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*J Immunol* 2006; 177:7761-7771; doi: 10.4049/jimmunol.177.11.7761
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IL-10 Inhibits Lipopolysaccharide-Induced CD40 Gene Expression through Induction of Suppressor of Cytokine Signaling-3

Hongwei Qin,1 Cynthia A. Wilson, Kevin L. Roberts, Brandi J. Baker, Xueyan Zhao, and Etty N. Benveniste

Costimulation between T cells and APCs is required for adaptive immune responses. CD40, an important costimulatory molecule, is expressed on a variety of cell types, including macrophages and microglia. The aberrant expression of CD40 is implicated in diseases including multiple sclerosis, rheumatoid arthritis, and Alzheimer’s disease, and inhibition of CD40 signaling has beneficial effects in a number of animal models of autoimmune diseases. In this study, we discovered that IL-10, a cytokine with anti-inflammatory properties, inhibits LPS-induced CD40 gene expression. We previously demonstrated that LPS induction of CD40 in macrophages/microglia involves both NF-κB and LPS-induced production of IFN-β, which subsequently activate STAT-1. IL-10 inhibits LPS-induced IFN-β gene expression and subsequent STAT-1 activation, but does not affect NF-κB activation. Our results also demonstrate that IL-10 inhibits LPS-induced recruitment of STAT-1α, RNA polymerase II, and the coactivators CREB binding protein and p300 to the CD40 promoter, as well as inhibiting permissive histone H3 acetylation (AcH3). IL-10 and LPS synergize to induce suppressor of cytokine signaling (SOCS)-3 gene expression in macrophages and microglia. Ectopic expression of SOCS-3 attenuates LPS-induced STAT activation, and inhibits LPS-induced CD40 gene expression, comparable to that seen by IL-10. These results indicate that SOCS-3 plays an important role in the negative regulation of LPS-induced CD40 gene expression by IL-10. The Journal of Immunology, 2006, 177: 7761–7771.

Interleukin-10 is a key physiological negative regulator of macrophage activation (1). The critical anti-inflammatory role of IL-10 was demonstrated by studies on IL-10-deficient mice, which develop chronic enterocolitis in the absence of endogenous IL-10 (2). Numerous studies have shown that IL-10 plays an important role in shaping the development of the immune response by blocking class II MHC expression, inhibiting Th1 effector cell development, and decreasing expression of proinflammatory cytokines and chemokines (3–5). IL-10 uses both transcriptional and translational-mediated mechanisms to suppress cytokine expression (6, 7). The intracellular mechanism(s) by which IL-10 mediates its anti-inflammatory effects remains largely unknown. IL-10 signaling occurs via a high-affinity cell surface receptor complexed with inhibitory IkB proteins. The activation process is mediated by the IkB kinase (IKK) complex; activation of IKK by a stimulus such as LPS leads to the phosphorylation and degradation of IkB proteins, and subsequent activation of NF-κB (14). However, there are also MyD88-independent pathways that occur in response to LPS through adaptors named Toll-IL-1R (TIR) domain-containing adaptor-inducing IFN-β (TRIF)/TRIF-containing adaptor molecule-1, and TRIF-related adaptor molecule to induce expression and activation (by phosphorylation) of the transcription factor IFN regulatory factor-3 (IRF-3), which leads to IFN-β expression. This endogenous production of IFN-β subsequently induces activation of the JAK-STAT pathway, particularly STAT-1α, and a number of IFN-β-dependent genes (15).

CD40 is a costimulatory molecule important for Ag presentation, and is expressed by a wide variety of cells including B cells, macrophages, microglia, and dendritic cells (16, 17). Aberrant CD40 expression has been implicated in participating in many human diseases, particularly those of autoimmune origin (16, 17). Blocking the interaction between CD40 and its ligand, CD154, with anti-CD154 or anti-CD40 Ab is beneficial in several animal models of autoimmune and neurodegenerative diseases (18, 19). We have recently shown that LPS induces CD40 gene expression in macrophages and microglia (20). LPS-induced CD40 gene expression involves activation of NF-κB, induction and activation of NF-κB and MAPKs (13). NF-κB transcription factors are present in the cytoplasm in an inactive state, complexed with inhibitory IkB proteins. The activation process is mediated by the IkB kinase (IKK) complex; activation of IKK by a stimulus such as LPS leads to the phosphorylation and degradation of IkB proteins, and subsequent activation of NF-κB (14). However, there are also MyD88-independent pathways that occur in response to LPS through adaptors named Toll-IL-1R (TIR) domain-containing adaptor-inducing IFN-β (TRIF)/TRIF-containing adaptor molecule-1, and TRIF-related adaptor molecule to induce expression and activation (by phosphorylation) of the transcription factor IFN regulatory factor-3 (IRF-3), which leads to IFN-β expression. This endogenous production of IFN-β subsequently induces activation of the JAK-STAT pathway, particularly STAT-1α, and a number of IFN-β-dependent genes (15).

Abbreviations used in this paper: IKK, IkB kinase; TIR, Toll-IL-1R; TRIF, TIR domain-containing adaptor-inducing IFN-β; IRF, IFN regulatory factor-3; GAS, IFN-γ activation site; Pol II, polymerase II; AcH3, histone H3 acetylation; SOCS, suppressor of cytokine signaling; SH, Src homology; ChIP, chromatin immunoprecipitation; CBP, CREB binding protein; MFI, mean fluorescence intensity; RPA, RNase protection assay; EAE, experimental autoimmune encephalomyelitis; MS, multiple sclerosis; UN, untreated.
IRF-3, and subsequent endogenous production of IFN-β that induces STAT-1α activation. Activated NF-κB binds to NF-κB elements and STAT-1α binds to IFN-γ activation site (GAS) elements in the CD40 promoter, which coordinate control CD40 expression (20). Furthermore, LPS induces acetylation and phosphorylation of histones H3 and H4, and the recruitment of RNA polymerase II (Pol II) on the CD40 promoter in a time-dependent manner (20).

Suppressor of cytokine signaling (SOCS)-3 is one of eight cytokine-inducible inhibitors of the JAK-STAT signaling pathway (21). SOCS-3 has a central Src homology (SH)2 domain, a N-terminal domain, and a C-terminal 40-aa module called the SOCS box (21). The SOCS-3 SH2 domain binds to JAK-proximal sites on cytokine receptors and inhibits JAK activity, and has been shown to specifically inhibit signaling by the IL-6 family of cytokines as well as IL-10 (10, 22–24). SOCS-3 deficiency is embryonic lethal due to defects in placental development (25). Forced expression of SOCS-3 in mouse arthritis models suppressed the induction and/or development of disease (21, 26), whereas intracellular administration of a cell-penetrating SOCS-3 suppressed cytokine-mediated signal transduction associated with acute inflammation (27), thus indicating that SOCS-3 is a negative regulator of inflammatory diseases. A number of stimuli are able to induce SOCS-3 expression in a cell type-specific manner; these stimuli include erythropoietin, IFN-α, IFN-β, LPS, Oncostatin M, IL-4, IL-10, IL-8, IGF, LIF, and IL-6 (28–32). LIF activation of STAT-3 leads to induction of SOCS-3 promoter activity, and subsequent expression of SOCS-3 (32). IL-4 induction of SOCS-3 gene expression in B cells occurs by activation of the p38 MAPK pathway (30), whereas IL-8 induction of SOCS-3 occurs in a STAT-independent manner (28). These findings indicate that SOCS-3 is induced by activation of disparate intracellular signaling pathways.

CD40 expression can be inhibited by a number of mediators, including cytokines, neurotrophins, neuropeptides, and statins (17, 33–36). TGF-β inhibits CD40 expression by destabilization of the CD40 message, whereas IL-4 inhibits CD40 gene transcription through a mechanism involving activated STAT-6 (34, 35). The neuropeptides vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide inhibit IFN-γ signal transduction cascades, specifically STAT-1α phosphorylation, leading to inhibition of IFN-γ-induced CD40 expression (33). We have recently demonstrated that the SOCS-1 protein inhibits both IFN-γ and IFN-β-induced CD40 gene expression in macrophages/microglia by preventing STAT-1α activation and recruitment to the CD40 promoter (37, 38). Having established that LPS is a potent inducer of CD40, we wished to investigate possible inhibitors of this pathway, and focused on IL-10. In this study, we demonstrate that IL-10 inhibits LPS-induced CD40 gene expression at the transcriptional level by inhibiting LPS-induced IFN-β expression and subsequent STAT-1α activation. Chromatin immunoprecipitation (ChIP) assays demonstrate that IL-10 inhibits LPS-induced recruitment of STAT-1α, RNA Pol II, CREB binding protein (CBP) and p300 to the CD40 promoter, and also inhibits permissive histone H3 modifications on the CD40 promoter. However, IL-10 has no effect on LPS-induced activation of the NF-κB signaling pathway. IL-10 and LPS function synergistically to enhance SOCS-3 expression in macrophages and microglia. Ectopic overexpression of SOCS-3 attenuates LPS-induced CD40 activation, and inhibits LPS-induced CD40 gene expression. These results indicate that IL-10 inhibits LPS-induced CD40 gene expression through the inhibition of LPS-induced IFN-β gene expression and induction of SOCS-3.

Materials and Methods

Recombinant proteins and reagents

Escherichia coli LPS was purchased from Sigma-Aldrich, and human IL-10 was purchased from R&D Systems. Rat-anti-mouse PE-conjugated CD40 Ab (clone 3/23) and rat-PE-conjugated IgG isotype control were purchased from BD Pharmingen. Abs against phospho-NF-κB p65Ser536, phospho-IRκBα, phospho-IEκBα, phospho-IκB-STAT-1α(Ser72) and phospho-IRF-3(Ser396) were purchased from Cell Signaling Technology. Abs against NF-κB p65, STAT-1α, STAT-3, CBP, p300, IRF-3, and actin were obtained from Santa Cruz Biotechnology. Abs against AcH3 and AcH4 were obtained from Upstate Biotechnology. Ab against RNA Pol II was obtained from Covance, and Ab against SOCS-3 was purchased from Zymed Laboratories.

Cells

Primary microglia from C57BL/6J mice (The Jackson Laboratory) were prepared as described previously (39, 40). The murine microglia cell line EOC13 and the murine macrophage cell line RAW264.7 were maintained as described previously (34).

Stable transfection of SOCS-3

SOCS-3 stable transfectants were created by transfecting RAW264.7 cells with the pcDNA3 expression vector containing N-terminal myc-tagged cDNA of human SOCS-3 (41) (a gift from Dr. A. Yoshimura, Kurume University, Kurume, Japan). There is 97.5% homology between the 221-aa protein sequences of human and murine SOCS-3 (32). The Lipofectamine Plus (Invitrogen Life Technologies) method was used for transfection. RAW264.7 cells stably transfected with pcDNA3 plasmid alone were used as a negative control. Cells were selected in G418 sulfate (100 μg/ml) and screened for SOCS-3 expression by immunoblotting for c-myc expression.

Stable transfection of dominant-negative STAT-3

Dominant-negative STAT-3 (STAT-3F) stable transfectants were created by transfecting RAW264.7 cells with the STAT-3F construct that has a phenylalanine substitution at Tyr255 (a gift from Drs. M. Hibi, T. Hirano, and K. Nakajima, Osaka University Medical School, Osaka, Japan) (42). The Lipofectamine Plus (Invitrogen Life Technologies) method was used for transfection, and RAW264.7 cells stably transfected with pcDNA3 plasmid alone were used as a negative control. Cells were selected in G418 sulfate (100 μg/ml) and screened for SOCS-3 expression/function by inhibition of IL-10-induced STAT-3 phosphorylation.

Immunofluorescence flow cytometry

Cells were plated at 2 × 10^5 cells/well into 12-well plates, treated with medium, LPS, IL-10, or LPS plus IL-10 for up to 36 h, and then incubated with 100 μl of 2.4G2 hybridoma supernatant (which contains rat anti-mouse FcγR Ab) for blocking FcγR. Cell surface CD40 protein expression was determined by incubating cells with 10 μg/ml PE-conjugated anti-mouse CD40 Ab, and then analyzed on the FACStar (BD Biosciences) as described previously (43). Negative controls were incubated with IgG isotype-matched Ab. Fold induction of CD40 expression was calculated by dividing the mean fluorescence intensity (MFI) value of treated samples by the value of untreated samples. Cytofix/Cytoperm Plus Fixation/Permeabilization Kit (with BD GolgiStop protein transport inhibitor containing monensin; BD Biosciences) was used to detect intracellular CD40 protein expression.

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

Total cellular RNA was isolated from unstimulated LPS, IL-10, or LPS plus IL-10-treated cells. The riboprobes for murine CD40, IFN-β, SOCS-3, and GAPDH were prepared as described previously (37, 43). Twenty micrograms of total RNA was hybridized with the riboprobes at 42°C overnight. The hybridized mixture was treated with RNase A/T1 (1:200) and then analyzed by 5% denaturing (8 M urea) PAGE. Values for CD40, IFN-β, and SOCS-3 mRNA expression were normalized to GAPDH mRNA levels for each experimental condition.

Immunoblotting

Fifty micrograms of cell lysate was separated on 10% SDS-PAGE, and probed with phospho-STAT-1α, phospho-STAT-3, or phospho-IRF-3 Abs as described previously (44). Membranes were stripped at 50°C in buffer containing 100 mM 2-ME, 2% SDS, and 62.5 mM Tris-HCl (pH 6.7) with occasional shaking, and reprobed for total STAT-1α, STAT-3, IRF-3, or actin. SOCS-3 protein expression was analyzed using 15% SDS-PAGE gel and polyvinylidene difluoride membranes.
SOCS-3 promoter constructs, transient transfection, and luciferase assays

The 1556-bp (−1429 to +127) murine SOCS-3 promoter was amplified from genomic DNA using the forward primer (5'-CCAAGCTACTCTGTGTAGTCA-3') and the reverse primer (5'-GGGAATCAGGAACGAAGGACCTG-3'), and cloned into the pGL3 basic vector. The promoter sequence was confirmed by automatic sequencing. A total of 0.2 μg of the SOCS-3 promoter construct was transiently transfected into 5 × 10⁵ RAW264.7 cells in 6-well plates using the Lipofectamine Plus (Invitrogen Life Technologies) method as described previously (43). Transfected cells were treated with LPS, IL-10, or LPS plus IL-10 for 8 h, and the luciferase activity of each sample was normalized to the total protein concentration of each well. Luciferase activity from the untreated sample was arbitrarily set at 1 for calculation of fold induction.

ELISA

Supernatants were collected from unstimulated LPS, IL-10, or LPS plus IL-10-stimulated RAW264.7 cells, and assayed by ELISA for secretion of IFN-β (PBL Biomedical Laboratories). IFN-β levels were normalized to total protein levels. The activated cell-based ELISA (FACE) NF-κB p65 (Ser536 and Ser638) profiler kit was purchased from Active Motif. A total of 5 × 10⁴ RAW264.7 cells were stimulated with LPS, IL-10, or LPS plus IL-10 in 96-well plates for 30 min, and then cells were fixed. Each well was incubated with a primary Ab specific for phospho-Ser536, phospho-Ser638, or total NF-κB p65 for 1 h. After washing with PBS, samples were incubated with secondary HRP-conjugated Ab, followed by an incubation with developing solution. The levels of NF-κB p65 phosphorylation were normalized by both the levels of total NF-κB p65 protein and total cell number in each well.

ChIP assays

ChIP analysis was done following a protocol provided by Upstate Biotechnology with modifications as described previously (20, 44). RAW264.7 cells were unstimulated or incubated with LPS, IL-10, or LPS plus IL-10 for up to 6 h, fixed with 1% formaldehyde for 15 min at room temperature, and nuclei were isolated. Chromatin was sheared by sonication, and samples were precleared for 2 h at 4°C with salmon sperm DNA–protein A/G Sepharose. Chromatin solutions were precipitated overnight at 4°C with 5 μg of Abs or isotype-matched control IgG. Input and immunoprecipitated chromatin were incubated at 65°C overnight to reverse cross-links. After proteinase K digestion, DNA was extracted by the Qiagen Miniprep Kit. Purified DNA was analyzed by PCR with Taq polymerase. The primer pair 5'-CTACAGGCTCTGATTGGAGC-3' and 5'-TGCGAGAAGGGCGGTCTC-3' was used to amplify a 250-bp region in the mouse CD40 promoter containing functional NF-κB and GAS elements. Densitometry was used to quantify the PCR results, and all results were normalized by the respective input values.

Statistical analysis

Levels of significance for comparison between samples were determined by the Student t test distribution.

Results

IL-10 inhibits LPS-induced CD40 expression in macrophages and microglia

We have recently demonstrated that optimal induction of CD40 gene expression in response to LPS requires activation of NF-κB

FIGURE 1. IL-10 inhibits LPS-induced CD40 expression in macrophages and microglia. A, RAW264.7 or EOC13 cells were treated with medium, LPS (10 ng/ml), IL-10 (10 ng/ml), or LPS plus IL-10 at varying concentrations (0.1–100 ng/ml) for 8 h, then total RNA was isolated and analyzed by RPA for CD40 and GAPDH mRNA. The basal level of the untreated sample was set at 1.0, and fold induction upon treatment was compared with that. Representative of three experiments. B, RAW264.7 cells were treated in the absence or presence of LPS (10 ng/ml), IL-10 (10 ng/ml), or LPS plus IL-10 for 36 h, then stained with either PE-conjugated anti-CD40 or PE-conjugated isotype-matched control Ab. Cells were subjected to FACS analysis. Samples were analyzed by measuring MFI. Fold induction of intracellular CD40 protein expression was calculated and shown as the mean ± SD of three experiments. **p < 0.01 compared with LPS alone. C, RAW264.7 cells were incubated with GolgiStop (0.67 μl/ml) along with medium, LPS (10 ng/ml), IL-10 (10 ng/ml), or LPS plus IL-10, then permeabilized and fixed to detect intracellular CD40. Cells were stained with either PE-conjugated anti-CD40 or PE-conjugated isotype-matched control Ab, and subjected to FACS analysis. Samples were analyzed by measuring MFI. Fold induction of intracellular CD40 protein expression was calculated and shown as the mean ± SD of three experiments. *p < 0.05 compared with LPS alone. D, Primary murine microglia were treated with medium, LPS (10 ng/ml), IL-10 (10 ng/ml), or LPS plus IL-10 for 8 h, then total RNA was isolated and analyzed by RPA for CD40 and GAPDH mRNA expression. Fold induction was calculated as described above. Representative of three experiments. E, Primary murine microglia were treated with medium, LPS, IL-10, or LPS plus IL-10 for 36 h, and then cells were subjected to FACS analysis for CD40 protein expression. Fold induction of CD40 protein was calculated and shown as the mean ± SD of three experiments. **p < 0.001 compared with LPS alone.
LPS-induced CD40 surface protein expression

**FIGURE 2.** IL-10 inhibits LPS-induced IFN-β gene expression and STAT-1α activation. A, RAW264.7 cells were incubated in the absence or presence of LPS (10 ng/ml), IL-10 (10 ng/ml), or LPS plus IL-10 for 4 h, then total RNA was isolated and analyzed by RPA for IFN-β and GAPDH mRNA. Representative of three experiments. B, RAW264.7 cells were treated with LPS, IL-10, or LPS plus IL-10 for up to 12 h, then supernatants were collected and subjected to ELISA analysis for IFN-β protein expression. Data are presented as the mean ± SD of three experiments. *, p < 0.05 and **, p < 0.001 compared with LPS alone. C, RAW264.7 cells were incubated with medium, LPS, IL-10, or LPS plus IL-10 for 30 min, then cell lysates were prepared and subjected to immunoblotting with anti-phospho-IRF-3Ser396 Ab, and stripped and reprobed with anti-IRF-3 and anti-actin as loading controls. Representative of three experiments. D, RAW264.7 cells were incubated with medium (UN), LPS, IL-10, or LPS plus IL-10 for up to 6 h, then cell lysates were prepared and subjected to immunoblotting with anti-phospho-STAT-1α(Tyr701) and anti-phospho-STAT-1αSer727 Abs, and stripped and reprobed with anti-STAT-1α and anti-actin as loading controls. Representative of three experiments. E, RAW264.7 cells were treated with LPS, IL-10, or LPS plus IL-10 for 30 min, then fixed cells were subjected to the FACE ELISA NF-κB p65 Profiler Kit for determination of phospho-Ser536 NF-κB p65, phospho-Ser568 NF-κB p65, and total p65 protein expression levels. Data are presented as the mean ± SD of three experiments. There is no significant difference between LPS alone and LPS plus IL-10 treatment.

and endogenous IFN-β production, which subsequently activates STAT-1α in both macrophages and microglia (20). As an anti-inflammatory cytokine, IL-10 suppresses the inflammatory response to many microbial stimuli by negative regulation of macrophage activation (1). To determine whether IL-10 had an inhibitory effect on LPS-induced CD40 gene expression, we initiated experiments to examine the effect of IL-10 (0.1–100 ng/ml) in the macrophage cell line, RAW264.7, and the microglial cell line, EOC13. These cells were incubated with medium, LPS, IL-10, or LPS plus IL-10 for 8 h, and then CD40 mRNA expression was analyzed. In RAW264.7 and EOC13 cells, LPS-induced CD40 mRNA was inhibited by addition of IL-10 in a dose-dependent manner (Fig. 1A). LPS-induced CD40 surface protein expression was also inhibited by IL-10 (71.2 ± 6.3% inhibition) in the RAW264.7 cells (Fig. 1B). We next determined whether the inhibitory effect of IL-10 on CD40 surface expression may be due in part to retention of CD40 intracellularly. The Cytofix/Cytoperm Plus Fixation/Permeabilization Kit (BD Biosciences) was used to detect CD40 retained in the intracellular site. As shown in Fig. 1C, IL-10 did not promote the retention of CD40 intracellularly. Another possible explanation for reduced CD40 surface expression upon IL-10 treatment is that IL-10 may promote the shedding of CD40 from the cell surface. This was also tested experimentally, and IL-10 did not influence the rate of CD40 shedding in RAW264.7 cells (data not shown). Taken together, the retention of CD40 intracellularly and/or shedding of CD40 does not contribute to the inhibitory effect of IL-10 on CD40 surface protein expression.

We next examined the effect of IL-10 on CD40 expression in primary murine microglia. LPS-induced CD40 mRNA was strongly inhibited by IL-10 (81.0 ± 5.1% inhibition) (Fig. 1D), as was LPS-induced CD40 protein (73.2 ± 3.5% inhibition) (Fig. 1E). These data demonstrate that IL-10 inhibits LPS induction of CD40 expression in macrophages and primary microglia.

To assess whether inhibition of LPS-induced CD40 mRNA levels by IL-10 was due to destabilization of the CD40 message, 11/2 experiments were performed. RAW264.7 cells were incubated with LPS or LPS plus IL-10 for 8 h, then actinomycin-D (5 μg/ml) was added for an additional 8 h. RNA was isolated at various time points (0–8 h) and analyzed for CD40 mRNA levels. The inclusion of IL-10 inhibited LPS-induced CD40 mRNA expression by 74% (data not shown). There was no significant difference between the 11/2 of CD40 mRNA from LPS or LPS plus IL-10-treated samples (~8 h) (data not shown). These results indicate that IL-10 has no effect on CD40 message stability, suggesting that the inhibitory effect of IL-10 is not mediated at the posttranscriptional level.

**IL-10 inhibits LPS-induced IFN-β gene expression and STAT-1α activation**

To investigate the molecular mechanism by which IL-10 inhibits LPS-induced CD40 expression, the effect of IL-10 on LPS-induced IFN-β expression and subsequent STAT-1α activation was examined. RAW264.7 cells were treated with LPS, IL-10, or LPS plus
activated by phosphorylation and subsequently initiates adaptor protein TRIF to induce IRF-3 expression, which is also induced by phosphorylation of STAT-1 (15, 20, 45). The effect of IL-10 on LPS-induced phosphorylation of IRF-3, with no effect on LPS-induced NF-κB activation (data not shown).

The NF-κB signaling pathway plays an important role in LPS-induced CD40 gene expression (20). Thus, the effect of IL-10 on LPS-induced activation of the NF-κB signaling pathway was tested by immunoblotting for the phosphorylation of IKKαβ, IkBα, and NF-κB. Our results indicate that IL-10 has no effect on LPS-induced NF-κB activation (data not shown). ELISA (FACE) was performed to specifically monitor the phosphorylation status of NF-κB p65, which is indicative of NF-κB activation. RAW264.7 cells were stimulated with LPS, IL-10, or LPS plus IL-10 for 30 min, and then phosphorylation of NF-κB p65 Ser536 and Ser468 was determined. LPS did not induce phosphorylation of NF-κB p65 on Ser536, but did promote phosphorylation of NF-κB p65 Ser468 (Fig. 2E). The Ser468 phosphorylation level of NF-κB p65 induced by LPS was not affected by IL-10 treatment in RAW264.7 cells (Fig. 2E). These results suggest that the inhibitory effect of IL-10 on LPS-induced CD40 gene expression is due to inhibition of LPS-induced IFN-β expression and subsequent activation of STAT-1α, with no effect on LPS-induced NF-κB activation.

**FIGURE 3.** IL-10 inhibits LPS-induced recruitment of RNA Pol II, STAT-1α, and coactivators CBP and p300 to the CD40 promoter and regulates histone modifications. A, RAW264.7 cells were incubated in the absence or presence of LPS (10 ng/ml), IL-10 (10 ng/ml), or LPS plus IL-10 for up to 6 h, then the cells were cross-linked with formaldehyde. Soluble chromatin was subjected to immunoprecipitation with Abs against RNA Pol II, STAT-1α, NF-κB p65, or normal rabbit IgG. The basal level of the untreated sample was set at 1.0, and fold induction upon treatment was compared with that. Representative of four experiments. B, The mean fold induction ± SD of STAT-1α recruitment to the CD40 promoter upon LPS or IL-10 plus LPS treatment from three separate experiments is shown as an example to illustrate the inhibitory effect of IL-10 on transcriptional events occurring at the CD40 promoter. *, p < 0.05 and **, p < 0.001 compared with LPS alone. C, RAW264.7 cells were treated with LPS, IL-10, or LPS plus IL-10 for up to 6 h, then the cells were cross-linked with formaldehyde. Soluble chromatin was subjected to immunoprecipitation with Abs against CBP, p300, and histone acetylation (Ac-H3, Ac-H4). The basal level of the untreated sample was set at 1.0, and fold induction upon treatment was compared with that. Representative of three experiments.

IL-10 for 4 h, then IFN-β mRNA expression was analyzed by RPA. IL-10 inhibited LPS induction of IFN-β mRNA by ~90% (Fig. 2A), and also strongly inhibited LPS-induced IFN-β protein expression (Fig. 2B). Previous studies have demonstrated that TLR4 possesses a MyD88-independent pathway that uses the adaptor protein TRIF to induce IRF-3 expression, which is activated by phosphorylation and subsequently initiates IFN-β gene transcription (15, 20, 45). The effect of IL-10 on LPS-induced IRF-3 phosphorylation and IRF-3 total protein expression was investigated in RAW264.7 cells. Our results indicate that IL-10 inhibits LPS-induced phosphorylation of IRF-3, while having no effect on total levels of the IRF-3 transcription factor (Fig. 2C). This inhibitory effect on IRF-3 phosphorylation may contribute to IL-10 inhibition of IFN-β expression.

The effect of IL-10 on LPS-mediated STAT-1α activation was tested by immunoblotting. LPS induces phosphorylation of STAT-1α Tyr701 or STAT-1α Ser727 at 2–6 h after LPS treatment (Fig. 2D, lanes 3–5), which we have previously shown is due to IFN-β induction by LPS (20). IL-10 alone does not induce phosphorylation of STAT-1α Tyr701 and STAT-1α Ser727 (Fig. 2D, lanes 6–9), but partially inhibits LPS-induced STAT-1α tyrosine phosphorylation at all time points tested (Fig. 2D, lanes 10–13). As well, LPS-induced STAT-1α serine phosphorylation is strongly inhibited at 2–6 h. Preincubation with IL-10 for 1 h, then exposure to LPS for 1, 2, 4, or 6 h promotes a greater extent of inhibition of both STAT-1α tyrosine and serine phosphorylation than the simultaneous addition of LPS plus IL-10 (data not shown).
IL-10 inhibits recruitment of STAT-1α, RNA Pol II, and the coactivators CBP and p300 to the CD40 promoter, and modifies AcH3 in response to LPS

To further investigate the inhibitory effect of IL-10 on CD40 transcription, we examined the effect of IL-10 on CD40 promoter activity. We were not able to observe IL-10 inhibition of LPS-induced CD40 promoter activity in transient transfection assays performed in RAW264.7 cells (data not shown). This result is similar to those of Zhou et al. (7), in which they did not detect inhibition of IL-12 p40 promoter activity by IL-10 in either transient or stable-transfection assays with the IL-12 p40 promoter reporter plasmid. This finding prompted us to investigate the effect of IL-10 on events occurring at the endogenous CD40 promoter.

We previously described a time-dependent recruitment of the transcription factors NF-κB and STAT-1α, and RNA Pol II to the CD40 promoter after LPS stimulation, as well as permissive AcH3 and AcH4 modifications of the CD40 promoter (20). We examined the effect of IL-10 on the recruitment of transcription factors to the endogenous CD40 promoter after LPS stimulation in vivo. RAW264.7 cells were incubated in the absence or presence of LPS, IL-10, or LPS plus IL-10 for up to 6 h, and ChIP assays were performed using Abs against RNA Pol II, STAT-1α, NF-κB p65, or normal rabbit IgG (as a negative control). PCR analysis of the positive control (input) indicates that the soluble chromatin samples obtained from each time point had equal amounts of chromatin fragments containing the CD40 promoter (Fig. 3A). The recruitment of LPS-induced RNA Pol II to the CD40 promoter was inhibited by IL-10 at all time points (50–60% inhibition) (Fig. 3A). Recruitment of LPS-induced STAT-1α to the CD40 promoter was also inhibited by IL-10, especially between 0.5–6 h (Fig. 3, A and B). However, IL-10 had little effect on LPS-induced NF-κB recruitment to the CD40 promoter (Fig. 3A), which is consistent with the lack of effect on LPS-induced NF-κB phosphorylation (Fig. 2E). These results indicate that the LPS-induced recruitment of RNA Pol II and STAT-1α to the CD40 promoter is inhibited by IL-10.

Covalent histone modifications are indicators of the recruitment of histone modifying complexes such as histone acetyltransferases, histone deacetylases, and histone methyltransferases, and also have functional roles in gene transcriptional regulation (46, 47). The N-terminal tails of core histones have several basic amino acid residues that are subject to modifications such as acetylation, methylation, phosphorylation, and ubiquitination (48). Histone acetylation of lysine residues requires the activities of histone acetyltransferases such as CBP/p300, which have a critical role in relaxing the compact structure of nucleosomes (49). The effect of IL-10 on the recruitment of coactivators CBP and p300 to the CD40 promoter was analyzed. CBP and p300 were weakly associated with the CD40 promoter in untreated RAW264.7 cells, and LPS stimulation promoted recruitment of both CBP and p300 at 2–6 h (Fig. 3C). IL-10 inhibited LPS-induced recruitment of CBP and p300 to the CD40 promoter at all time points tested (Fig. 3B). As described previously, LPS-induced AcH3 and AcH4 on the CD40 promoter is critical for LPS-induced CD40 gene expression (20), and, as such, the effect of IL-10 on LPS-induced histone modifications was also examined. Histone H4 was weakly acetylated on the CD40 promoter
STAT-3 activation is critical for IL-10 and LPS-induced SOCS-3 gene expression

To test whether STAT-3 is required for LPS and IL-10 induction of SOCS-3 expression, a STAT-3DN mutant that has a phenylalanine substitution at Tyr705 (STAT-3F) was used (42). STAT-3F cannot be phosphorylated, and outcompetes endogenous STAT-3, thus preventing STAT-3 activation (42). The STAT-3F construct was introduced into RAW264.7 cells by stable transfection (37), and RAW264.7 cells stably transfected with STAT-3F were established (RAW-STAT-3F). RAW-STAT-3F and RAW-pcDNA3 cells were treated with LPS, IL-10, or LPS plus IL-10 for 4 h, and then phosphorylation of STAT-3 was analyzed by FACS. Fold induction of CD40 protein was calculated and shown as the mean ± SD of three experiments. *, p < 0.05 and **, p < 0.001 compared with pcDNA3. F. RAW264.7 cells stably transfected with pcDNA3 or STAT-3F were treated with LPS (10 ng/ml) for 24 h, then total RNA was isolated and analyzed by RPA for CD40 and GAPDH mRNA. Representative of three experiments.

Ectopic expression of SOCS-3 inhibits LPS-stimulated STAT activation and CD40 gene expression

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IL-10 inhibits lipopolysaccharide-induced CD40 expression

RAW-SOCS-3 cells were used. Three independent clones were selected to confirm protein expression of SOCS-3 by immunoblotting with anti-c-myc Ab (Fig. 6A). Cells were incubated with medium or LPS for up to 6 h, and protein lysates were subjected to SDS-PAGE analysis to detect the phosphorylation status of STAT-1α and STAT-3 in RAW-pcDNA3 and RAW-SOCS-3-C14. LPS-induced STAT-1α and STAT-3 phosphorylation was strongly inhibited at all time points in RAW-SOCS-3-C14 cells compared with RAW-pcDNA3 cells (Fig. 6B).

Interestingly, SOCS-3 overexpression had no effect on LPS-induced degradation of IκBα in RAW-SOCS-3-C14 cells compared with RAW-pcDNA3 cells, which indicates that SOCS-3 overexpression does not affect LPS-induced NF-κB activation. Total STAT-1α, STAT-3, and actin expression levels were examined in these cells as loading controls. To confirm the lack of effect of SOCS-3 on LPS-induced NF-κB activation, the induction of a NF-κB-dependent gene, TNF-α, was analyzed upon LPS treatment in RAW-pcDNA3 and RAW-SOCS-3 (clone 14) cells. SOCS-3 overexpression has no influence on LPS-induced TNF-α mRNA expression (Fig. 6C), supporting the idea that SOCS-3 has no effect on LPS-induced NF-κB signaling. LPS-induced CD40 mRNA expression was suppressed (50–70% inhibition) at 8 h of LPS treatment in three clones of RAW-SOCS-3 cells compared with RAW-pcDNA3 cells (Fig. 6D). In addition, LPS-induced IRF-1 mRNA expression was strongly inhibited in the three clones of RAW-SOCS-3 cells compared with RAW-pcDNA3 cells (Fig. 6D). Further studies showed that ectopic SOCS-3 expression partially inhibited LPS-induced CD40 protein expression (30–60% inhibition) at 36 h of LPS treatment (Fig. 6E). The extent of CD40 protein inhibition was comparable to that of CD40 mRNA inhibition in the three individual SOCS-3 clones (Fig. 6, D and E).

To further study the inhibitory role of SOCS-3 in LPS-induced CD40 gene expression, RAW-STAT-3F cells were used. As previously shown in Fig. 5B, SOCS-3 mRNA expression was inhibited in RAW-STAT-3F cells compared with RAW-pcDNA3 cells. If the SOCS-3 protein is involved in negative regulation of LPS-induced CD40 expression, CD40 expression should be enhanced in cells with lower SOCS-3 levels, i.e., the RAW-STAT-3F cells. To test this hypothesis, RAW-pcDNA3 and RAW-STAT-3F cells were treated with LPS for 24 h, and then CD40 mRNA was analyzed by RPA. As shown in Fig. 6F, LPS-induced CD40 mRNA expression in STAT-3F cells was higher (12.4 ± 3.4-fold) than in RAW-pcDNA control cells (3.5 ± 0.5-fold). These results indicate that SOCS-3 is important in the negative regulation of LPS-induced CD40 gene expression.

Discussion

As an anti-inflammatory cytokine, IL-10 has numerous effects on the regulation of immune responses, such as down-regulation of proinflammatory cytokines including TNF-α, IL-6, and IL-12, chemokines, and costimulatory molecules (1). Despite the biological importance of IL-10, limited information is available about the mechanism(s) by which it inhibits gene transcription. Several studies have shown that the mechanism(s) of IL-10-mediated inhibition of LPS-induced proinflammatory gene expression involves inhibition of the NF-κB or p38 MAPK pathways, as well as destabilization of RNA message (51, 52). Experiments using cycloheximide treatment suggest that new protein synthesis is required for IL-10 to inhibit LPS-induced IL-12 p40 and TNF-α expression in macrophages (53). Recently, microarray studies have been used to identify proteins that may contribute to IL-10-mediated inhibition of gene transcription (8, 54). The combination of IL-10 and LPS resulted in synergistic induction of SOCS-3, IL-1RA, Bcl-3, metallothionein-2, and NFIL-3 (8, 54). The introduction of Bcl-3 into macrophages revealed that Bcl-3 inhibits LPS-induced TNF-α production by interaction with NF-κB p50 on the TNF-α promoter (54). The inhibitory effect of SOCS-3 on LPS-induced TNF-α, IL-12, IL-6, NO and GM-CSF gene expression is complex, and appears to be gene and cell-type specific. SOCS-3 inhibits LPS-induced gene expression via a multitude of mechanisms, including inhibition of NF-κB and p38 MAPK pathways, by NF-κB-independent mechanisms, by inhibiting IFN-β-induced STAT-1α activation, and at the posttranscriptional level (22, 55–57). As mentioned previously, the ability of SOCS-3 to inhibit TLR4-induced NF-κB activation is controversial.

One mechanism by which IL-10 inhibits gene expression has been reported for IL-10 suppression of LPS-induced IL-12 p40 gene expression (7). IL-10 abolished recruitment of RNA Pol II to the p40 promoter, but only modestly reduced binding of C/EBPβ and NF-κB complexed to the IL-12 p40 promoter (7). We speculated that IL-10 may inhibit events occurring after nucleosome remodeling of the p40 promoter, but did not provide any additional data to confirm this point. Our previous studies led to the identification of STAT-1α and NF-κB as transcriptional activators for LPS-induced CD40 expression (20). In this study, we examined the effect of IL-10 on molecular events occurring at the CD40 promoter during activation by LPS. Our results demonstrate that IL-10 inhibits LPS-induced IFN-β induction and the subsequent activation of STAT-1α, which in concert with decreased recruitment of the coactivators CBP and p300, inhibition of permissive histone H3 modifications, and inhibition of RNA Pol II recruitment to the CD40 promoter, leads to inhibition of CD40 gene transcription (Fig. 7, A and B). However, IL-10 had no effect on LPS-mediated recruitment of the transcription factor NF-κB to the CD40 promoter or acetylation of histone H4. These results demonstrate that for CD40 inhibition, IL-10 inhibits the LPS-induced STAT-1α activation pathway and transcriptional events occurring on the CD40 promoter, but does not affect the LPS-induced NF-κB component involved in CD40 expression.

Studies on IL-10-deficient mice have demonstrated that IL-10 plays an essential role in the endogenous anti-inflammatory response of the host (1, 58). STAT-3 deletion leads to overactivation of innate immune responses, impaired Ag-specific T cell responses, and aggressive and fatal enterocolitis, which mimics the loss of IL-10 itself. This suggests that IL-10 signal transduction proceeds through STAT-3 (59–61). It has been reported that IL-10 and STAT-3 are critical for the regulation of LPS-induced IL-12 p40 gene expression in bone marrow derived-dendritic cells (58). It has also been proposed that IL-10-induced SOCS-3 may inhibit LPS-induced p38 MAPK signaling and thereby interfere with TNF-α mRNA translation (57). SOCS-3 is essential for IL-10 inhibition of LPS-induced TNF-α, inducible NO synthase, and NO production (10). Studies on the SH2 domain, SOCS box, kinase inhibitory region, and two tyrosine residues (Tyr204 and Tyr221) of SOCS-3 defined that SOCS-3 is an important mediator of IL-10 inhibition of macrophage activation, and that SOCS-3 interferes with distinct LPS-stimulated signals through differing mechanisms (10). Our results provide new information on the involvement of SOCS-3 and STAT-3 in the inhibition of LPS-induced CD40 gene expression. We have shown that IL-10 and LPS induce STAT-3 activation, which subsequently leads to SOCS-3 gene expression (Figs. 4 and 5). LPS-induced STAT-3 activation and SOCS-3 expression may be due to LPS induction of endogenous IL-10 and IL-6 expression (data not shown), which is consistent with the results of previous studies (58). Our results also demonstrate that ectopic expression of SOCS-3 attenuates LPS-induced STAT activation,
and partially inhibits LPS-induced CD40 gene expression, comparable to that seen by IL-10. The inactivation of STAT-3 leads to a reduction of LPS-induced SOCS-3 expression, and the subsequent elevation of LPS-induced CD40 expression (Figs. 5B and 6F). LPS-induced endogenous IFN-β expression is also blocked by ectopic expression of SOCS-3 (our unpublished data), which suggests that SOCS-3 negatively regulates LPS-induced CD40 gene expression by attenuating IFN-β production and subsequent STAT-1α activation (Fig. 7B).

There are limited studies on the expression or function of SOCS family members in the CNS during neuroinflammatory disease. In the myelin oligodendrocyte glycoprotein-experimental autoimmune encephalomyelitis (EAE) model of multiple sclerosis (MS), SOCS-1 and SOCS-3 mRNA transcripts increased significantly in the cerebellum and spinal cord at the height of disease, and then declined (62). SOCS-3 has been suggested to have neuroprotective effects, because antisense knockdown of SOCS-3 expression increases stroke size (63). SOCS-1 is also rapidly induced by LPS and negatively regulates innate immune responses triggered by LPS (64, 65). Our previous studies indicated that SOCS-1 plays an important role in the negative regulation of IFN-γ and IFN-β-induced CD40 gene expression (37, 38). Using a mimic of SOCS-1 (TKip), Mujtaba et al. (66) demonstrated that TKip has a protective effect in EAE by inhibiting STAT-1α activation. Furthermore, targeted expression of SOCS-1 in oligodendrocytes protected these cells against injury mediated by IFN-γ (67). Because macrophages and microglia are cell types in the CNS that contribute to neuroinflammation, the expression of SOCS proteins, especially SOCS-1 and SOCS-3, may serve as endogenous attenuators of macrophage/microglial activation and gene expression. Similarly, SOCS-1 expression in myelinating oligodendrocytes may protect these cells from the harmful effects of CNS inflammation. As such, the molecular mechanism of SOCS gene expression in macrophages, microglia, and oligodendrocytes is under investigation in our laboratory.

Bacterial endotoxins such as LPS can lead to the activation of innate immunity within the CNS by engagement of TLR4 on cells including microglia and macrophages, which can then trigger responses leading to neurotoxicity and neurodegeneration within the brain (68, 69). A number of inhibitors of macrophage/microglia activation have been identified, including IL-10. Exogenous administration of IL-10 in the CNS ameliorates EAE progression (70). As an essential anti-inflammatory cytokine produced by T cells and activated macrophages, IL-10 has elicited considerable clinical interest for the treatment of chronic inflammatory conditions such as MS and systemic lupus erythematosus (1, 71, 72). IFN-β is also an immunosuppressive cytokine that inhibits class II MHC expression (73), inhibits Th1 development, and also promotes the expression of IL-10 (74). IFN-β is currently used for therapeutic treatment of patients with MS, although efficacy is attenuated over time (75). Our previous study documents that IFN-β also has “proinflammatory” actions such as induction of CD40 expression, which is then attenuated by SOCS-1 expression (38). As we have shown in this study, IL-10 also functions to inhibit CD40 gene expression, in part through the SOCS-3 protein. We speculate that IFN-β has a complex role in regulating neuroinflammatory events, acting in part to promote expression of CD40, but then inducing expression of SOCS-1 to inhibit such expression. IFN-β also induces IL-10, and IL-10 promotes SOCS-3 expression, which can also feedback to inhibit LPS-induced CD40 expression. The balance between LPS-induced signals, IFN-β and IL-10 induction, and subsequent expression of both SOCS-1 and SOCS-3 will dictate the extent of negative regulation of immune and inflammatory responses within the CNS, which is relevant to the pathogenesis of autoimmune and neurodegenerative diseases.

Acknowledgments
We thank Dr. Akihiko Yoshimura (Kurume University, Kurume-shi, Japan) for the gift of the human SOCS-3 cDNA; Drs. M. Hibi, T. Hirano, and K. Nakajima (Osaka University Medical School, Osaka, Japan) for the STAT-3F construct; and Dr. Susan Nozell for help with Fig. 7.
Disclosures
The authors have no financial conflict of interest.

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