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Regulation of Suppressor of Cytokine Signaling 3 (SOCS3) mRNA Stability by TNF-α Involves Activation of the MKK6/p38^{MAPK}/MK2 Cascade[1]

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The potential of some proinflammatory mediators to inhibit gp130-dependent STAT3 activation by enhancing suppressor of cytokine signaling (SOCS) 3 expression represents an important molecular mechanism admitting the modulation of the cellular response toward gp130-mediated signals. Thus, it is necessary to understand the mechanisms involved in the regulation of SOCS3 expression by proinflammatory mediators. In this study, we investigate SOCS3 expression initiated by the proinflammatory cytokine TNF-α. In contrast to IL-6, TNF-α increases SOCS3 expression by stabilizing SOCS3 mRNA. Activation of the MAPK kinase 6 (MKK6)/p38^{MAPK}-cascade is required for TNF-α-mediated stabilization of SOCS3 mRNA and results in enhanced SOCS3 protein expression. In fibroblasts or macrophages deficient for MAPK-activated protein kinase 2 (MK2), a downstream target of the MKK6/p38^{MAPK} cascade, basal SOCS3-expression is strongly reduced and TNF-α-induced SOCS3-mRNA stabilization is impaired, indicating that MK2 is crucial for the control of SOCS3 expression by p38^{MAPK}-dependent signals. As a target for SOCS3 mRNA stability-regulating signals, a region containing three copies of a pentameric AUUUA motif in close proximity to a U-rich region located between positions 2422 and 2541 of the 3′ untranslated region of SOCS3 is identified. One factor that could target this region is the zinc finger protein tristetraprolin (TTP), which is shown to be capable of destabilizing SOCS3 mRNA via this region. However, data from TTP-deficient cells suggest that TTP does not play an irreplaceable role in the regulation of SOCS3 mRNA stability by TNF-α. In summary, these data indicate that TNF-α regulates SOCS3 expression on the level of mRNA stability via activation of the MKK6/p38^{MAPK} cascade and that the activation of MK2, a downstream target of p38^{MAPK}, is important for the regulation of SOCS3 expression. *The Journal of Immunology,* **2007, 178: 2813–2826.**

After its discovery, the signaling cascade mediated by the activation of members of the tyrosine kinase family Jak and the subsequent activation of STAT factors has been recognized as one of the key events in the signal transduction of many different stimuli such as ILs, IFNs, or growth factors (1, 2). These proteins are involved in the regulation of different biological processes such as the pathogen-specific immune response, inflammation, cellular proliferation, and the differentiation and induction of apoptosis. Considering the importance of the Jak/STAT signaling cascade, it is very conceivable that tight and balanced control of the activation status of the signal-transducing molecules involved is indispensable. Deregulation results in inefficient or even detrimental inflammatory responses and/or contributes to malignant cellular transformation and promotion of growth. Thus, intensive research on the negative regulation of Jak/STAT-mediated signal transduction has been initiated to understand the regulation of cytokine signaling in more detail. One of the major breakthroughs was the discovery of eight structurally related proteins nowadays known as suppressor of cytokine signaling (SOCS)[3] (3–8). Originally conceived as classical feedback inhibitors induced by cytokines and other mediators, SOCS proteins attenuate Jak/STAT signaling by blocking the activity of Jak tyrosine kinases and the activation of STAT factors either through direct interaction with Jak (9) or through interaction with the activated receptor (10). All of these proteins contain a central Src homology 2 (SH2) domain and a C-terminal SOCS box that may target signaling intermediates to proteasomal degradation.

As suggested by targeted gene disruption experiments, SOCS proteins are responsible for the specific regulation of the activity of certain cytokines. For instance, SOCS1$^{-/-}$ deficient mice suffer from fatal inflammatory disease (11–13) and are hypersensitive to IFN-γ and other cytokines (i.e., IL-12) involved in the regulation of T cell homeostasis. In contrast to SOCS1, our understanding of the physiological role of SOCS3 is incomplete because SOCS3-deficient animals die in utero due to placental insufficiency (14).

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[3] Abbreviations used in this paper: SOCS, suppressor of cytokine signaling; ARE, adenosine/uridine-rich element; ΔCT, delta cycle threshold; DIG, digoxygenin; Epo, erythropoietin; HA, hemagglutinin; MEF, mouse embryonic fibroblast; MK2, MAPK-activated protein kinase 2; MKK6, MAPK kinase 6; SDHA, succinate dehydrogenase; SH2, Src homology 2; siRNA, small interfering RNA; TTP, tristetraprolin; Tet, tetracycline; UTR, untranslated region.

However, recent reports shed new light on the role of SOCS3 for the regulation of hepatic and macrophage function and give evidence that SOCS3 plays a nonredundant role in IL-6- and gp130-mediated signal transduction in macrophages. In these reports SOCS3 deficiency in either hepatocytes or macrophages and neutrophils correlates with prolonged IL-6-induced STAT3 activation, whereas the activation of STAT3 or STAT1 through IL-10- or IFN-γ-induced signal transduction remains unaffected (15–17). Moreover, the findings of Yasukawa et al. (16) indicate that in the absence of SOCS3, IL-6 elicits anti-inflammatory properties and acts similarly as IL-10 to attenuate TNF-α and IL-12 production induced by LPS in macrophages. In this context it is interesting to note that several proinflammatory stimuli such as bacterial products (e.g., LPS and CpG) (18–20) or cytokines such as TNF-α (18) have been identified as inducing SOCS3 expression in human and rat liver macrophages but also in a macrophage cell line, thereby inhibiting IL-6- or IFN-γ-mediated activation of STAT3 or STAT1, respectively, This implies that bacterial components (e.g., LPS and CpG) (18–20) or proinflammatory cytokines (e.g., TNF-α) largely modify the biological responses to other cytokines such as IL-6 or IFN-γ. Thus, with respect to the biological role of IL-6 or IFN-γ, it is essential to understand the mechanisms involved in the control of SOCS3 expression by proinflammatory mediators such as TNF-α.

p38^{MAPK} belongs to the family of MAPKs and is strongly activated by bacterial products such as LPS and CpG but also by proinflammatory cytokines such as TNF-α and IL-1β. Activation of p38^{MAPK} is crucial for cellular stress response, cytokine signaling, and inflammatory cytokine production (for review see Ref. 21). As a positive feedback, p38^{MAPK} supports the production of IL-1β, TNF-α, and other cytokines. Consequently, disturbance of the p38^{MAPK} cascade results in reduced production of these factors. Several reports provide clear evidence that the p38^{MAPK} pathway regulates cytokine expression on the level of either protein translation or mRNA stabilization or both. Control of mRNA stability is mediated by *cis*-acting sequences within the 5′ or 3′ untranslated regions (UTRs) or, in some cases, within the coding sequence. The best-characterized regulatory elements are the adenosine/uridine-rich elements (ARE) within the 3′ UTR of the mRNA of cytokines, growth factors, and protooncogenes. Often, these mRNAs contain several copies of the pentameric AUUUA motif originally described by Shaw and Kamen (22) with nearby U-rich regions. It is now well accepted that AREs act as potent destabilizing sequences. Particularly, they target mRNA for rapid deadenylation in vivo (23–25) and promote 3′-5′ exonuclease decay by the exosome in vitro (26, 27). A large body of evidences indicates that the function of these elements is exerted by *trans*-acting RNA-binding proteins such as AUF-1 (28), HuR (29), or tristetraprolin (TTP) (30, 31), either stabilizing or destabilizing mRNA. The activity, at least of some of these factors, can be modified in response to external stimuli allowing a dynamic regulation of the expression of the respective gene product. In particular, activation of the p38^{MAPK} cascade has been shown to control the stability of mRNAs coding for proteins such as TNF-α (32), IL-8 (33), or cyclooxygenase-2 (34). Although the substrate of p38^{MAPK} responsible for this activity remains to be identified, previous reports indicate that the MAPK-activated protein kinase 2 (MK2) is one of the key molecules mediating mRNA stability in response to p38^{MAPK} activation (33, 35).

Several reports suggest that the activation of p38^{MAPK} is also important for SOCS3-induction by proinflammatory mediators such as TNF-α, LPS, or CpG (18, 19) or by other cytokines such as IL-6 (36) and IL-4 (37). Up to now it is not clear how p38^{MAPK} regulates the expression of SOCS3. Activation of the MAPK kinase 6 (MKK6)/p38^{MAPK} may represent one, if not the, key event for the induction of SOCS3 expression by proinflammatory mediators. The major intention of the present study was to elucidate the mechanisms by which TNF-α controls the expression of SOCS3 and to evaluate the role of the MKK6/p38^{MAPK} cascade in this context.

Materials and Methods

Materials

CDP-Star ready-to-use substrate, digoxygenin (DIG) oligonucleotide 3′-end labeling kit, DIG easy hybridization granules, positively charged nylon-membrane, Taq polymerase, human IL-6, murine and human TNF-α, and recombinant murine erythropoietin (Epo) were purchased from Roche; doxycycline was from Promega; polyvinylidene difluoride Hybond membrane was from Amersham Biosciences; restriction enzymes were from New England Biolabs; oligonucleotides were obtained from MWG-Biotec; DMEM, DMEM nutritional mix F12, Opti-MEM, and Lipofectamine 2000 were from Invitrogen Life Technologies; murine IL-6 was from Peprotech; human GM-CSF was from Biochrom; actinomycin D was from Calbiochem/Merck; FCS was from Perbio Science; and SYBR Green was from Applied Biosystems. The following Abs were used: anti-DIG-alkaline phosphatase Fab (Roche); mAb against GAPDH (BioDesign); mAb against the hemagglutinin (HA) tag (Roche); polyclonal SOCS3 Ab (IBL); polyclonal MKK6 Ab (Upstate/Biomol); polyclonal p38^{MAPK} Ab (StressGen Biotechnologies); polyclonal phospho-p38 as well as Ab specific for MK2 (Cell Signaling); and mAbs against the Flag tag (Sigma-Aldrich).

Cultivation of RAW 264.7 murine macrophages, NIH 3T3 cells, MK2$^{-/-}$, and MK2$^{+/+}$ macrophage cell lines or mouse embryonic fibroblast (MEF) cells

RAW 264.7 cells were cultivated in DMEM including 1000 mg/L glucose with GlutaMAX (Invitrogen Life Technologies) supplemented with 10% heat inactivated FCS. NIH 3T3 cells were grown in DMEM with 4500 mg/L glucose plus GlutaMAX supplemented with 10% heat inactivated FCS. MEF cells isolated from MK2-deficient animals or respective wild-type animals (38) were cultured in DMEM with 4500 mg/L glucose plus GlutaMAX supplemented with 10% heat-inactivated FCS. Murine macrophage cell lines were established from MK2-deficient animals or respective wild-type animals by retroviral infection with a virus encoding both v-Raf and v-Myc oncogenes as described recently (39). Cells were cultivated in DMEM with 1000 mg/L glucose supplemented with 10% heat inactivated FCS. If not stated otherwise, the experiments with macrophages were performed after an incubation period of 4 h in serum-free medium.

Preparation and cultivation of human peripheral blood derived macrophages

Human PBMCs were isolated from freshly collected leukocyte-rich buffy coats obtained from healthy blood donors (University of Düsseldorf Blood Transfusion Service, Düsseldorf, Germany) by density gradient centrifugation over Ficoll-Paque gradient (Amersham Biosciences). Monocytes (3×10^6 cells/well) were allowed to adhere to plastic 6-well plates for 2 h at 37°C in RPMI 1640 medium supplemented with antibiotics and 10% human non-A/non-B serum. After incubation, nonadherent cells were removed and the wells were washed three times with culture medium. Monocytes were differentiated into macrophages in RPMI 1640 medium supplemented with antibiotics, 10% human non-A/non-B serum, and recombinant human GM-CSF (5 ng/ml). Culture medium (2 ml/well) was replaced every 2 days. Cells were used after 6 days of culture. Medium was changed to serum- and GM-CSF-free culture medium for 4 h before experiments were performed.

Preparation and cultivation of primary bone marrow derived macrophages

Mice deficient for TTP, their genotyping, and maintenance of the colony have been described elsewhere (40). Bone marrow-derived macrophages were prepared as described previously (41) and used after 2 wk in culture. At ~16 h before the treatments, the culture medium was changed to RPMI 1640 that contained 1% (v/v) FBS.

Vectors

Standard cloning procedures were performed as outlined by Sambrook and Russel (42). Full-length murine SOCS3 cDNA including the 5′ and 3′ UTRs of the murine SOCS3 mRNA was generated by RT-PCR from total

Table I. *Northern oligo probes (5′ to 3′) designed for detection of the HA tag, murine SOCS3 mRNA, and GAPDH*

Probe	Sequence
NB-SOCS3-cs188-as	ATA AGA AAG GTG CCC GCG GGC
NB-SOCS3-cs226-as	TCA ACG TGA AGA AGT GGC GCT
NB-SOCS3-cs555-as	AAG ATG CTG GAG GGT GGC CAC
NB-SOCS3-cs419-as	ACT TCG GAC GAG GGT TCC GTG
NB-SOCS3-cs621-as	AAT GGG TCC AGG CAG CTG GGT
NB-GAPDH-cs006-as	AAA TCC GTT CAC ACC GAC CTT
NB-GAPDH-cs391-as	ATT TCT CGT GGT TCA CAC CCA
NB-GAPDH-cs793-as	AGA TGC CCT TCA GTG GGC CCT
NB-GAPDH-5′021-as	TCT ACG GGA CGA GGC TGG CAC
NB-GAPDH-3′071-as	AAT TGT GAG GGA GAT GCT CAG
HA-as	AGC ATA GTC TGG GAC GTC ATA TGG ATA

RNA isolated from LPS-stimulated RAW 264.7 cells and cloned into the TOPO cloning vector (Invitrogen Life Technologies). To distinguish between endogenous SOCS3 mRNA and exogenous mRNA, a sequence coding for the HA tag was introduced into the SOCS3 cDNA directly downstream of the start codon (see Fig. 2). The β-globulin encoding-sequence of the pTet-BBB vector (43) was removed by using *Bst*X1 and *Eco*RI restriction, and new restriction sites for *Eco*RV and *Spe*I were introduced by the ligation of oligonucleotides. pTet-HA-SOCS3 was generated by the introduction of the HA-tagged SOCS3 construct into the newly introduced *Eco*RV/*Spe*I site. The deletion mutant HA-SOCS3 Δ109, lacking 109 nucleotides within the 3′ UTR of the SOCS3 mRNA, was generated by *Stu*I/*Spe*I restriction of the TOPO-HA-SOCS3 construct and the subsequent reinsertion of an oligonucleotide representing the last 40 nucleotides of the 3′ UTR of the murine SOCS3 mRNA. cDNA encoding for TTP was generated by RT-PCR from total RNA isolated from LPS-stimulated RAW 264.7 cells using 5′-ATGGATCTCTCTGCCATCTACGAGAGC-3′ as the sense primer and 5′-TCACTCAGAGACAGAGATACGATTG-3′ as the antisense primer. The resulting PCR-product was introduced into the pGEM-T Easy cloning vector (Promega) and sequenced. Thereafter, the TTP encoding fragment was introduced into the pRcCMV expression vector (Invitrogen Life Technologies) using the *Hin*dIII and *Not*I restriction sites.

pGL3-SOCS3–2757Luc containing the promoter region −2757 to +929 of the murine SOCS3 gene fused to the luciferase-encoding sequence was provided by S. Melmed (Cedars-Sinai Medical Center, Los Angeles, CA) and has been described previously (44). The Epo/gp130 receptor construct was described previously (45). To control the correct sequence of the respective constructs, vectors were submitted to the laboratories of MWG Biotec for sequencing. The expression vector for Flag-tagged dominant negative $p38^{MAPK}$ (KRSPA-flag-p38-dn) was previously described in (46). The pcDNA3-flag-MKK6-ca vector encoding for a constitutively active mutant of MKK6 was from Dr. R. Davis (University of Massachussetts Medical School, Worcester, MA). The pTet-Off expression vector encoding the tetracycline (Tet)-sensitive Tet-Off factor was purchased from Clontech.

For silencing the expression of TTP, a mix of three different siRNAs (siRNA 1, 5′-AGUGACUGCCCGGUCAGAU-3′; siRNA 2, 5′-AGACG GAACUCUGUCACAA-3′; and siRNA 3, 5′-AGACUGAGCUAUGU CGGAC-3′) was used. For control, siRNA directed against GFP (5′-AGC AGAAGAACGGCAUCAA-3′) was used. siRNA oligonucleotides were synthesized by MWG Biotec.

Transfection procedure and reporter gene assay

For reporter gene assays, RAW 264.7 and NIH 3T3 cells were transfected using Lipofectamine 2000. Briefly, the cells were grown on 6-well plates for RAW 264.7 or 12-well plates for NIH 3T3 toward 80–95% confluency. RAW 264.7 cells were preincubated with 1 ml of Opti-MEM 1 h before transfection. Six microliters of Lipofectamine 2000 and 3.2 μg of DNA in total were prediluted in 250 and 50 μl of Opti-MEM, respectively. For NIH 3T3 cells we used half of these amounts. Diluted reagent and DNA were mixed, incubated for 20 min at room temperature, and then added to the cells. After 6 h the medium was changed and incubation was continued in the respective medium. Cell lysis and luciferase assays were conducted using the Dual luciferase kit (Promega) as described in the manufacturer's instructions.

Luciferase activity values were normalized to transfection efficiency monitored by the cotransfected *Renilla* expression vector (Promega). All expression experiments were done at least in duplicate. Data are presented as the percentage of the respective control experiment. Error bars are SD. Results were analyzed using the Student's *t* test; $p < 0.05$ was considered statistically significant.

For higher transfection efficiency, cells were transfected using Lipofectamine 2000 and a modified transfection procedure. In brief, 5 μl of Lipofectamine 2000 was diluted in 250 μl of Opti-MEM and 2.8 μg of DNA was diluted in 50 μl of Opti-MEM. Diluted reagent and DNA were mixed and incubated for 20 min at room temperature. Meanwhile, a subconfluent flask (75-cm^2 flask) of NIH 3T3 cells or mouse embryonic fibroblasts were trypsinized and, after centrifugation, resuspended in their respective culture medium supplemented with 10% FCS; then 300 μl of resuspended cells were added to the transfection mixture. Afterward, 1 ml of culture medium was added and cells were seeded on a 60-mm culture dish. After 16 h incubation at 37°C, the medium was replaced by DMEM containing 4500 mg/L glucose supplemented with 10% FCS. Cell culture was continued for another 24–72 h as indicated in the figure legends. Thereafter, experiments were performed as outlined in the results section.

Immunoblotting and immunodetection

For immunoblot analyses cells were grown in a 60-mm culture dish and stimulated with the respective cytokine at the concentrations indicated. Cells were washed twice with PBS supplemented with 0.1 mM Na_3VO_4 and solubilized in 1 ml of lysis buffer (1% Triton, 20 mM Tris-HCl (pH 7.4), 136 mM NaCl, 2 mM EDTA, 50 mM β-glycerophosphate, 20 mM sodium pyrophosphate, 1 mM Na_3VO_4, 4 mM benzamidine, 0.2 mM Pefabloc, 5 μg/ml aprotinin, 5 μg/ml leupeptin, and 10% glycerol) at 4°C. Fifty micrograms of protein was subjected to electrophoresis with an 8% SDS gel. The electrophoretically separated proteins were transferred onto polyvinylidene difluoride membranes by the semidry Western blotting method. Nonspecific binding was blocked with 5% nonfat dry milk powder in TBST (20 mM Tris-HCl (pH 7.4), 137 mM NaCl, and 0.1% Tween 20) for 30 min. The blots were incubated overnight at 4°C or for 2 h at room temperature with primary Abs diluted in TBST (1/1000). After extensive washing with TBST, blots were incubated with goat anti-rabbit IgG or goat anti-mouse IgG secondary Abs conjugated to HRP and diluted in TBST (1/5000) for 1 h. After further rinsing in TBST, the immunoblots were developed with the ECL system (Amersham Biosciences) following the manufacturer's instructions.

RNA isolation and Northern blot

Total RNA was isolated with the total RNA kit from Qiagen as described in the manufacturer's instructions. All buffers used were made with DEPC-treated water. The Northern blot presented in Fig. 11 was performed as described (41) using cDNA probes specific for murine TTP, SOCS3, and GAPDH. To specifically detect mRNA coding for the HA tag, a detection method based on DIG-labeled oligonucleotides was established. In brief, RNA (10 μg) was separated by electrophoresis with 2% denaturing formaldehyde and 0.8% agarose gel in 20 mM MOPS (pH 7.0), 2 mM EDTA, and 5 mM sodium acetate. RNA was blotted onto positively charged nylon membranes (Roche) by capillary transfer. Oligonucleotides used for the detection of specific mRNAs were labeled with DIG using the respective labeling kit according to the manufacturer's instructions (Roche). Blots were hybridized overnight at 50°C using DIG easy hybridization buffer as outlined by the manufacturer (Roche). The HA tag was detected using a single specific oligonucleotide antisense probe, while a mixture of five antisense oligonucleotides derived from the coding sequence of murine GAPDH mRNA was used for detection of GAPDH mRNA. To analyze the expression of SOCS3 mRNA, a mixture of five antisense oligonucleotide derived from the coding sequence of murine SOCS3 mRNA was used for hybridization (Table

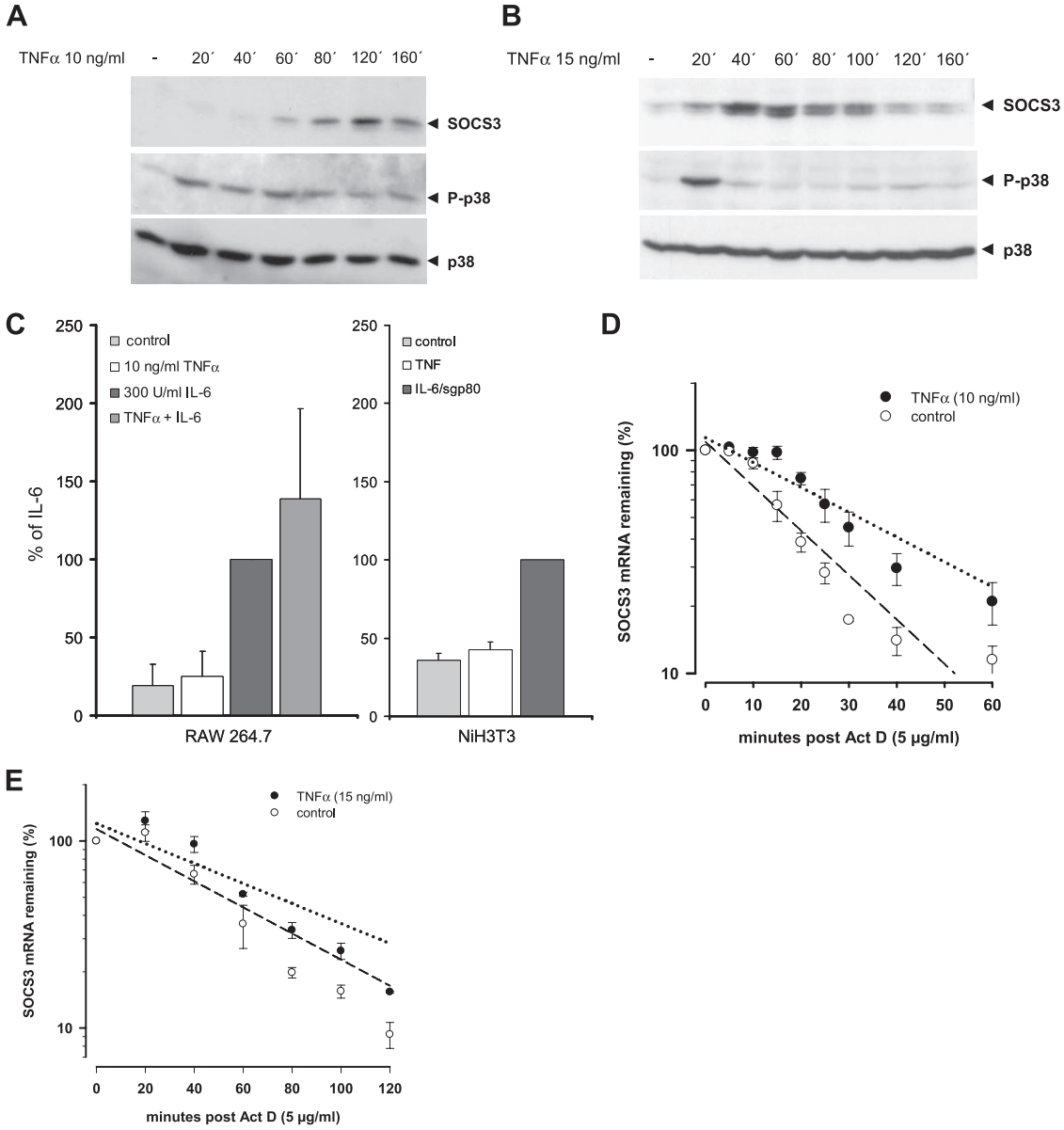

FIGURE 1. Regulation of SOCS3 expression by TNF-α. *A* and *B*, MEFs (*A*) and RAW 264.7 (*B*) cells were stimulated with TNF-α (10 ng/ml or 15 ng/ml, respectively) for the times indicated. Thereafter, protein lysates were prepared and 80 μg of protein per lane of protein was subjected to immunoblot analysis using Abs specific for SOCS3, phosphorylated p38^{MAPK} (P-p38), and p38^{MAPK} (p38). *C*, RAW 264.7 cells or NIH 3T3 cells were transfected with 3 μg of vector for a luciferase reporter gene driven by a 2757-bp SOCS3 promoter fragment. An expression vector for *Renilla* luciferase was cotransfected for monitoring transfection efficiency. Two days after transfection, RAW cells were stimulated with IL-6 (300 U/ml) and/or TNF-α (10 ng/ml) and NIH 3T3 cells were stimulated with IL-6 (300 U/ml) plus soluble gp80 (5 μg/ml) and/or TNF-α (10 ng/ml) as indicated. Luciferase activity in cellular extracts of these cells was determined after an incubation period of 16 h and normalized to *Renilla* luciferase activity as described in *Materials and Methods*. Results are expressed as percentage (means $\pm$ S.D.) of IL-6-induced luciferase activity. *D* and *E*, RAW 264.7 cells (*D*) or peripheral blood-derived human macrophages (*E*) were preincubated with IL-6 (300 U/ml) for 60 min. Thereafter, cells were washed and cultured in medium supplemented with 4 μmol/ml actinomycin D (Act D) in the presence or absence of TNF-α (10 ng/ml). Cells were harvested after the time periods indicated, and total RNA was isolated and subjected to real time PCR for SOCS3 mRNA expression as described in *Materials and Methods*. Semiquantitative PCR results were obtained using the ΔCT method. Relative SOCS3 mRNA levels at the various times after the addition of actinomycin D were expressed as fractions of the normalized value at time 0 (when actinomycin D was introduced), which was set at 100%. Exponential decay curves were calculated and the time to 50% mRNA decay was estimated as outlined in *Materials and Methods*.

I). After hybridization, blots were washed twice at room temperature with washing buffer A (2$\times$ SSC, 0.1% SDS) and twice at 50°C using washing buffer B (0.2$\times$ SSC, 0.1% SDS). Chemiluminescent detection of the DIG-labeled oligonucleotide hybridized to the respective mRNA was performed with an anti-DIG Ab diluted in blocking solution (Roche) and CDP-Star according to the manufacturer's instructions (Roche).

Real-time PCR

Total cellular RNA was isolated by using the RNeasy Miniprep kit (Qiagen). One microgram of total RNA was reverse-transcribed with a QuantiTect reverse Transcriptase kit (Qiagen) using oligo(dT), which included DNase I digestion. cDNA was diluted one-fifth and 2.5 μl of the diluted cDNA was added as a template to 25 μl of SYBR Green PCR master mix according to the manufacturer's instructions (Applied Biosystems). The primers for human (sense, 5′-CAGCAAGCAGGAGTATGACG-3′; antisense, 5′-AAAGCCATGCCAATCTCATC-3′) and mouse (sense, 5′-CCCTGTGCTGCTCACCGA-3′; antisense, 5′-ACAGTGTGGGTGACCCCGTC-3′) β-actin, human (sense, 5′-GGAGTTCCTGGACCAGTACG-3′; antisense, 5′-TTCTTGTGCTTGTGCCATGT-3′) and mouse (sense, 5′-GCTCCAAAAGCGAGTACCAGC-3′; antisense, 5′-AGTAGAATCCGCTCTCCTGCAG-3′) SOCS3, mouse TTP

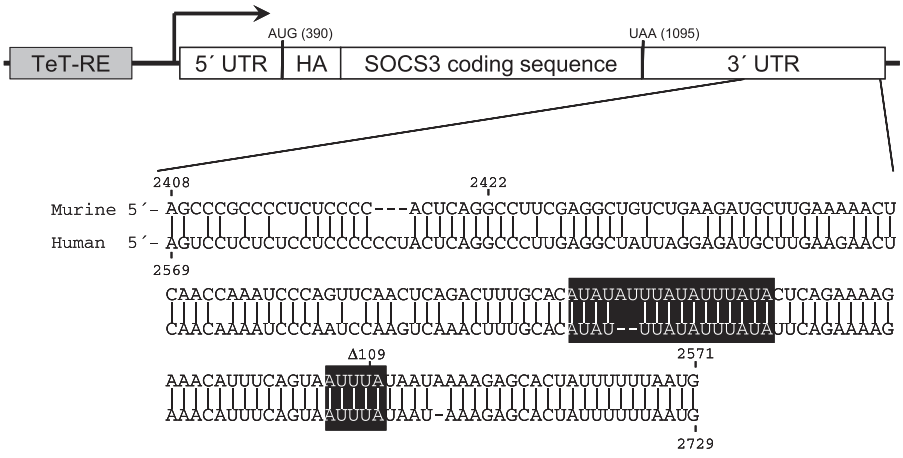

FIGURE 2. Schematic of the Tet-inducible HA-SOCS3 construct established to study the regulation of SOCS3 mRNA stability. The artwork shows the Tet-inducible HA-SOCS3 construct encoding for the murine SOCS3 gene under the control of a Tet-sensitive promoter. The sequences below the schematic represent the last 163 nucleic acids of the 3′ UTR of the murine SOCS3 mRNA and its homology to the 3′ UTR of the human SOCS3 mRNA. Black boxes indicate the position of the AUUUA pentameric motifs within the 3′ UTR.

(sense, 5′-TTTCCCCTTCTGCCTTCTCT-3′; antisense, 5′-TGGTGCTGGG GGTAGTAGAC-3′), and the flavoprotein subunit of the murine succinate dehydrogenase (SDHA) (sense, 5′-TGGGGAGTGCCGTGGTGTCA-3′; antisense, 5′-GTGCCGTCCCCTGTGCTGGT-3′) were purchased from MWG Biotec. The specificity of RT-PCR was controlled by no template and no reverse-transcriptase controls.

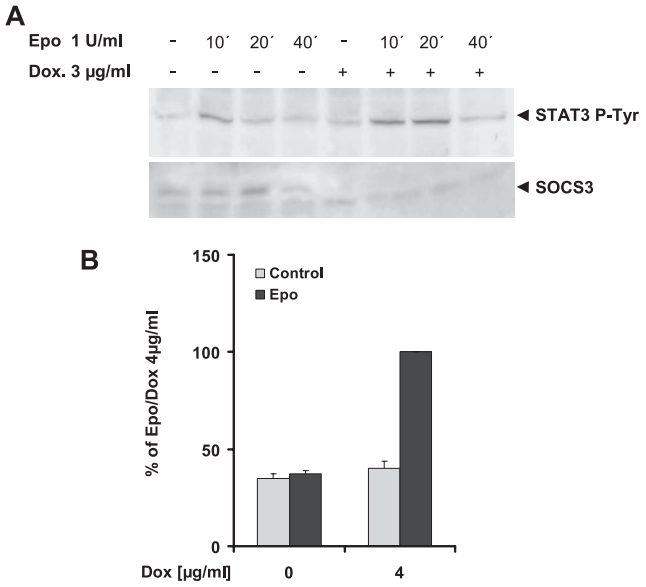

FIGURE 3. Expression of HA-SOCS3 inhibits tyrosine phosphorylation of STAT3 and blocks transcriptional activation of the SOCS3 promoter. *A*, NIH 3T3 cells were cotransfected with expression vectors for doxycycline-inducible SOCS3 (pTet-HA-SOCS3) and for the Epo/gp130 chimeric receptor composed of the extracellular domain of the erythropoietin receptor and the transmembrane and cytoplasmic domain of the gp130 signal transducing receptor subunit (pSVL-EG). This approach allows selective activation of gp130-dependent signaling in transfected NIH 3T3 cells by stimulation with Epo. Twenty-four hours after transfection, cells were pretreated with doxycycline (Dox; 3 μg/ml) for 2 h and then stimulated with erythropoietin (1 U/ml) for the time periods indicated. After preparation of cellular lysates 60 μg of protein were subjected to Western blot analysis using Abs specifically recognizing STAT3 when tyrosine phosphorylated (P-Tyr) at tyrosine residue 705 (*top lane*) or Abs specifically raised against SOCS3 (*bottom lane*). *B*, NIH 3T3 cells were cotransfected with the pTet-HA-SOCS3 vector, a SOCS3 promoter reporter gene construct (pGL3-SOCS3–2757Luc), and the expression vector of the Epo/gp130 chimeric receptor (pSVL-EG). Twenty-four hours after transfection, the expression of HA-SOCS3 was blocked by the addition of doxycycline (Dox; 4 μg/ml) for 120 min and finally stimulated with Epo (1 U/ml) as indicated. Cellular extracts were prepared for the determination of luciferase activity. Data shown are the averages of triplicate determinations of firefly luciferase activity normalized to *Renilla* luciferase activity as outlined in *Materials and Methods* and the legend to Fig. 1.

Semiquantitative PCR results were obtained using the delta cycle threshold (ΔCT) method (32). Because PCR efficiencies for all three reactions were similar (~0.95–0.98) for all experiments despite the experiments using MK2$^{-/-}$ and MK2$^{+/+}$ macrophages, threshold values were normalized to β-actin. Because β-actin levels were substantially lower in MK2$^{-/-}$ macrophages, SDHA mRNA levels were determined as control gene which did not significantly differ between MK2$^{-/-}$ and MK2$^{+/+}$ macrophages and threshold values were normalized to SDHA.

Determination of mRNA half-life and statistics

To determine the decay of SOCS3 mRNA, either semiquantitative PCR results or relative signal intensities of the Northern blot were used. Blots were scanned and digitally processed using the Kodak station 4000 MM and the ChemiImager software Total Lab 100 from Nonlinear Dynamics. Signals from HA-SOCS3 mRNA by either the SOCS3 or by the HA probe were normalized to GAPDH signals, and the respective control without doxycycline was set to 100%. The relative transcript abundance in the presence of actinomycin D with or without TNF-α was calculated by setting the values of the SOCS3 mRNA levels induced by IL-6 to 100%. Percentages were plotted against time, exponential decay curves were calculated, and the time to 50% mRNA decay was deduced from the respective curves. Data are presented as average values $\pm$ SD of $n \geq 3$ independent experiments. Statistics were performed using a *t* test (SigmaStat software).

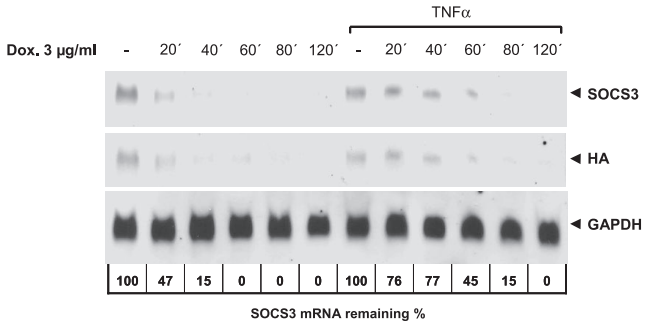

FIGURE 4. Influence of TNF-α on SOCS3 mRNA stability. NIH 3T3 cells were transfected with 0.4 μg of the pTet-HA-SOCS3 expression vector. Twenty-four hours later, cells were pretreated with doxycycline (Dox; 3 μg/ml) and then stimulated with TNF-α or left untreated for the times indicated. Total RNA-extracts were prepared and 10 μg of RNA was subjected to Northern blot analysis using DIG-labeled oligonucleotides specifically binding to SOCS3 (*top panel*), the sequence of the HA tag within HA-SOCS3 (*middle panel*), or GAPDH (*bottom panel*) as outlined in *Material and Methods*. The values below the GAPDH panel represent the SOCS3 mRNA amount normalized to the amounts of GAPDH and expressed relative to the initial amount of SOCS3 mRNA at time 0, which was set to 100%.

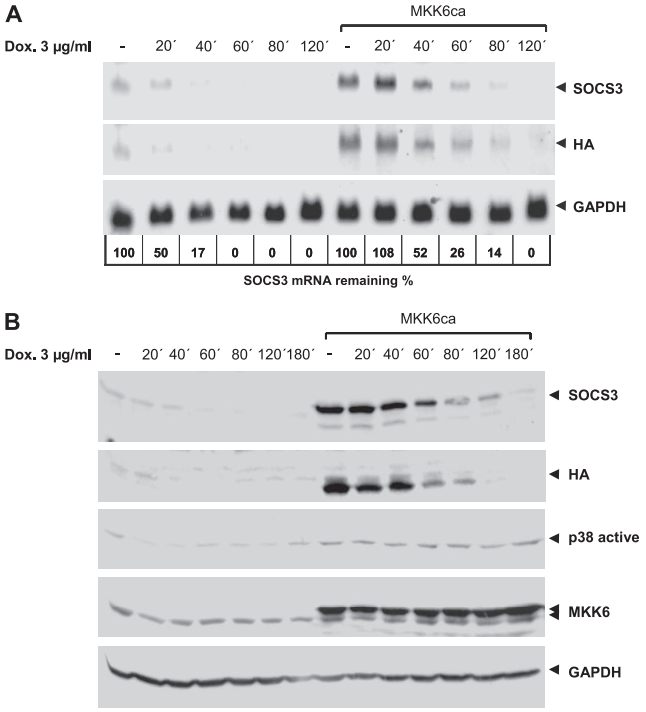

FIGURE 5. Regulation of SOCS3 mRNA stability by constitutive activation of the MKK6/p38^{MAPK} cascade. NIH 3T3 cells were cotransfected with 0.4 μg of the pTet-HA-SOCS3 expression vector, 0.6 μg of the pTet-Off vector, and 1 μg of a vector encoding a constitutively active mutant of MKK6 (pcDNA3-MKK6ca). Twenty-four hours after transfection, cells were treated with doxycycline (Dox; 3 μg/ml) as indicated. *A*, After the incubation period the depicted cells were lysated for RNA extraction, total RNA extracts were prepared, and 10 μg of RNA was subjected to Northern blot analysis for detection of SOCS3 mRNA (*top panel*), mRNA containing the HA tag (*middle panel*), or mRNA of GAPDH (*bottom panel*). The values below the GAPDH panel represent the SOCS3 mRNA amount normalized to the amounts of GAPDH and expressed relative to the initial amount of SOCS3 mRNA at time 0, which was set to 100%. *B*, Cells were lysated and 80 μg of protein was analyzed by immunoblotting using Abs specific for SOCS3 (*top panel*), the HA tag with the HA-SOCS3 mRNA (*second panel from top*), activated (phosphorylated) p38^{MAPK} (*third panel from top*), MKK6 (*fourth panel from top*) and GAPDH (*bottom panel*). To discriminate between endogenous and constitutively active MKK6 (MKK6ca), the latter contains a Flag epitope that leads to slightly slower migration of constitutively active MKK6 when compared with the endogenous MKK6 (*fourth panel from top*).

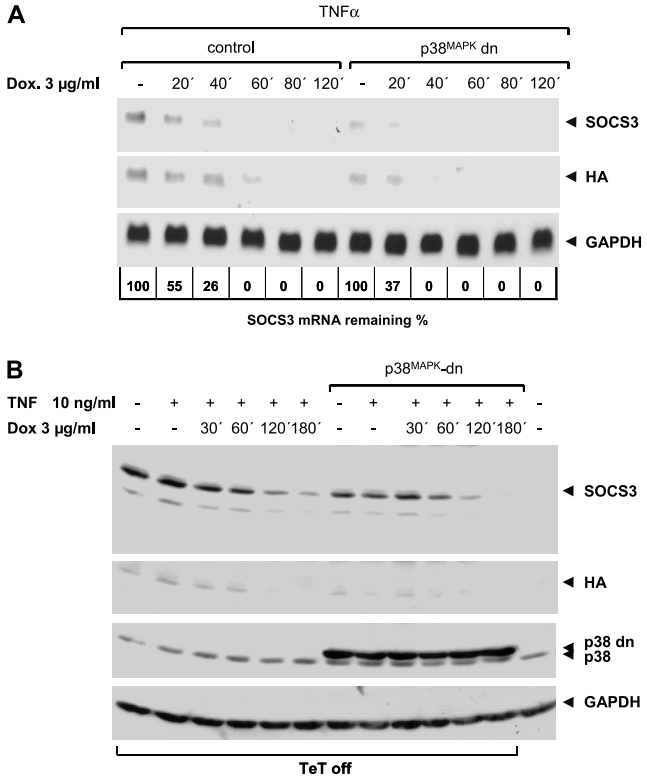

FIGURE 6. Role of p38^{MAPK} for the SOCS3 mRNA-stabilizing activity of TNF-α. *A* and *B*, NIH 3T3-cells were cotransfected with 0.4 μg of pTet-HA-SOCS3, 0.6 μg of the pTet-Off, vector and an expression vector coding for a dominant negative mutant of p38^{MAPK} (p38^{MAPK} dn; 1 μg). Twenty-four hours after transfection, cells were treated with 3 μg/ml doxycycline (Dox) and TNF-α (10 ng/ml) as indicated. *A*, After the respective incubation period, RNA was prepared as outlined in *Materials and Methods* and analyzed by Northern blotting using DIG-labeled oligonucleotides specifically binding to SOCS3 (*top panel*), the sequence of the HA tag within HA-SOCS3 (*middle panel*), or GAPDH (*bottom panel*). The values below the GAPDH panel represent the SOCS3 mRNA amount normalized to the amounts of GAPDH and expressed relative to the initial amount of SOCS3 mRNA at time 0, which was set to 100%. *B*, Cells were lysed and 80 μg of protein was analyzed by immunoblotting using Abs specific for SOCS3 (*top panel*), the HA-tag within HA-SOCS3 mRNA (*second panel from top*), the p38^{MAPK} (*third panel from top*); and GAPDH (*bottom panel*).

Results

Regulation of SOCS3 mRNA expression by TNF-α and gp130-induced signal transduction

Previously, we could demonstrate that TNF-α induces the expression of SOCS3 mRNA in primary human or rat liver macrophages as well as in macrophage cell lines (18). Correspondingly, TNF-α time-dependently induces the SOCS3 protein in murine fibroblasts and the murine macrophage cell line RAW 264.7 as demonstrated in Fig. 1, *A* and *B*. Apart from evidence that activation of the MKK6/p38^{MAPK} is required (18), the molecular mechanisms responsible for TNF-α-induced SOCS3 expression remained unclear. In contrast to the induction of SOCS3 by most of the other cytokines, TNF-α-induced SOCS3 expression is at least not associated with the increased activation of STAT3, because TNF-α inhibits rather than activates STAT3 (18). Instead, regulation of SOCS3 by mediators such as TNF-α could occur by the activation of other transcription factors. Therefore, the influence of TNF-α on the transcriptional activity of the SOCS3 promoter was analyzed. A reporter gene construct encoding the firefly luciferase gene under the control of the murine SOCS3 promoter comprising the sequence from position -2757 to $+924$ was transiently transfected in RAW 264.7 macrophages or in NIH 3T3 murine fibroblasts. Fig. 1*C* shows that IL-6 but not TNF-α activates the SOCS3 promoter in both cell lines. Moreover, IL-6-mediated activation of the SOCS3 promoter reporter is not significantly altered by costimulation with TNF-α. Although these data do not exclude the possibility that TNF-α may act via responsive elements upstream of position -2757 of the 5′ regulatory region of the SOCS3 gene promoter, they point to the possibility that transcription-independent mechanisms may play a role in the regulation of SOCS3 expression by TNF-α.

To evaluate whether the regulation of RNA stability might be involved in the control of SOCS3 mRNA expression by TNF-α, we analyzed the decay of preinduced SOCS3 mRNA in the presence or absence of TNF-α. SOCS3 mRNA synthesis was induced by the preincubation of either RAW 264.7 macrophages or primary human PBMC-derived macrophages with IL-6, and the decay was monitored by real-time PCR after removing IL-6 and

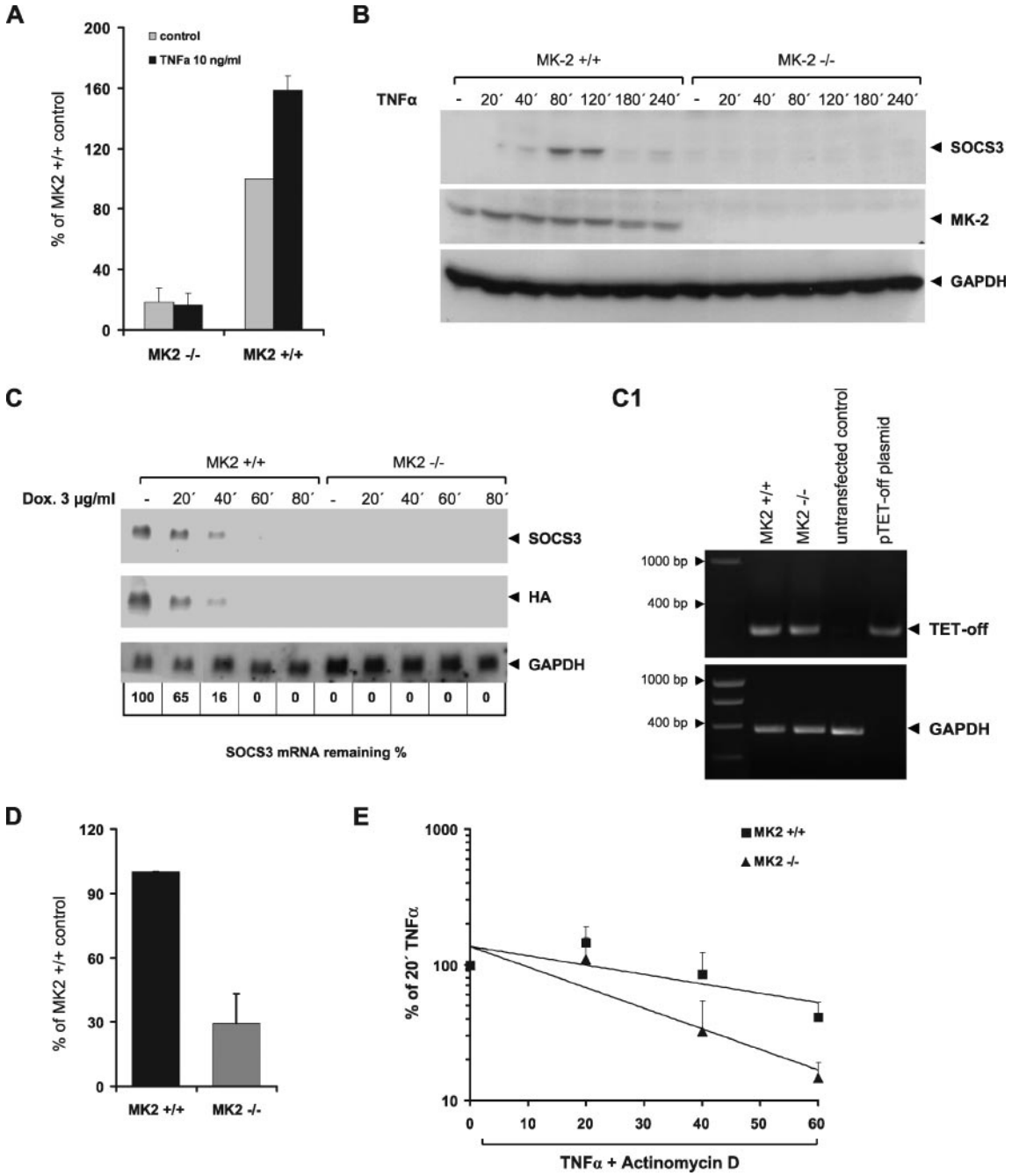

FIGURE 7. MK2 is crucial for the regulation of basal and TNF-α-induced SOCS3 mRNA and protein expression. MEFs derived from MK2-deficient mice or the respective wild-type littermates were treated with TNF-α (10 ng/ml) as indicated. *A*, Thereafter, cells were harvested and total RNA was prepared and subjected to real-time analysis for SOCS3 mRNA expression as outlined in the legend to Fig. 1. *B*, Eighty micrograms of protein was analyzed by immunoblotting using Abs specific for SOCS3 (*top panel*), MK2 expression (*middle panel*), and GAPDH (*bottom panel*). *C*, $MK2^{-/-}$ or $MK2^{+/+}$ murine embryonic fibroblasts were cotransfected with 0.4 μg of the pTet-HA-SOCS3 expression vector and 0.6 μg of the pTet-Off vector. Twenty-four hours after transfection, cells were treated with doxycycline (Dox; 3 μg/ml) as indicated. Thereafter, cells were lysated for RNA extraction, total RNA extracts were prepared, and 10 μg of RNA was subjected to Northern blot analysis for the detection of SOCS3 mRNA (*top panel*), mRNA containing the HA tag (*middle panel*), or mRNA of GAPDH (*bottom panel*). The values below the GAPDH panel represent the SOCS3 mRNA amount normalized to the amounts of GAPDH and expressed relative to the initial amount of SOCS3 mRNA at time 0, which was set to 100%. To prove equal transfection of $MK2^{+/+}$ and $MK2^{-/-}$ cells, mRNA from the respective control experiments was analyzed by RT-PCR for the expression of the cotransfected Tet-Off repressor mRNA (*C1*). *D* and *E*, $MK2^{+/+}$ and $MK2^{-/-}$ macrophages were analyzed for either basal SOCS3 mRNA expression (*D*) or the decay of SOCS3 mRNA in cells treated with TNF-α (15 ng/ml) for 20 min and subsequently treated with actinomycin D (5 μg/ml) for the times indicated (*E*). Total RNA was isolated and subjected to real-time PCR for SOCS3 mRNA expression as described in *Materials and Methods*. Semiquantitative PCR results were obtained using the ΔCT method. Relative SOCS3 mRNA levels at the various times after treatment with actinomycin D were expressed as fractions of the normalized value of actinomycin D at time 0, which was set to 100%.

blocking transcription with actinomycin D. As shown in Fig. 1*D* the decay in RAW 264.7 macrophages was less pronounced in the presence of TNF-α, resulting in a significant ($p = 0.01$) prolongation of the half-life of the SOCS3 transcript (17.4 $\pm$ 2.6 min under control conditions vs 33.1 $\pm$ 6.9 min in the presence of TNF-α as calculated by exponential decay regression analysis).

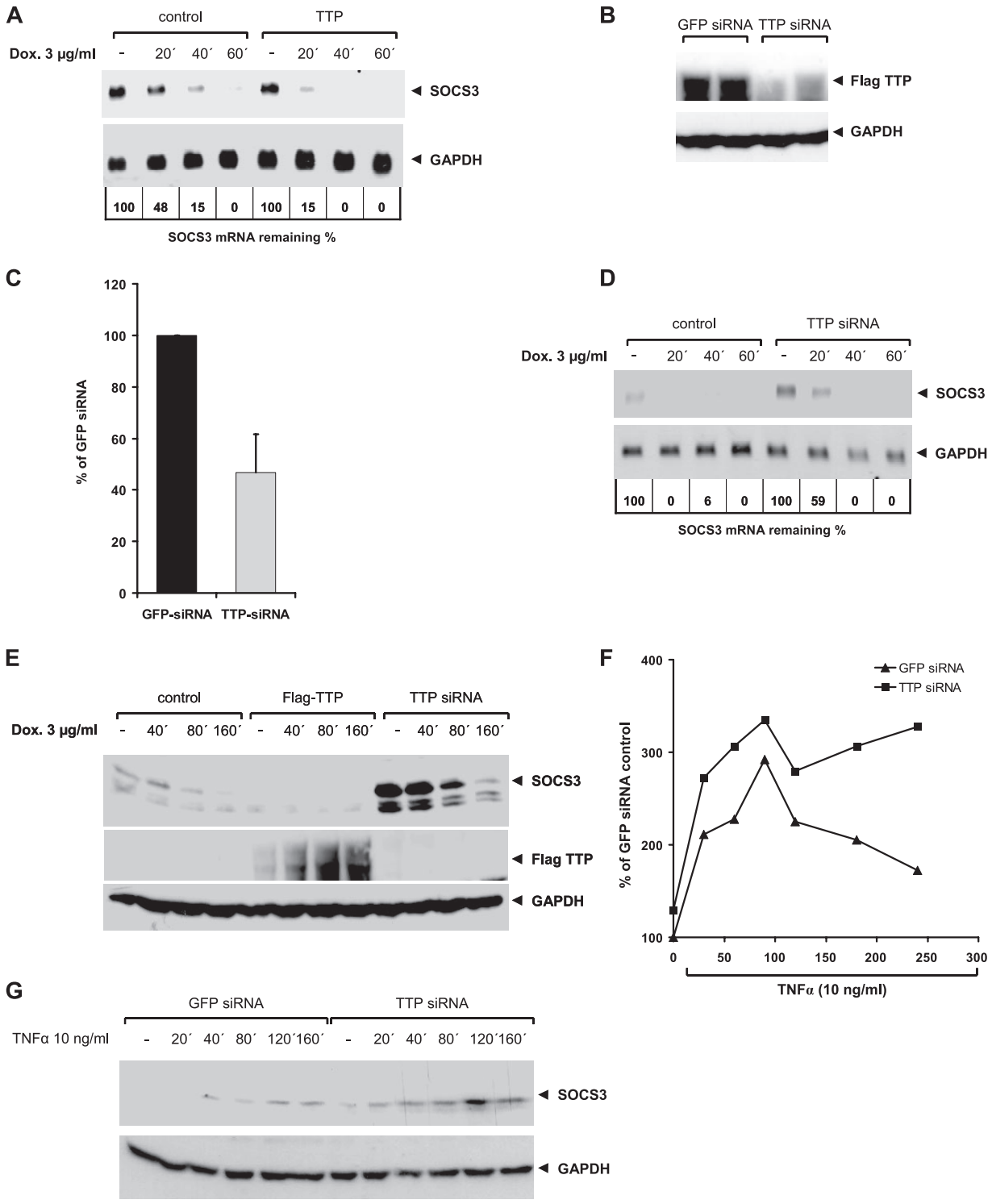

FIGURE 8. Influence of TTP on SOCS3 mRNA and protein expression. *A*, NIH 3T3 cells were cotransfected with 0.4 μg of pTet-HA-SOCS3, 0.6 μg of a vector encoding the Tet-Off factor, and 1.5 μg of an expression vector coding for tristetraprolin (rcCMV- TTP). After 24 h the cells were treated with 3 μg/ml doxycycline (Dox) as indicated and lysates for isolation of total mRNA were prepared. Ten micrograms of mRNA was subjected to Northern blot analysis for the detection of HA-SOCS3 mRNA (*top panel*) or mRNA of GAPDH (*bottom panel*) as described in *Materials and Methods*. The values below the GAPDH panel represent the SOCS3 mRNA amount normalized to the amounts of GAPDH and expressed relative to the initial amount of SOCS3 mRNA at the time 0, which was set to 100%. *B* and *C*, Cells were transfected with an expression vector encoding for Flag-tagged TTP and 20 nM TTP siRNA or GFP siRNA for control (*B*) or with 20 nM TTP siRNA or GFP siRNA for control alone (*C*). Cell culture was continued for 72 h and cells were harvested for preparation of the total protein lysate (*B*) or total RNA (*C*). *B*, 80 μg of protein was analyzed by Western blotting for the expression of Flag-tagged TTP and for GAPDH as loading control. *C*, RNA was subjected to real-time PCR for the expression of TTP mRNA as described in *Materials and Methods* and the legend to Fig. 1. Relative TTP mRNA amounts in the presence of TTP siRNA are expressed as fractions of the normalized value of the GFP siRNA control, which was set to 100%. *D*, NIH 3T3 cells were cotransfected with 0.4 μg of pTet-HA-SOCS3, 0.6 μg of a vector encoding the Tet-Off factor, and 20 nM of TTP siRNA or GFP siRNA for control. After 72 h the cells were treated with 3 μg/ml doxycycline (Dox) as indicated and lysates for the isolation of total mRNA were prepared. Ten micrograms of mRNA was subjected to Northern blot analysis for the detection of SOCS3 mRNA (*top panel*) or the mRNA of GAPDH (*bottom panel*) as described above. *E*, NIH 3T3 cells were cotransfected with 0.4 μg of pTet-HA-SOCS3,

Similarly, the half-life of SOCS3 mRNA was significantly ($p < 0.01$) prolonged in primary human macrophages (48.9 ± 4.4 min in the absence vs 70.4 ± 3.8 min in the presence of TNF-α) (Fig. 1*E*). These observations indicate that stabilization of SOCS3 mRNA is involved in the regulation of SOCS3 expression by TNF-α in the RAW 264.7 murine macrophage cell line as well as in PBMC-derived human macrophages. Moreover, these data implicate that there is a significant cell-specific difference in basal SOCS3 half-life between RAW 264.7 cells and PBMC-derived macrophages, albeit the reason for this remains unclear.

To study the modulation of SOCS3 mRNA stability by TNF-α in more detail, an expression system based on a modified pTet splice vector system described by Xu et al. (43) was established. pTet-HA-SOCS3 codes for the full-length murine SOCS3 mRNA under the control of a Tet-sensitive (Tet-Off) promoter. An HA tag encoding sequence was introduced into the SOCS3 mRNA to distinguish exogenous from endogenous SOCS3 mRNA (Fig. 2). This experimental approach allows the study of SOCS3-mRNA stability in the context of the native full-length SOCS3 mRNA, including both the 5′ and 3′ UTRs as well as the coding region.

To test whether induction of the Tet-regulated HA-tagged SOCS3 is functional and affects signal transduction initiated by gp130, Epo/gp130 chimeric receptors consisting of the extracellular part of the Epo receptor and the transmembrane and the cytoplasmic part of the signal-transducing gp130 receptor subunit of the IL-6 receptor complex were cotransfected with the pTet-HA SOCS3 expression vector to selectively activate gp130-dependent signaling in transfected NIH 3T3 cells by stimulation with Epo (10). As shown in Fig. 3*A* (*upper panel*, *right*), gp130-induced STAT3 tyrosine phosphorylation is enhanced and prolonged upon doxycycline-mediated inhibition of HA-SOCS3 expression. In line with these findings, gp130-dependent activation of a cotransfected reporter gene construct encoding a STAT3-dependent fragment of the SOCS3 promoter in NIH 3T3 cells is also efficiently suppressed upon the induction of HA-SOCS3 expression in response to the withdrawal of doxycycline (Fig. 3*B*). These experiments show that an active SOCS3 protein is expressed upon the deprivation of doxycycline in the experimental setting.

The influence of TNF-α-induced signal transduction on the stability of exogenous SOCS3 mRNA was analyzed in NIH 3T3 fibroblasts transfected with the pTet-HA SOCS3 expression vector after blocking the de novo synthesis of this mRNA by adding doxycycline. The half life of the exogenous HA-tagged SOCS3 mRNA (Fig. 4) was significantly ($p < 0.001$) prolonged in the presence of TNF-α (22.0 ± 5.6 min vs 41.3 ± 8.2 min) (Fig. 4), indicating that TNF-α also stabilizes exogenous SOCS3 mRNA.

Influence of the MKK6/p38^{MAPK} cascade on the stability of the SOCS3 mRNA

Several reports point to a crucial role of the MKK6/p38^{MAPK} in the regulation of mRNA stability of various proteins such as COX2 (34), TNF-α (32), and IL-8 (33). Evidence is given from inhibitor studies that the activation of p38^{MAPK} is also important for TNF-α-, LPS-, and CpG-induced SOCS3 expression in macrophages (18–20). Considering these observations, it was of interest whether the MKK6/p38^{MAPK} cascade has any impact on SOCS3 mRNA stability. Therefore, the regulation of HA-SOCS3 mRNA decay was analyzed in the presence of a constitutively active MKK6 mutant. Fig. 5*A* shows that MKK6-induced constitutive activation of the p38^{MAPK} cascade results in a strong increase in HA-SOCS3 mRNA (Fig. 5*A*) and protein (Fig. 5*B*). Additionally, the mRNA decay is reduced ($p < 0.001$) to 42.3 ± 3.1 min, which is comparable to the decay observed in Fig. 4 in the presence of TNF-α (41.3 ± 8.2 min). Because the expression of the HA-tagged SOCS3 mRNA is not under the control of the endogenous SOCS3 promoter, these data suggest that the enhanced accumulation of HA-tagged SOCS3 mRNA in response to constitutive p38^{MAPK} activity is independent of transcriptional regulation.

To further evaluate the contribution of p38^{MAPK} to TNF-α-dependent regulation of the SOCS3 mRNA turnover, the effect of inhibiting p38^{MAPK} activity was analyzed by cotransfection of a dominant negative mutant of p38^{MAPK}. TNFα-dependent stabilization of HA-SOCS3 mRNA is significantly reduced, leading to a mRNA half-life of 25.4 ± 5.7 min ($p < 0.05$) upon the antagonization of p38^{MAPK} activity (Fig. 6*A*). This effect is also reflected on the protein level (Fig. 6*B*). The reduction of the initial amount of SOCS3 mRNA (to 30%) and protein (to 50%) in the absence of p38^{MAPK} activity are most likely due to enhanced mRNA decay during the synthesis of HA-SOCS3. In summary, these data suggest that the decelerated degradation of SOCS3 mRNA by the activation of the MKK6/p38^{MAPK} cascade is a potent mechanism by which TNF-α exerts its enhancing effect on SOCS3 mRNA accumulation.

MK2, a downstream target of the MKK6/p38^{MAPK} cascade is crucial for basal and TNFα-induced SOCS3 expression

Previous work identified MK2 as the executive kinase for the MKK6/p38^{MAPK} cascade to posttranscriptionally regulate mRNA expression (35). Using mouse embryonic fibroblasts isolated from either MK2-deficient or wild-type animals TNF-α-mediated SOCS3 mRNA and protein expression was analyzed. In MK2 deficient cells TNF-α fails to induce the expression of SOCS3 mRNA (Fig. 7*A*, *right bars*) and protein (Fig. 7*B*, *right section*). Moreover, basal SOCS3 mRNA levels are strongly diminished in MK2-deficient cells when compared with the respective wild-type controls (compare gray bars in Fig. 7*A*). Similar results were obtained for the exogenous HA-tagged SOCS3 mRNA (Fig. 7*C*), which is independent of its genuine transcriptional regulation but still subject to mechanisms controlling mRNA stability. MK2$^{-/-}$ MEF cells fail to accumulate relevant amounts of the HA-SOCS3 mRNA, whereas in MK2$^{+/+}$ cells SOCS3 mRNA is built up well and mRNA decay after the addition of doxycycline occurs at a rate that is similar to that observed in NIH 3T3 cells. Of note, the observed differences between the MK2$^{-/-}$ and the MK2$^{+/+}$ cells are not due to different transfection efficiencies, because the mRNA levels for the cotransfected Tet-Off regulator were found to

0.6 μg of a vector encoding the Tet-Off factor, and 1.5 μg of an expression vector coding for Flag-tagged TTP (pRcCMV-flag-TTP) plus 20 nM GFP siRNA or the respective empty expression vector (pRcCMV) plus either 20 nM TTP siRNA or GFP siRNA for control. After 72 h, 3 μg/ml doxycycline (Dox) was added as indicated and total protein lysates were prepared. Eighty micrograms of protein was analyzed by immunoblotting using Abs specific for SOCS3 (*top panel*), the Flag tag (*middle panel*), or GAPDH (*bottom panel*). To demonstrate the effect of TTP siRNA on endogenous SOCS3 mRNA (*F*) and protein (*G*), NIH 3T3 cells were transfected with 20 nM TTP siRNA or GFP siRNA for control. *F*, Cells were stimulated with TNF-α as indicated and for total mRNA was prepared and subjected to real time PCR to monitor expression of SOCS3 mRNA as described in *Materials and Methods* and the legend to Fig. 1. The amount of SOCS3 mRNA is expressed relative to the amount in the control experiment in the absence of TNF-α. *G*, Protein lysates were prepared and 80 μg of protein per lane was analyzed by immunoblotting for the expression of SOCS3 and for GAPDH as loading control.

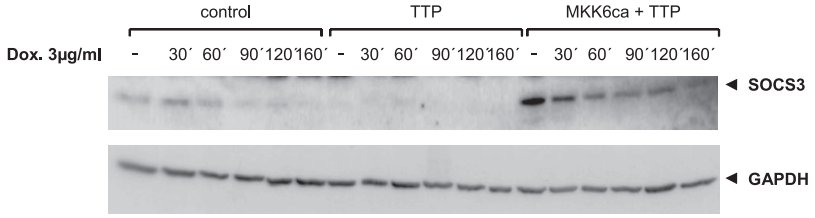

FIGURE 9. Constitutive activation of the MKK6/p38^{MAPK} cascade neutralizes the effect of TTP on SOCS3 expression. NIH 3T3 cells were cotransfected with 0.4 μg of pTet-HA-SOCS3, 0.6 μg of pTet-OFF, an expression vector encoding for TTP (0.8 μg), and a vector encoding a constitutively active mutant of MKK6 (pcDNA3-MKK6ca). For control experiments the respective empty expression vectors were cotransfected instead of TTP or constitutively active MKK6 (MKK6ca) encoding an expression vector. After 24 h the cells were treated with 3 μg/ml doxycycline (Dox) as depicted and cellular lysates for immunoblot analysis were prepared as outlined in *Materials and Methods*. Eight micrograms of protein was analyzed by immunoblotting using Abs specific for SOCS3 (*top panel*) or GAPDH (*bottom panel*).

be equal in both cell lines (Fig. 7*C1*). Similarly as found for MK2-deficient fibroblasts, macrophages derived from the same MK2-deficient mice show strongly reduced basal SOCS3 mRNA expression when compared with the respective control macrophage cell line (Fig. 7*D*). Moreover, SOCS3 mRNA is less efficiently stabilized (24.7 $\pm$ 7.0 min for MK2$^{-/-}$ vs 58.0 $\pm$ 15.7 min for MK2$^{+/+}$) by treatment with TNF-α in MK2-deficient macrophages when compared with TNF-α-treated macrophages derived from the wild-type littermates (Fig. 7*E*). These data further strengthen the hypothesis that SOCS3 mRNA is less stable in the absence of functional MK2. In summary, our data suggest that MK2 is important for the regulation of basal as well as TNFα-dependent stabilization of SOCS3 mRNA in fibroblasts and macrophages.

Expression of TTP destabilizes SOCS3 whereas down-regulation of TTP by siRNA stabilizes and enhances SOCS3 protein expression

The mechanism of the regulation of SOCS3 mRNA stability by p38^{MAPK} is unclear. It is very likely that an integral component of the mRNA-stabilizing activity is a protein controlled by activation of p38^{MAPK} and MK2 that binds to the respective mRNA. Several reports investigating the regulation of mRNA stability by the p38^{MAPK}/MK2 cascade point to a crucial role of the zinc finger protein TTP (31). Because the results of the present study indicate that activation of the MKK6/p38^{MAPK}/MK2 cascade is important for TNF-α-induced SOCS3 expression, the question was addressed of whether or not TTP has an influence on SOCS3 mRNA stability. As shown in Fig. 8*A*, cotransfection of TTP results in an accelerated decay of the HA-SOCS3 mRNA with a decreased mRNA half-life of 14.3 $\pm$ 2.5 min ($p < 0.05$). In line with this observation, the suppression of TTP expression by TTP siRNA leads to enhanced accumulation and a slower decay ($p < 0.05$) of the HA-SOCS3 mRNA with an RNA half-life of 32.3 $\pm$ 5.5 (Fig. 8*D*). These effects of either TTP expression or suppression of TTP by TTP siRNA are also reflected when analyzing the HA-SOCS3 protein (Fig. 8*E*). Interestingly, although the expression of TTP has little effect on the steady-state HA-SOCS mRNA amount in the absence of doxycycline, HA-SOCS3 (Fig. 8*A*) protein expression is almost completely abolished (Fig. 8*E*). These differences are

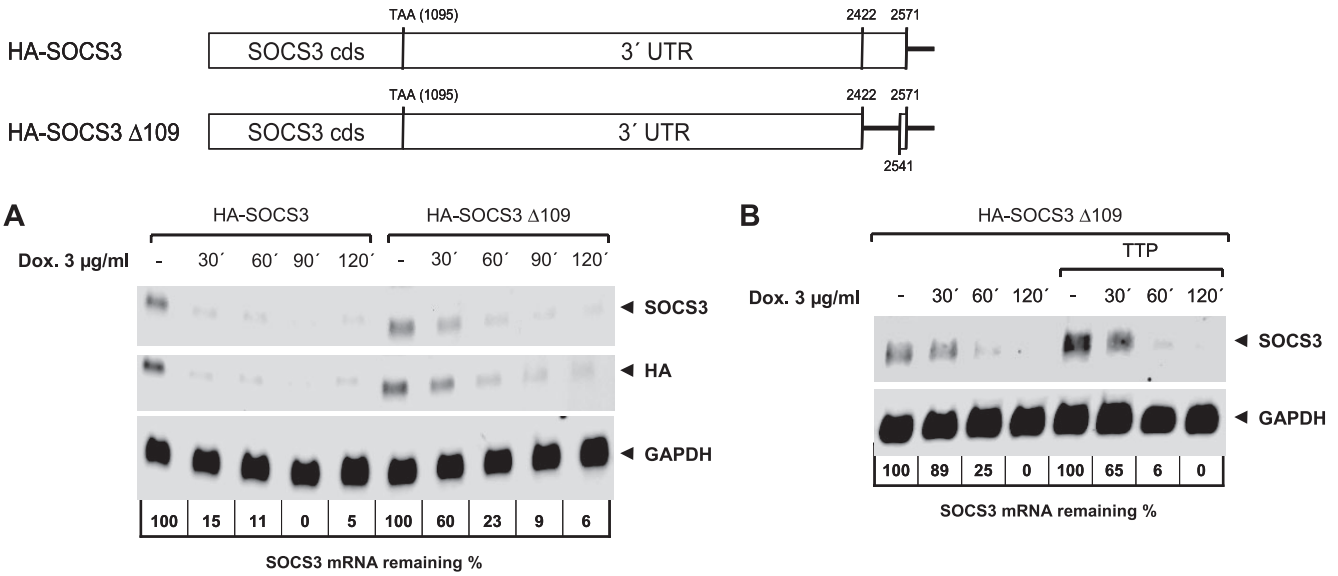

FIGURE 10. Role of the 3′ UTR for SOCS3 mRNA stability. The HA-SOCS3-mRNA deletion mutant HA-SOCS3 Δ109 was generated as described in *Materials and Methods* and is schematically depicted. *A* and *B*, One microgram of either pTet-HA-SOCS3 or pTet-HA-SOCS3 Δ109 was cotransfected into NIH 3T3 cells together with 0.6 μg of the pTet-Off expression vector (*A*), and expression vectors (0.4 μg) encoding the deletion mutant HA-SOCS3 Δ109 were cotransfected with 0.6 μg of pTet-Off and either 1.5 μg of the TTP expression vector (rcCMV-TTP) or a control vector into NIH 3T3 cells (*B*). After 24 h the cells were treated with 3 μg/ml doxycycline (Dox) as indicated and lysates for the isolation of total mRNA were prepared. Ten micrograms of mRNA was subjected to Northern blot analysis for the detection of SOCS3 mRNA (*top panels*), HA (*middle panel* in *A*), or GAPDH (*bottom panels*) as described in the *Materials and Methods*. The values below the GAPDH panels represent the SOCS3 mRNA amount expressed relative to the initial amount of SOCS3 mRNA at time 0.

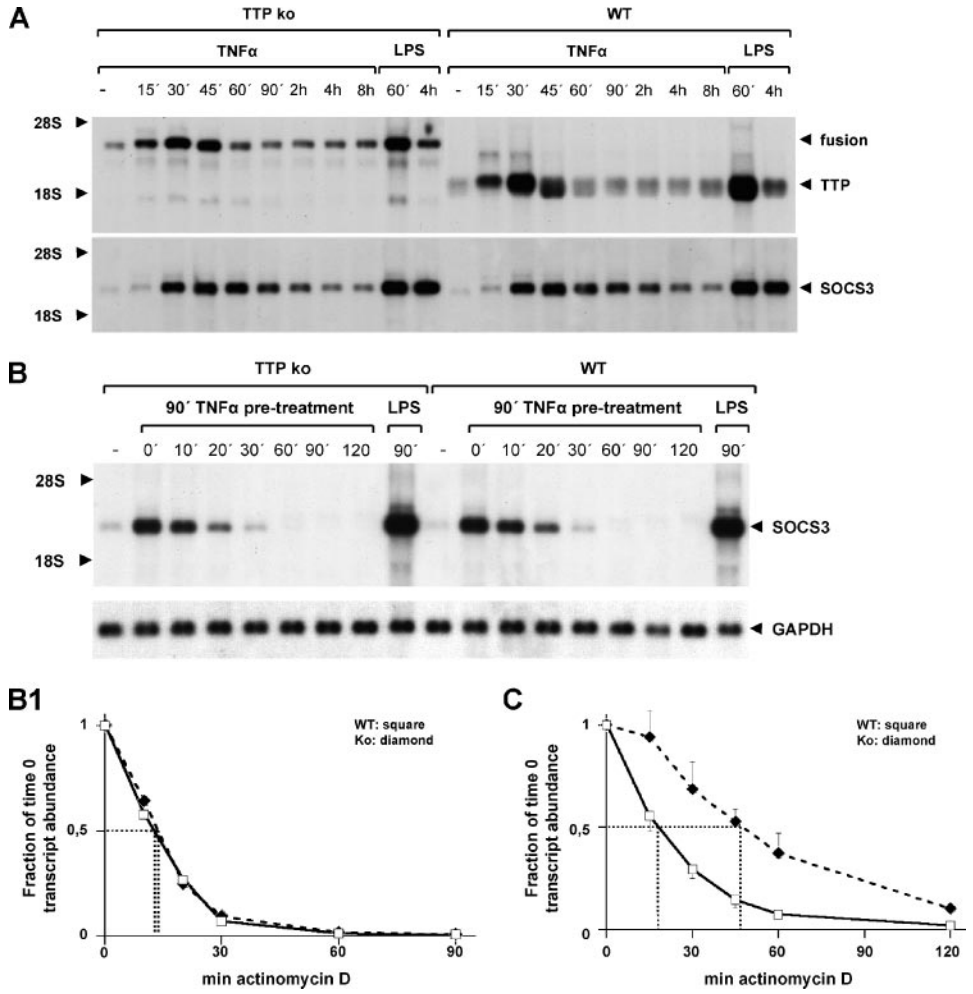

FIGURE 11. TNF-α-induced SOCS3 mRNA expression in TTP-deficient bone marrow-derived macrophages. Primary macrophages from littermate 430TO (TTP knockout) and 431TO (wild type) were prepared and cultured as outlined in *Materials and Methods*. Approximately 16 h before treatment the culture medium was replaced by RPMI 1640 containing 1% (v/v) FBS. *A*, TTP and SOCS3 mRNA expression after TNF-α or LPS stimulation. The TTP knockout (TTP ko) and wild-type (WT) cells were either left untreated (time 0) or treated with 10 ng/ml TNF-α or 1 μg/ml LPS for the times indicated. Total cellular RNA was prepared and 8 μg of RNA per lane was subjected to Northern blotting with cDNA probes specific for TTP and SOCS3. The positions of the wild-type TTP, TTP-neo fusion in TTP knockout cells, SOSCS3 mRNAs, and ribosomal RNAs are indicated by arrowheads. *B*, Demonstration of the decay of SOCS3 mRNA after actinomycin D treatment. After stimulating TTP knockout (TTP ko) or wild-type (WT) macrophages with 10 ng/ml TNF-α for 90 min, the cells were treated with actinomycin D (5 μg/ml) and subsequently lysed for RNA isolation at the indicated times. Control cells were also treated with 1 μg/ml LPS for 90 min. Eight micrograms of RNA was subjected to Northern blotting and hybridized with a SOCS3 cDNA probe. Therefore, blots were stripped and reprobed with GAPDH cDNA-specific probe. The positions of the SOCS3-mRNA, GAPDH-mRNAs, and ribosomal RNAs is indicated. *B1*, Comparison of SOCS3 mRNA decay after 90 min of TNF-α stimulation followed by actinomycin D treatment in wild-type (□) and TTP-deficient (◆) macrophages. Values were determined by a phosphorimaging device and normalized to the GAPDH mRNA amount in each RNA preparation and related to the initial amount of SOCS3 mRNA a time 0. Representative data for two independent experiments are shown. *C*, The effect of TTP deficiency on the stability of TNF-α mRNA in the presence of actinomycin D is shown. Macrophages isolated from TTP-deficient (Ko, knockout) (◆) or wild-type (WT) (□) animals were stimulated with LPS (1 μg/ml) for 3 h and subsequently treated with actinomycin D (5 μg/ml) for the times indicated. RNA was isolated and 8 μg of RNA was analyzed for TNF-α and GAPDH mRNA by Northern blotting. Data were processed as described for Fig. 10*B*. Each point represents the mean ± SD from four identical experiments. mRNA half-life was 17.7 ± 2.7 min (mean ± SD) for the wild-type cells and 46.9 ± 9.6 min for the TTP knockout cells.

further indicative of a rapid turnover of SOCS3-mRNA. However, we cannot exclude that TTP additionally controls expression of SOCS3 at other posttranscriptional levels e.g., protein translation. Supporting the hypothesis that TTP antagonizes TNF-α-induced SOCS3 protein expression by destabilizing SOCS3 mRNA, the suppression of endogenous TTP by TTP-siRNA increases the amount of endogenous SOCS3 mRNA (Fig. 8*F*) and protein (Fig. 8*G*). The observation that constitutive activation of the MKK6/p38^{MAPK} cascade completely neutralizes the destabilizing effect of TTP on HA-SOCS3 (Fig. 9) indicates that the impact of TTP on the decay of SOCS3 could be antagonized by enhanced activation of the MKK6/p38^{MAPK} cascade.

Role of the 3′ UTR for SOCS3 mRNA stability

A common feature of the UTR of genes regulated by mRNA stability is the presence of AREs. The best characterized regulatory elements are those AREs containing two to five or even more copies of the pentameric motif AUUUA originally described by Shaw and Kamen (22). The SOCS3 mRNA contains three copies of a pentameric AUUUA motif in close proximity of the 3′ end (Fig. 2). To evaluate the regulatory role of this motif, a HA-SOCS3 construct lacking the ARE containing sequences between position 2422 and 2531 (HA-SOCS3 Δ109) within the 3′ UTR of the SOCS3 mRNA was generated (Fig. 10). SOCS3 mRNA lacking

the ARE motifs (HA-SOCS3 Δ109) exerts a significantly slower turnover ($p < 0.001$) with a half-life of 45.3 ± 8.1 min when compared with nontruncated HA-SOCS3 mRNA (22.0 ± 5.6 min). These observations indicate that this region within the 3′ UTR has a destabilizing effect on SOCS3 mRNA (Fig. 10*A*). Furthermore, deletion of the ARE confers SOCS3 mRNA insensitive to the action of a cotransfected TTP (Fig. 10*B*). These results suggests that the destabilizing effect of TTP on SOCS3 mRNA requires the region located in between positions 2422 and 2541 within the 3′ UTR of the SOCS3 mRNA.

SOCS3 mRNA stability is not altered in macrophages isolated from TTP-deficient mice

As outlined above, repression of TTP by TTP siRNA enhances the expression and stability of endogenous as well as exogenous SOCS3 mRNA and protein, whereas enhanced expression of TTP restricts SOCS3 expression. To further substantiate the hypothesis that TTP destabilizes SOCS3 mRNA, macrophages isolated from TTP-deficient animals or the respective wild-type littermates were analyzed for TNF-α-induced SOCS3 expression. Surprisingly, time-dependent induction of SOCS3 mRNA in TTP-deficient macrophages does not substantially differ from that observed in control cells (Fig. 11*A*, *lower panel*). Moreover, the decay of SOCS3 mRNA in macrophages from TTP$^{-/-}$ mice pretreated with TNF-α (10 ng/ml) for 90 min does not substantially differ from that observed in macrophages isolated from the wild-type littermates (Fig. 11*B* and processed data in *B1*). In contrast to TNF-α-induced SOCS3 mRNA, the decay of the LPS-induced TNF-α mRNA is largely delayed in macrophages isolated from TTP-deficient animals when compared with the wild-type cells (Fig. 11*C*).

Similar to the data from macrophages, the decay of SOCS3 mRNA in TTP-deficient mouse embryonic fibroblasts did not significantly differ from that found in the respective wild-type cells (data not shown). Considering the results from the TTP knockdown in cell culture using TTP siRNA (Fig. 8), one would have expected a substantial increase of TNF-α-induced SOCS3 mRNA expression or at least a delayed decay in the absence of TTP. Instead, the data presented in Fig. 11 indicate that the lack of TTP function can be compensated for during mouse development but not during immediate knockdown of the protein in cell culture. Thus TTP might not be the only factor destabilizing SOCS3 mRNA and thereby accounting for the regulation of SOCS3 expression by TNF-α in vivo.

Discussion

From recent studies it has been suggested that SOCS3 is not only a negative feedback inhibitor of Jak/STAT signaling but rather acts as a modulator of cytokine function with immunomodulatory implications, playing a crucial role in chronic inflammatory and autoimmune diseases. It has been demonstrated that proinflammatory mediators such as TNF-α, LPS, and CpG strongly induce the expression of SOCS3 in human or rat liver macrophages but also in macrophage cell lines or fibroblasts (18–20), thereby affecting cytokine signaling via the Jak/STAT signaling cascade. However, the mechanisms involved in the regulation of SOCS3 expression by proinflammatory mediators such as, for example, TNF-α are not understood.

As suggested from inhibitor studies or studies using dominant negative mutants of MKK6 or p38^{MAPK}, the activation of p38^{MAPK} plays an important role in the induction of SOCS3 expression by proinflammatory stimuli such as TNF-α, LPS, or CpG in macrophages and fibroblasts (18, 19). Moreover, in hepatocytes IL-6-induced SOCS3 expression is impaired by blocking p38^{MAPK} activity (36) and in B cells IL-4-induced expression of SOCS3 depends on p38^{MAPK} activation (37). However, the molecular mechanisms upstream and downstream of p38^{MAPK} activation are still obscure. Until now, the regulation of SOCS3 expression is known to occur on at least at four different levels: 1) at the level of transcription regulated by transcription factors of the STAT family (7, 44, 47) but also other factors such as Sp3 (48) acting via a GC-rich motif close to the TATA box (48, 49); 2) at the level of protein degradation (50) that is thought to be accelerated by tyrosine phosphorylation of SOCS3 (51) or crosstalk with SOCS2 (52); 3) at the level of translation by using alternative translation start points under stress conditions (53); and 4) on the level of SOCS3-mRNA stability as shown here.

The results of the present study show that TNF-α does not induce transcriptional activation of a SOCS3 promoter reporter gene harboring the sequence from position −2757 to +924 of the SOCS3 gene in macrophages (Fig. 1*A*). These data suggest that at least this part of the 5′ regulatory region of the SOCS3 gene is not controlled by TNF-α-induced signals, indicating that transcriptional regulation might play a minor role for TNF-α-mediated SOCS3 expression. The observation that TNF-α delayed the decay of preinduced SOCS3 mRNA in the RAW 264.7 murine macrophages indicates that deceleration of the decay of SOCS3 mRNA is an important regulatory target for signals activated by TNF-α (Fig. 1*B*). This interpretation of our observations was further substantiated by the fact that TNF-α (Fig. 4) decelerates the decay of HA-tagged SOCS3 mRNA. Moreover, constitutive activation of the MKK6/p38^{MAPK} cascade through coexpression of a constitutively active mutant of MKK6 results in enhanced and prolonged expression of the HA-tagged SOCS3 mRNA and protein (Fig. 5, *A* and *B*), indicating that activation of the MKK6/p38^{MAPK} cascade is suitable for stabilizing SOCS3 mRNA. Accordingly, the TNF-α-dependent prolonged expression of SOCS3 mRNA and protein was sensitive to cotransfection of a dominant negative p38^{MAPK} mutant (Fig. 6, *A* and *B*). In summary, these data indicate that TNF-α mediates its stabilizing effects on SOCS3 mRNA through activation of the MKK6/p38^{MAPK} pathway. Several studies indicate that activation of MK2, the downstream target of the MKK6/p38^{MAPK} cascade, plays a crucial role for mRNA stabilization of cytokine mRNAs such as, e.g., TNF-α mRNA. As implicated from experiments using MEF cells and a macrophage cell line derived from MK2-deficient mice, the present study provides substantial evidence that MK2 is also a key regulator of SOCS3 mRNA stability. MK2 deficiency does not only result in an impaired TNF-α-induced SOCS3 expression but also in reduced basal SOCS3 mRNA levels (Fig. 7).

The precise mechanism of how p38^{MAPK} regulates mRNA stability is unsolved. Recent evidence suggests that the mRNA-stabilizing effects of p38^{MAPK} are predominantly due to the inhibition of mRNA deadenylation rather than stabilization of the mRNA body (54). Still, it is not known which factors, despite the well known members of the MKK6/p38^{MAPK}/MK2 cascade, own further key positions in p38^{MAPK}-dependent regulation of mRNA stability. It is very likely that an integral component of the mRNA-stabilizing activity is a mRNA-binding protein controlled by p38^{MAPK}.

The best characterized elements influencing mRNA stability are A/U-rich elements within the 3′ UTR of the mRNA of cytokines, growth factors, or protooncogenes. Often, these mRNAs contain several copies of the pentameric AUUUA motif (22) with nearby U-rich regions. These A/U-rich elements act as potent destabilizing sequences by targeting mRNA for rapid deadenylation in vivo (23–25) and promoting 3′-5′ exonuclease decay by the exosome in vitro (26, 27). The murine as well as the human SOCS3 mRNA contain three copies of this pentameric AUUUA motif in close proximity to a U-rich region (Fig. 2) located between the positions 2422 and 2541 within the 3′ UTR. Deletion of these nucleotides led to a prolonged

half-life of SOCS3 mRNA (Fig. 10*A*), further indicating the capability of this sequence of nucleotides to act as a destabilizing element.

The function of A/U-rich elements is controlled by *trans*-acting RNA-binding proteins that either protect mRNA from degradation or accelerate the degradation of the respective mRNA. In recent years much progress in identifying the mRNA-binding proteins involved in the regulation of mRNA stability has been made. Proteins such as the RNA-binding protein homologous to human A–D or R Ag (HuA–D/R), which are also known as embryonic-lethal normal vision (ELAV1–4), have been identified as prolonging mRNA half-life upon binding to the respective A/U-rich regions within the 3′ UTR (29, 55). Additionally, destabilizing proteins such as A/U-rich element binding factor AUF-1 (28), TTP (31), K homology-type splicing regulatory protein (56), and butyrate-response factor (57) accelerate degradation of there target mRNA via binding to A/U-rich elements.

Particularly for the zinc finger protein TTP there is increasing evidence that the $p38^{MAPK}$ cascade is crucial for the control of its mRNA destabilizing activity (32, 58). Recently, Hitti et al. (39) demonstrated that MK2 represents the key component for $p38^{MAPK}$-dependent regulation of TTP. TTP targets A/U-rich element-containing mRNA, such as the mRNA of TNF-α or IL-6, toward degradation (31) by binding to the *cis*-acting 3′ UTR of the respective mRNA. TTP is the only *trans*-acting factor shown to be capable of regulating A/U-rich element-dependent mRNA turnover in the intact animal. Most likely due to enhanced expression of TNF-α, mice lacking TTP develop erosive arthritis, cachexia, alopecia, dermatitis, autoantibodies, and myeloid hyperplasia spontaneously (40). Moreover, TTP regulates its own expression in a posttranscriptional manner through binding to an ARE within its own UTR (59). As shown in particular for TNF-α mRNA, phosphorylation of TTP by MK2 enables TTP to bind 14-3-3 proteins, which excludes TTP from the site of mRNA degradation and in turn leads to a preformed, stabilized mRNA. Alternatively, the affinity of TTP to the ARE within the 3′ UTR of TNF-α mRNA could be regulated by the $p38^{MAPK}$-cascade (32).

Considering the observation that the mRNA-stabilizing activities of $p38^{MAPK}$ are predominantly exerted by the inhibition of mRNA deadenylation and subsequent degradation, TTP seemed to be a favorite to represent the link between MK2 activity and the regulation of SOCS3 mRNA levels. This idea was supported by the following observations. First, TTP expression enhances SOCS3 mRNA degradation (Fig. 8*A*). Second, siRNA-mediated knockdown of TTP strongly enhances the amount of endogenous and exogenous SOCS3 mRNA (Fig. 8, *D* and *F*) and, even more pronounced, of SOCS3 protein (Fig. 8, *E* and *G*). Furthermore, deletion of the region, which contains three copies of the pentameric AUUUA motif, rendered SOCS3 mRNA insensitive toward TTP expression (Fig. 10*B*). All of these observations indicate that, in analogy to the regulation of TNF-α-mRNA by MK2 (39), TTP represents the link between TNF-α-induced MK2 activation and enhanced SOCS3 mRNA expression. However, the amounts of TNF-α-induced SOCS3 mRNA (Fig. 11*A*) as well as the decay of TNF-α-induced SOCS3 mRNA (Fig. 11*B*) are not substantially altered in macrophages isolated from TTP-deficient animals. Therefore, one must conclude that in contrast to LPS-induced TNF-α mRNA (Fig. 11*C* and Ref. 31), TTP does not play an irreplaceable role for the control of SOCS3-mRNA levels by TNF-α and can be substituted by another mechanism, at least during mouse development. Presuming the importance of the MKK6/$p38^{MAPK}$/MK2 cascade for the control of SOCS3 mRNA, it is therefore likely that other RNA-binding factors controlled by the activation state of the MKK6/$p38^{MAPK}$/MK2, such as the heterogeneous nuclear ribonucleoprotein or the poly(A)-binding protein-1, may represent a more important link between $p38^{MAPK}$/MK2 activation and SOCS3 expression. Future experimental work is required to further elucidate this relationship.

The fact that MK2 is critical for both TNF-α-induced SOCS3 expression as shown here (Fig. 7), as well as TNF-α expression itself (39), is interesting because it implies that the enhanced expression of TNF-α would be always accompanied by suppression of Jak/STAT signaling through SOCS3. Despite this, it is interesting to note that there is some concordance between MK2-deficient mice and mice with a macrophage-selective gene knockout of SOCS3. MK2-deficient mice show an increased resistance to endotoxic shock, owing to an impaired inflammatory response as well as a decreased production of cytokines such as TNF and IL-6 upon LPS-stimulation (38). Contrariwise, they also show increased susceptibility to bacterial infection and exacerbation of pathology in a genetic model of inflammatory bowel disease (38, 60). In macrophages lacking the SOCS3 gene or carrying a mutation of the SOCS3-binding site in gp130, the LPS-induced production of TNF and IL-12 is suppressed by both IL-10 and IL-6, whereas in respective wild-type littermates only IL-10 is capable of suppressing TNF-α and IL-12 release in response to LPS (16). Thus, both MK2 as well as macrophage-restricted SOCS3 deficiency resulted in a kind of immunosuppressive phenotype. Because MK2 is crucial for the regulation of basal as well as TNF-α-induced SOCS3 expression, one may speculate that the lack of SOCS3 might somehow contribute to the phenotype observed in MK2-deficient animals.

Disclosures

The authors have no financial conflict of interest.

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