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Curcumin abrogates LPS-induced proinflammatory cytokines in RAW 264.7 macrophages. Evidence for novel mechanisms involving SOCS-1, -3 and p38 MAPK

Morgana Rodrigues Guimarães^a, Fábio Renato Manzoli Leite^{a,1}, Luís Carlos Spolidorio^b, Keith Lough Kirkwood^c, and Carlos Rossa Jr^{a,*}

^a Department of Diagnosis and Surgery, Faculdade de Odontologia de Araraquara, Univ Estadual Paulista (UNESP), Araraquara, SP, Brazil

^b Department of Physiology and Pathology, Faculdade de Odontologia de Araraquara, Univ Estadual Paulista (UNESP), Araraquara, SP, Brazil

^c Department of Craniofacial Biology, College of Dental Medicine, Medical University of South Carolina (MUSC), Charleston, SC, USA

Abstract

Curcumin is the active compound in the extract of *Curcuma longa* rhizomes with anti-inflammatory properties mediated by inhibition of intracellular signalling. SOCS and MAPKs are involved in the signalling events controlling the expression of IL-6, TNF- α and PGE₂, which have important roles on chronic inflammatory diseases. The aim was to assess if these pathways are involved in curcumin-mediated effects on LPS-induced expression of these cytokines in macrophages. RAW 264.7 murine macrophages were stimulated with *Escherichia coli* LPS in the presence and absence of non-cytotoxic concentrations of curcumin. Curcumin potently inhibited LPS-induced expression of IL-6, TNF- α and COX-2 mRNA and prevented LPS-induced inhibition of SOCS-1 and -3 expression and the inhibition of the activation of p38 MAPK by modulation of its nuclear translocation. In conclusion, curcumin potently inhibits expression of LPS-induced inflammatory cytokines in macrophages via mechanisms that involve modulation of expression and activity of SOCS-1 and SOCS-3 and of p38 MAPK.

Keywords

Curcumin; Innate immunity; Cell signalling; Cytokines; NF- κ B; MAPK; SOCS

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*Corresponding author at: Department of Diagnosis and Surgery, Faculdade de Odontologia de Araraquara, Univ Estadual Paulista (UNESP), Rua Humaitá, 1680 – Centro, 14801-903 Araraquara, SP, Brazil. Tel.: +55 16 3301 6509; fax: +55 16 3301 6369. crossajr@foar.unesp.br, krlinhos@uol.com.br (C. Rossa Jr).

¹Current address: School of Dentistry, Univ Federal de Pelotas (UFPEL), Rua Gomes Carneiro, 1 – Centro, 96001-970 Pelotas, RS, Brazil. Tel.: +55 53 3921 1268.

Competing interests

None declared.

Ethical approval

This is an in vitro study using commercially-available cell lines. No animal or human participation. There are no ethical considerations.

1. Introduction

Curcumin is a constituent of the spice turmeric, one of the principal ingredients in curry powder. Many studies have evaluated the effect in vivo of curcumin in the treatment of conditions associated with an inflammatory/immune response component, including cancer,^{1–3} rheumatoid arthritis,⁴ Crohn's disease,⁵ inflammatory bowel disease,⁶ colitis⁷ and psoriasis.⁸ Understanding of the biological mechanisms involved in the effects of curcumin is improved by in vitro studies demonstrating inhibition of inflammatory cytokines such as TNF- α , IL-8, IL-1, IL-6, MMP-9, NO, COX-2 by curcumin in various cell types^{9–14}; however, information on the macrophage-specific effects of curcumin are relatively scarce. Macrophages are associated with innate immunity and are important for the initiation and modulation of adaptive immunity, having a central role in inflammatory diseases.

Among the intracellular signalling pathways involved in cytokine production, the transcription factor NF- κ B and mitogen-activated protein kinase (MAPK) cascades were shown to play fundamental roles. NF- κ B is one of the most important inducible transcription factors in mammals and has been shown to play a pivotal role in the mammalian innate immune response¹⁵ and chronic inflammatory conditions.¹⁶ MAPKs, which include JNK, ERK, and p38, are well known stress-activated kinases implicated in the regulation of many genes, particularly those of the inflammatory and immune response.¹⁷ NF- κ B is considered the primary target of curcumin, whereas MAPKinase activation is also affected by curcumin in some cell types. Besides NF- κ B and MAPK, curcumin has been shown to modulate other signalling pathways involving protein tyrosine kinases as well as serine/threonine kinases, such as JAK/STAT/SOCS and PKC.^{18–21}

The suppressor of cytokine signalling (SOCS) family of proteins²² represent an important endogenous negative regulatory mechanism of cytokine signalling that also affects pro-inflammatory cytokine expression. As an endogenous mechanism, SOCS proteins are²² essential to normal immune physiology, but they are also involved in the development of immunological disorders including inflammatory diseases such as atherosclerosis, arthritis, airway inflammation, diabetes and cancer. This has encouraged studies on the control of cytokine-related pathological conditions by mimicking the functions or modulating the expression of SOCS proteins.²³ SOCS-1, also called SSI-1 (STAT-induced STAT inhibitor-1) was initially identified as an intracellular negative-feedback molecule that inhibits the overactivation of the JAK-STAT-mediated signal cascade initiated by various stimuli, including IFN- γ , IL-6, IL-4.^{24–26} Recent reports indicate an important role for SOCS-1 on the downregulation of LPS signal transduction alongside with direct effects on negative feedback inhibition of NF- κ B signalling.^{22,27}

SOCS-3 may have profound effects on the regulation of immunity and inflammation by affecting the activation, development and homeostatic functions of all lineages involved in immune and inflammatory responses.²⁸ SOCS-3 is strongly induced by interleukin-1, -6, -10 and IFN- γ , and its main role seems to be the attenuation of inflammatory cytokine signalling. Previous studies have demonstrated that SOCS-1 deficiency results in severe synovial inflammation and joint destruction^{29–31} while SOCS-3 has also been implicated in

the modulation of arthritis pathogenesis, being able to suppress the induction and development of joint inflammation in mouse models.^{31,32}

While numerous in vitro and in vivo studies report on the anti-inflammatory role of curcumin, particularly on NF- κ B activation, this is the first study to evaluate the effect of curcumin on the kinetics of SOCS-1 and SOCS-3 expression and on the activation of MAPKinases in macrophages stimulated by LPS and the effect of this modulation on expression of IL-6, TNF- α and PGE₂.

In this study, we assess the effects of curcumin on the modulation of macrophage response to bacterial LPS, which is a well characterized, widely used and efficient model of evaluation of inflammatory response in vitro.^{33–37} Curcumin suppressed LPS-induced expression of TNF- α , IL-6 and PGE₂; and this effect was paralleled by preventing the LPS-induced inhibition of SOCS-1 and -3 expression and the inhibition of the activation of p38 MAPKinase by modulation of its nuclear translocation.

2. Materials and methods

2.1. Cell line and materials

The murine macrophage cell line RAW 264.7 (ATCC #TIB-71) was cultured in alpha-MEM supplemented with 100 U/mL penicillin, 100 μ g/mL streptomycin, and 10% heat-inactivated foetal bovine serum and maintained in a humidified atmosphere at 37 °C and 5% CO₂. LPS from *Escherichia coli* (serotype O55:B8) and curcumin were purchased from Sigma. *E. coli* LPS was diluted in PBS (pH 7.4) at 1 mg/mL (stock concentration) and used at a final concentration of 1 μ g/mL (1:1000 dilution from the stock LPS solution). Curcumin was diluted in DMSO and used at the indicated concentrations. The concentration of LPS used for the stimulation, the experimental periods and the cell density used were optimized in preliminary experiments aimed at obtaining the maximum cell response (data not shown). NE-PER Nuclear and Cytoplasmic Extraction Reagent was purchased from Pierce (Thermo-Fisher Scientific). Rabbit polyclonal antibodies against phosphorylated forms of p38 MAP kinases, p65, ERK1/2, as well as to beta-actin, Lamin A/C and GAPDH were from Cell Signalling, as well as the secondary HRP-conjugated antibodies. Rabbit polyclonal antibodies for SOCS-1 and SOCS-3 were from Santa Cruz Biotechnology.

2.2. Cell viability

The effect of curcumin on cell viability was determined using a commercial kit (Cell Titer 96 Aqueous; Promega Corp.) according to the manufacturer's instructions. This kit evaluates cell viability by the activity of mitochondrial dehydrogenase enzymes that reduce a tetrazolium containing substrate solution to formazan, generating a colorimetric reaction. Briefly, 1×10^5 cells were plated in each well of 96-well plates, allowed to attach for 18 h, washed once with phosphate-buffered saline (PBS) and then de-induced for 6 h in culture medium with reduced concentration (0.3%) of FBS. Curcumin was added to the culture medium in various concentrations for a dose-response experiment and the cells incubated for 24 h. Controls were represented by the same volume of the DMSO vehicle. 20 μ L of reagent containing the tetrazolium salt (MTS) substrate was added to each well and incubated 2 h

and the results obtained by measuring the absorbance at 490 nm on a microplate reader (Bio-Rad Inc., model 550). The relative number of viable cells in the wells treated with curcumin was estimated in relation to the vehicle-treated control wells.

2.3. Evaluation of cytokine gene expression (RT-qPCR)

A total of 3×10^5 cells were grown for 24 h in each well of six-well plates, de-induced by incubation for 6 h in culture medium containing 0.3% foetal bovine serum and stimulated with *E. coli* lipopolysaccharide (1 µg/mL) for 24 h, both with and without a 30 min pretreatment with 5 or 10 µM of curcumin. Control wells were treated with the same volume of the DMSO vehicle used to dilute these compounds. Total RNA was isolated from the cells with Trizol reagent (Invitrogen Corp.) according to the manufacturer's instructions. The RNA was quantitated by spectrophotometry and 500 ng were reverse-transcribed into cDNA. The relative abundance of the transcripts of the candidate inflammatory genes were determined by real-time reverse transcription-PCR (RT-PCR) using Taqman chemistry and pre-designed sets of primers and probes (TaqMan Gene Expression Assays, Applied Biosystems) on a StepOne Plus Real-Time PCR System (Applied Biosystems). The reactions were carried out in a 96-well plate on a final reaction volume of 30 µL that included Taqman Universal PCR Master Mix (Applied Biosystems), Taqman Gene Expression Assays (Applied Biosystems) for each target gene: TNF-α (tumour necrosis factor alpha), NM_013693; IL-6 (interleukin 6), NM031168; PTGS-2 (prostaglandin-endoperoxide synthase-2), NM011198; GAPDH (glyceraldehyde-3-phosphate dehydrogenase), NM008084; and cDNA template (corresponding to 300 ng of total RNA). Optimized thermal cycling conditions were: 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. For each sample, analyses of gene expression were performed in duplicate. Three independent experiments were performed. To normalize the amount of total RNA present in each reaction, the expression of GAPDH, which was not altered by the experimental conditions, was used as a housekeeping gene. To compare the expression levels among different samples, the relative expression level of the genes was calculated using the comparative (CT) method using the thermocycler's software.

2.4. Determination of cytokine gene expression at the protein level (ELISA)

Cell culture supernatants were collected from the same wells from which total RNA was harvested immediately thereafter. In brief, cells were stimulated with *E. coli* lipopolysaccharide for 24 h, both with and without a 30 min pretreatment with 5 or 10 µM of curcumin. Control wells were treated with the same volume of the DMSO vehicle used to dilute these compounds. These culture supernatants were cleared by centrifugation (5 min, 12,000 rpm at 4 °C), aliquoted and immediately stored at -80 °C until used. These samples were thawed only once and used in ELISA assays to quantify the secreted PGE₂, IL-6 and TNF-α. These assays were performed according to the manufacturer's instructions (R&D Systems) and the target protein concentration was normalized to the total protein content determined by the Lowry method (DC assay, Bio-Rad).

2.5. Activation of signalling pathways (Western-blot)

3×10^5 cells were grown for 24 h in each well of six-well plates, routinely deinduced for 6 h and subsequently treated with 5 or 10 μM of curcumin 30 min before the stimulation with bacterial LPS. Control experiments were performed with the addition of the same volume of DMSO vehicle 30 min before LPS stimulation. Samples (cell lysates) were harvested 10, 30 and 60 min for the short-term experiments (activation of signalling pathways) and for 12, 24 and 48 h in the experiments to evaluate SOCS-1 and -3 expression. Whole-cell lysates were harvested by scraping the cells in sodium dodecyl sulfate sample buffer (62.5 mM Tris HCl buffer, pH 6.8, 10% glycerol, 50 mM dithiothreitol, 2% sodium dodecyl sulfate, 0.01% bromophenol blue) on ice, followed by sonication for 10 s and heat-denaturation at 95 °C for 5 min. For preparation of nuclear and cytoplasmic extracts, the cells were washed and scraped into phosphate-buffered saline 10, 30 and 60 min after LPS stimulation, and pelleted by centrifugation. For cytoplasmic/nuclear fractions, 1×10^6 cells were lysed in NE-PER (Nuclear and Cytoplasmic Extraction Reagent, Pierce) according to the manufacturer's protocol. Total protein content was quantified by Lowry method (DC Assay, Bio-Rad). For western blotting, 30 μg of total protein were separated on 10% Tris-HCl polyacrylamide gels run at 100 V for 60 min and subsequently electrotransferred to nitrocellulose membranes for another 60 min in a semidry apparatus at 110 mA/gel. The membranes were blocked (Tris-buffered saline with 5% nonfat dry milk, 0.1% Tween-20) for 1 h at room temperature and then probed with the primary antibodies overnight at 4 °C. The presence of the primary antibodies was detected by using horseradish peroxidase-conjugated secondary antibodies and a chemiluminescence system (SuperSignal West Pico Chemiluminescent Substrate; Pierce, Rockford, IL, USA). Digital images of the radiographic films exposed to the membranes were obtained on a gel documentation system (GelDoc XT; Bio-Rad). Expression levels of beta-actin were determined to verify equal loading of proteins, GAPDH and Lamin A/C were used as cytoplasmic and nuclear protein controls, respectively.

3. Results

3.1. Cytotoxicity of curcumin

Initially, we performed a dose-response evaluation of the cytotoxic effects of curcumin. Since the biological effects of curcumin may be related to its cytotoxic effects, we intended to determine and use the highest non-cytotoxic concentration of curcumin. RAW 264.7 cells were treated with the indicated concentrations of curcumin (5–100 μM) for 24 h. The results of the MTS-based assay of DMSO vehicle-treated cells were set to 100% and the results for cells treated with curcumin are presented as relative increase or decrease. Concentrations of curcumin up to 10 μM produced no change in cell viability, whereas higher concentrations of curcumin (25 μM) caused significant cytotoxicity (Fig. 1). We then used 10 μM of curcumin as the maximum concentration for the subsequent experiments. We also used 5 μM of curcumin to assess possible dose-related effects.

3.2. Curcumin inhibits LPS-induced cytokine expression

LPS stimulation induced a significant increase on the expression of PTGS-2 (the murine homolog to human cyclooxygenase-2) mRNA by the macrophages. This increase was inhibited by pre-treatment of the cells with curcumin in a concentration-dependent manner;

but statistical significance was only observed with 10 μ M of curcumin. In the absence of LPS stimulation, curcumin induced a slight increase of PTGS-2 mRNA (Fig. 2). Detectable levels of PGE₂ in the cell culture supernatants were detected only upon LPS stimulation and were completely abrogated by curcumin (at 5 and 10 μ M) (Fig. 3).

Curcumin induced TNF- α and IL-6 mRNA expression in the absence of LPS stimulation. However, in cells stimulated with LPS curcumin dose-dependently inhibited the expression of IL-6, whereas inhibition of TNF- α mRNA was observed only with 10 μ M of curcumin (Fig. 2). IL-6 and TNF- α protein were undetectable in the absence of LPS stimulation; and pre-treatment of the cells with 10 μ M of curcumin significantly inhibited LPS-induced secretion of IL-6 and TNF- α (Fig. 3).

3.3. Curcumin prevents the decrease of SOCS-1 and -3 associated with stimulation with LPS and inhibits LPS-induced activation p38 MAPKinase

Considering the relevance of SOCS and MAPKinase signalling pathways for LPS-induced cytokine gene expression, we next evaluated the modulation of these pathways by curcumin in macrophages. As a positive control, we assessed the effects of curcumin on phosphorylation of p65 as indicative of NF- κ B activation status; which is documented as a major target of curcumin. In the vehicle control experiments, equivalent volumes of the DMSO vehicle replaced curcumin. Macrophages were treated with curcumin (5 and 10 μ M) for 30 min followed by stimulation with LPS (1 μ g/mL) for 10, 30 and 60 min. LPS induced activation of NF- κ B, p38 and ERK within 10 min. This activation was sustained, in all signalling pathways for at least 60 min. Pre-treatment of the cells with curcumin inhibited LPS-induced activation of p38 MAPK as well as of p65 (NF- κ B) in a concentration-dependent manner starting 30 min after stimulation (Fig. 4).

The experiments assessing subcellular localization of the signalling intermediates show that LPS induced the nuclear translocation of p38 within 10 min and this induction was maintained at 30 min. Pre-treatment with 10 μ M of curcumin inhibited nuclear translocation of p38 at both 10 and 30 min after LPS stimulation, whereas when curcumin was used at 5 μ M this inhibition was observed only at 30 min (Fig. 5). Curcumin pre-treatment did not affect subcellular localization of ERK, but was associated with increased phosphorylation of ERK in the cytoplasmic compartment.

In the experiments to evaluate the modulation of SOCS-1 and SOCS-3, macrophages were treated with curcumin (5 and 10 μ M) for 30 min followed by stimulation with LPS (1 μ g/mL) for 12, 24 and 48 h. Curcumin (10 μ M) inhibited constitutive expression of SOCS-3 at 12 and 24 h, but prevented the LPS-induced inhibition at 24 h. When used at 5 μ M, curcumin did not alter constitutive expression of SOCS-3, but this lower concentration also prevented the LPS-induced inhibition at 12 h. SOCS-1 expression was increased by both concentrations of curcumin at 24 h and this increase was sustained at 48 h when cells were treated with 10 μ M of curcumin. Curcumin treatment also increased SOCS-1 expression 12 h after LPS stimulation and dose-dependently prevented the LPS-induced inhibition on SOCS-1 expression after 48 h (Fig. 6).

4. Discussion

In this study, we show for the first time, that curcumin prevented the LPS-induced inhibition of SOCS-1 and -3 and blocked the phosphorylation and nuclear translocation of p38 MAPK in murine macrophages. The modulation of signalling pathways can be related to potent inhibitory effect of curcumin on the expression of PGE₂, IL-6 and TNF- α induced by LPS and observed in this work.

Curcumin was recently shown to dose-dependently inhibit the expression of TNF- α and IL-1 β by LPS-stimulated RAW 264.7 cells.³⁸ In this study, cells were treated with similar (5 and 10 μ M) and higher (20 and 30 μ M) concentrations of curcumin in comparison to the present report. The authors used a 2 h pre-treatment with curcumin before stimulation with LPS. The nature of LPS used was also different from the LPS used in the present study: LPS from *E. coli* that we used is a known activator of TLR4; whereas LPS from the periodontopathogenic microorganism *Porphyromonas gingivalis* used in the study by Chen et al.³⁸ activates preferentially TLR2. These differences on the curcumin treatment and source of LPS used can account for the weak inhibition of both TNF- α and IL-1 β with lower concentrations of curcumin reported by Chen et al.³⁸ In our study, concentrations above 20 μ M of curcumin were toxic for this cell line; moreover even lower concentrations of curcumin (5 μ M) markedly decreased TNF- α protein production by RAW 264.7 cells stimulated with TLR4-activating LPS from *E. coli*. The concentrations of curcumin used in the present study were similar to those of Abe et al.³⁹ who showed a marked inhibition of IL-1 β , TNF- α , IL-8 and MIP-1 α in LPS-stimulated human monocytes and alveolar macrophages; and higher than the maximum of 1 μ M used by Jain et al.⁴⁰ who reported a significant but less potent inhibition of IL-6 and TNF- α in human monocytes cultured in high glucose.

Curcumin has been shown to inhibit AP-1 and MAPKinases besides its prime target, NF- κ B.^{21,41} In human intestinal epithelial cell line HT29 stimulated with TNF- α and IL-1 β , curcumin was able to inhibit NF- κ B and MAPKinases, such as p38 and JNK, but there was no inhibition of ERK1/2.⁴² On the other hand, inhibition of all three MAPKinases was observed in human esophageal cells stimulated by low pH,²¹ as well as in an adipocyte cell line treated with palmitate⁴³ and in human microvascular endothelial cells stimulated with VEGF.⁴⁴ Our data indicates that curcumin inhibits LPS-induced p38 MAPK activation in macrophages by reducing both its phosphorylation and nuclear translocation. Interestingly, curcumin at 10 μ M had contrasting effects on the kinetic of LPS-induced activation of ERK MAPKinases: an initial dose-dependent increased activation 10 min after stimulation, was followed by a very marked inhibitory effect 30 and 60 min after LPS stimulation. This delayed inhibition may be attributed to cross-talk between these pathways resulting in a shift of the LPS/TLR4 signal to other signalling pathways due to the inhibition of NF- κ B and p38 MAPK, in a compensatory activation mechanism. The fact that curcumin inhibits p38 while simultaneously not affecting or even increasing the activation of ERK MAPKinases suggests that curcumin exerts its effects relatively downstream on the MAPK cascade, perhaps at the MAP2K level (MKK3/6).

There is paucity information regarding the modulation of SOCS proteins by curcumin. In one of these few studies, the effect of curcumin on SOCS-3 levels was analyzed in ovarian and endometrial cancer cells and compared to non-neoplastic cells.⁴⁵ The level of SOCS-3 was elevated in cancer cells compared to normal cells and its expression was inhibited following curcumin treatment. The authors correlated this marked decrease in SOCS-3 expression following curcumin treatment of cancer cells to the decrease on STAT3 activation.⁴⁵ Our data show that higher dose of curcumin inhibits SOCS-3 expression in unstimulated macrophages and dose-dependently prevents the LPS-induced inhibition, which correlates with the potent inhibition of IL-6 production observed. In the absence of LPS stimulation, curcumin, particularly at 10 μ M, increased expression of SOCS-1, which is consistent with the inhibition of NF-kB signalling,²² a major effect of curcumin. On the other hand, expression of SOCS-3 was reduced by curcumin at 10 μ M in macrophages not stimulated by LPS, which is consistent with the finding in cancer cells.

Importantly, the marked effect of curcumin on cytokine expression may be dependent on this simultaneous inhibition of multiple signalling pathways. A novel observation is the rescue of SOCS-1 expression in LPS-stimulated macrophages. Recent data suggest that SOCS-1 regulates NF-kB signalling.^{22,46} The NF-kB component p65 was identified as an interaction partner for SOCS-1 but not for other members of the SOCS family. SOCS-1 bind to p65 within nucleus leading to its polyubiquitination and proteasomal degradation. Thus, SOCS-1 limits prolonged p65 signalling and terminates expression of NF-kB inducible genes by regulating the duration of NF-kB signal within the cell nucleus.⁴⁶

According to these data, it is plausible to speculate that the increase of SOCS-1 by curcumin may be an additional mechanism involved in the modulation of NF-kB activation and NF-kB-inducible genes such as those evaluated in this study and, therefore, can be related to the anti-inflammatory effects of curcumin.

It will be interesting to define the specific target(s) of curcumin in the TLR4 signalling pathway. This prominent role of curcumin on the activation of innate immunity may have potential to be explored in the modulation of host response in chronic conditions associated with infectious stimuli, such as periodontal diseases and lower digestive tract diseases.

5. Conclusion

In summary, we have shown that curcumin modulates TLR signalling in macrophages and effectively inhibits cytokine gene expression induced by TLR stimulation indicating its therapeutic potential as a host modulatory agent in chronic infectious conditions.

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Fig. 1.

Cytotoxicity of curcumin in RAW 264.7 cells. RAW macrophages were grown in 96-well plates, de-induced in medium containing 0.3% FBS and treated with the indicated concentrations of curcumin (5–100 μ M) for 24 h. Cell viability was determined by an MTS-based assay 2 h after the addition of the mitochondrial enzymatic substrate MTS. The average value of the absorbance reading of DMSO-vehicle treated cells was set to 100% and the results for cells treated with curcumin are presented as relative increase or decrease. Bars represent means and vertical lines standard deviations of 3 independent experiments.

Fig. 2.

Curcumin inhibits LPS-induced pro-inflammatory cytokine expression at the mRNA level. RAW 264.7 cells were de-induced in 0.3% FBS, treated with curcumin (5 or 10 μM) or the same volume of DMSO vehicle for 30 min and then stimulated with *E. coli* LPS (1 μg/mL) or with the same volume of PBS vehicle (control) for 24 h. Total RNA was harvested and used for RT-qPCR performed with pre-designed primers and probes for the indicated target genes and to the housekeeping GAPDH using TaqMan reagents. The results were analyzed by the delta-Ct method and expression of target genes was normalized to GAPDH expression. Data is presented as fold change to DMSO-vehicle treated and unstimulated (control) cells. (*) indicates significant increase, $p < 0.05$, in comparison to control, (+) indicates significant decrease, $p < 0.05$, in comparison to LPS. Bars indicate means and vertical lines standard error of mean of three independent experiments analyzed in duplicate.

Fig. 3.

Effective inhibition of LPS-induced pro-inflammatory cytokine expression at protein level by curcumin. RAW 264.7 cells were de-induced in 0.3% FBS, treated with curcumin (5 or 10 μ M) or the same volume of DMSO vehicle for 30 min and then stimulated with *E. coli* LPS (1 μ g/mL) or with the same volume of PBS vehicle (control) for 24 h. Cell culture supernatants were harvested, aliquoted and used in ELISA tests to quantify the expression of the target cytokines. These tests were performed according to the manufacturer's instructions and the results for each sample were normalized to the concentration of total proteins determined by a Lowry-based microassay (DC assay, Bio-Rad). (*) indicates significant difference, $p < 0.05$, in comparison to control, (+) indicates significant difference, $p < 0.05$, in comparison to LPS. Bars indicate means and vertical lines standard error of mean of three independent experiments analyzed in duplicate.

Fig. 4.

Modulation of LPS-induced activation of NF-κB and all three MAPKs by curcumin. RAW 264.7 cells were de-induced in 0.3% FBS, treated curcumin (5 or 10 μM) or the same volume of DMSO vehicle for 30 min and then stimulated with *E. coli* LPS (1 μg/mL) or with the same volume of PBS vehicle (control) for 10, 30 and 60 min. Cell lysates were harvested at the indicated time points in SDS-containing sample buffer supplemented with protease and phosphatase inhibitor cocktails (Complete and Phos-Stop, Roche). Total concentration of protein was determined with a Lowry-based microassay (DC assay, Bio-Rad) and 30 μg of each sample were diluted in LDS-containing running buffer supplemented with DTT, heat-denatured, electrophoresed in 10% Tris–Acrylamide gels and transferred to 0.2 μm nitrocellulose membranes in a semi-dry transfer apparatus. The activation of the signalling pathways was assessed by the detection of phosphorylated forms of p65 (NF-κB), p38, JNK and ERK MAPKs. Expression levels of constitutive housekeeping beta-actin are shown to confirm equal protein loading. The images are representative of three independent experiments.

Fig. 5.

Curcumin prevents nuclear localization of p38 MAPK in early periods after LPS stimulation of macrophages. RAW macrophages were de-induced and treated with curcumin or vehicle 30 min previously to the stimulation with *E. coli* LPS for the indicated periods, as described in Fig. 4. Cell lysates from 1×10^6 macrophages were harvested in NEPER buffer and the cytoplasmic and nuclear fractions were separated according to the supplier's protocol (Pierce, Thermo-Fisher Scientific). The protein fractions were separated by SDS-PAGE electrophoresis and blotted onto 0.2 μ m nitrocellulose membranes as described. Primary antibodies against phosphorylated (active) p38 and ERK MAPKs were used to verify the nuclear translocation of the active forms of these signalling intermediates. Antibodies against GAPDH and lamin A/C were used as controls for the effectiveness of the subcellular fractionation of the proteins. Images are representative of three independent experiments.

Fig. 6.

Curcumin modulates expression of SOCS1 and SOCS3. RAW macrophages were de-induced and pre-treated with curcumin (5 and 10 μ M) for 30 min previously to stimulation with LPS from *E. coli* (1 μ g/mL) for 12, 24 and 48 h. Cell lysates were collected at each experimental period, quantitated by the Bradford method and 30 μ g of total protein were subjected to SDS-PAGE electrophoresis, followed by blotting onto 0.2 μ m nitrocellulose membranes as described in the methods section. The membranes were probed with primary antibodies against SOCS1 and SOCS3 and GAPDH (not shown) was used as a housekeeping normalization protein. The images (A) are representative of three independent experiments, and the graphs (B) depict the mean and standard deviations of the densitometric analysis for the bands corresponding to SOCS1 and SOCS3 after

normalization for the intensity of bands corresponding to GAPDH. * indicates a significant difference ($p < 0.01$).