-regulation. Its inhibitory effect seems to involve the ability of STAT-6 to compete with STAT-1α for binding to GAS elements within the CD40 promoter. Thus, numerous mechanisms exist to suppress CD40 expression.

The studies applied here confirm the essential role of IFN-γ and the JAK-STAT pathway in CD40 up-regulation in macrophages. Subsequent to IFN-γ exposure, macrophages up-regulate the expression of TNF-α as well as TNFR1 and RIP, all important for TNF-α-mediated NF-κB activation. NF-κB can then enter the nucleus and bind to κB elements within the CD40 promoter. Additionally, STAT-1α is activated and binds to GAS elements in the promoter region of CD40. In SOCS-1-overexpressing cells, IFN-γ is unable to induce STAT-1α phosphorylation, TNF-α secretion, TNFR1 and RIP mRNA up-regulation, and NF-κB DNA-binding activity, making it a potent inhibitor of CD40 gene expression.

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