LPS and TNFα induce SOCS3 mRNA and inhibit IL-6-induced activation of STAT3 in macrophages

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Received 10 November 1999

Edited by Marco Baggioloni

Abstract Recent findings indicate that cytokine signaling can be modulated by other mediators of simultaneously activated signal transduction pathways. In this study we show that LPS and TNFα are potent inhibitors of IL-6-mediated STAT3 activation in human monocyte derived macrophages, rat liver macrophages and RAW 264.7 mouse macrophages but not in human hepatoma cells (HepG2) or in rat hepatocytes. Accordingly, LPS and TNFα were found to induce the expression of SOCS3 mRNA in each of the investigated type of macrophages but not in HepG2 cells. Using a specific inhibitor, evidence is presented that the p38 MAP kinase might be involved, especially for the inhibitory effect of TNFα.

Key words: Cytokine cross-talk; Lipopolysaccharide resistance; Inflammation; Cytokine signaling

1. Introduction

Interleukin-6 is a multifunctional cytokine with pro- as well as anti-inflammatory properties (for review, see Ref. [1]). IL-6 regulates its target genes by binding to its respective receptor complex, thereby activating tyrosine kinases of the Janus (Jak) family, followed by tyrosine phosphorylation, dimerization and nuclear translocation of mainly signal transducer and activator of transcription (STAT)3 (for review, see Ref. [2]). Recently, it became evident that the Jak/STAT pathway can be modulated by mediators of other simultaneously activated signal transduction pathways.

Thus, it has been shown that lipopolysaccharide (LPS) strongly enhances the interferon (IFN)-γ-induced activation of STAT1 [3]. On the other hand, activation of the mitogen-activated protein (MAP) kinases Erk-1 and Erk-2 by fibroblast growth factor or the phorbol ester PMA inhibits the IL-6-induced STAT activation ([4], Terstegen/Graeve, unpublished work). Moreover, activation of protein kinase A or protein kinase C [5–7] as well as stimulation by transforming growth factor β, granulocyte/macrophage-colony-stimulating factor or angiotensin II [7–10] inhibits the activation of the Jak/STAT pathway by various cytokines.

Pre-incubation of monocytes with IL-10 attenuated the expression of IFNα/β-inducible genes through inhibition of STAT1 activation. It was further shown that IL-10 induces the expression of the suppressor of cytokine signaling (SOCS)-3 [11]. Since binding of SOCS proteins to tyrosine kinases of the Janus family inhibits downstream activation of STATs [12] it was suggested that IL-10 inhibits IFN-induced STAT activation via induction of SOCS3.

In this study we explored the effect of two mediators of inflammation, LPS and tumor necrosis factor α (TNFα) on IL-6 signaling in monocytic and hepatic cells. We found the IL-6-induced activation of STAT3 to be inhibited by pre-incubation of macrophages with LPS or TNFα. This inhibition is paralleled by increased SOCS3 mRNA levels. Inhibitor studies indicate the involvement of the p38 mitogen-activated protein kinase (MAP kinase), especially for the inhibitory effect of TNFα.

2. Materials and methods

2.1. Materials

Recombinant human TNFα and LPS were purchased from Boehringer Mannheim (Mannheim, Germany). Oligonucleotides were obtained from MWG Biotech (Ebersberg, Germany), SB 202190 [13] from Calbiochem (Bad Soden, Germany). RPMI 1640 and L-glutamine were from Biowhittaker (Verviers, Belgium); S-MEM spinner medium and Dulbecco's modified Eagle's medium (DMEM) from Life Technologies (Eggenstein, Germany). Fetal calf serum was from Seromed (Berlin, Germany). Recombinant human IL-6 was prepared as described [14]. Lymphoflot was from Biotest (Dreieich, Germany), Percoll from Sigma (Deisenhofen, Germany) and Nycodenz from Nycomed (Oslo, Norway).

2.2. Isolation and cultivation of rat liver macrophages

Liver macrophages (KC, Kupffer cells) were isolated from 1 year old male Wistar rats by collagenase-pronase perfusion and separated by a single Nycodenz gradient and centrifugal elutriation [15]. Cells were cultured in a 60 mm dish in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS) and 1% gentamicin for up to 48 h in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. Culture medium was changed after 24 h. Purity of KC was ≥ 97% as assessed 24 h after seeding by their typical light microscopic appearance, by immunological staining for the specific macrophage marker protein ED2 [16], and by their ability to phagocytose fluorescent 1.1 μm latex particles.

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Abbreviations: DMEM, Dulbecco's modified Eagle's medium; EMSA, electrophoretic mobility shift assay; GAPDH, glycerin aldehyde-3-phosphate dehydrogenase; IFN, interferon; LPS, lipopolysaccharide; MAP kinase, mitogen-activated protein kinase; STAT, signal transducer and activator of transcription; SOCS, suppressor of cytokine signaling; TNFα, tumor necrosis factor α

0014-5793/99/$20.00
PII: S0014-5793(99)01662-2

2.3. Isolation and cultivation of parenchymal cells from rat liver

Isolated parenchymal cells were prepared from livers of 5–8 weeks old male Wistar rats by a collagenase perfusion technique as described [17]. Cells were plated on collagen-coated culture dishes and maintained in Krebs-Henseleit medium supplemented with 6 mmol/l glucose. After 2 h medium was removed and the culture was continued for 24 h in DMEM containing 5% fetal calf serum, 100 mg/l penicillin/streptomycin, 100 nmol/l insulin, 100 nmol/l dexamethasone, 30 nmol/l Na-selenite and 1 mg/l aprotinin.

2.4. Isolation and cultivation of macrophages derived from human blood

Human monocytes were isolated from buffy coats, kindly provided by the local blood bank (Transfusionsmedizin, RWTH Aachen, Germany) with a Lymphoflot 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 S-MEM spinner medium to remove contaminating lymphocytes. Experiments were performed after 4 days of cultivation. All solutions and materials contacting monocytes/macrophages were LPS tested by the manufacturers or by ourselves with the limulus amoebocyte limusate test (LAL-Test, Kappes, Laborservice, Augsburg, Germany). Serum from healthy human volunteers was sterile filtered and heat inactivated. Serum was only used for cell culture when IL-6 and TNFα were below 11.3 pg/ml and 8.1 pg/ml as measured by Immulite (Biermann, Bad Nauheim, Germany) according to the manufacturer's instructions. Serum from a minimum of four volunteers was pooled.

2.5. Cultivation and stimulation of cells

The murine peritoneal macrophage cell line RAW 264.7 and the human hepatoma cell line HepG2 were grown in RPMI 1640 or in DMEM/nut mix F12 medium, respectively, supplemented with 10% fetal calf serum, streptomycin (100 mg/l) and penicillin (60 mg/l). Medium was changed and adjusted to 5 ml 24 h before experiments were carried out.

Cells grown in a 60 mm (KC) or 100 mm (RAW 264.7, MC) dish were stimulated with LPS, TNFα or IL-6 at the concentrations indicated. SB 202190 was dissolved in dimethyl sulfoxide and added to the culture medium 40 min before stimulation at concentrations as indicated in the figure legends. Nuclear extracts were prepared as described by Andrews and Faller [18]. Protein concentrations were determined with a Bio-Rad protein assay.

2.6. Electrophoretic mobility shift assay (EMSA)

EMSAs were performed as described previously [19] using a double-stranded ^{32}P-labeled mutated m67SIE-oligonucleotide from the c-fos promoter (m67SIE: 5′-GAT CCG GGA GGG ATT TAC GGG GAA ATG CTG-3′) [20]. 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, 0.5 mM EDTA) at 20 V/cm for 4 h. Gels were fixed in 10% methanol, 10% acetic acid and 80% water for 1 h, dried and autoradiographed.

2.7. Total RNA isolation and Northern blot analysis

Total RNA was isolated using RNeasy mini kit (Qiagen, Hilden, Germany) as described by the manufacturer. 10 μg of total RNA were separated on 1% denaturating agarose gels and transferred to Nitro-Plus transfer membrane (MSI, Westboro, WI, USA). The membranes were pre-hybridized at 68°C for 2 h in 10% dextran sulfate, 1 M sodium chloride, 1% SDS, and hybridized over night in the same solution with cDNA fragments labeled with Random Primed DNA Labeling kit (Boehringer Mannheim GmbH, Germany). Blots were exposed to Kodak X-OMAT AR-5 film at −70°C with intensifying screens. Suitably exposed autoradiograms were then analyzed by den-

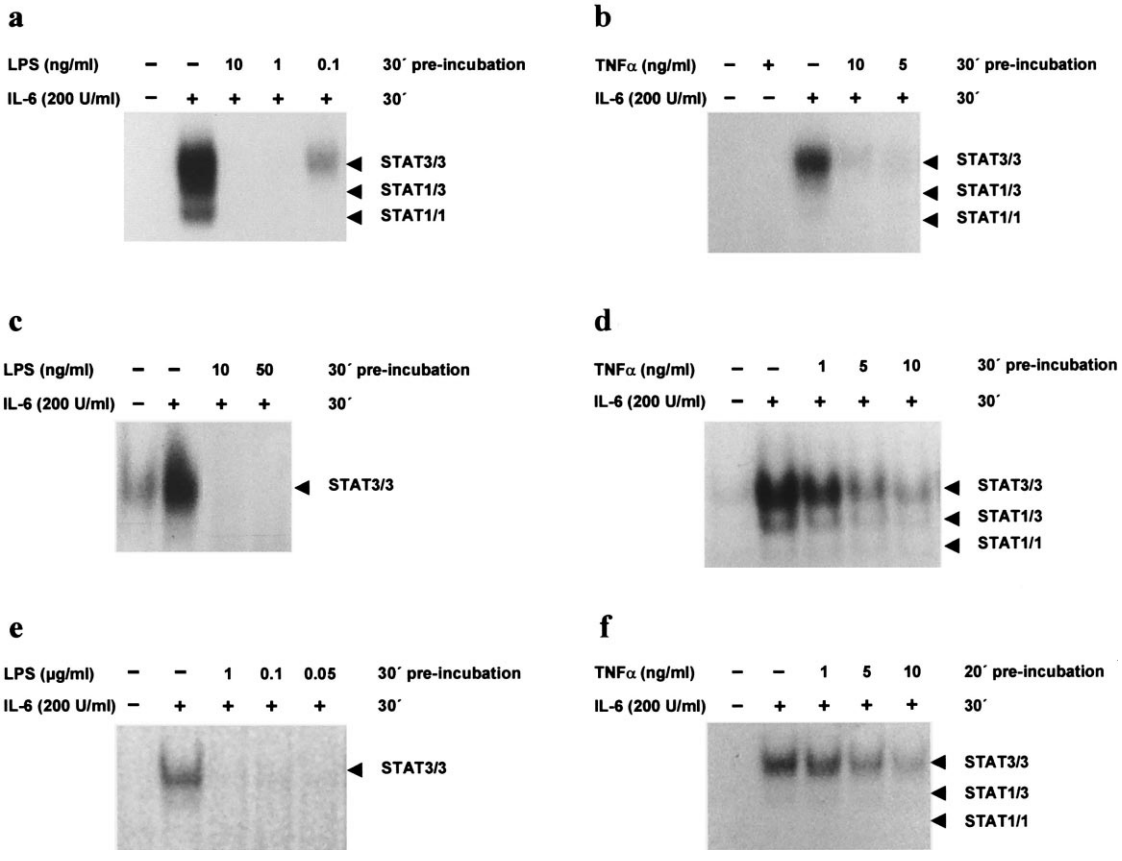

Fig. 1. Effect of LPS or TNFα pre-treatment on IL-6 induced STAT3 activation in macrophages. Human macrophages (a,b), rat liver macrophages (c,d) and the mouse macrophage cell line RAW 264.7 (e,f) were pre-treated with either LPS or TNFα as indicated and subsequently stimulated with IL-6. After the respective incubation period cells were harvested and nuclear extracts prepared and analyzed as described in Section 2. 5 μg of nuclear extracts were mixed with a ^{32}P-labeled oligonucleotide (mutated SIE probe of the c-fos promoter 5′-GAT CCG GGA GGG ATT TAC GGG GAA ATG CTG-3′) and EMSAs were performed. The DNA-protein complexes formed were separated from the free probe by electrophoresis on a native 4.5% gel.

sitometry scanning (PDI, New York, USA). Glycerin aldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe was kindly provided by Dr. Burchart-Graeve (RWTH Aachen, Germany) and SOCS3 cDNA probe by Dr. Hilton (The Walter and Eliza Hall Institute of Medial Research, Parkville, Vic., Australia).

3. Results

Recent findings of several groups indicate that cytokine signaling can be modulated by other mediators of simultaneously activated signal transduction pathways [3–12]. Therefore, we investigated whether IL-6-induced signaling is influenced by LPS or TNFα, important pro-inflammatory mediators. Pre-incubation of human macrophages isolated from peripheral blood (Fig. 1a,b), rat liver macrophages (Fig. 1c,d) or RAW 264.7 murine peritoneal macrophages (Fig. 1e,f) with either LPS or TNFα dose dependently suppressed IL-6-induced activation of STAT3 as assessed by an EMSA with an oligonucleotide from the c-fos promoter [20].

Since SOCS3 is known to be a cytokine-inducible suppressor of cytokine signal transduction [12] we asked whether there is a correlation between inhibition of STAT3 activation and induction of SOCS3 expression. As shown in Fig. 2 LPS (Fig. 2a,c, e) as well as TNFα (Fig. 2b,d,f) induced the expression of SOCS3 mRNA in all three types of macrophages. Thus, it is possible that inhibition of IL-6 signal transduction by LPS or TNFα might be due to the de novo synthesis of SOCS3.

Most interestingly, the inhibition of IL-6-stimulated activation of STAT3 by LPS or TNFα was not observed in primary hepatocytes isolated from rat liver (Fig. 3b,d) or the human hepatoma cell line HepG2 (Fig. 3a,c) even at high concentrations of LPS or TNFα. Likewise, induction of SOCS3 mRNA by LPS or TNFα was found to depend on the cell type investigated, since neither TNFα nor LPS were able to induce SOCS3 mRNA in HepG2 cells (Fig. 3e). These data strongly suggest that inhibition of IL-6 signaling by LPS or TNFα is a specific feature of macrophages.

Activation of members of the MAP kinase family has been shown to have an inhibitory effect on cytokine signaling ([4], Terstegen/Graeve, unpublished work). Hence, we were interested to find out whether MAP kinases are involved in the inhibition of IL-6-induced STAT3 activation by LPS or TNFα. As shown in Fig. 4 pre-incubation of RAW 264.7 mouse macrophages with a specific inhibitor of the p38 MAP kinase (SB202190) almost completely blocks the inhibition of STAT3 activation by TNFα (Fig. 4a,b), whereas the LPS effect was only partially affected (Fig. 4c). Accordingly in

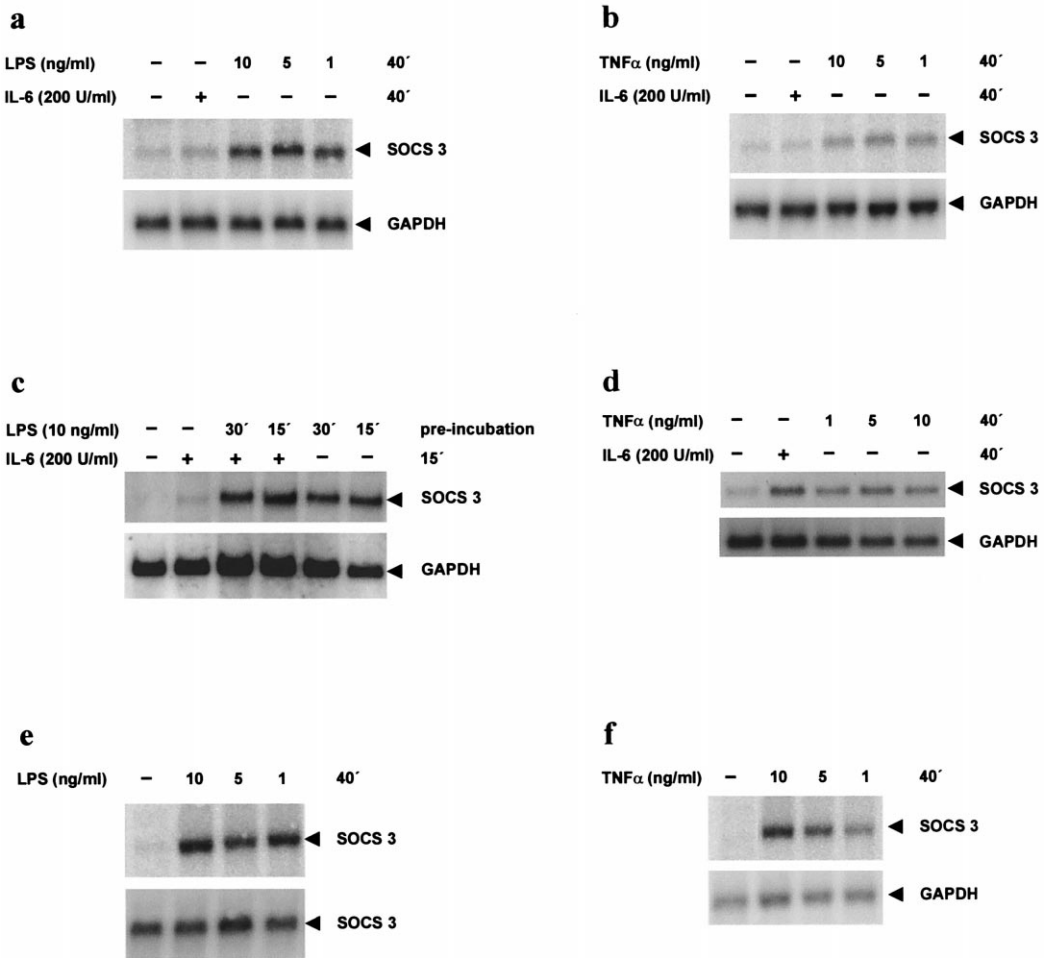

Fig. 2. Induction of the SOCS3 mRNA expression in macrophages by LPS or TNFα. Human macrophages (a,b), rat liver macrophages (c,d) and RAW 264.7 mouse peritoneal macrophages (e,f) were treated with IL-6, LPS or with TNFα as indicated. After the respective incubation period cells were harvested for isolation of total RNA and subjected to Northern blot analysis for SOCS3 and GAPDH (loading control) as described in Section 2.

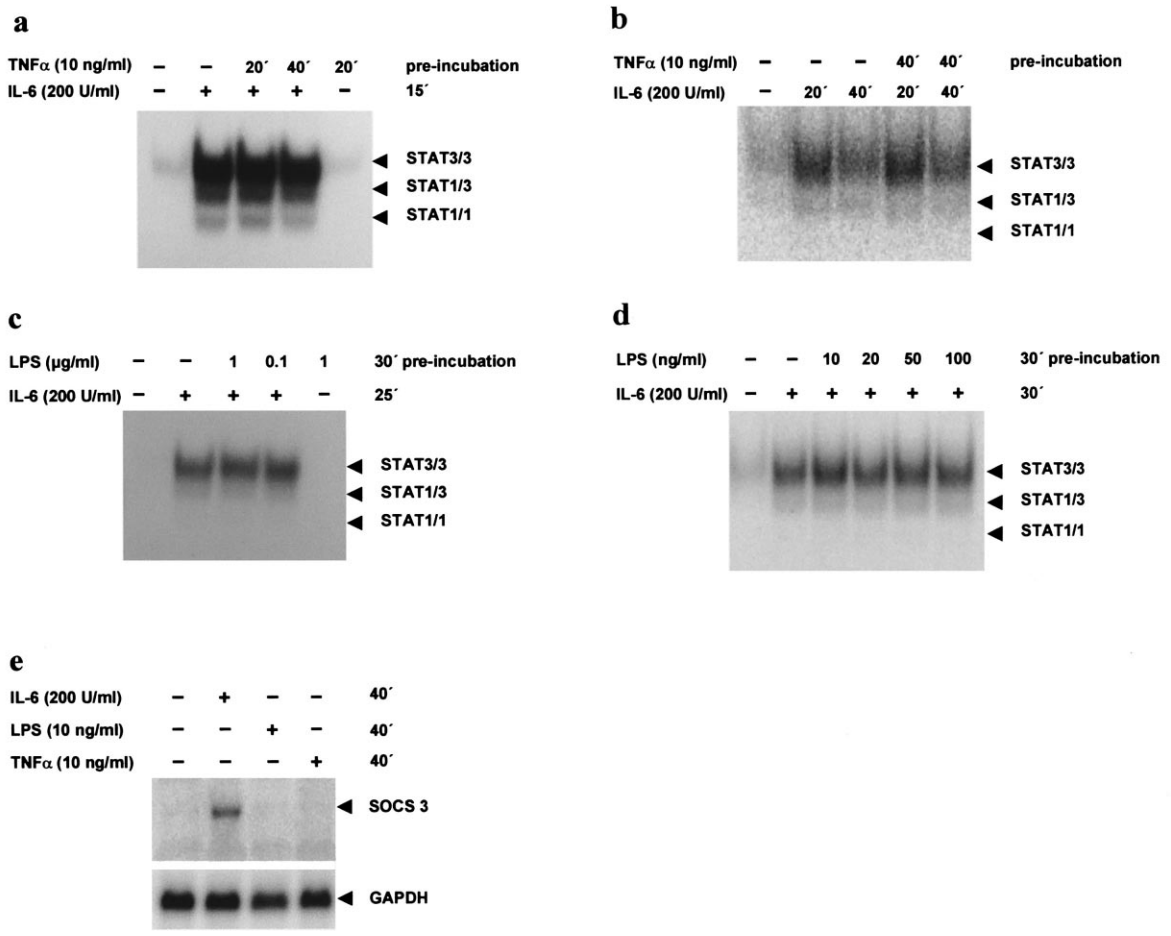

Fig. 3. Effect of LPS or TNFα on SOCS3 mRNA expression and on IL-6-induced STAT3 activation in human hepatoma cells (HepG2) and in rat hepatocytes. HepG2 cells (a,c,e) and rat hepatocytes (b,d) were pre-treated with either TNFα or LPS and stimulated with IL-6 as indicated. After the respective incubation period cells were harvested and nuclear extracts were prepared. EMSAs were performed as described in the legend to Fig. 1. For (e) HepG2 cells were treated with IL-6, TNFα and LPS as indicated. Thereafter cells were harvested for the isolation of total RNA, subsequently subjected to Northern blot analysis for SOCS3 and GAPDH as described in Section 2.

RAW 264.7 cells (Fig. 5a) and liver macrophages the TNFα (Fig. 5b) mediated SOCS3 mRNA expression was reduced to basic expression levels by the inhibition of p38, whereas LPS-induced SOCS3 mRNA expression in RAW 264.7 mouse macrophages was only partially attenuated. Thus, one may conclude that the p38 MAP kinase plays an important role in the inhibitory effect of TNFα (or LPS) on IL-6 signaling.

4. Discussion

This study demonstrates that pre-treatment of different types of macrophages with the pro-inflammatory mediators TNFα or LPS largely decreased or even completely abolished STAT3 activation after IL-6-stimulation. This inhibition closely correlates with the induction of SOCS3 mRNA by LPS or TNFα. Since neither LPS nor TNFα influenced IL-6-induced STAT3 activation in HepG2 cells or primary hepatocytes from rat liver, this effect seems to be macrophage-specific.

Both, LPS and TNFα have shown to be potent activators of the p38 MAP kinase as well as other members of the MAP kinase family ([13,21,22], for review [23]). Using a specific inhibitor of the p38 MAP kinase, we obtained evidence that p38 is involved, particularly in the inhibition of IL-6 signaling by TNFα (Fig. 4). Most interestingly, inhibition of the p38 activity not only neutralized the TNFα effect on IL-6-induced STAT3 activation but also suppressed induction of SOCS3 mRNA by TFNα, suggesting that inhibition of STAT3 activation and induction of SOCS3 mRNA are functionally linked. On the other hand our data indicate that p38 activation by LPS does only partially contribute to the inhibitory influence of LPS on activation of STAT3 by IL-6. Accordingly increased SOCS3 expression due to stimulation with LPS was largely reduced after pre-treatment with SB202190 but was still comparable to that observed after treatment with 10 ng/ml TNFα. Therefore, besides p38 activation, additional signaling events are required to explain the LPS-mediated increase in SOCS3 mRNA levels and its suppressive action on IL-6 signal transduction.

LPS has recently been described to induce expression of SOCS3 but not SOCS1 or SOCS2 mRNA and protein in a macrophage cell line. This was shown to correlate not only with the inhibition of IFNγ-dependent long term activation of Jak1 and STAT1 but also with the suppression of Jak2 and STAT5 activation by granulocyte/macrophage-colony-stimulating factor [24]. The authors also showed that inhibition of IFNγ signaling does not occur until 4 h of LPS pre-incubation. This was discussed to be a possible mechanism respon-

sible for the LPS tolerance of macrophages after long term exposure to endotoxins. In contrast, short term pre-treatment with LPS even enhanced the transcriptional response to IFNγ as well as STAT1 serine- and tyrosine-phosphorylation [3].

Our data show that LPS as well as TNFα inhibit IL-6-induced STAT3 activation already after a pre-incubation period of 30 min (Fig. 1), suggesting that early inhibition of IL-6 signaling can not be considered in the context of the emergence of LPS tolerance in macrophages. With respect to this observation it is important to note that IL-6 shows distinct anti-inflammatory properties. Thus, as an example IL-6 was shown to inhibit the synthesis of TNFα and IL-1 both in vivo and in vitro in response to several stimuli [25–27]. Moreover, it was shown that IL-6 suppressed the macrophage-colony-stimulating factor-induced proliferation and differentiation of both tissue and bone marrow macrophages [28]. It is attractive to speculate that parts of the IL-6 signaling cascade are inhibited after short term exposure of macrophages to the pro-inflammatory mediators LPS and TNFα in order to enforce inflammatory response to pathogens whereas at the same time anti-inflammatory properties of IL-6 are suppressed. In such a scenario the early SOCS3 expression would promote the activation of macrophages, whereas its long term expression would lead to a desensitization towards other mediators like IFNγ. Further studies are needed to establish the role and the pathophysiological implications of the inhibition of IL-6 signaling by LPS or TNFα in macrophages.

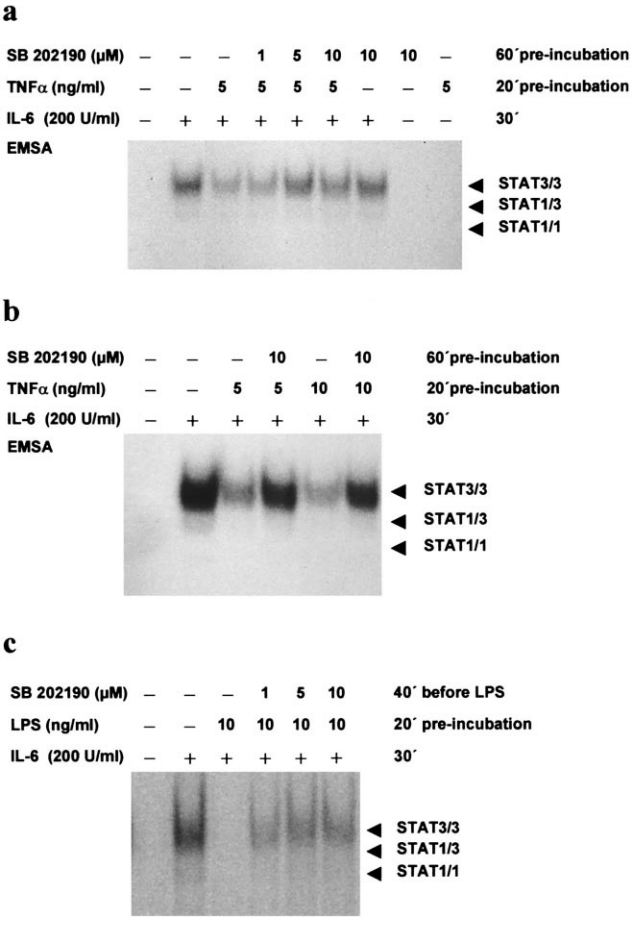

Fig. 4. Involvement of the p38 MAP kinase in the inhibition of IL-6 signaling by LPS or TNFα in macrophages. Following a pre-incubation period of 40 min with SB 202190 at the concentrations indicated, RAW 264.7 mouse macrophages were pre-treated with either TNFα (a,b) or LPS (c) as indicated and then stimulated with IL-6. After the respective incubation period cells were harvested and nuclear extracts were prepared. EMSAs were performed as described in the legend to Fig. 1.

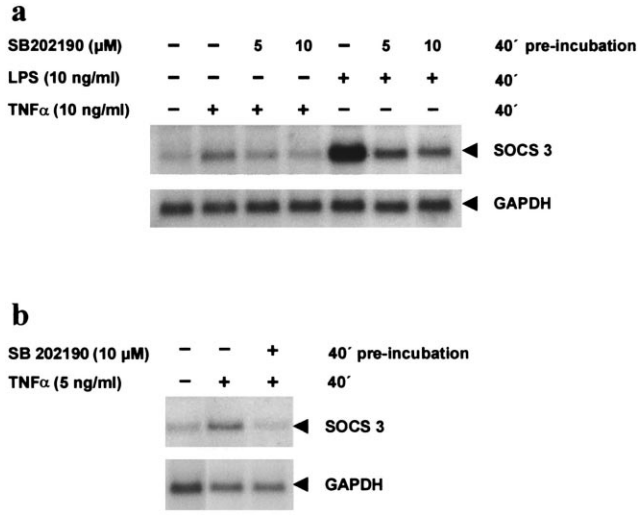

Fig. 5. Involvement of the p38 MAP kinase in LPS or TNFα induced SOCS3 mRNA expression in macrophages. After pre-incubation for 40 min with SB 202190 at the concentrations indicated, RAW 264.7 mouse macrophages (a) and rat liver macrophages (b) were treated either with TNFα or with LPS as depicted. Thereafter cells were harvested for isolation of total RNA, subsequently subjected to Northern blot analysis for SOCS3 and GAPDH as described in Section 2.

Inhibition of IL-6 signaling by LPS or TNFα was not observed in the human hepatoma cell line HepG2 and in primary hepatocytes from rat liver. The lack of the inhibitory effect of LPS on IL-6 signaling in HepG2 cells or hepatocytes might be due to the absence of LPS binding protein, since this protein was shown to be required for stimulation of HepG2 cells by LPS [29]. To our knowledge no differences have been described so far between HepG2/hepatocytes and macrophages in respect to the signal transduction of TNFα. However, the fact that TNFα is capable to induce SOCS3 mRNA expression and inhibit STAT3 activation in macrophages but not in HepG2 or hepatocytes suggests that the signaling of TNFα is different in these two cell types.

Acknowledgements: Buffy coats were kindly provided by W. Bauch from the local blood bank. We thank S. Belau for the determination of TNFα and IL-6 concentrations in human serum, P. Gatsios, L. Terstegen, S. Thiel and A. Martens for their helpful discussion. J.G.B. is recipient of an DFG fellowship. This work was supported by grants from the Deutsche Forschungsgemeinschaft (Bonn) and the Fonds der Chemischen Industrie (Frankfurt).

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