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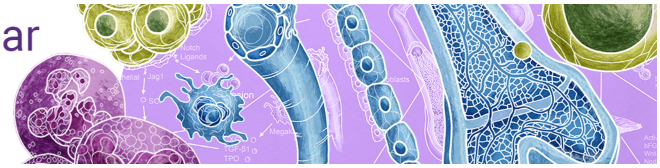

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Activation of STAT3 by IL-6 and IL-10 in Primary Human Macrophages Is Differentially Modulated by Suppressor of Cytokine Signaling 3[1]

Claudia Niemand,*† Ariane Nimmesgern,*‡ Serge Haan,* Patrick Fischer,* Fred Schaper,* Rolf Rossaint,† Peter C. Heinrich,* and Gerhard Müller-Newen[2]*

On human macrophages IL-10 acts as a more potent anti-inflammatory cytokine than IL-6, although both cytokines signal mainly via activation of the transcription factor STAT3. In this study we compare IL-10 and IL-6 signaling in primary human macrophages derived from blood monocytes. Pretreatment of macrophages with PMA or the proinflammatory mediators LPS and TNF-α blocks IL-6-induced STAT3 activation, whereas IL-10-induced activation of STAT3 remains largely unaffected. Although LPS induces the feedback inhibitor suppressor of cytokine signaling 3 (SOCS3) in macrophages, inhibition of IL-6 signal transduction by LPS occurs rapidly and does not depend on gene transcription. We also found that pretreatment of macrophages with IL-10 inhibits subsequent STAT3 activation by IL-6, whereas IL-10-induced STAT3 activation is not affected by preincubation with IL-6. This cross-inhibition is dependent on active transcription and might therefore be explained by different sensitivities of IL-10 and IL-6 signaling toward the feedback inhibitor SOCS3, which is induced by both cytokines. In contrast to the IL-6 signal transducer gp130, which has been previously shown to recruit SOCS3 to one of its phosphotyrosine residues (Y759), peptide precipitation experiments suggest that SOCS3 does not interact with phosphorylated tyrosine motifs of the IL-10R. Taken together, different sensitivities of IL-10 and IL-6 signaling toward mechanisms that inhibit the Janus kinase/STAT pathway define an important mechanism that contributes to the different anti-inflammatory potencies of these two cytokines. ***The Journal of Immunology*****, 2003, 170: 3263–3272.**

Cytokines play a pivotal role in the coordination and regulation of immune responses. They are pleiotropically acting factors, meaning that a single cytokine has the potential to elicit different biological responses on different cell types. Despite their pleiotropy, cytokines can be classified according to their predominant biological activities. In this respect TNF-α and IL-1β are regarded as proinflammatory cytokines that sustain the inflammatory response. IL-10 is a strong anti-inflammatory cytokine due to its ability to efficiently suppress the release of proinflammatory cytokines such as TNF-α by macrophages (1) as well as to induce the synthesis of IL-1β receptor antagonist and soluble TNF receptors (2). IL-6 exerts both pro- and anti-inflammatory activities, such as the stimulation of T cell proliferation and their differentiation into cytotoxic T cells, the stimulation of Ab production, and the induction of acute phase protein synthesis in hepatocytes (3, 4). TNF-α release by macrophages is only weakly suppressed by IL-6 (5).

During the initial response to infectious and noninfectious stimuli, macrophages become activated and release proinflammatory cytokines to stimulate the immune response that leads to the symptoms of inflammation. Subsequently, anti-inflammatory cytokines are produced to limit the extent of the proinflammatory activities. Dysbalance between the release of pro- and anti-inflammatory cytokines is one cause of chronic inflammatory diseases and acute sepsis (6, 7). Since macrophages express the receptors for IL-10 and IL-6, they respond to both cytokines (5). IL-10 and IL-6 exhibit different characteristics with respect to their biological activities, although these cytokines mainly act via the same signal transduction pathway. Both cytokines engage receptors that recruit Janus kinases (Jaks)[3] and activate STAT transcription factors, predominantly STAT3 (8, 9).

IL-10 signals via two receptors that belong to the IFN receptor family (class II cytokine receptors) (10). IL-10RI is constitutively associated with Jak1 and is mainly involved in downstream signaling (11–13). IL-10RII acts as an accessory subunit that recruits the second kinase, tyrosine kinase 2 (Tyk2), to the receptor complex (14, 15). Upon binding of IL-10 to the IL-10R subunits, the Jaks are activated by transphosphorylation. The activated Jaks phosphorylate IL-10RI, thereby creating docking sites for STAT transcription factors such as STAT3, which is primarily recruited in macrophages (16, 17). The intracellular part of the IL-10R1 contains only two tyrosine motifs. Each of the single motifs is sufficient for full STAT3 activation (18). STAT3 becomes activated at the receptor by tyrosine phosphorylation and subsequently translocates into the nucleus to induce STAT3-responsive genes.

IL-6 binds to the IL-6R α-subunit (IL-6Rα; gp80) and the signal transducer gp130. Both receptor subunits belong to the family of hemopoietic receptors (class I cytokine receptors). The IL-6Rα is

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[3] Abbreviations used in this paper: Jak, Janus kinase; MAPK, mitogen-activated protein kinase; SHP2, SH2-domain containing tyrosine phosphatase 2; SOCS, suppressor of cytokine signaling; Tyk, tyrosine kinase.

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not involved in the cytoplasmic signal transduction events. Signaling is triggered by the IL-6/IL-6Rα-induced homodimerization of gp130 (19, 20). Gp130 constitutively associates with Jak1, Jak2, or Tyk2 and contains five tyrosine motifs for the phosphorylation-dependent recruitment of signaling molecules (21–23). Similar to IL-10 signal transduction, activation of gp130 leads to Jak activation, receptor phosphorylation, and, finally, activation of the transcription factor STAT3. Moreover, one of the tyrosine motifs in gp130 (Y759 and surrounding amino acids) has been identified to be crucial for recruitment of the SH2-domain containing tyrosine phosphatase 2 (SHP2) (24, 25) and the feedback inhibitor suppressor of cytokine signaling 3 (SOCS3) (26, 27). Both SHP2 and SOCS3 attenuate IL-6 signaling.

SHP2 is a tyrosine phosphatase that contains two SH2 domains (28). Upon binding to a phosphotyrosine motif, the phosphatase becomes activated (29) and might be involved in dephosphorylation of Jaks and receptors. Moreover, SHP2 acts as an adapter protein that links gp130 to the mitogen-activated protein kinase (MAPK) pathway (30). SOCS3 is a member of the SOCS family (31). Mechanisms by which SOCS proteins modulate signaling include inactivation of Jaks, blocking access of the STAT proteins to receptor binding sites, and enabling ubiquitination of signaling proteins and their subsequent targeting to the proteasome (31). SOCS3 exerts its inhibitory effect on IL-6 signaling by binding to pY759 of gp130 via its SH2 domain (26, 27). Therefore, it is a very efficient inhibitor of IL-6 signaling. IL-6 as well as IL-10 induce SOCS3 (32–35).

In animal models of sepsis, Jak1 and the transcription factor STAT3 proved to be most important mediators of anti-inflammatory signals in macrophages (12, 36). In an LPS sepsis model in mice, a macrophage-specific STAT3 knockout leads to elevated TNF-α concentrations in body fluids and increased lethality (36). Since IL-10 and IL-6 both signal via activation of Jak1/STAT3, the discrepancy in their biological effects is unexpected. Several attempts have been made to explain the specific anti-inflammatory ability of IL-10 in contrast to IL-6. One possibility is the existence of an as yet unknown mechanism in addition to STAT3 activation (35). A certain C-terminal part of IL-10RI has been identified by Riley et al. (5) to be important for this special anti-inflammatory activity of IL-10 in mice.

In this study using primary human macrophages we compared STAT3 activation induced by IL-10 and IL-6. We found that STAT3 activations by IL-10 and IL-6 are affected differentially by mechanisms that suppress the Jak1/STAT3 pathway. These findings might explain the different biological effects of these cytokines despite their ability to signal via similar pathways.

Materials and Methods

Materials

Recombinant human LPS, IL-10, and TNF-α were purchased from Roche (Mannheim, Germany). PMA, Percoll, RPMI 1640, and Ficoll 400 were obtained from Sigma-Aldrich (Deisenhofen, Germany). SMEM Spinner medium was obtained from Life Technologies (Eggenstein, Germany). Human serum off the clot was purchased from PAA Laboratories (Colbe, Germany). The phosphotyrosine peptides corresponding to receptor sequences were synthesized by JERINI (Berlin, Germany). The gp130-derived peptides were described previously (26). The sequences of the IL-10R peptides are biotin-(βA)$_2$-VAFQGpY$_{446}$LRQTR, biotin-(βA)$_2$-ALAKGpY$_{496}$LKQDPLE and biotin-(βA)$_2$-KKTKpY$_{254}$AFSPRNS, where βA means β-alanine. All peptides were purified by HPLC and analyzed by mass spectrometry.

Antibodies

The following primary Abs were used: anti-STAT3 rabbit polyclonal Ab (Santa Cruz Biotechnology, Santa Cruz, CA); phosphotyrosine-specific STAT3(P-Tyr-705) rabbit polyclonal Ab, rabbit polyclonal Abs against the phosphorylated and nonphosphorylated MAPKs ERK1/2, c-Jun N-terminal kinase, and p38 (New England Biolabs, Beverly, MA); anti-phosphotyrosine mouse mAb 4G10 (Upstate Biotechnology, Lake Placid, NY); and anti-SHP2 rabbit polyclonal Ab (Santa Cruz Biotechnology). Secondary Abs were obtained from DAKO (Hamburg, Germany).

Cells and cell culture

Human monocytes were isolated from buffy coats (provided by the local blood bank, Transfusionsmedizin, Aachen, Germany) with a Ficoll gradient, followed by hypertonic density centrifugation in Percoll. After 30-min cultivation in RPMI supplemented with 5% human serum and 1% L-glutamine, the monocytes became adherent and were washed three times with SMEM Spinner medium to remove contaminating lymphocytes. Experiments were performed after 4 days of cultivation. All solutions and materials contacting monocytes/macrophages were proven to be LPS free.

EMSA

EMSAs were performed as described previously using a double-stranded ^{32}P-labeled mutated m67SIE-oligonucleotide from the c-Fos promoter (m67SIE, 5′-GATCC GGGAG GGATT TACGG GAAAT GCTG-3′). The protein-DNA complexes were separated on a 4.5% polyacrylamide gel containing 7.5% glycerol in 0.25-fold TBE (20 mM Tris, 20 mM boric acid, and 0.5 mM EDTA) at 20 V/cm for 4 h. Gels were fixed in 10% methanol, 10% acetic acid, and 80% water for 10 min, dried, and analyzed by autoradiography.

Immunoprecipitation

Cells were washed twice with PBS and solubilized in 1 ml of lysis buffer (0.5% Nonidet P-40, 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM NaF, 1 mM EDTA, 1 mM Na_3VO_4, 0.25 mM PMSF, 5 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 15% glycerol) for 30 min at 4°C. Insoluble material was removed by centrifugation, and the cell lysates were incubated with specific Abs overnight at 4°C. The immune complexes were bound to protein A-Sepharose (2.5 mg/ml in lysis buffer) for 1 h at 4°C. After centrifugation, the Sepharose beads were washed three times with washing buffer (0.05% Nonidet P-40, 50 mM Tris-HCl (pH 7.4), 100 mM NaCl, 1 mM NaF, 1 mM EDTA, 1 mM Na_3VO_4, and 15% glycerol). The samples were boiled in gel electrophoresis sample buffer, and the precipitated proteins were separated on SDS-polyacrylamide gels (7.5 or 10% acrylamide)

Phosphotyrosine peptide precipitation assay

COS-7 cells were transfected with SOCS3 (pcDNA3-hSOCS3) as well as STAT3 (pSVL-STAT3) cDNA using the fuGENE6 (Roche, Mannheim, Germany) transfection reagent. Approximately 0.15 μmol of the biotinylated phosphotyrosine peptides (JERINI) were immobilized by incubation with 2.5 mg of NeutrAvidin-coupled Sepharose (Pierce, Bonn, Germany). For SOCS3 and STAT3 precipitation, transfected cells were lysed in lysis buffer (50 mM Tris-HCl (pH 8), 150 mM NaCl, 10% glycerol, 0.5% Nonidet P-40, and 0.1 mM EDTA) supplemented with pepstatin A (2 μg/ml), leupeptin (5 μg/ml), aprotinin (5 μg/ml), PMSF (1 mM), and Na_3VO_4 (1 mM). Equal amounts of expressed SOCS3 and STAT3 in each sample were obtained by mixing the total cell lysates before the precipitation experiment. SOCS3 and STAT3 were precipitated by incubation of the total cell lysates with the immobilized peptides at 4°C overnight. Precipitates were then washed three times with 500 μl of lysis buffer. The precipitated proteins were resolved by SDS-PAGE and transferred to an Immobilon polyvinylidene difluoride membrane (Millipore, Eschborn, Germany) using a semidry electroblotting apparatus. SOCS3 and STAT3 were detected with specific Abs for SOCS3 (M20; Santa Cruz Biotechnology) and STAT3 (C20; Santa Cruz Biotechnology), respectively. A polyclonal goat anti-rabbit HRP-conjugated secondary Ab (DAKO) was used to visualize the immunoreactive bands by ECL techniques.

Immunoblotting and immunodetection

The electrophoretically separated proteins were transferred to a polyvinylidene difluoride membrane by the semidry Western blotting method. Nonspecific binding sites were blocked with 10% BSA in TBS-N (20 mM Tris-HCl (pH 7.4), 137 mM NaCl, and 0.1% Nonidet P-40) for 15 min. The blots were incubated with the respective primary Abs in TBS-N for 1 h at room temperature. After extensive rinsing with TBS-N, blots were incubated with secondary Abs (goat anti-rabbit IgG or goat anti-mouse IgG conjugated to HRP) for 1 h and after an additional washing step they were developed using the ECL detection system (Amersham Pharmacia Biotech, Arlington Heights, IL).

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Total RNA isolation and Northern blot analysis

Total RNA was isolated using the RNeasy mini kit (Qiagen, Hilden, Germany) as described by the manufacturer. Ten micrograms of total RNA was separated on a 1% denaturing agarose gel and transferred to a Nitro-Plus transfer membrane (MSI, Westboro, MA). The membrane was prehybridized at 68°C for 2 h in 10% dextran sulfate, 1 M sodium chloride, and 1% SDS and hybridized overnight in the same solution with cDNA fragments labeled with a random primed DNA labeling kit (Roche). Blots were exposed to Kodak X-OMAT AR-5 films (Eastman Kodak, Rochester, NY) at −79°C with intensifying screens. Suitably exposed autoradiograms were then analyzed by densitometry scanning (PDI, New York, NY).

ELISA

Macrophages were cultured as described above. Cells were incubated with 10 ng/ml LPS and different concentrations of IL-6 or IL-10 for 24 h. Culture supernatants were harvested, and TNF-α concentrations were determined by a TNF-α ELISA (DIACLONE, Besançon, France).

RT-PCR

RT-PCR was performed with 1 μg of total cell mRNA using the OneStep RT-PCR kit from Qiagen (Hilden, Germany). PCR amplification was performed using primer pairs specific for SOCS-1 (upstream primer, 5′-GAGAG CTTCG ACTGC CTCTT-3′; downstream primer, 5′-AGGTA GGAGG TGCGA GTTCA-3′), SOCS-3 (upstream primer, 5′-CTCAA GACCT TCAGC TCCAA-3′; downstream primer, 5′-TTCTC ATAGG AGTCC AGGTG-3′) and GAPDH (upstream primer, 5′-TGATG ACATC AAGAA GGTGG-3′; downstream primer, 5′-TTACT CCTTG GAGGC CATGT-3′); the predicted products for SOCS-1, SOCS-3, and GAPDH were 562, 554, and 244 bp, respectively. The PCR products were separated on a 2% agarose gel and visualized by ethidium bromide staining.

Results

Comparison of IL-10- and IL-6-induced STAT3 activation and anti-inflammatory activities in primary human macrophages

STAT3 is the major transcription factor activated in response to IL-10 and IL-6 in monocytic cells (36). To determine whether the two cytokines activate STAT3 to the same extent, primary human macrophages were stimulated with various amounts of IL-10 or IL-6. Activation of STAT3 was analyzed by monitoring its tyrosine phosphorylation in whole cell lysates by Western blotting. Furthermore, DNA-binding activity was determined in nuclear extracts by EMSA. Fig. 1*A* shows that STAT3 is activated in a concentration-dependent manner after stimulation with IL-10 and IL-6, respectively. Although the outcome of the experiments differs to some extent depending on the charge of macrophages used, 10 ng/ml IL-10 and 20 ng/ml IL-6 elicited comparable responses (Fig. 1*A*). Next, the time courses of STAT3 activation in primary human macrophages in response to IL-10 and IL-6 were compared (Fig. 1*B*). For both cytokines, STAT3 activation was transient, reaching a maximum 20–30 min after stimulation. Compared with IL-6, STAT3 activation in response to IL-10 was prolonged ~20 min.

It is well known that macrophages respond to activation by endotoxins with the release of the proinflammatory cytokine TNF-α. To characterize the anti-inflammatory potential of IL-10 and IL-6 on primary human macrophages, cells were challenged with LPS in the presence of various amounts of IL-10 or IL-6. TNF-α release into the medium was analyzed by a TNF-α ELISA. As shown in

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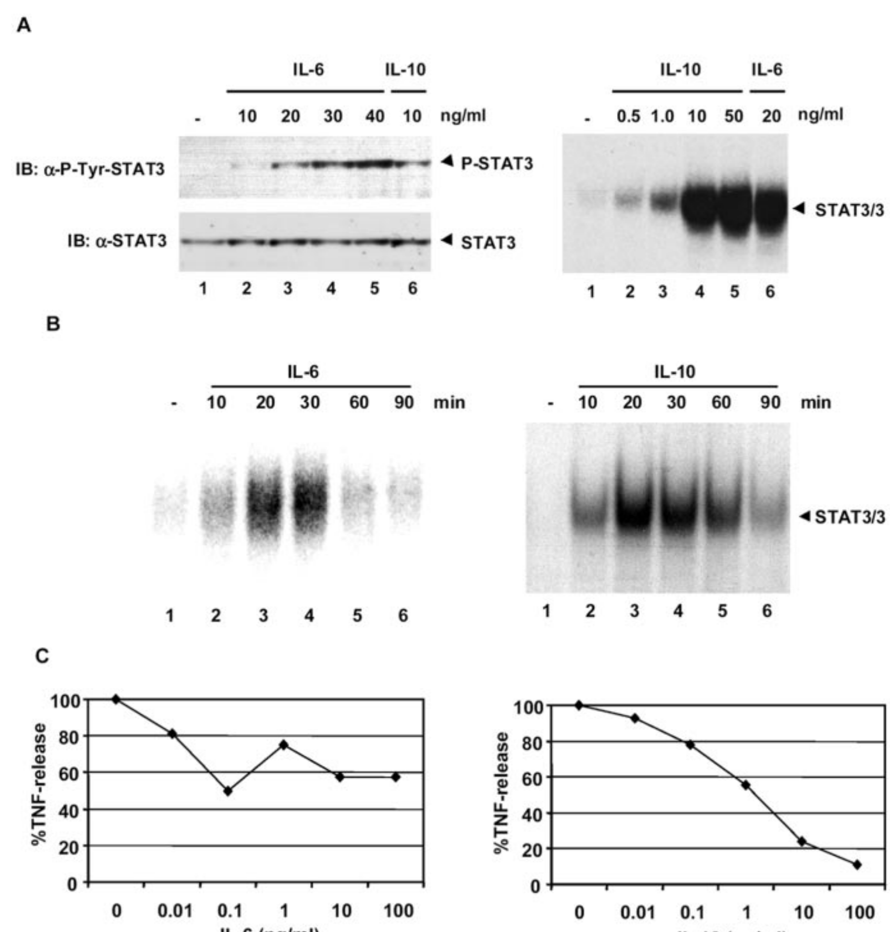

FIGURE 1. STAT3 activation and suppression of TNF-α release by IL-6 and IL-10. *A*, Human macrophages were stimulated with different concentrations of IL-6 or IL-10 for 10 min (Western blot, *left panel*) or 20 min (EMSA, *right panel*) as indicated. Cell lysates and nuclear extracts were prepared and analyzed for STAT3 tyrosine phosphorylation (*left panel*) and STAT3 homodimer (STAT3/3) DNA-binding activity (*right panel*), respectively. *B*, Macrophages were stimulated with 20 ng/ml IL-6 or 10 ng/ml IL-10 for different periods of time as indicated. Nuclear extracts were prepared and analyzed by an EMSA for STAT3 DNA-binding activity. *C*, Macrophages were treated with 10 ng/ml LPS and varying amounts of IL-10 or IL-6 as indicated. Supernatants were harvested after 24 h, and TNF-α concentrations were determined by ELISA. The graph indicates the percentage of TNF-α levels compared with those in cells treated with LPS alone.

Fig. 1*C*, IL-10 treatment resulted in a much more efficient suppression of TNF-α release than stimulation with IL-6. A concentration of 10 ng/ml IL-10 led to 80% reduction of TNF-α production, whereas 20 ng/ml IL-6 reduced TNF-α levels by only ~40%. These data are in line with the findings of a previous study performed with murine macrophages by Riley et al. (5). Thus, the initially equivalent STAT3 activation by IL-10 and IL-6 resulted in different anti-inflammatory activities. Taken together, IL-10 and IL-6 induce largely similar STAT3 activation in primary human macrophages regarding dose-response and time course, but exhibit different potencies in the suppression of TNF-α release after LPS challenge. These first experiments also show that the macrophages isolated in our laboratory exhibit the characteristics of this cell type that are important for this study, such as TNF-α release upon LPS challenge, strong inhibition of TNF-α release by IL-10, and activation of STAT3 in response to IL-10 and IL-6.

IL-10- and IL-6-induced STAT3 activation is differentially perturbed by various proinflammatory stimuli

In previous reports it has been shown that STAT3 activation in response to IL-6 is perturbed by pretreatment of macrophages with PMA or proinflammatory mediators, such as LPS and TNF-α (33, 37–39). The EMSA results presented in Fig. 2*A* confirm that pretreatment of macrophages for 20 min with PMA or with the proinflammatory mediators LPS and TNF-α completely blocked STAT3 activation in response to IL-6 (*lanes 4*, *6*, and *8*). However, STAT3 activation in response to IL-10 was not inhibited by any of these agents (Fig. 2*A*, *lanes 5*, *7*, and *9*). The different pretreatments by themselves did not induce any significant activation of STAT3 (data not shown).

Since it is known that LPS induces the feedback inhibitor of cytokine signaling, SOCS3, we investigated whether SOCS3 induction or induction of another protein might be required for the

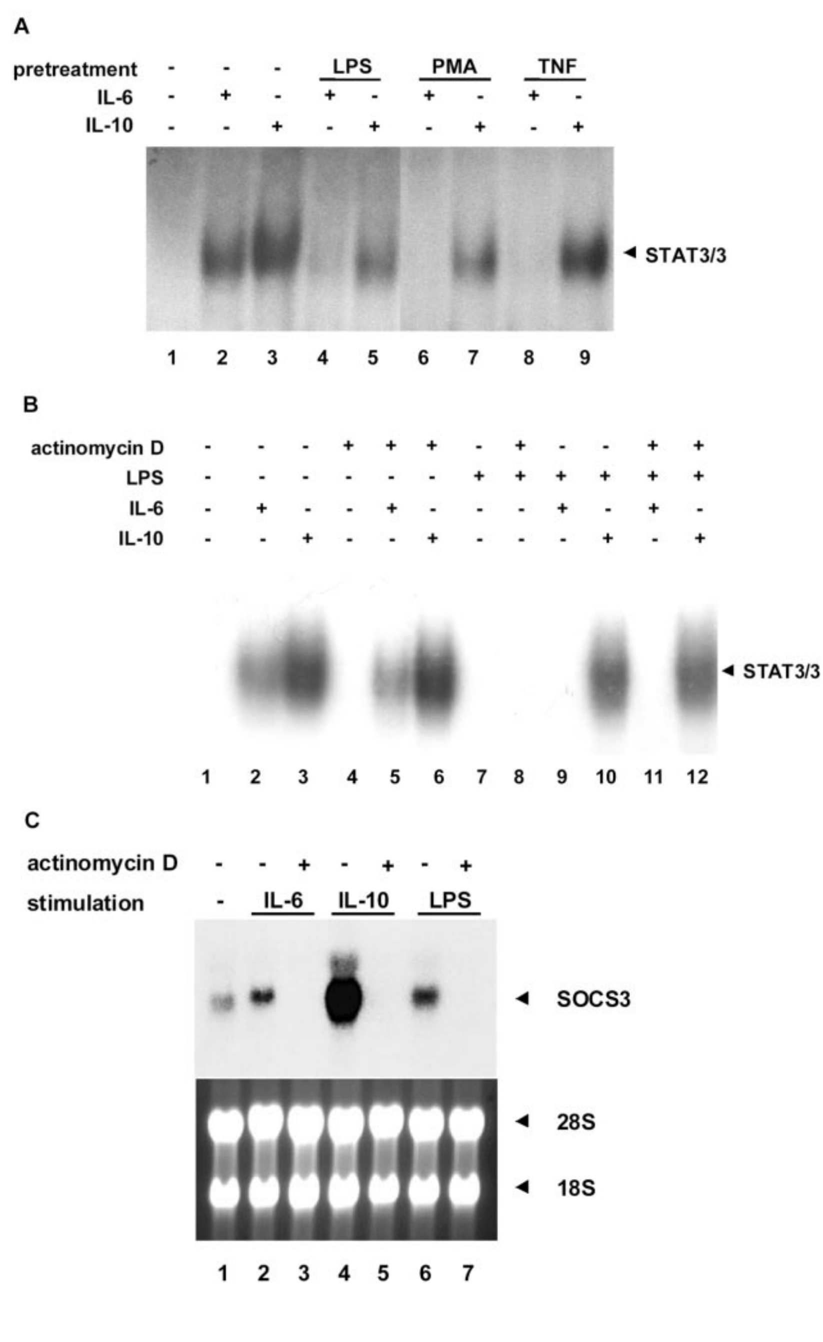

FIGURE 2. Proinflammatory mediators suppress STAT3 activation induced by IL-6, but not STAT3 activation induced by IL-10 stimulation. *A*, Macrophages were pretreated as indicated with 10 ng/ml LPS, 10^{-7} M PMA, or 10 ng/ml TNF-α for 20 min. Subsequently, cells were stimulated with 20 ng/ml IL-6 or 10 ng/ml IL-10 for 30 min. Nuclear extracts were prepared and analyzed with an EMSA for STAT3 DNA-binding activity. *B*, Cells were pretreated with 50 μg/ml actinomycin D for 15 min, then stimulated with 10 ng/ml LPS for 20 min and subsequently with 20 ng/ml IL-6 or 10 ng/ml IL-10 for 15 min. Nuclear extracts were prepared, and STAT3 DNA-binding activities were measured by EMSA. *C*, Human macrophages were pretreated with 50 μg/ml actinomycin D for 15 min and then incubated with 20 ng/ml IL-6, 10 ng/ml IL-10, or 10 ng/ml LPS for 75 min as indicated. After the incubation period, cells were harvested, and total RNA was prepared and subjected to Northern blot analysis for SOCS3 mRNA expression (*upper panel*). Equal loading of the gel was verified by ethidium bromide staining and comparing the intensities of the 28S and 18S rRNA bands (*lower panel*).

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suppression of IL-6 signaling. Therefore, macrophages were pretreated with actinomycin D, a potent inhibitor of transcription. Actinomycin D itself had no influence on STAT3 activation by IL-6 or IL-10 (Fig. 2*B*, *lanes 1–6*). Treatment of macrophages with actinomycin D and LPS did not lead to any activation of STAT3 (Fig. 2*B*, *lanes 7* and *8*). Inhibition of IL-6 induced STAT3 activation by LPS (Fig. 2*B*, *lane 9*) was not affected by preincubation of the cells with actinomycin D (Fig. 2*B*, *lane 11*).

To compare the strength of SOCS3 induction by IL-6, IL-10, and LPS and to evaluate the inhibitory activity of actinomycin D on SOCS3 gene transcription, macrophages were stimulated in the presence or the absence of actinomycin D. After 75 min, RNA was prepared. The Northern blot presented in Fig. 2*C* documents that IL-6, IL-10, and LPS induce SOCS3 mRNA (*lanes 2*, *4*, and *6*). The SOCS3 induction is potently inhibited by actinomycin D (*lanes 3*, *5*, and *7*). Interestingly, IL-10 induced SOCS3 much more strongly than IL-6 or LPS. A low level of SOCS3 mRNA was observed even in unstimulated macrophages (Fig. 2*C*, *lane 1*).

Thus, inhibition of gene transcription is not sufficient to avoid suppression of the STAT3 signal. Therefore, we conclude that induction of SOCS3 is not required for LPS-mediated inhibition of IL-6-induced STAT3 activation. In addition, our results show that compared with IL-6, IL-10-induced STAT3 activation is by far less influenced by proinflammatory mediators.

PMA, LPS, and TNF-α very rapidly inhibit IL-6-induced STAT3 activation

To learn more about the mechanism of the LPS-mediated suppression of IL-6-induced STAT3 activation we studied the time course of inhibition. Macrophages were incubated with IL-6 or IL-10, and after 15 min nuclear extracts were prepared. STAT3 DNA-binding activity was measured by EMSA (Fig. 3*A*, *lanes 1* and *2*). Pretreatment of macrophages with LPS for 5 min, simultaneous addition of LPS and IL-6, or even addition of LPS 5 min after IL-6 stimulation led to a strong inhibition of STAT3 activation (Fig. 3*A*, *lanes 3*, *5*, and *7*). Ten minutes after IL-6 stimulation, LPS was not able to interfere with STAT3 activation (Fig. 3*A*, *lane 9*). Therefore, ~15 min of LPS action was sufficient to inhibit STAT3 activation by IL-6. This is indicative of a rapid inhibitory mechanism that does not depend on de novo protein synthesis. Again, in all settings STAT3 activation by IL-10 remained unaffected by LPS pretreatment (Fig. 3*A*, *lanes 4*, *6*, *8*, and *10*).

The influence of simultaneous challenge of macrophages with PMA or TNF-α and IL-6 was also investigated (Fig. 3*B*). Both mediators suppressed IL-6-signaling even when they were added together with IL-6. These data confirm that LPS, PMA, and TNF-α trigger a rapid mechanism that suppresses STAT3 activation after IL-6 stimulation, which is independent of SOCS3-induction.

IL-6 and IL-10 activate SHP2 phosphatase as well as ERK1, ERK2, and MAPKs

Stimulation of cells with IL-6 leads to activation of the Jak/STAT pathway and phosphorylation of the SH2 domain-containing phosphatase SHP2. SHP2 is an adapter molecule for the activation of ERK1 and -2 by IL-6 and seems to play a negative regulatory role in IL-6-induced Jak/STAT signaling (40). Phosphorylation of

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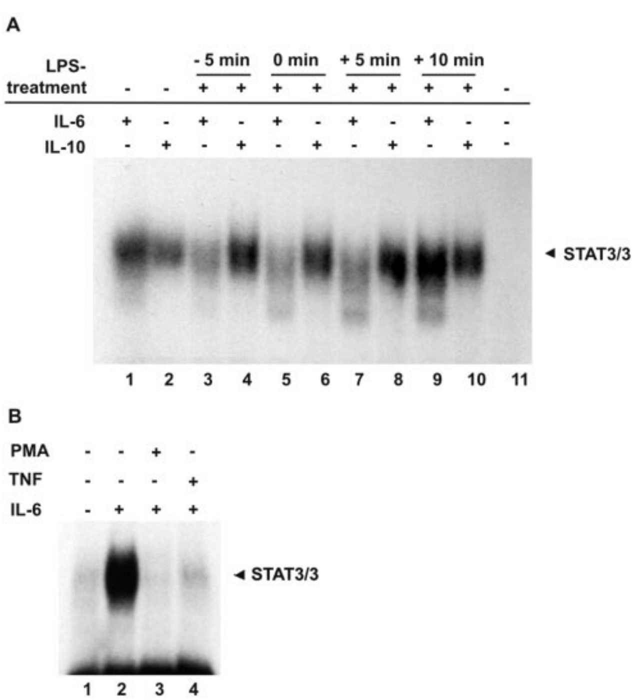

FIGURE 3. LPS, PMA, and TNFα rapidly suppress IL-6-induced STAT3 activation. *A*, Cells were treated with 10 ng/ml LPS, 20 ng/ml IL-6, or 10 ng/ml IL-10 as indicated. LPS was added 5 min before, at the same time as, 5 min after, or 10 min after cytokine stimulation. Twenty minutes after cytokine addition cells were lysed, and nuclear extracts were prepared and analyzed for STAT3 DNA-binding activity by an EMSA. *B*, Macrophages were treated with 20 ng/ml IL-6 and 10^{-7} M PMA or with 20 ng/ml IL-6 and 10 ng/ml TNF-α for 20 min. Nuclear extracts were prepared and analyzed for STAT3 DNA-binding activity by EMSA.

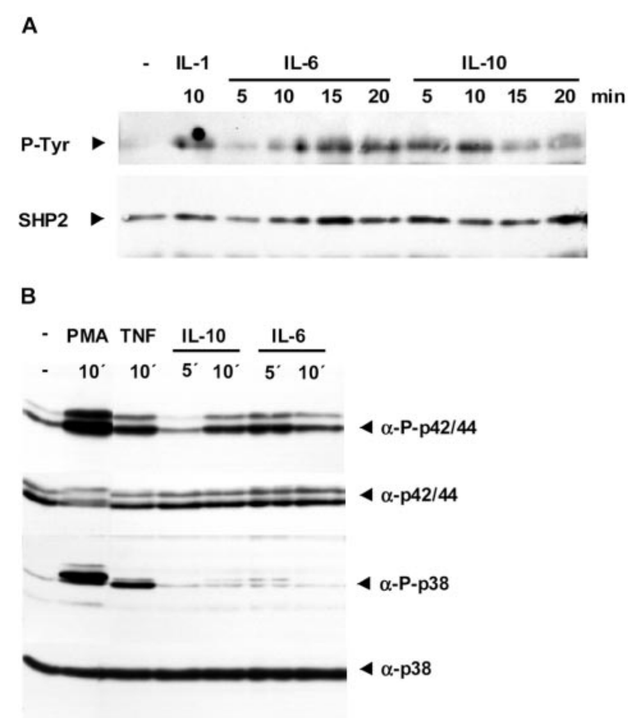

FIGURE 4. Analysis of SHP2 phosphorylation and MAPK activation in response to IL-10 and IL-6. *A*, Cells were treated with 100 U/ml IL-1β, 20 ng/ml IL-6, or 10 ng/ml IL-10 for the time periods indicated. SHP2 was immunoprecipitated from all cell lysates with a human SHP2-specific Ab. SHP2 phosphorylation was determined by immunoblotting with a phosphotyrosine-specific Ab. Equal loading was controlled using an SHP2-specific Ab (*lower panel*). *B*, Macrophages were treated with 10^{-7} M PMA, 10 ng/ml TNF-α, 20 ng/ml IL-6, or 10 ng/ml IL-10 for the times indicated. Cell lysates were prepared and analyzed by Western blot using specific Abs against phosphorylated and total p42/44 or p38.

SHP2 in response to IL-6 as well as to other factors (platelet-derived growth factor and epidermal growth factor) requires a distinct tyrosine motif of the receptor (24). The Y759STV motif required for SHP2 recruitment to gp130 fits well in the consensus sequence YXXV/I/L. Although the IL-10R1 does not contain such a motif, competition between SHP2 and STAT3 for the phosphorylated tyrosine motifs is conceivable. To test this hypothesis we examined macrophages for SHP2- and ERK1 and -2 activation after stimulation with IL-6 or IL-10.

Macrophages were treated with IL-1β, IL-6, or IL-10 for various periods of time (Fig. 4*A*). IL-1β served as a positive control for the induction of SHP2-phosphorylation (41). SHP2 was immunoprecipitated from cell lysates, and tyrosine phosphorylation was detected using a phosphotyrosine-specific Ab. Fig. 4*A* shows that besides the well-established SHP2 activators, IL-1β and IL-6, IL-10 also rapidly activates SHP2 in primary human macrophages. Cell lysates were also analyzed by Western blotting for activation of MAPKs. PMA and TNF-α treatments served as positive controls. Compared with TNF-α stimulation, PMA led to a more prominent activation of p42/44 and p38 isoforms. Weak ERK1 and -2 phosphorylation was detected after IL-10 as well as after IL-6 stimulation (Fig. 4*B*). No significant p38 activation could be detected in response to IL-10 or IL-6.

Feedback inhibitors induced by IL-10 and IL-6 differentially interfere with IL-10 and IL-6 signaling

The transient activation of STAT3 by IL-10 or IL-6 (see Fig. 1*C*) is probably due to the induction of SOCS proteins. These feedback inhibitors act by inhibiting the activity of associated Jaks or by blocking STAT recruitment sites at the receptor (31). Both IL-10 and IL-6 induce SOCS3 in primary human macrophages (see Fig. 2*C*). We investigated to what extent the induction of feedback inhibitors by IL-6 interferes with IL-10 signaling and vice versa.

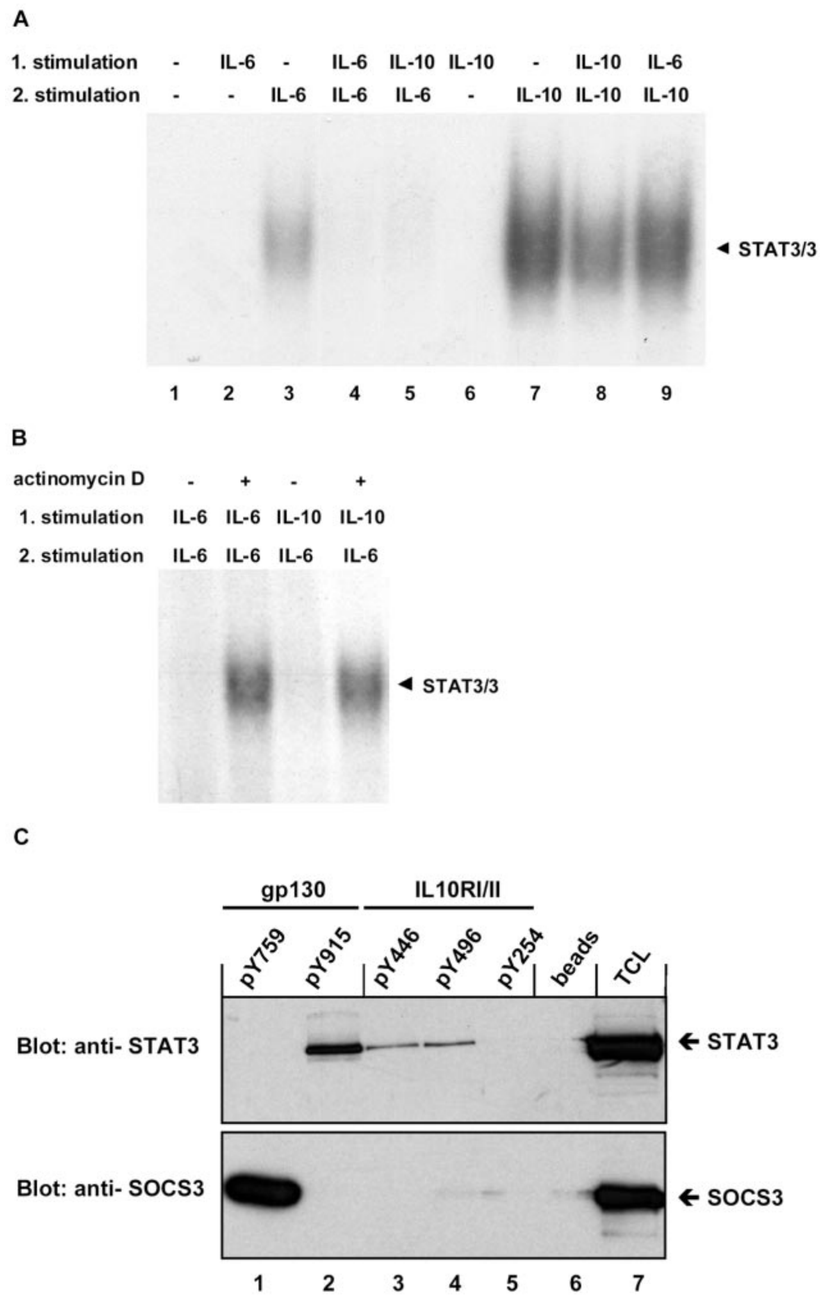

FIGURE 5. Low sensitivity of IL-10 signaling toward inhibition by SOCS3. *A*, Cells were first stimulated with 20 ng/ml IL-6 or 10 ng/ml IL-10 for 15 min; thereafter, the culture medium was changed. After incubation for 45 min without stimulation, the cells were treated again with 20 ng/ml IL-6 or 10 ng/ml IL-10 for 15 min. Subsequently, the cells were harvested, and nuclear extracts were prepared. STAT3 DNA-binding activity was determined by EMSA. *B*, Macrophages were stimulated twice as described in *A*. Where indicated cells were pretreated with 50 μg/ml actinomycin D for 15 min before stimulation with the cytokines. *C*, SOCS-3 does not associate with IL-10R phosphotyrosine motifs. Lysates from COS7 cells transiently transfected with SOCS3 and STAT3 were incubated with immobilized phosphopeptides comprising the tyrosine motifs of the cytoplasmic part of the IL-10RI (biotin-(βA)$_2$-VAFQGpY$_{446}$LRQTR and biotin-(βA)$_2$-ALAKGpY$_{496}$LKQDPLE) and IL10RII (biotin-(βA)$_2$-KKTKpY$_{254}$AFSPRNS) chains. Phosphopeptides corresponding to the gp130 motifs pY759 (biotin-βA-TSSTVQpYSTVVHSG) and pY915 (biotin-βA-PQTVRQGGYMPQ) were used as positive controls for SOCS3 and STAT3 binding, respectively. NeutrAvidin beads were used to detect nonspecific binding of the expressed proteins. Total cell lysates (TCL) show the expression of SOCS3 and STAT3 proteins. Peptide precipitates were analyzed by Western blotting and probed with SOCS3 and STAT3 Abs.

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To induce SOCS3, macrophages were pretreated with IL-6 or IL-10 for 15 min, and after another 45 min the effects of the prestimulation on a second IL-6 and IL-10 stimulation were analyzed. The pretreatment with IL-6 or IL-10 alone did not lead to any detectable activation of STAT3 after 1 h (Fig. 5*A*, *lanes 2* and *6*), whereas the second stimulus alone resulted in normal STAT3 activation (Fig. 5*A*, *lanes 3* and *7*). Pretreatment of cells with IL-6 or IL-10 led to a total inhibition of STAT3 activation in response to IL-6 (Fig. 5*A*, *lanes 4* and *5*). This finding might be easily explained by the induction of SOCS3 by IL-10 and IL-6 and the high sensitivity of IL-6 signaling to suppression by SOCS3. However, neither pretreatment of cells with IL-6 or IL-10 resulted in a similar strong inhibition of STAT3 activation by IL-10 (Fig. 5*A*, *lanes 8* and *9*).

To support that the above-described effects were due to induction of suppressor proteins, the dependence of the inhibition of IL-6 signaling on gene expression was studied. In contrast to LPS-dependent inhibition (Fig. 2*B*), the inhibitory effects of both IL-6 and IL-10 pretreatment on IL-6-induced STAT3 activation were abrogated in the presence of actinomycin D (Fig. 5*B*), indicating that gene transcription is required for the observed inhibitory effects of IL-6 as well as IL-10 pretreatment on IL-6 signal transduction. Compared with IL-6 signal transduction, IL-10 signaling seems to be much less sensitive to the inhibitory activity of SOCS3.

To unravel the molecular basis for the different sensitivities of IL-10 and IL-6 signaling to inhibition by SOCS3, we performed precipitation experiments using biotinylated peptides corresponding to phosphotyrosine motifs of the IL-10R chains and gp130. The biotinylated phosphotyrosine peptides bound to avidin-Sepharose were incubated with lysates of COS-7 cells that were cotransfected with SOCS3 and STAT3. Avidin-Sepharose-precipitated proteins were analyzed by Western blotting using Abs against STAT3 and SOCS3. The gp130 peptides comprising pY759 and pY915 behaved as previously described (26). pY759, the SOCS3 recruitment site of gp130, precipitated SOCS3, but no STAT3, whereas pY915 precipitated STAT3, but no SOCS3 (Fig. 5*C*, *lanes 1* and *2*). The phosphotyrosine motifs of the IL-10RI previously identified to be essential for STAT activation (18) indeed precipitated STAT3, whereas the single tyrosine residue of IL-10RII was not involved in STAT3 activation. Interestingly, none of the cytoplasmic IL-10R phosphotyrosine motifs seemed to be a SOCS3 recruitment site, since none of the IL-10R-derived phosphopeptides precipitated SOCS3 (Fig. 5*C*, *lanes 3–5*). Avidin-Sepharose beads alone precipitated neither STAT3 nor SOCS3 (*lane 6*). STAT3 and SOCS3 were readily detected in total lysates of the transfected cells (*lane 7*).

Feedback inhibitors mediate IL-10 signal attenuation

The studies presented here suggest that SOCS3, although strongly induced by IL-10, is not the major feedback inhibitor for IL-10 signal transduction. On the other hand, STAT3 is transiently activated by IL-10 as well as by IL-6 (Fig. 1*C*). How is the IL-10 signal attenuated? To analyze whether gene transcription is required for IL-10 signal attenuation, a time course of STAT3 activation was measured in the presence of actinomycin D. Fig. 6*A* shows that as a result of inhibition of transcription by actinomycin D, the IL-10-induced STAT3 activation was more intense and decreased only weakly over time. Thus, as in the case of IL-6, an inducible inhibitor seemed to be responsible for the attenuation of IL-10 signaling.

The analysis of inhibitory feedback mechanisms of IL-10 signaling led us to assume that SOCS proteins different from SOCS3 might be responsible for IL-10 signal attenuation. SOCS1 could be a candidate for the feedback inhibition of IL-10 signal transduction, since it is known to inactivate Jaks independently from specific recruitment to cytokine receptors (42–44). To test this hypothesis we compared the effects of IL-10 and IL-6 on gene expression of SOCS1 and SOCS3 by RT-PCR (Fig. 6*B*).

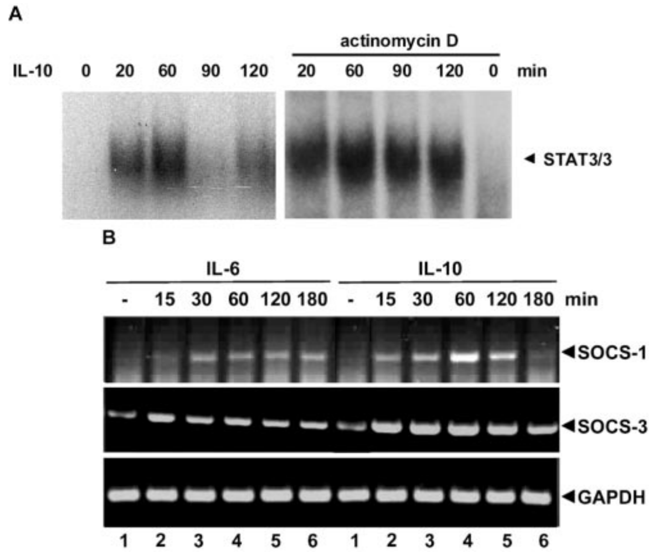

FIGURE 6. IL-6 as well as IL-10 induce SOCS1 and SOCS3 gene expression. *A*, Human macrophages were stimulated with 10 ng/ml IL-10 for the time periods indicated. The cells used for the preparation of the probes in the *right panel* were pretreated with 50 μg/ml actinomycin D for 15 min before stimulation with IL-10. Nuclear extracts were prepared, and STAT3 DNA-binding activity was determined by EMSA. *B*, Macrophages were stimulated with 20 ng/ml IL-6 or 10 ng/ml IL-10 for different periods of time as indicated. Total RNA was prepared, and mRNA for SOCS1, SOCS3, and GAPDH were amplified by RT-PCR as described in *Materials and Methods*. RT-PCR products were analyzed by agarose gel electrophoresis and ethidium bromide staining.

Macrophages were stimulated with IL-10 and IL-6 from 15 min up to 3 h. Subsequently, RNA was prepared, and SOCS mRNA was specifically amplified by RT-PCR. Whereas expression levels of GAPDH mRNA did not change upon stimulation, SOCS1 and SOCS3 mRNA were induced by IL-10. The RT-PCR experiments confirmed the findings from the Northern blot (Fig. 2*C*) that SOCS3 induction in response to IL-10 was stronger than in response to IL-6 and that low levels of SOCS3 mRNA were present in unstimulated macrophages. Most interestingly, IL-10 is also a very strong inducer of SOCS1. The results of these analyses showed a transient SOCS1 mRNA induction by IL-10, with a maximal production after 60 min. This time course inversely paralleled the time course of IL-10-induced STAT3 activation. Therefore, SOCS1 might be responsible for feedback inhibition of IL-10 signal transduction.

Discussion

IL-10 and IL-6 mainly signal via activation of the transcription factor STAT3. Although both cytokines use essentially the same signaling pathways to mediate their physiological responses, distinct biological activities on macrophages can be observed. Compared with the anti-inflammatory activity of IL-10 the suppression of TNF-α release of activated murine macrophages by IL-6 is relatively weak (5). The functional role of STAT3 in deactivation of macrophages by IL-10 has been firmly established (5, 36). On primary human macrophages we found that doses of IL-10 and

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Table I. *Sequence comparison of receptor phosphotyrosine motifs known to recruit SOCS3 and phosphotyrosine motifs of the IL-10 receptor chains*[a]

Receptor	pY Location	Sequence	Ref. No.
h-gp130	pY759	S T V Q pY S T V V H	26, 27
m-Leptin receptor	pY985	P S V K pY A T L V S	49, 50
m-Leptin receptor	pY1077	K S V C pY L G V T S	49
h-Epo receptor	pY401	A S F E pY T I L D P	48
h-Epo receptor	pY429	P H L K pY L pY L V V	51
h-G-CSF receptor	pY729	D Q V L pY G Q L L G	53
h-IL-10RI	pY446	A F Q G pY L R Q T R	
h-IL-10RI	pY496	L A K G pY L K Q D P	
h-IL-10RII	pY254	K K T K pY A F S P R	

[a] Underlined characters highlight conserved residues. h, human; m, mouse; Epo, erythropoietin.

IL-6 that lead to similar initial STAT3 activation result in a superior anti-inflammatory activity of IL-10, as measured by the decrease in TNF-α release upon LPS challenge (Fig. 1*C*). Since the characteristics of STAT3 activation by IL-10 and IL-6 in respect to dose dependence and time course (Fig. 1, *A* and *B*) are largely the same, we investigated whether proinflammatory stimuli differentially interfere with IL-10 and IL-6 signal transduction.

In previous studies it has been demonstrated that pretreatment of macrophages with PMA or proinflammatory stimuli such as LPS, TNF-α, or IL-1β leads to the inhibition of IL-6-induced STAT3 activation (33, 37, 38, 45). We (33) and others (46, 47) demonstrated that LPS and TNF-α induce SOCS3, the major feedback inhibitor of IL-6-induced Jak/STAT signaling. This finding suggested a crucial role for SOCS3 for the inhibition of IL-6-mediated STAT3 activation by proinflammatory stimuli. Only recently, in the study by Ahmed et al. (37), it was shown that gene transcription is not required for the inhibitory activity of proinflammatory stimuli on IL-6 signal transduction in primary human macrophages. Activation of p38 seemed to be most important for the suppression of IL-6 signaling. Using different cell lines, Sengupta et al. (38) established a crucial role of the MAPK ERK1 and -2 in response to PMA stimulation for blocking STAT3 activation. We confirmed that LPS, PMA, and TNF-α inhibit STAT3 activation in response to IL-6 (Fig. 2*A*). The inhibitory activity was rapidly established (within 10 min in the case of LPS) and did not depend on de novo gene expression (Figs. 2*B* and 3*A*). We also showed that TNF-α and PMA (which mimics many effector functions of LPS) activated p38 as well as ERK1 and -2 in primary human macrophages (Fig. 4*B*). Interestingly, STAT3 activation in response to IL-10 appeared more robust, since it was largely unaffected by pretreatment of macrophages with LPS, PMA, or TNF-α (Figs. 2*A* and 3*A*). Therefore, IL-10-induced STAT3 activation is not efficiently inhibited by activated ERK1, ERK2, or p38. This finding might be one explanation for the superior anti-inflammatory activity of IL-10.

IL-6 signal transduction is most sensitive to the feedback inhibitor SOCS3, since SOCS3 is recruited to one of the five phosphorylated tyrosine residues in the signal transducer gp130 (Y759) (26, 27). To establish the role of SOCS3 in inhibition of IL-6 or IL-10 signaling, induction of SOCS3 by IL-6, IL-10, and LPS was compared (Fig. 2*C*). Amounts of IL-6 (20 ng/ml) and IL-10 (10 ng/ml) were used that lead to similar initial STAT3 activation. We found that under these conditions IL-10 is a much more potent inducer of SOCS3 mRNA than IL-6 or LPS, indicating that IL-10-induced STAT3 activation might be much less sensitive to the inhibitory activity of SOCS3. It should also be noted that small amounts of SOCS3 mRNA were detected by Northern blotting as well as RT-PCR even in unstimulated macrophages. Therefore, one cannot exclude that induced translation of SOCS3 from this preformed mRNA is a mechanism for the observed rapid inhibition of IL-6 signal transduction by proinflammatory stimuli. This hypothesis could not be proven by blocking protein synthesis, since cycloheximide treatment interferes with IL-6 signaling in macrophages (37).

Cross-stimulation experiments with IL-6 and IL-10 were performed to characterize the role of inducible feedback inhibitors in signal attenuation (Fig. 5*A*). Whereas prestimulation of primary human macrophages with both IL-6 or IL-10 completely inhibited IL-6-mediated STAT3 activation, STAT3 activation in response to IL-10 remained largely unaffected by IL-6 or IL-10 pretreatment. Since IL-6 and IL-10 only moderately activated ERK1 and -2 and failed to significantly activate p38 in macrophages, these kinases seem to play a minor role in suppression of IL-6 signal transduction in the cross-stimulation experiment. Indeed, inhibition of IL-6 signal transduction by IL-6 or IL-10 pretreatment was dependent on active gene expression, suggesting a functional role for SOCS3 (Fig. 5*B*). Why is IL-10 signaling rather insensitive to the feedback inhibitor SOCS3? Accumulating data suggest that recruitment of SOCS3 to a phosphorylated tyrosine motif of a cytokine receptor is a prerequisite for its inhibitory activity (26, 27, 48–51). From these data a consensus motif for SOCS3 recruitment can be defined (L/V/FXpYXXL/V(V/L); see Table I). Neither the two tyrosine motifs in IL-10R1 (which fit the STAT3 consensus sequence pYXXQ) nor the one in IL-10R2 (TKpYAFSP) has any similarity to known SOCS3 recruitment sites. Accordingly, the phosphotyrosine peptide precipitation assay (Fig. 5*C*) clearly shows that none of the IL-10R phosphotyrosine motifs acts as a SOCS3 recruitment

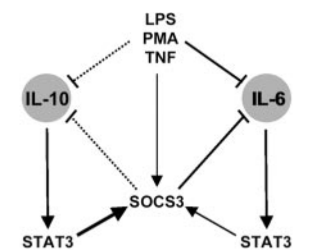

FIGURE 7. Different sensitivities of IL-10- and IL-6-induced signal transduction toward inhibitory mechanisms in primary human macrophages. Doses of IL-10 and IL-6 that lead to a similar activation of STAT3 result in a much stronger induction of SOCS3 in response to IL-10. IL-10 signaling, however, is much less sensitive to the inhibitory activity of SOCS3. This is explained by the lack of SOCS3 recruitment motifs in the IL-10R. LPS, PMA, and TNF are also inducers of SOCS3, but, in addition, inhibit IL-6 signal transduction by a more rapid mechanism that involves p38, ERK1, and ERK2 MAP kinases. IL-10 signal transduction is also less sensitive to these inhibitory mechanisms. The robustness of IL-10-induced STAT3 activation might substantially contribute to the superior anti-inflammatory activity of this cytokine.

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site. Therefore, the different sensitivities of IL-10 and IL-6 signaling to inhibition by SOCS3 are probably due to different SOCS3 recruitment capabilities of the receptors.

We observed that STAT3 activation by both IL-10 and IL-6 is transient (Fig. 1*B*). The transiency of IL-6-induced STAT3 activation is easily explained by the induction of SOCS3 by IL-6 and the sensitivity of IL-6 signaling to SOCS3 inhibitory activity. How is transiency of IL-10 signaling achieved? We found that gene expression is required for a transient STAT3 activation by IL-10, since in the presence of actinomycin D STAT3 remains activated for a prolonged period (Fig. 6*A*). Since it is known that SOCS1 acts independently from specific recruitment to a receptor chain, but is a potent inhibitor of Jak/STAT signaling by directly binding to Jaks (42–44), we compared the induction of SOCS1 by IL-10 and IL-6. Indeed, whereas in unstimulated macrophages no SOCS1 mRNA could be detected, stimulation with IL-10 led to a strong induction of SOCS1 mRNA (Fig. 6*B*). Compared with this, the SOCS1 induction by IL-6 is rather weak. Therefore, SOCS1 might be the major feedback inhibitor responsible for the transiency of the IL-10 signal.

The data presented in Fig. 5*C* showing that SOCS3 is not recruited to phosphotyrosine motifs of the IL-10R complex strongly support our hypothesis that IL-10 signaling is less sensitive than IL-6 signaling to inhibition by SOCS3. Since SOCS3 does not inhibit IL-10 signaling, SOCS3 is strongly up-regulated in response to IL-10 (Fig. 2*C*). We propose that due to lack of inhibition by SOCS3, concomitantly SOCS1 is strongly up-regulated (see RT-PCR, Fig. 6*B*) until it reaches levels that lead to inhibition of Jaks at the IL-10R complex. IL-6 signal transduction, however, is extremely sensitive to inhibition by SOCS3 (26), and therefore signaling is blocked before larger amounts of SOCS1 accumulate.

The tyrosine phosphatase SHP2 that is recruited to the IL-6 signal transducer gp130 has also been proposed to play a functional role in IL-6 signal attenuation (40). Binding of SHP2 to gp130 is dependent on phosphorylation of tyrosine 759 (24, 25), the same tyrosine residue that recruits SOCS3. Unexpectedly, SHP2 is activated upon IL-10 stimulation of macrophages (Fig. 4*A*), although the IL-10R chains lack an SHP2 binding consensus motif (VXpYT/V/IXV/L/I). Despite the fact that the detailed mechanism of SHP2 recruitment by IL-10 remains to be elucidated, involvement of SHP2 in IL-10 signaling provides the link for IL-10-induced activation of ERK1 and -2 (Fig. 4*B*) (52), since the role of SHP2 as an adapter for the MAPK cascade has been established (30).

Several mechanisms might contribute to the superior anti-inflammatory activity of IL-10. Riley et al. (5) postulated that besides the activation of STAT3 a carboxyl-terminal serine residue in the IL-10R1 by an unknown mechanism might be involved in down-modulating TNF-α release by macrophages. In this study we established that IL-10-induced STAT3 activation in primary human macrophages is largely unaffected by the inhibitory activities of proinflammatory stimuli or induction of the feedback inhibitor SOCS3 (summarized in Fig. 7). Therefore, mechanisms that lead to inhibition of cytokine-mediated signal transduction and cross-talks between different pathways have to be taken into consideration to obtain a deeper understanding of the biological activity of a cytokine at the molecular level.

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