Research article

Nitric oxide induces SOCS-1 expression in human monocytes in a TNF-α-dependent manner

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In contrast to the thoroughly characterized mechanisms of positive regulation within cytokine signaling pathways, our knowledge of negative feedback loops is comparatively sparse. We and others have previously reported that IRAK-M down-regulates inflammatory responses to multiple stimuli. In particular, we could show that the nitric oxide (NO) donor, GSNO, induces IRAK-M overexpression in human monocytes. Here we study the expression of another important negative regulator of cytokine signaling, SOCS-1, in human monocytes exposed to GSNO. The NO donor induced significant levels of SOCS-1 mRNA and protein, 6 h and 16 h after stimulation, respectively. Monocytes stimulated with GSNO for longer periods (24 h and 48 h) failed to express IL-6 and IP-10 upon LPS challenge. In addition, and in line with previous reports of NO-mediated induction of TNF-α, we have found that exposure to this cytokine induces SOCS-1 mRNA in human monocytes. A blocking antibody against TNF-α impaired SOCS-1 expression upon GSNO treatment and re-instated IL-6 and IP-10 mRNA levels after LPS challenge in cultures pretreated with the NO donor. We conclude that NO stimulates SOCS-1 overexpression in a pathway at least partially regulated by TNF-α.

Keywords: Nitric oxide, SOCS-1, TNF-α, monocyte, inflammation

INTRODUCTION

Nitric oxide (NO) regulates a wide range of biological activities in the nervous, vascular and immune systems, and is implicated in a number of different pathologies.[1–8] This highly reactive molecule is produced from L-arginine by an enzyme termed NO synthase (NOS). An inducible isoform of NOS (iNOS), mainly activated by immune stimuli, has been identified in several cell types.[9–14] NO stimulates anti-tumoral and anti-microbial activity in activated rat and murine macrophages, thus playing an important role in innate defense mechanisms in rodents. Expression of iNOS and concomitant increases in NO production has also been reported in humans during bacterial infections, in septic patients and in other inflammatory diseases.[14–24] However, and

despite expressing iNOS, activated human monocytes generate, at least *in vitro,* much lower amounts of NO than murine macrophages challenged with lipopolysaccharide (LPS) or cytokines.[25,26] Thus, while in mouse and rat models of inflammation activated macrophages represent the major source of NO, *in vitro* studies indicate that in humans NO is mainly produced by local resident cells such as renal mesangial cells and colon epithelial cells, not by monocytes/macrophages.[15–19,26]

In spite of this fundamental difference between human and rodents, it is well documented that human monocytes/macrophages are exposed to low concentrations of NO in the blood in either normal or pathological conditions. For instance, smooth muscle and endothelial cells produce low amounts of this molecule under normal conditions to maintain the vascular tone.[27–29] Nitric oxide produced by these cells can diffuse into the blood and interact with mononuclear blood cells. In addition, macrophages that migrate to sites of inflammation are exposed to NO produced by resident cells.[30,31] This is, for example, the case of septic shock patients, in whom average serum concentrations of nitrite/nitrate (NO_2^-/NO_3^-), the stable products of NO decomposition, rise to ~70 μM.[32]

It has been demonstrated that NO up-regulates negative signaling pathways in human monocytes,[33,34] as mononuclear cells become refractory to LPS and/or cytokine stimulation after exposure to NO donors.[35–37] In our efforts to unveil the molecular basis of these pathways, we have previously reported that NO induces expression of IRAK-M,[34] a catalytically disabled member of the IRAK family that exerts a negative control on the progression of inflammatory responses.[38–41] In addition, we and others were able to show that NO prevents activation of the IRAK cascade and the subsequent translocation of NF-κB to the nucleus.[33,34]

Over the past 5 years, further molecules have been implicated in the down-regulation of inflammatory responses. A critical regulator of cytokine signaling, SOCS-1, was identified as an intracellular negative-feedback molecule that inhibits overactivation of the JAK–STAT-mediated signal cascade through binding to JAK.[42–44] On the other hand, overexpression of SOCS-1 impairs production of LPS-induced molecules such as IP-10 and IL-6.[45,46] Interestingly, SOCS-1 knockout mice show spontaneously elevated serum levels of pro-inflammatory cytokines and suffer from multiple inflammatory diseases, from which they die around 3 weeks after birth.[42,44] These symptoms coincide with hallmarks of LPS-diseases in wild-type mice.[47,48] Moreover, endotoxin tolerance is strikingly impaired in macrophages from SOCS-1^{−/−} mice, thus suggesting that SOCS-1 down-regulates TLR signaling in parallel to IRAK-M.[47,48] Indeed, SOCS-1 overexpression suppressed LPS-induced IκB phosphorylation and NF-κB activity.[48]

Additionally, inhibition of LPS-induced IL-6 production has been demonstrated in RAW cells that overexpress SOCS-1; this occurs through a mechanism that involves JAK2 and STAT5.[46] Other authors have suggested that SOCS-1 limits the extent of TLR signaling indirectly, by inhibiting type I IFN signaling, but not the main NF-κB pathway.[45]

Based on these findings, we hypothesized that NO modulates the expression of SOCS-1 in human monocytes. Furthermore, and since NO is known to induce TNF-α release from monocytes and macrophages,[34,49–52] we also examined whether TNF-α is implicated in the regulation of SOCS-1 by NO. Using the NO donor S-nitrosoglutathione (GSNO), we show here for the first time that the exposure of human monocytes to NO induces both SOCS-1 mRNA and protein expression, and that this process involves the release of TNF-α.

MATERIALS AND METHODS

Reagents

S-Nitrosoglutathione (GSNO) and glutathione (GSH) were obtained from Biosensing (Berlin, Germany). Dr Chris Galanos (Max Planck Institute für Immunobiologie, Freiburg, Germany) generously provided LPS from *Salmonella abortus.* All other reagents, of the highest quality commercially available, were obtained from Merck (Darmstadt, Germany), Boehringer (Mannheim, Germany), or Sigma (St Louis, MO, USA). The medium used for cell culture was Dulbecco's MEM (DMEM) from Gibco, UK. The goat anti-SOCS-1 polyclonal antiserum, the mouse anti-β-actin monoclonal antibody and the rabbit anti-TNF-α antibody were purchased from Santa Cruz (Los Angeles, CA, USA). CD-14-FITC was from eBioscience (San Diego, CA, USA).

PMBC isolation and cell culture

Peripheral mononuclear blood cells (PMBCs) were isolated from the blood of healthy donors by centrifugation on Ficoll–Hypaque Plus (Amersham Bioscience, The Netherlands). Cells were initially cultured for at least 2 h at a density of 10^6 cells/ml in DMEM supplemented with antibiotics (100 IU/ml penicillin and 100 μg/ml streptomycin). After this period, the supernatant was removed and the adherent cells were cultured in the same medium supplemented with antibiotics (100 IU/ml penicillin and 100 μg/ml streptomycin) and 10% heat-inactivated normal serum (pooled from healthy volunteers). Cells were treated for different periods with a range of GSNO concentrations (from 0.5 nM to 1 μM) as described below. In other experiments, cells were treated with GSNO

and/or TNF-α (2 ng/ml) in the presence or absence of an anti-TNF-α antibody (from 50–300 ng/ml) for 16 h. All reagents and medium used were endotoxin free, as tested using *Limulus* Amebocyte Lysate kit (Bio Whittaker, Walkersville, VA, USA). The homogeneity of cells was tested in every experiment by flow cytometry (anti-CD14–FITC staining).

RNA and protein isolation

Cells were washed once in PBS and their RNA and protein were isolated using TRI-Reagent (IMICO, Cincinnati, OH, USA). To obtain cDNA, RNA was treated with RNAse-free DNAse I (Amersham Biosciences), and reverse transcription was performed on 1 µg of this material using a poly(dT) oligonucleotide primer (Roche, Palo Alto, CA, USA) in a final volume of 20 µl. Protein fractions were isolated as recommended (Roche), and the final pellets were dissolved in a solution containing 1% SDS.

RT-PCR

The RT-PCR was performed using a 'One Step' protocol from Biotools (Kit Retrotools, Biotechnological and Medical Laboratories, SA, Spain).

mRNA quantification

Expression levels of SOCS-1, IP-10, TNF-α, and 18S were analyzed by real-time quantitative PCR (LightCycler, Roche Diagnostics, Indianapolis, IN, USA), using cDNA obtained from 1 µg total RNA. Reactions were performed using a Fast-Start DNA master SYBR Green system (Roche) and specific primers. All results were normalized with respect to the expression of the 18S gene, and the cDNA copy number of each gene of interest was determined using a 7-point standard curve. Standard curves were run with each set of samples, the correlation coefficients (r^2) for the standard curves being > 0.98. To confirm the specificity of the reaction products in each experiment, melting profiles were analyzed using the LightCycler. Profiles were recorded by maintaining the reaction at 80°C for 10 s and then raising the temperature to 95°C at a linear rate of 0.1°C/s, while measuring the emitted fluorescence. Analysis of the melting curves demonstrated that each pair of primers amplified a single product. In all cases, identity of this final product was verified by agarose gel electrophoresis; gels were stained with 0.5 µg/ml ethidium bromide and viewed under UV light to confirm that a single product of the predicted size was amplified. Each LightCycler PCR run consisted of 45 cycles with an initial denaturation step of

5 min at 95°C. Cycling profiles were as follows: for TNF-α: 95°C for 10 s, 64°C for 10 s, and 72°C for 19 s; for IL-6: 95°C for 10 s, 50°C for 10 s, and 72°C for 16 s; for IP-10: 95°C for 10 s, 48°C for 10 s, and 72°C for 10 s; for SOCS-1: 95°C for 10 s, 63°C for 10 s, and 72°C for 10 s, for IL-10: 95°C for 10 s, 68°C for 10 s, and 72°C for 10 s.

Primers

The sequences of the primers used are as follows:

TNF-α sense: 5′-GCC TCT TCT CCT TCC TGA TCG T-3′
TNF-α antisense: 5′-CTC GGC AAA GTC GAG ATA GTC G-3′
SOCS-1 sense: 5′-GCG CGA CAC GCA CTT CCG CAC A-3′
SOCS-1 antisense: 5′-TCG AAG AGG CAG TCG AAG CTC TCG-3′
IL-6 sense: 5′-TCG AAA TGT GGG ATT TTC CCA TGA GT-3′
IL-6 antisense: 5′-ACT CAT GGG AAA ATC CCA CAT TTC GA-3′
IP-10 sense: 5′-CTG ACT CTA AGT GGC ATT-3′
IP-10 antisense: 5′-TGA TGG CCT TCG ATT CTG-3′
IL-10 sense: 5′-ATG CCC CAA GCT GAG AAC CA-3′
IL-10 antisense: 5′ –TCT CAA GGG GCT GGG TCA GC-3′

These primers were synthesized, desalted and purified by IZASA (Barcelona, Spain). For 18S mRNA detection, we used the primers of QuantumRNA Classic 18S provided by Ambion.

Apoptosis assay

Cells were washed and resuspended in PBS. The apoptosis assay was performed using an Annexin V-FITC/PI Apoptosis Detection kit (Oncogene, Germany) according to the manufacturer's recommendations. The stained cells were analyzed in a BD FACScalibur flow cytometer (San Jose, CA, USA) equipped with a 25-mW argon laser. The proportion of apoptotic cells was quantified by plotting the Annexin V-FITC versus the PI fluorescence.

Western-blot analysis

Proteins were denatured by boiling in Laemmli buffer, resolved on 12% SDS–PAGE gels in Tris/glycine/SDS buffer (25 mM Tris, 250 mM glycine, 0.1% SDS), and transferred to Immun-Blot PVDF membranes (Bio-Rad, CA, USA) at 300 mA for 1.5 h at 4°C. After blocking for 1 h in 20 mM Tris-HCl, 150 mM NaCl, 0.2% Tween 20 (TBS-T) containing 5% non-fat milk, the membranes were washed three times in TBS-T alone and probed for 20 h with the antibody diluted in TBS-T (anti-SOCS-1; Santa Cruz, CA, USA). Following washing in TBS-T, the membranes were incubated with secondary HRP-conjugated (1:2000 dilutions) for 45 min and washed

three times in TBS-T. The bound antibodies were detected using ECL Plus reagents according to the manufacturer's instructions (Amersham–Pharmacia Biotech, The Netherlands).

IL-6 protein quantification by ELISA

Supernatants from cell cultures of human monocytes treated with different stimuli were used to analyze levels of IL-6 protein production. The assay was performed using a human IL-6 Instant ELISA (Bender MedSystem GmbH, Austria) according to the manufacturer's recommendations.

Data analysis

The number of experiments analyzed is indicated in each figure. Data were collected from a minimum of three experiments and used to calculate the mean ± SD. The statistical significance was calculated using the unpaired Student's *t*-test; differences were considered significant at *P* values < 0.05.

RESULTS AND DISCUSSION

Nitric oxide induces SOCS-1 gene expression in human monocytes

As S-nitrosoglutathione (GSNO) slowly and spontaneously releases NO, it is a potential *in vivo* storage and transport vehicle for this highly reactive molecule.[53–55] In a previous work, we showed that human monocytes exposed to this NO donor overexpressed the negative regulator of inflammation, IRAK-M.[34] This pseudokinase is also involved in endotoxin tolerance.[38–40] On the other hand, exposure to NO attenuates interactions
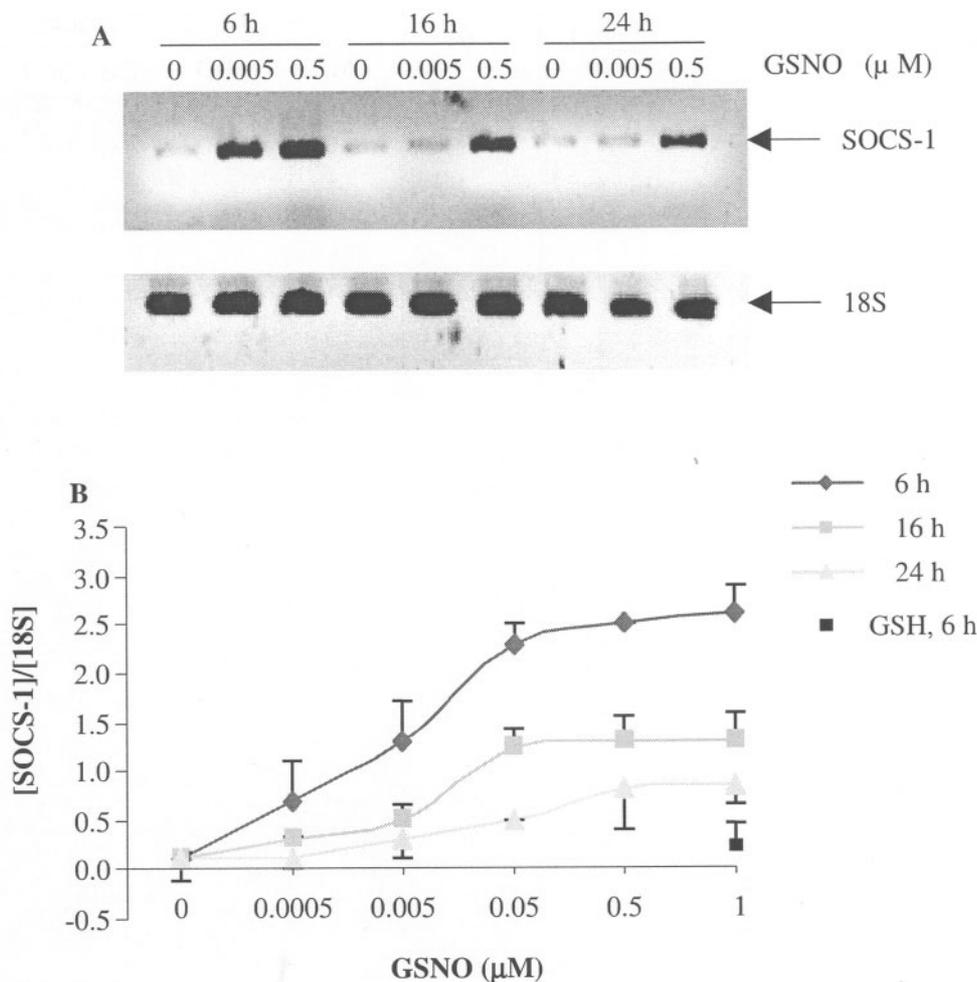

Fig. 1. SOCS-1 gene is overexpressed in human monocytes treated with low GSNO concentrations. Cultures of human monocytes were treated for 6, 16 or 24 h with the indicated concentrations of the nitric oxide donor, GSNO. Total RNA was isolated from the cells and cDNA was synthesized using a poly(dT) primer. (A) RT-PCR products were separated in an agarose gel and stained with ethidium bromide. (B) Levels of SOCS-1 and 18S mRNA expression were analyzed by real-time Q-PCR (LightCycler system). GSH (1 μM) was used as negative control. Data from one representative experiment are shown (*n* = 3).
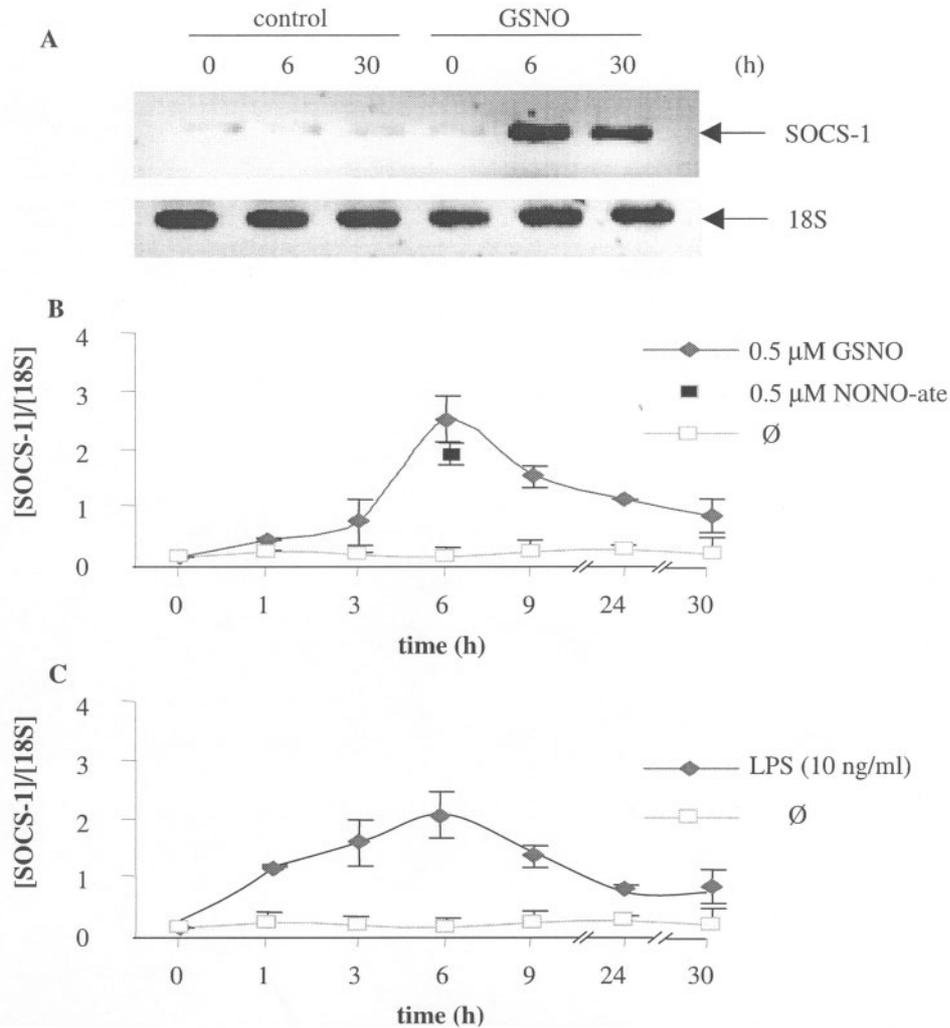
Fig. 2. Time course of SOCS-1 gene expression in GSNO-stimulated monocyte cultures. Human monocytes were treated with 0.5 μM GSNO for the times indicated. (A) RT-PCR products were separated on an agarose gel and stained with ethidium bromide. (Control: no treatment). (B) SOCS-1 and 18S mRNA levels were analyzed by Q-PCR (LightCycler system); the ratio between SOCS-1/18S is depicted. Positive control: cells treated for 6 h with 0.5 μM DETA-NONO-ate. (C) Human monocytes were treated with 10 ng/ml LPS for the indicated periods, the ratio of SOCS-1 and 18S mRNA expression levels was determined as in (B). The results of one representative experiment are shown ($n = 3$). Ø = Controls

between IRAK-1 and TRAF-6 and, as a consequence, the activation of NF-κB in murine macrophages and dendritic cells.[33] Thus, it appears that NO can disrupt the LPS signaling pathway by inhibiting various elements downstream of TLR engagement. Reports on the anti-inflammatory activity of the JAK-binding protein, SOCS-1, and its implication in endotoxin tolerance,[42–48] prompted us to analyze the effect of GSNO on SOCS-1 gene expression in human monocytes.

As hypothesized, cell treatment with GSNO (from 0.5 nM to 1 μM) resulted in a concentration-dependent induction of SOCS-1 mRNA (Fig. 1). Of particular note, even GSNO concentrations as low as 5 nM induced significant expression of the SOCS-1 gene. No SOCS-1 mRNA induction was detected when 1 μM GSH was used instead of GSNO, indicating that the observed

effect is most probably due to NO release. We also stress that no traces of endotoxin were detected in any of our solutions of NO donors, evaluated using *Limulus* Amebocyte Lysate kit.

We next examined the kinetics of SOCS-1 gene expression at a fixed GSNO concentration (0.5 μM). SOCS-1 mRNA levels were maximal 6 h after human monocytes exposure to GSNO, and remained significantly higher than background for about 30 h after stimulation (Fig. 2A,B). Similar results were obtained with an unrelated NO-donor; treatment for 6 h with 0.5 μM DETA-NONO-ate induced comparable levels of SOCS-1 mRNA (Fig. 2B). Finally, we compared the levels of SOCS-1 mRNA expression due to GSNO with those observed after stimulation with 10 ng/ml LPS (Fig. 2C). Comparison with Figure 2B reveals that maximum levels

of induction are similar upon treatment with LPS and NO donors. The time course of monocyte responses to these stimuli is markedly different, however, as endotoxin already induces high SOCS-1 levels 1 hour after challenging.

Low NO concentrations do not affect monocyte viability

As NO has been reported to induce programmed cell death in monocytes,[56–59] we wondered whether the observed effect on SOCS-1 expression levels could be related to apoptotic processes. In our hands, however, treatment with up to 1 μM GSNO did not affect culture viability as shown by the low percentage of AnnexinV/PI stained cells, which remained around 3% in all experiments (Fig. 3A). This apparent contradiction can be explained by the fact that much higher doses of GSNO and/or other (related/unrelated) NO donors were employed in most previous investigations. Moreover,
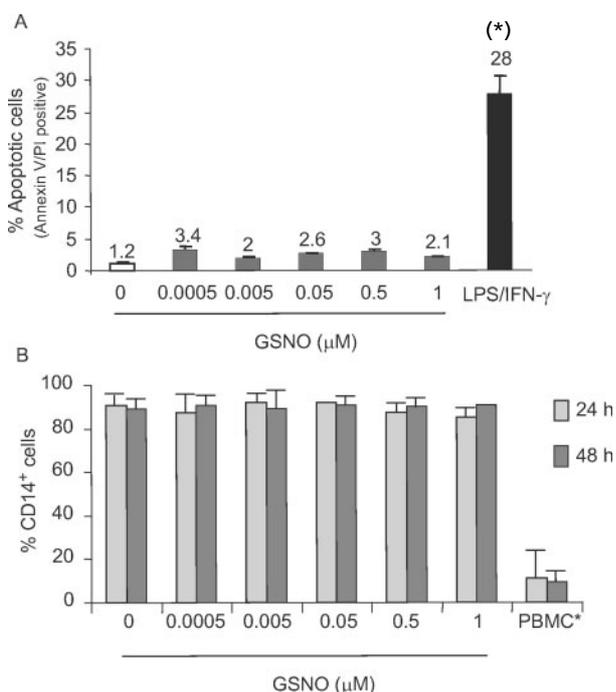
Fig. 3. Exposure to low NO concentrations does not compromise monocytes viability. (A) Human monocytes were treated with the indicated concentrations of GSNO, medium alone (0), or 10 μg/ml LPS and 100 U/ml IFN-γ (LPS/IFN-γ) for 24 h before they were harvested, washed and resuspended in PBS. Cells were double stained with Annexin V/PI and analyzed by flow cytometry. The proportion of cells stained for Annexin and PI is shown (*n* = 3). *P < 0.05 with respect to control cells (0). (B) Human monocytes were treated with the concentrations of GSNO indicated or medium alone for 24 h or 48 h before they were harvested, washed and resuspended in PBS. Cells were stained with anti-CD14–FITC and analyzed by flow cytometry. The proportion of cells stained for anti-CD14–FITC is shown (*n* = 3). *Fraction of cells collected just after isolation by centrifugation on Ficoll–Hypaque Plus.
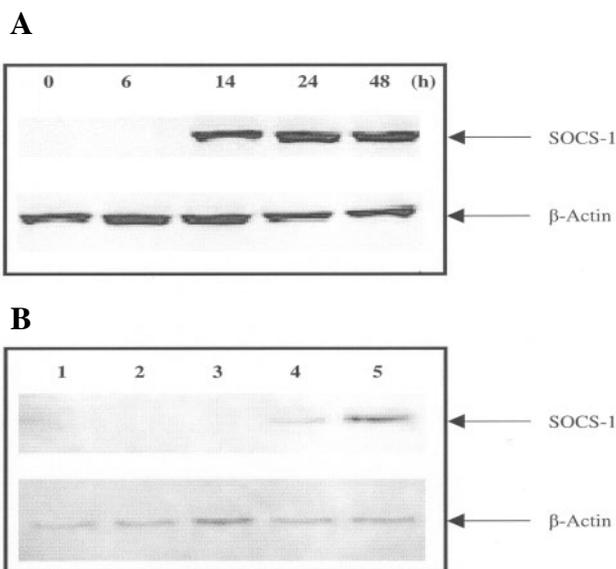
Fig. 4. SOCS-1 protein is present in GSNO-treated monocytes. (A) Human monocytes were culture in medium alone (0) or stimulated with 0.5 μM GSNO for 6, 14, 24 or 48 h. Total protein was isolated and quantified, and the expression of SOCS-1 was analyzed by Western blotting using 15 μg total protein and an anti-SOCS-1 polyclonal antiserum. An anti β-actin monoclonal was used as a control for loading. The data of a representative experiment are shown (*n* = 2). (B) Total protein was isolated and quantified from cultures of (1) human lymphocytes, (2) THP-1 cell line, and from human monocytes either untreated (3), or stimulated for either 16 h (lane 4) or 24 h (lane 5) with 10 ng/ml LPS; samples were analyzed by Western blotting using 15 μg total protein and an anti-SOCS-1 polyclonal antiserum. An anti β-actin monoclonal was used as a control for loading. The data of a representative experiment are shown (*n* = 3).

homogeneity of the human monocyte cultures was also unaffected by NO treatment as determined by flow cytometry (anti-CD14–FITC staining), which showed an average of 90% of the cells expressing the specific surface marker CD14 (Fig. 3B). These data suggest that the observed effect on SOCS-1 regulation occurs mainly in monocytes/macrophages (CD14 positive), while other cells subtypes have minimal representation, if any.

SOCS-1 protein is expressed in human monocytes exposed to NO

Having established that exposure of human monocytes to GSNO induces SOCS-1 up-regulation at the mRNA level, we examined cell extracts to determine whether this mRNA was also translated into protein. Cells stimulated with 0.5 μM GSNO produced SOCS-1 protein as determined by Western-blot analysis using a specific antiserum. In contrast to the significant SOCS-1 mRNA expression levels already seen after 6 h stimulation, larger amounts of the protein were first observed 14 h
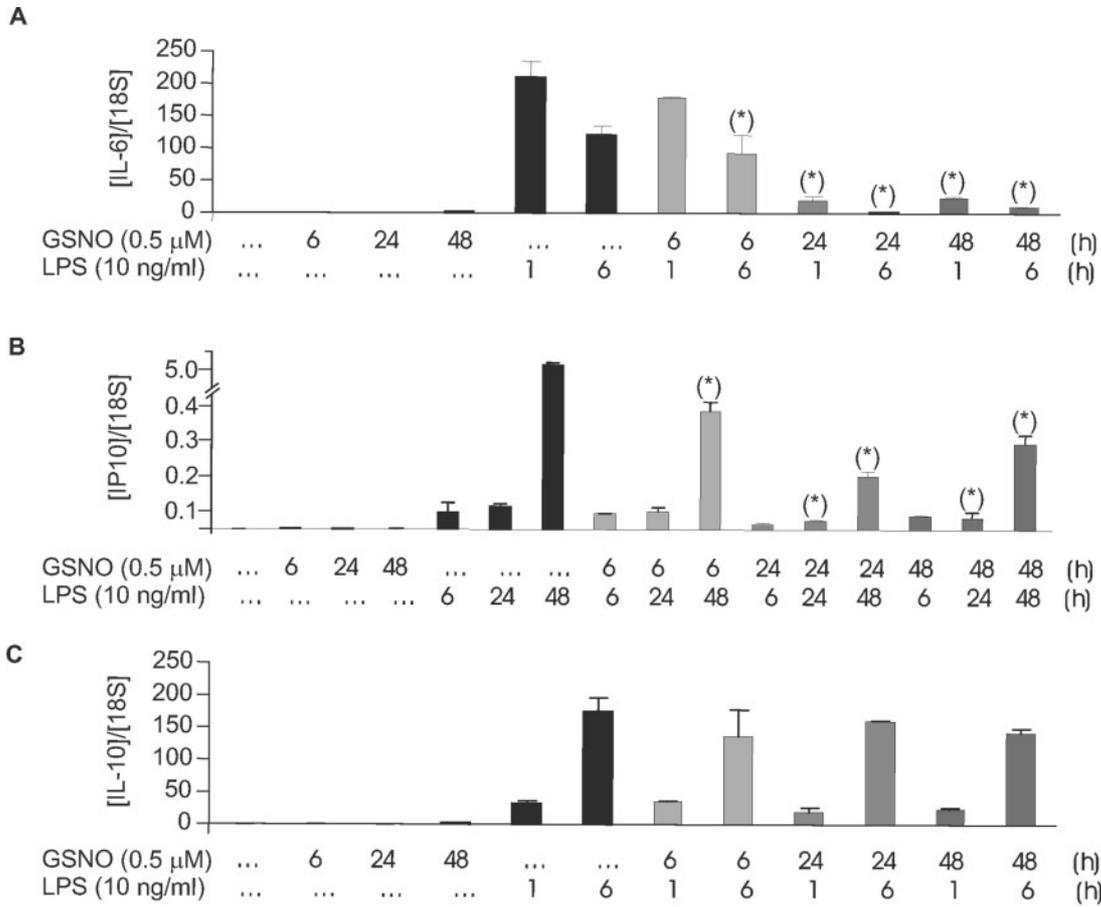
Fig. 5. IL-6, IP-10 and IL-10 mRNA expression after LPS treatment in human monocytes pretreated with GSNO. Human monocytes were pretreated or not with 0.5 μM GSNO for 6, 24 or 48 h and then treated with 10 ng/ml LPS for additional 1 or 6 h and 6, 24 or 48 h. The relative increases in mRNA levels of IL-6 (A), IP-10 (B) and IL-10 (C) with respect to 18S were determined by real-time Q-PCR as indicated in the caption to Figure 2. The result of a representative experiment is shown ($n = 4$). $*P < 0.05$ with respect to LPS stimulation without GSNO pretreatment.

after stimulation with GSNO, and were maintained for at least 48 h (Fig. 4A). Other controls are shown in Figure 4B. Similar results were obtained when other NO-donors were used (data not shown).

Interestingly, other researchers have demonstrated that exposure to NO promotes changes in monocytes by which they become refractory to subsequent stimulation by LPS and/or cytokines.[37,47] Based on our current findings, we are tempted to speculate that NO might contribute to this transient refractory state by inducing different negative regulators of inflammation such as IRAK-M and SOCS-1.

GSNO blocks LPS-induced IL-6 and IP-10 but not IL-10 mRNA expression

Previous reports have demonstrated that SOCS-1 blocks cytokine expression after LPS challenge. Overexpression of SOCS-1 inhibits IP-10 and IL-6 up-regulation following LPS-mediated TLR4 activation.[45,46] Having established that SOCS-1 protein is expressed upon GSNO treatment, we decided to study its influence on IL-6 and IP-10 mRNA levels in LPS-challenged cells. To this end, human monocytes were cultured in the presence of 0.5 μM GSNO for 6 h, 24 h or 48 h, before being stimulated for different times with 10 ng/ml LPS. Expression of LPS-induced IL-6 was markedly down-regulated in cells pretreated with GSNO for 24 h or 48 h (Fig. 5A). In a similar manner, expression of IP-10 due to endotoxin challenge was also significantly reduced after one or two days GSNO pre-treatment (Fig. 5B). This observation suggests a possible involvement of NO-induced SOCS-1 in regulating cytokine production, as its protein levels correlate with the measured effect on IL-6 and IP-10 induction (see above and Fig. 4). Further experiments using interfering RNA technology and/or knockout mice would help define the actual contributions of SOCS-1 expression on the observed effect.

We also determined mRNA levels of IL-10 induced upon LPS challenge in our experimental model. Interestingly, induction of this anti-inflammatory cytokine by LPS was not significantly down-regulated when cells were pre-treated with GSNO (Fig. 5C). Altogether, our data indicate that monocyte exposure to nitric oxide affects mainly the expression of LPS-induced pro-inflammatory cytokines.

GSNO blocks LPS-induced IL-6 protein expression

To confirm the results presented in the previous section, human monocytes were cultured in the presence of 0.5 μM GSNO for 24 h, before being stimulated for 1, 6, 16, 24 or 48 h with 10 ng/ml LPS. As shown in Figure 6, expression of LPS-induced IL-6 was also down-regu-
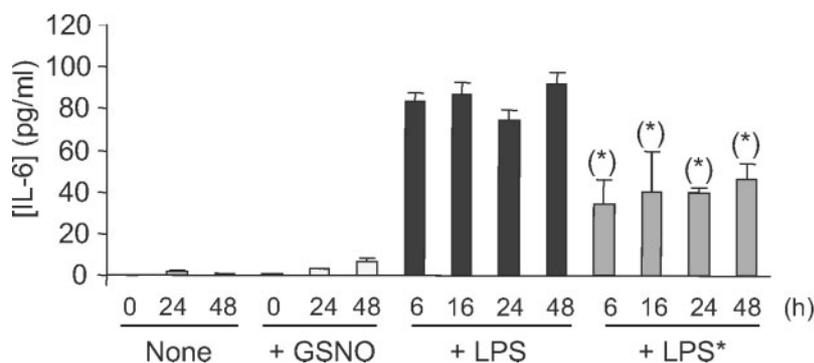

Fig. 6. Pretreatment with a NO donor decreases levels of IL-6 protein expression upon LPS stimulation. Human monocytes were cultured either in the presence of medium alone (none) or different stimuli: 0.5 μM GSNO alone (GSNO), 10 ng/ml LPS alone (LPS), or 0.5 μM GSNO for 24 h followed by 10 ng/ml LPS for the indicated times (LPS*). Levels of IL-6 protein in the supernatant of each culture were evaluated using a commercial ELISA. The result of a representative experiment is shown (*n* = 2). *P < 0.05 with respect to LPS stimulation without GSNO pretreatment.
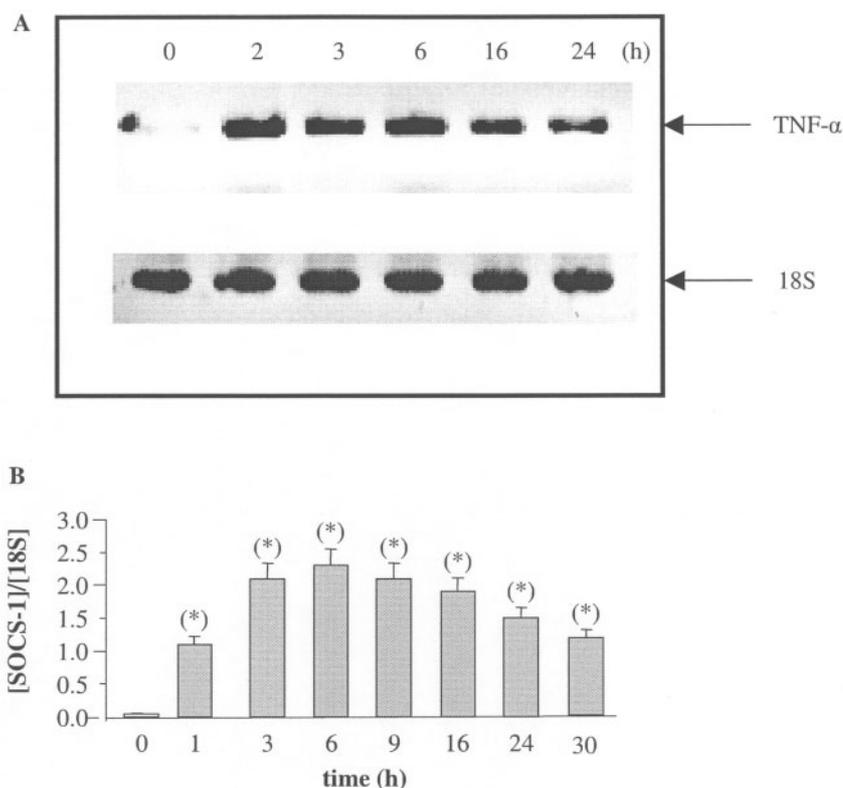

Fig. 7. GSNO induces expression of TNF-α mRNA and TNF-α induces SOCS-1 expression. (A) Cultures of human monocytes from healthy donors were treated with 0.5 μM GSNO or medium alone (0) for the indicated times. The expression of TNF-α gene was analyzed by RT-PCR from total RNA isolated from the cultures. The results of a representative experiment are shown (*n* = 2). (B) Human monocytes were treated with TNF-α (2 ng/ml) for the indicated times. The expression of SOCS-1 mRNA was evaluated by real-time Q-PCR (LightCycler system). The results of a representative experiment are shown (*n* = 4). *P < 0.05 with respect to the control (t = 0 h).
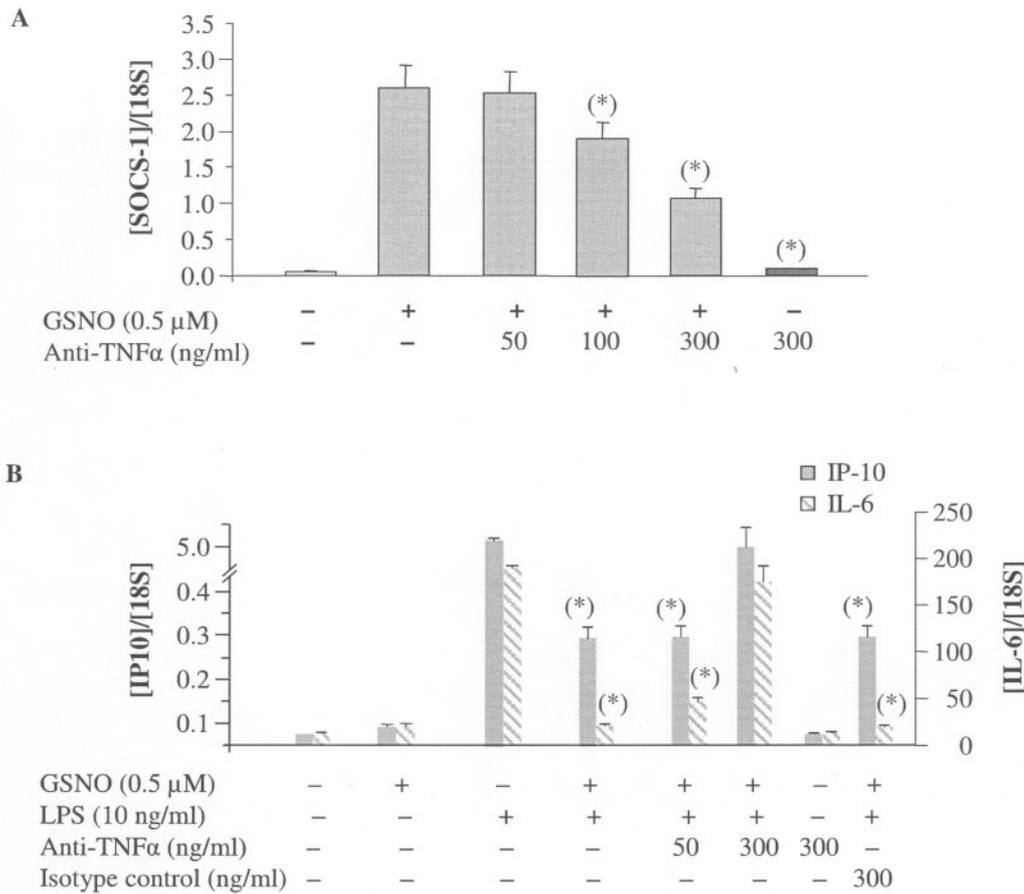
Fig. 8. GSNO-mediated stimulation of SOCS-1 is counteracted by an anti-TNF-α antibody. (A) Cultures of human monocytes were treated for 16 h with combinations of 0.5 μM GSNO and 0, 50, 100 or 300 ng/ml anti-TNF-α antibody. Subsequently, total RNA was isolated and cDNA synthesized. Quantitative analysis of the SOCS-1/18S ratio was carried out by real-time Q-PCR (LightCycler system). The results of a representative experiment are shown ($n = 3$). *$P < 0.05$ with respect to the GSNO alone treatment. (B) Human monocytes were pretreated (+) or not (–) with GSNO for 16 h. After this, cultures were stimulated with LPS for 1 h (striped bars) or 48 h (solid bars) in the presence or not of a blocking anti-TNF-α antibody. Quantitative analysis of the IP-10/18S and IL-6/18S ratios were carried out by real-time Q-PCR (LightCycler system). The results of a representative experiment are shown ($n = 3$). *$P < 0.05$ with respect to LPS alone.

lated at protein level in those cells pretreated with GSNO. Whilst LPS induced a clear up-regulation of IL-6 production, pretreatment with a NO-donor significantly reduced the presence of IL-6 in the supernatants of those cultures stimulated with LPS. In addition, we note that treatment with GSNO alone did not result in IL-6 accumulation.

TNF-α stimulates expression of SOCS-1

In a previous investigation, we established that NO induces TNF-α release from human monocytes.[34] Using RT-PCR we could detect a significant increase of TNF-α mRNA 2 h after stimulation with 0.5 μM GSNO (Fig. 7A). The same effect was observed when DETA-NONO-ate was used instead (data not shown). We then sought to determine whether exogenously added TNF-α

could directly influence SOCS-1 mRNA expression in these cells. As shown in Figure 7B, stimulation of human monocytes with TNF-α (2 ng/ml) was sufficient to induce SOCS-1 gene expression 1 h after treatment. In agreement with the kinetics of SOCS-1 induction by NO (see above and Fig. 2), maximum levels of mRNA were detected after 6 h challenge, and they remained high for at least 30 h.

Anti-TNF-α blocking antibody blocks GSNO-induced SOCS-1 expression and re-instates IL-6 and IP-10 levels after LPS stimulation

The rapid up-regulation of SOCS-1 expression in human monocytes exposed to TNF-α suggests that this cytokine may be involved in the pathway(s) that leads to the induction of SOCS-1 in response to NO. To determine

whether TNF-α was indeed involved in SOCS-1 up-regulation, we treated cultures of human monocytes for 16 h with 0.5 μM of GSNO in the presence of an anti-TNF-α blocking antibody (50–300 ng/ml). While GSNO-treated cultures expressed SOCS-1, mRNA levels of this gene were diminished in the presence of the antibody, in a dose-dependent manner (Fig. 8A). This effect was also observed at the protein level (data not shown). Moreover, addition of anti-TNF-α antibody to cultures of human monocytes pretreated with GSNO re-instated the IL-6 and IP-10 mRNA levels after LPS-stimulation (Fig. 8B).

CONCLUSIONS

We present here the first evidence that stimulation of human monocytes with NO results in a significant induction of SOCS-1, thus contributing to generate an endotoxin-tolerance state. In addition, our observations suggest and that this effect depends, at least partly, on the early NO-induced endogenous release of TNF-α by these cells. Since alternative mechanisms have been advanced to explain the ability of NO to inhibit/activate several important pathways,[60] we cannot rule out that other factors are implicated in the activation of SOCS-1 by NO in human monocytes. Nevertheless, our data support the contribution of TNF-α to this process. These findings will certainly help our understanding of the role of NO in inflammation and endotoxin tolerance. Further experiments using knockout mice and/or interfering RNA technology would help define the relative contributions of SOCS-1, IRAK-M and other factors to the generation of the endotoxin-tolerance state.

ACKNOWLEDGEMENTS

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