British Journal of Pharmacology (2000) 131, 1143–1153

www.nature.com/bjp

p38 MAP kinase and MKK-1 co-operate in the generation of GM-CSF from LPS-stimulated human monocytes by an NF-κB-independent mechanism

[1]Koremu K. Meja, [1,2]Paul M. Seldon, [1,3]Yasuyuki Nasuhara, [1,4]Kazuhiro Ito, [1]Peter J. Barnes, [1]Mark A. Lindsay & *,[1]Mark A. Giembycz

[1]Thoracic Medicine, National Heart and Lung Institute, Imperial College School of Medicine, Dovehouse Street, SW3 6LY

1 The extent to which the p38 mitogen-activated protein (MAP) kinase and MAP kinase kinase (MKK)-1-signalling pathways regulate the expression of granulocyte/macrophage colony-stimulating factor (GM-CSF) from LPS-stimulated human monocytes has been investigated and compared to the well studied cytokine tumour necrosis factor-α (TNFα).

2 Lipopolysaccharide (LPS) evoked a concentration-dependent generation of GM-CSF from human monocytes. Temporally, this effect was preceded by an increase in GM-CSF mRNA transcripts and abolished by actinomycin D and cycloheximide.

3 LPS-induced GM-CSF release and mRNA expression were associated with a rapid and time-dependent activation of p38 MAP kinase, ERK-1 and ERK-2.

4 The respective MKK-1 and p38 MAP kinase inhibitors, PD 098059 and SB 203580, maximally suppressed LPS-induced GM-CSF generation by >90%, indicating that both of these signalling cascades co-operate in the generation of this cytokine.

5 Electrophoretic mobility shift assays demonstrated that LPS increased nuclear factor κB (NF-κB) : DNA binding. SN50, an inhibitor of NF-κB translocation, abolished LPS-induced NF-κB : DNA binding and the elaboration of TNFα, a cytokine known to be regulated by NF-κB in monocytes. In contrast, SN50 failed to affect the release of GM-CSF from the same monocyte cultures.

6 Collectively, these results suggest that the generation of GM-CSF by LPS-stimulated human monocytes is regulated in a co-operative fashion by p38 MAP kinase- and MKK-1-dependent signalling pathways independently of the activation of NF-κB.

British Journal of Pharmacology (2000) **131,** 1143–1153

Keywords: Human blood monocyte; p38 MAP kinase; extracellular signal-regulated protein kinase; granulocyte/macrophage colony-stimulating factor; lipopolysaccharide

Abbreviations: AP-1, activator protein-1; ATF-2, activating transcription factor-2; BAL, bronchoalveolar lavage; ERK, extracellular signal-regulated kinase; GM-CSF, granulocyte/macrophage colony-stimulating factor; IL, interleukin; JNK, c-jun N-terminal kinase; K-FGF, Kaposi's fibroblast growth factor; LPS, lipopolysaccharide; MAP, mitogen-activated protein; MKK, MAP kinase kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NF-κB, nuclear factor κB; NLS, nuclear localization sequence; TNFα, tumour necrosis factor-α

Introduction

Granulocyte/macrophage colony-stimulating factor (GM-CSF) is a variably glycosylated cytokine produced by many cells including those of the human monocyte/macrophage lineage (Blanchard *et al*., 1991; Sallerfors & Olofsson, 1992). GM-CSF is implicated in a number of respiratory diseases including asthma (Broide *et al*., 1991; 1992a,b; Brown *et al*., 1991; Davies *et al*., 1995; Hallsworth *et al*., 1994; Lai *et al*., 1996; Lei *et al*., 1998; Nakamura *et al*., 1993; Sousa *et al*., 1993; Till *et al*., 1995; Woolley *et al*., 1995; Xing *et al*., 1996) where it is a potent pro-inflammatory stimulus. In particular, GM-CSF enhances the survival of human neutrophils and eosinophils (Brach *et al*., 1992; Lopez *et al*., 1986) in culture by inhibiting apoptosis, and is believed to be the main cytokine responsible for prolonging eosinophil longevity in the airways of asthmatic subjects (Park *et al*., 1998). Other pro-inflammatory effects attributed to GM-CSF include its ability to augment leukotriene C_4 and superoxide anion generation from eosinophils (Silberstein *et al*., 1986), promote the synthesis of cytokines from monocytes, including tumour necrosis factor-α (TNFα) and interleukin (IL)-1β, and stimulate non-haematopoietic cells, such as endothelial cells, to migrate and proliferate (Bussolino *et al*., 1989). Increased expression of GM-CSF has been detected in epithelial cells from bronchial biopsies and within the bronchoalveolar lavage (BAL) fluid of patients with asthma; indeed the number of GM-CSF$^+$ T-lymphocytes, eosinophils and monocytes in the BAL fluid of asthmatic subjects after allergen provocation is elevated when compared to normal individuals (Broide *et al*., 1991; 1992a,b; Davies *et al*., 1995; Hallsworth *et al*., 1994; Sousa *et al*., 1993; Woolley *et al*., 1995). These data are consistent with the finding that the circulating concentration of GM-CSF is increased in subjects with acute severe asthma (Brown *et al*., 1991), and that peripheral blood monocytes (Nakamura *et al*., 1993) and T-lymphocytes (Lai *et al*., 1996; Till *et al*., 1995) secrete more GM-CSF than

*Author for correspondence; E-mail: m.giembycz@ic.ac.uk
Current addresses: [2]Department of Biochemistry, University of Oxford, Oxford, U.K; [3]The First Department of Medicine, School of Medicine, Hokkaido University, Kita-ku, Sapporo, Japan; [4]Sankyo Corporation, 1484-10, Oomaki, Urawa City, Japan.

cells from normal subjects. In addition intrapulmonary transfer of the GM-CSF gene to rats leads to eosinophilia and monocytosis that is associated with irreversible fibrosis indicating a role in airway remodelling (Xing *et al*., 1996). In mice, this procedure contributes to the development of airways inflammation by prolonging leukocyte infiltration induced by IL-4 and IL-5 (Lei *et al*., 1998).

The possible contribution of GM-CSF to the pathogenesis of mucosal inflammation that characterizes asthma and other respiratory diseases provides a compelling rationale for targeting the generation and/or release of this cytokine with small molecule inhibitors. However, relatively little is known of the signalling pathways that control the expression of GM-CSF when compared to other pro-inflammatory genes such as TNFα, IL-1β, IL-6 and IL-8. GM-CSF expression can be controlled by both transcriptional and post-transcriptional mechanisms, in a cell type-specific manner. In T-lymphocytes, GM-CSF generation requires new protein synthesis (Bohjanen *et al*., 1990) whereas in murine peritoneal macrophages cycloheximide fails to suppress the elaboration of GM-CSF (Thorens *et al*., 1987) indicating that post-transcriptional mechanisms dominate the expression of this cytokine. Transcriptional regulation of the GM-CSF gene in T-lymphocytes and probably other cells is complex with the proximal promoter featuring several functional units, each expressing binding domains for several transcription factors (Shannon *et al*., 1997). The major inducible forms identified to date are the nuclear factor-κB (NF-κB) and activator protein-1 (AP-1) families of transcription factors (Jenkins *et al*., 1995; Kolenko *et al*., 1999; Miyatake *et al*., 1991; Schreck & Baeuerle, 1990; Tsuboi *et al*., 1991). An enhancer element has also been identified 3 kb upstream of the proximal promoter and binds the transcription factors NF-AT and AP-1 (Shannon *et al*., 1997).

The cell-specific control of cytokine gene expression is also reflected by differences in the relative importance of upstream regulatory elements, including the MAP kinase signalling cascades. Thus, TNFα production in T-lymphocytes is only partially dependent on activation of p38 MAP kinase (Hoffmeyer *et al*., 1999; Schafer *et al*., 1999), unlike lipopolysaccharide (LPS)-stimulated monocytes in which this enzyme and its proximal substrates play a more dominant role (Schafer *et al*., 1999). Collectively, therefore, these data indicate that it may be misleading to extrapolate mechanisms that regulate the expression of a specific gene across cell types.

In human monocytes, LPS promotes the phosphorylation of extracellular signal-regulated kinase (ERK)-1, ERK-2, p38 mitogen-activated protein (MAP) kinase and c-jun N-terminal kinase (JNK) (Dean *et al*., 1999; Foey *et al*., 1998; Liu *et al*., 1994; Scherle *et al*., 1998; Solomon *et al*., 1998) and activates the transcription factor, NF-κB (Frankenberger *et al*., 1994; Haas *et al*., 1990; Wang *et al*., 1995), but the extent to which any of these effects contribute to the generation of GM-CSF is unexplored. In this paper, we describe experiments designed to assess the role of the MKK-1 and p38 MAP kinase signalling cascades in LPS-induced GM-CSF generation from human peripheral blood monocytes and to determine if NF-κB is involved. In a limited number of experiments, TNFα was also measured as a positive control, since it is known that the expression of this cytokine is regulated, at least in part, by p38 MAP kinase (Lee *et al*., 1994; Schafer *et al*., 1999), MKK-1 (Scherle *et al*., 1998; van der Bruggen *et al*., 1999) and NF-κB (Cordle *et al*., 1993; Mackman *et al*., 1991; Muller *et al*., 1993; Vincenti *et al*., 1992; Ziegler-Heitbrock *et al*., 1993).

Methods

Isolation and purification of human mononuclear cells

Blood was collected from normal healthy individuals by antecubital venepuncture into acid citrate dextrose (in mM: disodium citrate 160, glucose 110 – pH 7.4) and mixed with 6% (w v^{-1}) Hespan to sediment erythrocytes. After standing at room temperature for 90 min, the leukocyte-rich plasma was removed and centrifuged at $312 \times g$ for 7 min. The cell pellet was resuspended in 7 ml of buffer A (in mM: KH_2PO_4 5, K_2HPO_4 5, NaCl 110 – pH 7.4) made 50% (v v^{-1}) with Percoll and layered over a discontinuous Percoll density gradient (63 and 73% v v^{-1}) in buffer A. Mononuclear cells were separated from the polymorphonuclear population by centrifugation at $1200 \times g$ for 25 min at 18°C and were recovered from the 50/63% (v v^{-1}) Percoll interface.

Mononuclear cells were washed three-times in Ca^{2+}/Mg^{2+}-free HBSS and suspended in Ca^{2+}/Mg^{2+}-free HBSS at a concentration of 10^6 cells ml^{-1}. Cells (5×10^5) were added to 24 well culture plates (Greiner Labortecnik Ltd, Dursley, Gloucestershire, U.K.) containing 500 μl Dutch-modified RPMI 1640 (RPMI 1640 supplemented with 10% FCS, 2 mM L-glutamine, 100 units ml^{-1} penicillin and 100 μg ml^{-1} streptomycin) and allowed to adhere to the plastic for 90 min at 37°C in a humidified incubator under a 5% CO_2 atmosphere. The purity of the adherent cell population was routinely >90%. Plates were agitated, non-adherent cells decanted and the resulting monocytes were cultured for various time (see text and Figure legends for details) in 1 ml supplemented Dutch-modified RMPI 1640 in the absence and presence of the drugs under investigation.

Measurement of GM-CSF and TNFα

The concentration of GM-CSF and TNFα released by LPS-stimulated monocytes into the culture supernatant was measured by immunospecific ELISAs as described previously (Seldon *et al*., 1998). The detection limit of the GM-CSF and TNFα assays is 16 and 8 pg ml^{-1} respectively.

Assessment of monocyte viability

Cell respiration, an index of viability, was assessed in vehicle- and drug-treated monocytes by measuring the mitochondria-dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) to formazan as described in Hirst *et al*. (1992).

Western immunoblot analysis

The phosphorylation state of p38 MAP kinase, ERK-1, ERK-2 and ATF-2 was assessed by Western immunoblot analysis using antibodies that recognize the activated form of the enzymes. Monocytes were lysed in buffer B (20 mM Tris base – pH 7.4, 1% v v^{-1} Triton X-100, 0.5% w v^{-1} Na-deoxycholate, 0.1% w v^{-1} SDS, 50 mM NaCl, 5 mM EDTA) supplemented with PMSF (500 μM), Na-orthovanadate (2 mM), leupeptin (10 μg ml^{-1}), aprotinin (25 μg ml^{-1}), pepstatin (10 μg ml^{-1}), NaF (1.25 mM) and Na-pyrophosphate (1 mM). Insoluble protein was removed by centrifugation at $12{,}000 \times g$ for 5 min and aliquots of the resulting supernatant were diluted 1 : 4 in Laemelli buffer (62.5 mM Tris-HCl – pH 6.8, 10% v v^{-1} glycerol, 1% w v^{-1} SDS, 1% v v^{-1} β-mercaptoethanol, 0.01% v v^{-1} bromophenol blue) and boiled for 5 min. Denatured proteins (20 – 60 μg) were

subsequently separated by SDS–PAGE upon 10% vertical slab gels and transferred to Hybond ECL membranes (Amersham) in buffer C (Tris base 50 mM – pH 8.3, glycine 192 mM, 20% v v^{-1} methanol). The nitrocellulose was incubated for 1 h in buffer D (25 mM Tris-base – pH 7.4, 150 mM NaCl, 0.1% v v^{-1} Tween 20, 5% w v^{-1} non-fat dry milk) and incubated overnight in buffer D containing 5% w v^{-1} BSA and the relevant primary antibody. After washing in buffer D, membranes were incubated for 90 min with a donkey anti-rabbit peroxidase-conjugated IgG antibody (Amersham) diluted 1 : 5000 in buffer D and then washed again. Antibody-labelled proteins were visualized by ECL and relevant bands were quantified by laser-scanning densitometry.

Semi-quantitative RT–PCR

Total RNA was extracted from 4×10^6 adherent monocytes using a Qiagen RNeasy mini kit according to the manufacturer's instructions and up to 1 μg was treated with DNaseI to remove contaminating genomic DNA. Five hundred nanograms of RNA were reverse transcribed in a total volume of 20 μl in 50 mM Tris-HCl (pH 8.3; 25°C) containing 10 mM $MgCl_2$, 500 μM spermidine, 10 mM DTT, 9 U AMV Reverse Transcriptase, 40 U RNase inhibitor, 0.5 μg random hexamers (Pharmacia, Uppsala, Sweden) and 1 mM deoxynucleotides. A RT-generated cDNA encoding the GM-CSF gene was amplified by PCR using specific primers designed from the reported primary sequences (Table 1) deposited with the GenBank database. To confirm the integrity of RNA and equal loading of sample, RT–PCR analysis of the GAPDH gene was routinely performed using primers synthesized from the sequence described in Maier *et al.* (1990). PCR amplification was conducted in a reaction volume of 25 μl using a Hybaid OmniGene thermal cycler (Hybaid, Teddington, Middlesex, U.K.) and 0.5 U *Taq* polymerase set for 25 (GAPDH) or 28 (GM-CSF) cycles at a denaturing temperature of 94°C for 30 s, specific annealing temperature (Table 1) and an extension temperature of 72°C for 30 s. The number of cycles was that necessary to achieve exponential amplification where product is proportional to starting cDNA. This parameter was determined empirically by performing PCR on an 'average' cDNA sample by combining cDNA from all samples within one experiment. PCR products were subsequently size-fractionated on 2% agarose/TAE gels, stained with ethidium bromide and visualized under UV light. To confirm identity with the published cDNA-sequence, the GM-CSF and GAPDH amplification products were cloned in to pGEM5Z®-vectors (Promega, Southampton, U.K.) and double-stranded sequencing was performed using the T7 Sequenase 2.0 system (Amersham).

After agarose gel electrophoresis, Southern blotting and hybridization were performed to confirm the identity of PCR products and to check for possible genomic contamination. To quantify product formation, aliquots of the PCR-product (5 μl) were 'dot-blotted' and hybridized with the appropriate radiolabelled cloned cDNA. After washing at high stringency the radioactivity associated with each 'dot-blot' was determined by Cérenkov counting. GM-CSF transcripts are expressed as ratio to GAPDH and relative values plotted as means ± s.e.mean.

Electrophoretic mobility shift assays

Nuclear proteins were prepared according to Osborn *et al.*, (1989) and used in binding reactions (25 μl total volume) containing 10 mM Tris HCl – pH 7.5, 4% v v^{-1} glycerol, 1 mM $MgCl_2$, 500 μM EDTA, 1 mM DTT, 50 mM NaCl and 80 μg ml^{-1} microwaved salmon sperm DNA. After incubation on ice for 10 min, 0.0175 pmol [^{32}P]-kinase-labelled double-stranded oligonucleotide probe was added. Consensus NF-κB probe containing the decameric NF-κB site (underlined), was 5′-AGT TGA GGG GAC TTT CCC AGG-3′ (sense strand). Binding reactions were performed on ice for 40 min and complexes were separated on 7% native acrylamide gels before drying and autoradiography. The specificity of NF-κB : DNA binding was assessed by adding a 100-fold excess of an unlabelled competitor consensus oligonucleotide prior to the probe. For supershift assays, nuclear extracts were incubated on ice for 2 h with antisera raised against the p50 and p65 subunits of NF-κB at 4 μg ml^{-1}, before the addition of radiolabelled oligonucleotide. Data were quantified by laser-scanning densitometry.

Drugs and analytical reagents

Percoll was obtained from Pharmacia/LKB (Milton Keynes, Buckinghamshire, U.K.), and LPS (from *Salmonella enteritidis*), FCS (F-9665; lot no. 48H3379), RPMI 1640 (R-7638), HBSS (H-6648), L-Glutamine (G-7513), penicillin/streptomycin (P-0781) and MMT were from the Sigma Chemical Company (Poole, Dorset, U.K.). ECL Western blotting reagents and Rainbow™ protein molecular weight markers were from Amersham International (Amersham, Buckinghamshire, U.K.). Anti-human p50 (codes *sc*7178, *sc*114X), p65 (codes *sc*109, *sc*109X) and actin (code *sc*1615) polyclonal antibodies, and horseradish peroxidase-conjugated secondary anti-rabbit antibodies were from Santa Cruz Biotechnology (London, U.K.). PhosphoPlus p38 MAP kinase (Thr180/Tyr182), ERK-1/ERK-2 (Thr202/Tyr204) antibody kits and anti-pATF-2 were purchased from New England Biolabs (Beverly, MA, U.S.A.). PD 098059, SN50/SN50-M, SB 203580 and RWJ 67657 were from Calbiochem-Novobiochem (Nottingham, U.K.), Alexis Corporation (Laüfelfingen, Switzerland), SmithKline-Beecham (King of Prussia, PA, U.S.A.) and R.W. Johnson Pharmaceutical Research Institute (Raritan, New Jersey, U.S.A.) respectively. The consensus NF-κB probe was from Promega (Southampton, U.K.). All other reagents were from BDH (Poole, Dorset, U.K.).

All cell culture reagents with the exception of FCS were endotoxin-free according to details provided by the manu-

Table 1 Primers and conditions used in RT–PCR experiments

Gene product	*GenBank accession number*	*Deoxyoligonucleotide sequences*	*Co-ordinates of PCR product in human cDNA sequence*	*Product size (base pairs)*	*Annealing temperature*
GM-CSF	P04141	Forward: 5′-CCA-TTC-TTC-TGC-CAT-GCC-TG-3′ Reverse: 5′-ATG-TTT-GAC-CTC-CAG-GAG-CCG-3′	116 to 615	500	58° for 30 s
GAPDH	J04038	Forward: 5′-TCT AGA CGG CAG GTC AGG TCC ACC-3′ Reverse: 5′-CCA CCC ATG GCA AAT TCC ATG GCA-3′	146 to 743	598	65° for 30 s

facturer. The concentration of endotoxin in the FCS (10% v v^{-1}) that was used in all experiments was 52 pg ml^{-1}. All drugs were dissolved at a stock concentration of 100 mM in DMSO (PD 098059, SB 203580, RWJ 67657) or RPMI 1640 (SN50, SN50-M) and diluted to the desired-working concentration in RPMI 1640. In none of the experiments did the solvent (always $<0.1\%$ v v^{-1}) significantly modify cytokine release, EMSAs or Western immunoblot analyses.

Data and statistical analyses

Data are expressed as the mean ± s.e.mean of '*n*' independent determinations. Concentration-response curves were analysed by least-squares, non-linear iterative regression with the 'PRISM' curve-fitting program (GraphPad Software Inc, San Diego, U.S.A.) and log transformed EC_{50}/IC_{50} values were interpolated from curves of best-fit. When statistical evaluation was required, data were analysed by Student's *t*-test for paired data or by one-way ANOVA/Newman-Keuls multiple comparison test. The null hypothesis was rejected when $P<0.05$.

Results

Effect of LPS on GM-CSF and TNFα generation

The basal amount of GM-CSF and TNFα released by human monocytes after they had been cultured for 18 h in enriched RPMI 1640 medium was very low (<100 pg ml^{-1}) or normally below the detection limit of the assays. LPS (3 ng ml^{-1}) evoked a time-dependent generation of GM-CSF and TNFα from human monocytes (Figure 1a). In the case of GM-CSF, there was a lag of approximately 4–6 h before it was detected in the supernatant. Thereafter, GM-CSF accumulated steadily and reached a plateau at approximately 18 h; no further change in the level of GM-CSF was seen over the remainder (28–30 h) of the experiment. The $t_{½}$ for LPS-induced GM-CSF generation was 14.3 ± 4.4 h. In contrast, TNFα was detected in the culture supernatant at much earlier time-points (1–2 h) after exposure of monocytes to LPS, accumulated more rapidly and peaked after approximately 10 h with a $t_{½}$ (3.5 ± 1.6 h) that was significantly ($P<0.005$) shorter than for the elaboration of GM-CSF. Again, no further change in the level of TNFα was seen over the remainder (38 h) of the experiment (Figure 1a). Based on these kinetic data, cytokines were measured in all further experiments 18 h after stimulation of monocytes with LPS.

The mean concentration-response relationship that described LPS (1 pg ml^{-1} to 100 ng ml^{-1})-induced GM-CSF generation at 18 h is shown in Figure 1b from which a pEC_{50} of 9.41 ± 0.07 was derived. The potency of LPS was very similar to its ability to promote the production of the well-studied cytokine, TNFα ($pEC_{50}=9.99\pm0.02$), although on a weight basis 4.8 fold more TNFα than GM-CSF was released (2.64 ± 0.28 *versus* 0.55 ± 0.11 ng ml^{-1}) from the same monocyte cultures (Figure 1b). Unless stated otherwise, LPS was used in all further experiments at a concentration of 3 ng ml^{-1}, which equated to the EC_{90} for cytokine generation.

Effect of LPS on GM-CSF mRNA expression

In the absence of LPS, mRNA transcripts for GM-CSF were not detected in adherent monocytes by RT–PCR after 28–30 cycles of amplification. After the addition of LPS (3 ng ml^{-1}) there was a brief lag (~0.5 h) after which GM-CSF mRNA expression increased rapidly in a time-dependent manner relative to the 'house-keeping' gene GAPDH (Figure 2). The accumulation of GM-CSF mRNA transcripts peaked at 2 h after stimulation, remained unchanged up to 12 h and had returned to near resting levels by 18 h (Figure 2). The $t_{½}$ for the increase in GM-CSF mRNA was 0.8 ± 0.4 h and preceded the appearance of GM-CSF protein by >4 h (Figure 2a).

Effect of cycloheximide and actinomycin D on GM-CSF release

Exposure of human monocytes to actinomycin D (5 μg ml^{-1}; 5 min) and cycloheximide (20 μg ml^{-1}; 20 min), inhibitors of transcription and translation respectively, prior to the addition of LPS (3 ng ml^{-1}) abolished the release of GM-CSF at 18 h.

Effect of LPS on the phosphorylation status of ERK-1, ERK-2 and p38 MAP kinase

The time-course of ERK-1, ERK-2 and p38 MAP kinase activation in LPS-stimulated human monocytes is shown in Figure 3. In resting monocytes, pERK-1, pERK-2 and pp38 MAP kinase were barely detectable. However, the dual phosphorylation status of each kinase increased rapidly and transiently in response to LPS, followed identical kinetics and temporally preceded the appearance of GM-CSF in the culture supernatant by 13.5 h. Maximal phosphorylation of each kinase occurred at 30 min ($t_{½}$'s ~15 min), which then declined gradually towards the resting level over the remainder (5.5–7.5 h) of the experiment. Re-probing the filters for actin confirmed equal loading of protein (Figure 3).

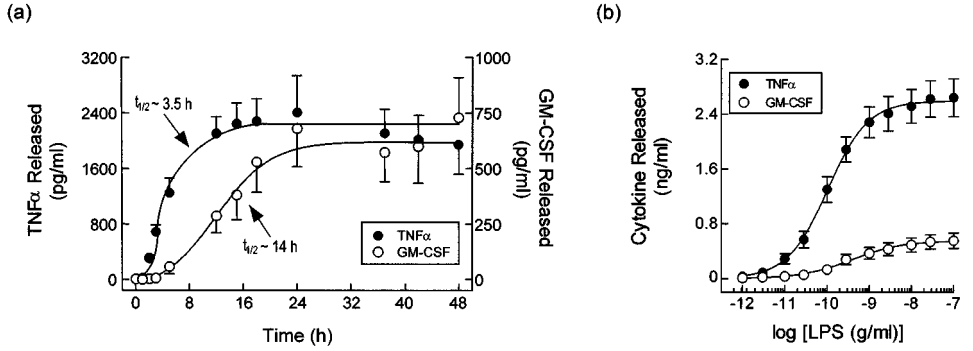

Figure 1 Time-course and concentration-dependence of LPS-induced GM-CSF and TNFα generation. Adherent monocytes were cultured in the presence of 3 ng ml^{-1} LPS (a) or 1 pg ml^{-1} to 100 ng ml^{-1} LPS (b) and the amount of GM-CSF and TNFα released into the culture supernatant was measured at the time-points indicated (a) or at 18 h (b) by sandwich ELISAs. Data points represent the mean ± s.e.mean of four determinations using monocytes from different donors.

Role of p38 MAP kinase and MKK-1 in LPS-induced GM-CSF generation

Figure 4a shows the effect of SB 203580 and RWJ 67657, inhibitors of the α- and β-isoforms of p38 MAP kinase (Kumar *et al*., 1997; Wadsworth *et al*., 1999), on LPS-induced GM-CSF production. SB 203580 and RWJ 67657 inhibited cytokine generation in a concentration-dependent manner with a potency (pIC_{50} values = 7.31 ± 0.13 and 9.56 ± 0.08 respectively) approximately 2.5-times greater than that required to suppress the production of TNFα (pIC_{50} values = 6.93 ± 0.12 and 9.11 ± 0.08 respectively). Moreover, SB 203580 at a concentration that abolished the ability of p38 MAP kinase to phosphorylate the transcription factor activating transcription factor-2 (ATF-2; Figure 4b), almost completely inhibited GM-CSF and TNFα generation (Figure 4a).

The MKK-1 inhibitor, PD 098059, which abolished basal and attenuated LPS-induced dual phosphorylation of ERK-1 and ERK-2 (Figure 5a), also suppressed (80.3 ± 3.6% at 30 μM) GM-CSF release in a concentration-dependent manner (pIC_{50} = 5.84 ± 0.17). However, in contrast to previous investigations where the MKK-1/2 inhibitors PD 098059, U-0126 and Ro-09-2210 were used (Scherle *et al*., 1998; van der Bruggen *et al*., 1999), TNFα generation was only marginally suppressed (36.9 ± 4.1% at 30 μM) under experimental conditions identical to those used to measure GM-CSF (Figure 5b).

The effect of SB 203580 and PD 098059 on the concentration-response relationships that described LPS-induced GM-CSF generation is shown in Figure 6. In both cases SB 203580 produced 4–16 fold shift to the right in the LPS concentration-response curves and an associated reduction in the maximal response (Figure 6a,b; Table 2). PD 098059 also antagonized GM-CSF generation where the maximum response was suppressed without any significant decrease in the LPS EC_{50} values (Figure 6c; Table 2). Consistent with the data presented in Figure 5b, PD 098059

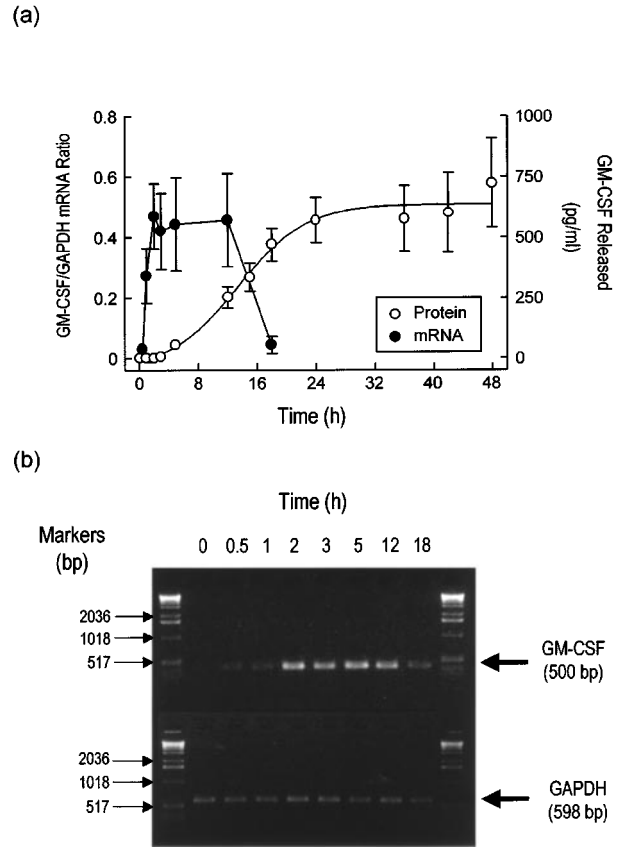

Figure 2 Time-course of LPS-induced GM-CSF mRNA expression. Adherent monocytes were cultured in the presence of 3 ng ml^{-1} LPS and at the appropriate time-points RNA was extracted and 0.5 μg reverse transcribed to generate cDNAs for GM-CSF and GAPDH using the primer pairs shown in Table 1. PCR was performed with reverse transcribed cDNA, the products subjected to electrophoresis on 2% agarose gels and DNA subsequently visualized after staining with ethidium bromide. PCR products were quantified by Southern blotting and standardized against the house-keeping gene GAPDH. RT–PCR product sizes for GM-CSF and GAPDH were 500 (28 cycles) and 598 bp (24 cycles) respectively. (a) and (b) show the mean data of four independent experiments and a representative gel (prior to Southern hybridization) respectively.

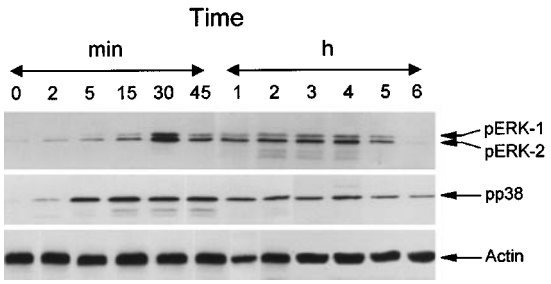

Figure 3 Time-course of LPS-induced ERK-1, ERK-2 and p38 MAP kinase phosphorylation. Adherent monocytes were cultured in the presence of LPS (3 ng ml^{-1}) and pERK-1, pERK-2 and pp38 MAP kinase was determined by Western blotting using antibodies that recognize the dual phosphorylated form of the enzymes. Equal loading of sample was confirmed by probing for the 'house-keeping' protein, actin. Data are representative of three independent determinations using monocytes from different donors.

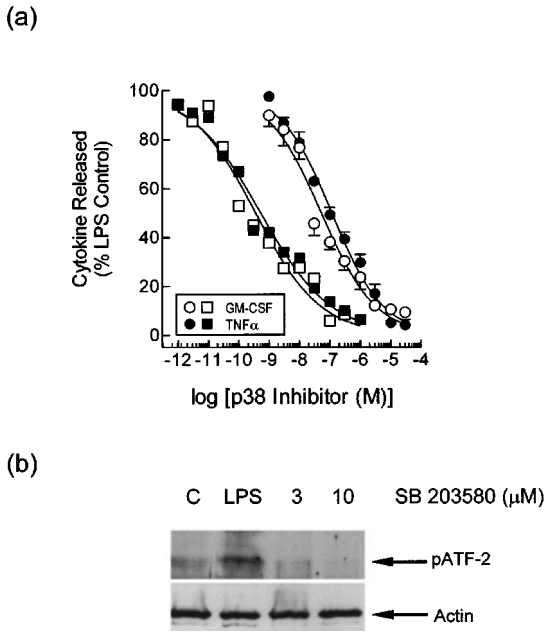

Figure 4 Inhibitory effect of SB 203580 and RWJ 67657 on LPS-induced GM-CSF and TNFα generation and on the phosphorylation of ATF-2. Adherent monocytes were pre-treated (20 min) with varying concentrations SB 203580 or RWJ 67657 as indicated and exposed to LPS (3 ng ml^{-1}). In (a) the amount of GM-CSF and TNFα released into the culture supernatant at 18 h was measured by sandwich ELISAs. Data points represent the mean ± s.e.mean of six determinations using monocytes from different donors. In (b) pATF-2 level was determined after 30 min by Western blotting. Equal loading of sample was confirmed by probing for actin. The results are representative of three experiments performed with monocytes from different donors.

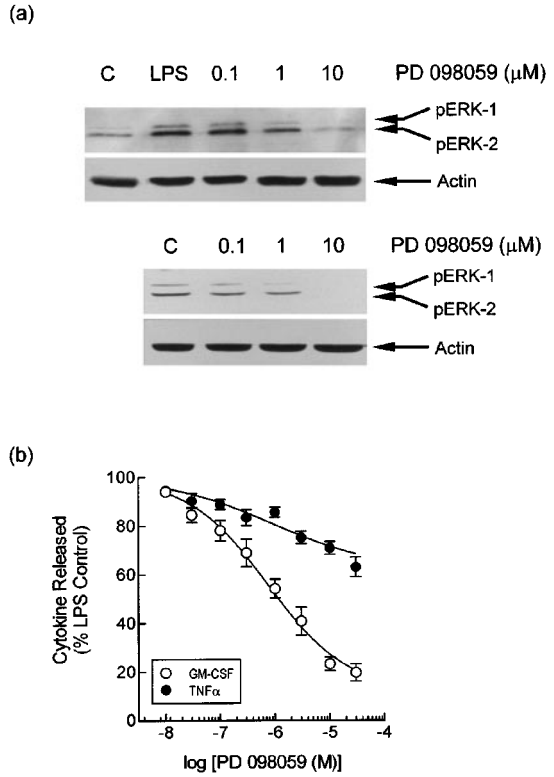

Figure 5 Effect of PD 098059 on LPS-induced cytokine generation and ERK-1 and ERK-2 phosphorylation. (a) Adherent monocytes were pre-treated (20 min) with PD 098059 (0.1, 1 and 10 μM) and exposed to LPS (3 ng ml^{-1}) or vehicle. pERK-1 and pERK-2 were determined after 30 min by Western blotting. Equal loading of sample was confirmed by probing for the 'house-keeping' protein, actin. The results are representative of three experiments performed with monocytes from different donors. (b) Adherent monocytes were pre-treated (20 min) with varying concentrations PD 098059 (10 nM to 30 μM) and exposed to LPS (3 ng ml^{-1}). The amount of GM-CSF and TNFα released in to the culture supernatant at 18 h was measured by sandwich ELISAs. Data points represent the mean$\pm$ s.e.mean of six determinations using monocytes from different donors.

(1 μM) was essentially inactive as an inhibitor of TNFα at all concentrations of LPS studied (Figure 6d). Increasing the concentration of PD 098059 to 10 μM, which abolished basal and LPS-induced ERK phosphorylation (Figure 5a), produced only a modest inhibition ($\sim$30% at 3 ng ml^{-1} LPS) of TNFα release (Figure 6d). Measurement of cell respiration, monitored by mitochondia-dependent reduction of MTT to formazan, indicated that SB 203580, RWJ 67657 and PD 098059 did not affect cell viability at the highest concentration examined.

Effect of LPS on NF-κB : DNA binding

Consistent with previous investigations where quiescent cells of the monocyte lineage were studied (Haas *et al*., 1990; Frankenberger *et al*., 1994; Griffin *et al*., 1989; Kaufman *et al*., 1992; Vallejo *et al*., 2000), a single NF-κB : DNA complex was always discernible in unstimulated human blood monocytes (see Figure 7a). LPS enhanced NF-κB : DNA binding in a time-dependent manner (data not shown) such that at 60 min the intensity of the complex had increased 2–3 fold above the basal level (Figure 7b,d). The induction of the complex was specific for the NF-κB consensus oligonucleotide, since an excess of unlabelled oligonucleotide competed-out the labelled probe (data not shown). Moreover, in resting and LPS-stimulated monocytes, complexes contained both p50 and p65 subunits of NF-κB, as evinced from the ability of antisera raised against the amino-terminus and the nuclear translocation sequence of these proteins respectively to retard the probe and reduce the intensity of the complex (Figure 7c).

Effect of SN50, SN50-M, PD 098059 and SB 203580 on LPS-induced NF-κB : DNA binding and cytokine generation

The effect of SN50, a synthetic, cell permeable peptide which carries the hydrophobic domain of the signal peptide from Kaposi's fibroblast growth factor (K-FGF) linked to the nuclear localization sequence (NLS) of the p50 subunit of murine NF-κB (Lin *et al*., 1995), was studied to determine the role of NF-κB in GM-CSF generation. Consistent with the study of Lin *et al*. (1995), SN50 (100 μM) abolished LPS-induced NF-κB : DNA binding but had no significant effect on the complex that was seen routinely in unstimulated cells (Figure 7a,b). However, SN50 failed to suppress LPS-induced GM-CSF generation (Figure 8a) under conditions where it abolished the release of TNFα ($pIC_{50} = 4.51 \pm 0.03$), with complete inhibition evident at 100 μM. SN50-M, a purportedly inactive control peptide that contains the same signal peptide from K-FGF linked to the NTS with lys$^{363}\rightarrow$Asn and Arg$^{364}\rightarrow$Gly substitutions, also suppressed TNFα generation (Figure 8b) but was less active than SN50 (37.6 ± 16.9% inhibition at 100 μM). Similarly, SN50-M (100 μM) weakly attenuated NF-κB : DNA binding but had no effect on the constitutive complex in unstimulated cells (Figure 7a,d).

Pre-treatment of monocytes with SB 203580 and PD 098059 at a concentration (10 μM) that abolished the LPS-induced phosphorylation of ATF-2 and ERK-1/2 respectively failed to attenuate LPS-induced NF-κB : DNA binding (Figure 9) indicating that inhibition of the p38 MAP kinase and MKK-1/ERK pathways does not prevent the release of NF-κB from IκB.

Discussion

LPS promoted the generation of GM-CSF from human monocytes by a mechanism that was abolished by cycloheximide and actinomycin D implicating *de novo* transcription and translation of the GM-CSF gene. These results are consistent with the regulation of GM-CSF expression from anti-CD3-stimulated human T-lymphocytes (Bohjanen *et al*., 1990) but differ from the post-transcriptional mechanisms that dominate the expression of GM-CSF from murine peritoneal macrophages (Thorens *et al*., 1987). Regulation of this gene is thus cell and/or stimulus-dependent. LPS also increased GM-CSF mRNA transcripts in an actinomycin D-sensitive manner providing further support for transcriptional regulation in human monocytes. As shown in Figure 1a, the kinetics of GM-CSF release were protracted when compared to the elaboration of TNFα, implying that LPS might indirectly transactivate the GM-CSF promoter through the rapid induction of another acute phase gene whose product acts in an autocrine/paracrine manner. Although this possibility was not investigated, evidence for an indirect mechanism was that 4 h elapsed after stimulation of monocytes with LPS before GM-CSF was detected in the culture supernatant. However, this interpretation is difficult to reconcile with the rapid appearance of GM-CSF mRNA transcripts ($t_{1/2} = 1$ h) and the equally rapid and transient activation ($t_{1/2} = 15$ min) of ERK-1, ERK-2 and p38 MAP kinase, which is required for GM-CSF production (see below). It is possible, therefore, the kinetics of GM-CSF release

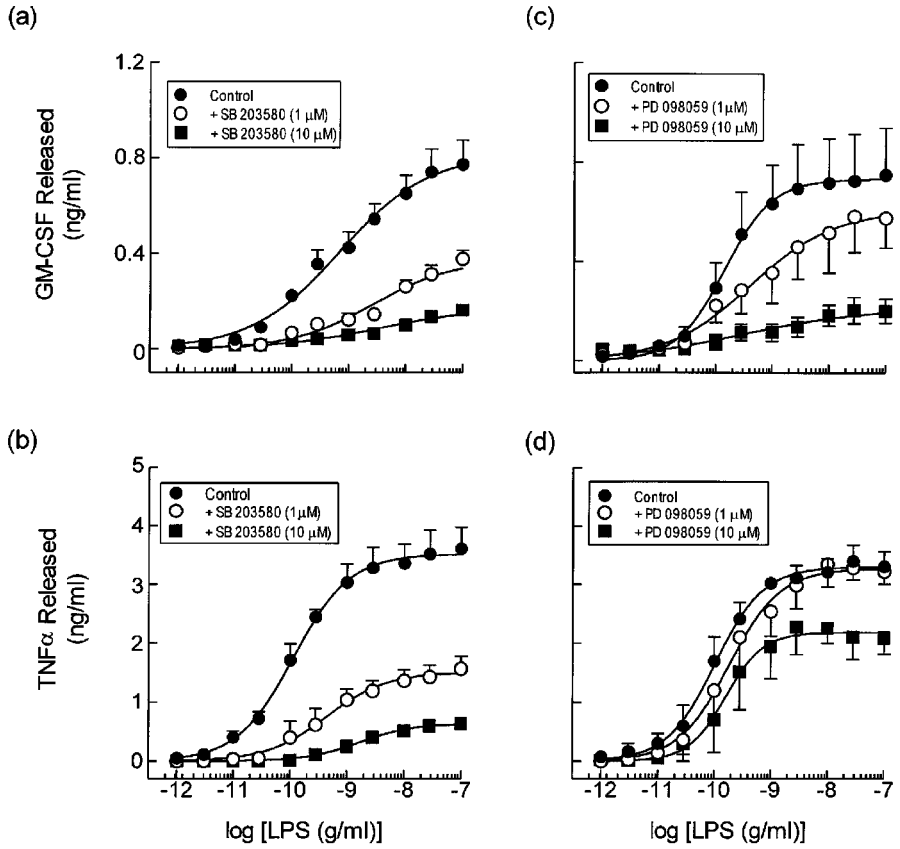

Figure 6 Effect of SB 203580 and PD 098059 on the concentration-response relationship that describes LPS-induced GM-CSF and TNFα generation. Adherent monocytes were pre-treated (20 min) with vehicle, 1 and 10 μM of SB 203580 or PD 098059 and exposed to LPS (1 pg ml^{-1} to 100 ng ml^{-1}). The amount of GM-CSF (a and c) and TNFα (b and d) released in to the culture supernatant at 18 h was measured by sandwich ELISAs. Data points represent the mean±s.e.mean of five and four determinations for the SB 203580 and PD 098059 experiments respectively using monocytes from different donors.

Table 2 Effect of SB 203580 and PD 098059 on LPS-induced GM-CSF and TNFα generation from human monocytes

	GM-CSF		*TNFα*	
	LPS pEC_{50}	*Response (% maximum)*	*LPS pEC_{50}*	*Response (% maximum)*
Control	9.22±0.19 (5)	100	9.97±0.13 (5)	100
SB 203580 (1 μM)	8.65±0.48 (5)	49.1±4.4 (5)	9.43±0.25 (5)	43.5±5.8 (5)
SB 203580 (10 μM)	8.27±0.18 (5)	21.1±2.3 (5)	8.78±0.16 (5)	17.8±3.2 (5)
Control	9.87±0.17 (4)	100	9.99±0.13 (4)	100
PD 098059 (1 μM)	9.47±0.44 (4)	76.7±16.2 (4)	9.68±0.27 (4)	94.7±6.4 (4)
PD 098059 (10 μM)	9.69±0.38 (4)	26.7±6.5 (4)	9.74±0.21 (4)	69.6±8.1 (4)

Adherent monocytes were pre-treated for 20 min with SB 203580 or PD 098059 at the concentrations indicated, exposed to LPS (1 pg ml^{-1} to 100 ng ml^{-1}) and incubated at 37°C for 18 h under a 5% CO_2 atmosphere. The amount of GM-CSF and TNFα released into the culture supernatant was measured by immunospecific ELISAs. Data are expressed as the mean±s.e.mean. Values in parentheses indicate the number of independent determinations.

from LPS-stimulated monocytes may not reflect the rate at which this cytokine is accumulated/stored intracellularly. Alternatively, the translation of GM-CSF mRNA or the release of GM-CSF protein may require a monocyte-derived autocrine factor that is generated relatively slowly in response to LPS.

The concentration-response relationships that described LPS-induced GM-CSF and TNFα generation were essentially identical, unlike the kinetics of gene expression suggesting that the signalling pathways that regulate these genes may be different. Consistent with previous studies, LPS promoted a rapid and transient phosphorylation of ERK-1, ERK-2 and p38 MAP kinase (Lee *et al*., 1994; Liu *et al*., 1994; Solomon *et al*., 1998) at a concentration that equated to the EC_{90} for cytokine generation. Significantly, the kinetics of this effect were rapid and preceded cytokine generation, which would be predicted if a causal relationship exists between the biochemical and functional responses. A role for p38 MAP kinase in GM-CSF generation was suggested from the inhibitory effect of SB 203580 and RWJ 67657, which displayed activity over a concentration range identical to that required to suppress TNFα, a cytokine known to be regulated by p38 MAP kinase (Wadsworth *et al*., 1999). Parallel studies established that SB 203580 effectively suppressed the activation of p38 MAP kinase in LPS-stimulated monocytes by monitoring the phosphorylation of ATF-2, a known substrate for this kinase. This finding confirms the results of a previous investigation (Scherle *et al*., 1998) and is consistent with the ability of SB 203580 and RWJ 67657 to prevent the phosphorylation of mitogen-activated protein kinase-activated protein kinase-2, another substrate of p38 MAP kinase in human monocytes (Dean *et al*., 1999; Wadsworth *et al*., 1999). Taken together, these data

are consistent with other studies in human monocytes where SB 203580 inhibits LPS-induced IL-10 and IL-1β release with identical potency (Foey *et al*., 1998; Lee *et al*., 1994). Moreover, the potency of SB 203580 ($IC_{50} \sim 100$ nM) in this respect was similar or even higher than that required to suppress a variety of p38 MAP kinase-dependent processes in unrelated cell types (see e.g. Detmers *et al*., 1998; Kankaanranta *et al*., 1999; Ridley *et al*., 1997).

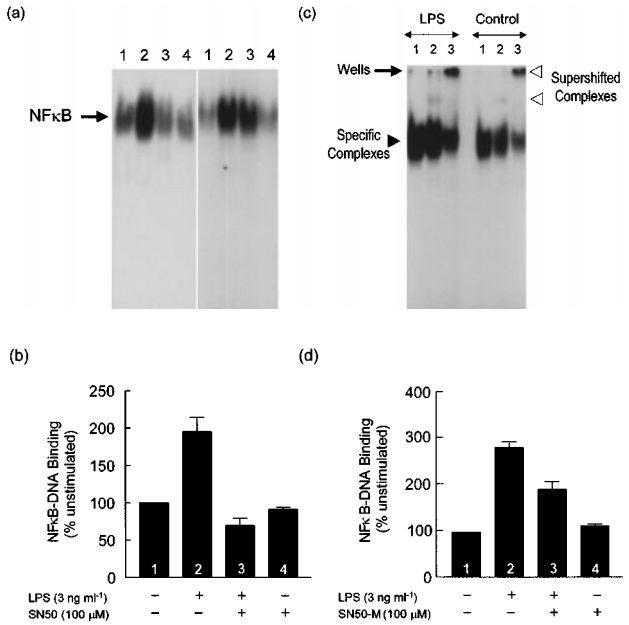

Figure 7 Effect of SN50/SN50-M on LPS-induced NFκB : DNA binding. Adherent monocytes were pre-treated (20 min) with SN50, SN50-M (both 100 μM) or vehicle and exposed to LPS (3 ng ml^{-1}) for 60 min. Nuclear extracts were prepared and subjected to EMSAs using a consensus NF-κB oligonucleotide probe. In (a) a representative autoradiogram of NF-κB : DNA binding is shown, and (b) and (d) depict the mean results $\pm$ s.e.mean of three independent experiments for SN50 and SN50-M respectively using monocytes from different donors. Key: lane/bar 1, unstimulated control; lane/bar 2, LPS; lane/bar 3, LPS + SN50; lane/bar 4, SN50/M. In some experiments, supershift analyses (c) were performed using antisera raised against the amino terminus of the nuclear translocation sequence of the p65 and p50 subunits of NF-κB. Specific and super-shifted complexes are indicated by the filled and open triangles respectively, and the arrow indicates the position of the wells. Key: lane 1, no antibody; lane 2, anti-p50; lane 3, anti-p65.

Activation of MKK-1 was also required for LPS-induced GM-CSF formation, based on the finding that PD 098059 suppressed cytokine release by $\sim$80 % at a concentration that abolished ERK-1 and ERK-2 phosphorylation. However, in contrast to the data obtained with SB 203580, which was an equi-effective and equi-active inhibitor of the release of both gene products, PD 098059 suppressed the elaboration of TNFα only at high concentrations (i.e. 37% at 30 μM). These data are at variance with results published by Scherle *et al*. (1998) who reported that U-0126, another inhibitor ($IC_{50} \sim 70$ nM) of MKK-1, markedly attenuated (by $\sim$90%) LPS-induced TNFα generation from human monocytes. Several explanations could account for this discrepancy. First, unlike PD 098059, which is 10 – 20 fold-selective for MKK-1 over MKK-2 (Alessi *et al*., 1995), U-0126 is equi-potent against these two enzymes (Favata *et al*., 1998). Thus, the greater than expected inhibitory action of U-0126 might be attributable to the inhibition of an ERK-independent, MKK-2 signalling pathway. Indeed, this would explain recent data published by van der Bruggen *et al*. (1999) where PD 098059 was found to suppress LPS-induced TNFα

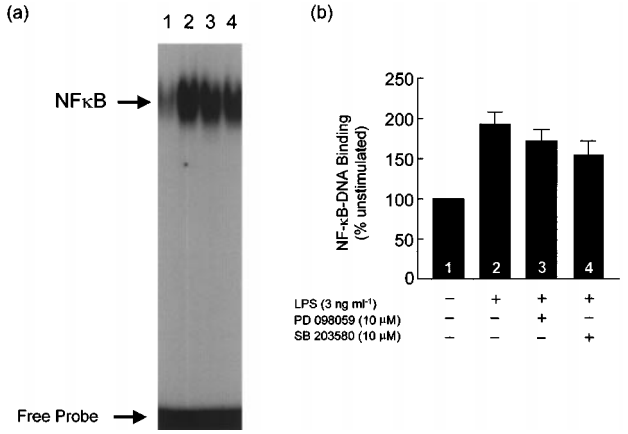

Figure 9 Lack of effect of PD 098059 and SB 203580 on LPS-induced NF-κB : DNA binding. Adherent monocytes were pre-treated (20 min) with PD 098059 (10 μM), SB 203580 (10 μM) or its vehicle and exposed to LPS (3 ng ml^{-1}) for 60 min. Nuclear extracts were prepared and subjected to EMSAs using a consensus NF-κB oligonucleotide probe. (a) shows an autoradiogram representative of nine independent experiments using monocytes from different donors. (b) The mean data ($\pm$ s.e.mean of 12 determinations) are given expressed as fold-change from the baseline level of NF-κB : DNA binding. Key: lane/bar 1, unstimulated control; lane/bar 2, LPS; lane/bar 3, LPS + PD 098059; lane/bar 4, LPS + SB 203580.

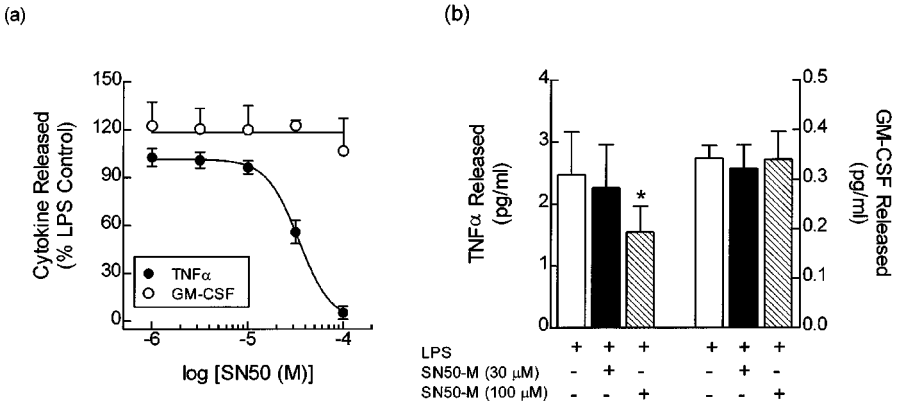

Figure 8 Effect of SN50/SN50-M on LPS-induced cytokine generation. Adherent monocytes were pre-treated (20 min) with varying concentrations SN50 (1 – 100 μM, (a) or SN50-M (30 and 100 μM, (b) and exposed to LPS (3 ng ml^{-1}). The amount of GM-CSF and TNFα released into the culture supernatant at 18 h was measured by sandwich ELISAs. Data points and bars represent the mean $\pm$ s.e.mean of six (SN50) and three (SN50-M) determinations using monocytes from different donors. *$P < 0.05$, significant inhibition of TNFα production.

generation from human monocytes only at a concentration (50 μM) that approaches the IC_{50} for the inhibition of other kinases such as MKK-2 (Alessi *et al*., 1995). Alternatively, at concentrations of U-0126 (1–10 μM) that profoundly attenuate TNFα production (Scherle *et al*., 1998), a significant inhibition ($\sim$5–35%) of other kinases can be expected including MKK-3 (IC_{50} = 38 μM) and MKK-6 (IC_{50} = 20 μM) (Favata *et al*., 1998). Knowledge of this loss in enzyme selectively is important because MKK-3 and MKK-6 lie upstream of p38 MAP kinase and would have a significant impact upon TNFα expression. Collectively, the results of the present investigation are consistent with LPS-induced TNFα gene expression being dependent, predominantly, on the activation of p38 MAP kinase whereas the formation of GM-CSF requires the co-operative activation of the p38 MAP kinase and MKK-1 signalling cascades. A similar conclusion was recently reached by Hoffmeyer *et al*. (1999) who found that p38 MAP kinase-, MKK-1- and JNK-signalling pathways co-ordinately regulate the TNFα gene in a human T-lymphocyte cell line. The results of that study are intriguing since they are contrary to what is established in other TNFα-producing cells, including human monocytes, and indicate that gene regulation is cell-type-specific. Thus, the ability of a single gene to be controlled by several, parallel signal transduction pathways implies a level of complexity that allows expression to be fine-tuned in response to the nature, duration and intensity of external stimuli.

In many LPS-sensitive cells including monocytes, TNFα generation appears to be regulated by the transcription factor NF-κB (Cordle *et al*., 1993; Mackman *et al*., 1991; Muller *et al*., 1993; Vincenti *et al*., 1992; Vallejo *et al*., 2000; Ziegler-Heitbrock *et al*., 1993). Given that the MAP kinase signalling pathways differed in their ability to regulate TNFα and GM-CSF expression in LPS-stimulated human monocytes, further studies were performed to determine if NF-κB is also involved in LPS-induced GM-CSF biosynthesis. Consistent with previous reports, LPS promoted a time-dependent activation of NF-κB. The specificity of activation was confirmed by the finding that NF-κB : DNA binding complexes were super-shifted with antibodies against the p50 and p65 subunits of NF-κB. Furthermore, the synthetic cell permeable peptide SN-50, which has been shown to specifically antagonize the nuclear translocation of NF-κB in a variety of diverse cells including endothelial LE II cells, fibroblasts and THP-1 cells (Lin *et al*., 1995; Xu *et al*., 1998), blocked the binding of NF-κB to DNA in LPS-stimulated human monocytes indicating cross-reactivity between species. Despite these results, NF-κB did not regulate GM-CSF expression as SN50, at a concentration that abolished LPS-induced NF-κB : DNA binding, failed to influence GM-CSF release, under conditions where the elaboration of TNFα was significantly inhibited. The inactive control peptide, SN50-M, also suppressed TNFα generation although on a molar basis it was much less effective than SN50. This property of SN-50M, which has been reported to affect other NF-κB-regulated processes including constitutive apoptosis of human neutrophils (Ward *et al*., 1999), is only seen at high (>30 μM) concentrations (this study; Ward *et al*., 1999 *c.f*. Kolenko *et al*., 1999; Maggirwar *et al*., 1998; Xu *et al*., 1998), and EMSAs suggest that this is due to weak antagonism of NF-κB : DNA binding. Regardless of the mechanism of action of SN50-M, the unambiguous findings that SN50 did not affect GM-CSF release under conditions where it abolished NF-κB : DNA binding provides compelling evidence that these events are unrelated.

The observation that NF-κB : DNA binding was unaffected by SB 203580 in LPS-stimulated human monocytes underscores the thesis that p38 MAP kinase does not inhibit κB-driven transcription by blocking the translocation of NF-κB to the nucleus (Beyaert *et al*., 1996; Vallejo *et al*., 2000; Wesselborg *et al*., 1997). However, Chen & Wang (1999) recently documented that SB 203580 prevented the induction of inducible nitric oxide synthetase by LPS in RAW 264.7 macrophages by reducing the formation of NF-κB : DNA complexes. The reason for these conflicting results is unclear, but may reflect cell-specific regulation of κB-sensitive genes by p38 MAP kinase.

A consistent finding of these experiments was the constitutive expression of NF-κB in the nucleus of unstimulated monocytes. Nuclear localization of NF-κB has been reported previously in monocytic cells, antibody-producing B-lymphocytes and human T-lymphocytes (Frankenberger *et al*., 1994; Haas *et al*., 1990; Kolenko *et al*., 1999; Vallejo *et al*., 2000; Wang *et al*., 1995), although its significance is unresolved. Previous investigations have eliminated cell differentiation and inadvertent contamination of the cultures with LPS (Frankenberger *et al*., 1994) and our results are consistent with the latter conclusion as spontaneous release of TNFα and GM-CSF was not normally detected. However, human monocytes are exquisitely sensitive to LPS and it is possible that the cultures were contaminated with trace amounts of LPS that activated NF-κB but not cytokine genes. Indeed, the FCS used in the present studies contained a low (52 pg ml^{-1}), but potentially biologically-active, concentration of endotoxin. Alternatively, the adhesion of monocytes to the culture plates or the tonic release an autocrine factor(s) equally could activate NF-κB. It has also been proposed that constitutive κB binding at the nucleus may represent a survival mechanism by protecting the cell against apoptosis (Kolenko *et al*., 1999).

In conclusion, the results of the present study suggest that LPS-induced GM-CSF generation from human peripheral blood monocytes requires the activation of p38 MAP kinase- and MKK-1-dependent signalling pathways that act in concert. However, unlike the regulation TNFα expression, no evidence was obtained implicating NF-κB in this response.

The work was supported by GlaxoWellcome, the Wellcome Trust (Grant no. 056814) and the National Asthma Campaign (U.K.).

References

ALESSI, D.R., CUENDA, A., COHEN, P., DUDLEY, D.T. & SALTIEL, A.R. (1995). PD 098059 is a specific inhibitor of the activation of mitogen-activated protein kinase kinase *in vitro* and *in vivo*. *J. Biol. Chem.,* **270,** 27489–27494.

BEYAERT, R., CUENDA, A., VANDEN BERGHE, W., PLAISANCE, S., LEE, J.C., HAEGEMAN, G., COHEN, P. & FIERS, W. (1996). The p38/RK mitogen-activated protein kinase pathway regulates interleukin-6 synthesis response to tumor necrosis factor. *EMBO. J.,* **15,** 1914–1923.

BLANCHARD, D.K., MICHELINI-NORRIS, M.B., PEARSON, C.A., McMILLEN, S. & DJEU, J.Y. (1991). Production of granulocyte-macrophage colony-stimulating factor (GM-CSF) by monocytes and large granular lymphocytes stimulated with *Mycobacterium avium* – M. intracellulare: activation of bactericidal activity by GM-CSF. *Infect. Immun.,* **59,** 2396–2402.

BOHJANEN, P.R., OKAJIMA, M. & HODES, R.J. (1990). Differential regulation of interleukin 4 and interleukin 5 gene expression: a comparison of T-cell gene induction by anti-CD3 antibody or by exogenous lymphokines. *Proc. Natl. Acad. Sci. U.S.A.,* **87,** 5283 – 5287.

BRACH, M.A., DEVOS, S., GRUSS, H.J. & HERRMANN, F. (1992). Prolongation of survival of human polymorphonuclear neutrophils by granulocyte-macrophage colony-stimulating factor is caused by inhibition of programmed cell death. *Blood,* **80,** 2920 – 2924.

BROIDE, D.H., GLEICH, G.J., CUOMO, A.J., COBURN, D.A., FEDERMAN, E.C., SCHWARTZ, L.B. & WASSERMAN, S.I. (1991). Evidence of ongoing mast cell and eosinophil degranulation in symptomatic asthma airway. *J. Allergy Clin. Immunol.,* **88,** 637 – 648.

BROIDE, D.H., LOTZ, M., CUOMO, A.J., COBURN, D.A., FEDERMAN, E.C. & WASSERMAN, S.I. (1992b). Cytokines in symptomatic asthma airways. *J. Allergy Clin. Immunol.,* **89,** 958 – 967.

BROIDE, D.H., PAINE, M.M. & FIRESTEIN, G.S. (1992a). Eosinophils express interleukin 5 and granulocyte macrophage-colony-stimulating factor mRNA at sites of allergic inflammation in asthmatics. *J. Clin. Invest.,* **90,** 1414 – 1424.

BROWN, P.H., CROMPTON, G.K. & GREENING, A.P. (1991). Proinflammatory cytokines in acute asthma. *Lancet.,* **338,** 590 – 593.

BUSSOLINO, F., WANG, J.M., DEFILIPPI, P., TURRINI, F., SANAVIO, F., EDGELL, C.J., AGLIETTA, M., ARESE, P. & MANTOVANI, A. (1989). Granulocyte and granulocyte-macrophage colony-stimulating factors induce human endothelial cells to migrate and proliferate. *Nature.,* **337,** 471 – 473.

CHEN, C.C. & WANG, J.K. (1999). p38 but not p44/42 mitogen-activated protein kinase is required for nitric oxide synthase induction mediated by lipopolysaccharide in RAW 264.7 macrophages. *Mol. Pharmacol.,* **55,** 481 – 488.

CORDLE, S.R., DONALD, R., READ, M.A. & HAWIGER, J. (1993). Lipopolysaccharide induces phosphorylation of MAD3 and activation of c-Rel and related NF-κB proteins in human monocytic THP-1 cells. *J. Biol. Chem.,* **268,** 11803 – 11810.

DAVIES, R.J., WANG, J.H., TRIGG, C.J. & DEVALIA, J.L. (1995). Expression of granulocyte/macrophage-colony-stimulating factor, interleukin-8 and RANTES in the bronchial epithelium of mild asthmatics is down-regulated by inhaled beclomethasone dipropionate. *Int. Arch. Allergy Immunol.,* **107,** 428 – 429.

DEAN, J.L., BROOK, M., CLARK, A.R. & SAKLATVALA, J. (1999). p38 mitogen-activated protein kinase regulates cyclooxygenase-2 mRNA stability and transcription in lipopolysaccharide-treated human monocytes. *J. Biol. Chem.,* **274,** 264 – 269.

DETMERS, P.A., ZHOU, D., POLIZZI, E., THIERINGER, R., HANLON, W.A., VAIDYA, S. & BANSAL, V. (1998). Role of stress-activated mitogen-activated protein kinase p38 in β2-integrin-dependent neutrophil adhesion and the adhesion-dependent oxidative burst. *J. Immunol.,* **161,** 1921 – 1929.

FAVATA, M.F., HORIUCHI, K.Y., MANOS, E.J., DAULERIO, A.J., STRADLEY, D.A., FEESER, W.S., VAN DYK, D.E., PITTS, W.J., EARL, R.A., HOBBS, F., COPELAND, R.A., MAGOLDA, R.L., SCHERLE, P.A. & TRZASKOS, J.M. (1998). Identification of a novel inhibitor of mitogen-activated protein kinase kinase. *J. Biol. Chem.,* **273,** 18623 – 18632.

FOEY, A.D., PARRY, S.L., WILLIAMS, L.M., FELDMANN, M., FOXWELL, B.M. & BRENNAN, F.M. (1998). Regulation of monocyte IL-10 synthesis by endogenous IL-1 and TNF-α: role of the p38 and p42/44 mitogen-activated protein kinases. *J. Immunol.,* **160,** 920 – 928.

FRANKENBERGER, M., PFORTE, A., STERNSDORF, T., PASSLICK, B., BAEUERLE, P.A. & ZIEGLER HEITBROCK, H.W. (1994). Constitutive nuclear NF-κB in cells of the monocyte lineage. *Biochem. J.,* **304,** 87 – 94.

GRIFFIN, G.E., LEUNG, K., FOLKS, T.M., KUNKEL, S. & NABEL, G.J. (1989). Activation of HIV gene expression during monocyte differentiation by induction of NF-κB. *Nature.,* **339,** 70 – 73.

HAAS, J.G., BAEUERLE, P.A., RIETHMULLER, G. & ZIEGLER-HEITBROCK, H.W. (1990). Molecular mechanisms in down-regulation of tumor necrosis factor expression. *Proc. Natl. Acad. Sci. U.S.A.,* **87,** 9563 – 9567.

HALLSWORTH, M.P., SOH, C.P., LANE, S.J., ARM, J.P. & LEE, T.H. (1994). Selective enhancement of GM-CSF, TNFα, IL-1β and IL-8 production by monocytes and macrophages of asthmatic subjects. *Eur. Respir. J.,* **7,** 1096 – 1102.

HIRST, S.J., BARNES, P.J. & TWORT, C.H.C. (1992). Quantifying proliferation of cultured human and rabbit airway smooth muscle cells in response to serum and platelet-derived growth factor. *Am. J. Respir. Cell. Mol. Biol.,* **7,** 574 – 581.

HOFFMEYER, A., GROSSE-WILDE, A., FLORY, E., NEUFELD, B., KUNZ, M., RAPP, U.R. & LUDWIG, S. (1999). Different mitogen-activated protein kinase signaling pathways cooperate to regulate tumor necrosis factor-α gene expression in T lymphocytes. *J. Biol. Chem.,* **274,** 4319 – 4327.

JENKINS, F., COCKERILL, P.N., BOHMANN, D. & SHANNON, M.F. (1995). Multiple signals are required for function of the human granulocyte/macrophage colony-stimulating factor gene promoter in T cells. *J. Immunol.,* **155,** 1240 – 1251.

KANKAANRANTA, H., DE SOUZA, P.M., SALMON, M., BARNES, P.J., GIEMBYCZ, M.A. & LINDSAY, M.A. (1999). SB 203580, an inhibitor of p38 mitogen-activated protein kinase, enhances constitutive apoptosis of human eosinophils. *J. Pharmacol. Exp. Ther.,* **290,** 621 – 628.

KAUFMAN, P.A., WEINBERG, J.B. & GREENE, W.C. (1992). Nuclear expression of the 50- and 65-kD Rel-related subunits of nuclear factor-κB is differentially regulated in human monocytic cells. *J. Clin. Invest.,* **90,** 121 – 129.

KOLENKO, V., BLOOM, T., RAYMAN, P., BUKOWSKI, R., HSI, E. & FINKE, J. (1999). Inhibition of NF-κB activity in human T lymphocytes induces caspase-dependent apoptosis without detectable activation of caspase-1 and -3. *J. Immunol.,* **163,** 590 – 598.

KUMAR, S., McDONNELL, P.C., GUM, R.J., HAND, A.T., LEE, J.C. & YOUNG, P.R. (1997). Novel homologues of CSBP/p38 MAP kinase: activation, substrate specificity and sensitivity to inhibition by pyridinyl imidazoles. *Biochem. Biophys. Res. Commun.,* **235,** 533 – 538.

LAI, C.K., HO, S.S., CHAN, C.H., LEUNG, R. & LAI, K.N. (1996). Gene expression of interleukin-3 and granulocyte macrophage colony-stimulating factor in circulating $CD4^+$ T cells in acute severe asthma. *Clin. Exp. Allergy.,* **26,** 138 – 146.

LEE, J.C., LAYDON, J.T., McDONNELL, P.C., GALLAGHER, T.F., KUMAR, S., GREEN, D., McNULTY, D., BLUMENTHAL, M.J., HEYS, J.R., LANDVATTER, S.W., STRICKLER, J.E., McLAUGHLIN, M.M., SIEMENS, I.R., FISHER, S.M., LIVI, G.P., WHITE, J.R., ADAMS, J.L. & YOUNG, P.R. (1994). A protein kinase involved in the regulation of inflammatory cytokine biosynthesis. *Nature.,* **372,** 739 – 746.

LEI, X.F., OHKAWARA, Y., STAMPFLI, M.R., GAULDIE, J., CROITORU, K., JORDANA, M. & XING, Z. (1998). Compartmentalized transgene expression of granulocyte-macrophage colony-stimulating factor (GM-CSF) in mouse lung enhances allergic airways inflammation. *Clin. Exp. Immunol.,* **113,** 157 – 165.

LIN, Y.Z., YAO, S.Y., VEACH, R.A., TORGERSON, T.R. & HAWIGER, J. (1995). Inhibition of nuclear translocation of transcription factor NF-κB by a synthetic peptide containing a cell membrane-permeable motif and nuclear localization sequence. *J. Biol. Chem.,* **270,** 14255 – 14258.

LIU, M.K., HERRERA-VELIT, P., BROWNSEY, R.W. & REINER, N.E. (1994). CD14-dependent activation of protein kinase C and mitogen-activated protein kinases (p42 and p44) in human monocytes treated with bacterial lipopolysaccharide. *J. Immunol.,* **153,** 2642 – 2652.

LOPEZ, A.F., WILLIAMSON, D.J., GAMBLE, J.R., BEGLEY, C.G., HARLAN, J.M., KLEBANOFF, S.J., WALTERSDORPH, A., WONG, G., CLARK, S.C. & VADAS, M.A. (1986). Recombinant human granulocyte-macrophage colony-stimulating factor stimulates *in vitro* mature human neutrophil and eosinophil function, surface receptor expression, and survival. *J. Clin. Invest.,* **78,** 1220 – 1228.

MACKMAN, N., BRAND, K. & EDGINGTON, T.S. (1991). Lipopolysaccharide-mediated transcriptional activation of the human tissue factor gene in THP-1 monocytic cells requires both activator protein 1 and nuclear factor κB binding sites. *J. Exp. Med.,* **174,** 1517 – 1526.

MAGGIRWAR, S.B., SARMIERE, P.D., DEWHURST, S. & FREEMAN, R.S. (1998). Nerve growth factor-dependent activation of NF-κB contributes to survival of sympathetic neurons. *J. Neurosci.,* **18,** 10356 – 10365.

MAIER, J.A., HLA, T. & MACIAG, T. (1990). Cyclooxygenase is an immediate-early gene induced by interleukin-1 in human endothelial cells. *J. Biol. Chem.,* **265,** 10805 – 10808.

MIYATAKE, S., SHLOMAI, J., ARAI, K. & ARAI, N. (1991). Characterization of the mouse granulocyte-macrophage colony-stimulating factor (GM-CSF) gene promoter: nuclear factors that interact with an element shared by three lymphokine genes - those for GM-CSF, interleukin-4 (IL-4), and IL-5. *Mol.Cell Biol.,* **11,** 5894 – 5901.

MULLER, J.M., ZIEGLER HEITBROCK, H.W. & BAEUERLE, P.A. (1993). Nuclear factor κB, a mediator of lipopolysaccharide effects. *Immunobiol.,* **187,** 233 – 256.

NAKAMURA, Y., OZAKI, T., KAMEI, T., KAWAJI, K., BANNO, K., MIKI, S., FUJISAWA, K., YASUOKA, S. & OGURA, T. (1993). Factors that stimulate the proliferation and survival of eosinophils in eosinophilic pleural effusion: relationship to granulocyte/macrophage colony-stimulating factor, interleukin-5, and interleukin-3. *Am. J. Respir. Cell Mol. Biol.,* **8,** 605 – 611.

OSBORN, L., KUNKEL, S. & NABEL, G.J. (1989). Tumor necrosis factor-α and interleukin-1 stimulate the human immunodeficiency virus enhancer by activation of the nuclear factor κB. *Proc. Natl. Acad. Sci. U.S.A.,* **86,** 2336 – 2340.

PARK, C.S., CHOI, Y.S., KI, S.Y., MOON, S.H., JEONG, S.W., UH, S.T. & KIM, Y.H. (1998). Granulocyte macrophage colony-stimulating factor is the main cytokine enhancing survival of eosinophils in asthmatic airways. *Eur. Respir. J.,* **12,** 872 – 878.

RIDLEY, S.H., SARSFIELD, S.J., LEE, J.C., BIGG, H.F., CAWSTON, T.E., TAYLOR, D.J., DEWITT, D.L. & SAKLATVALA, J. (1997). Actions of IL-1 are selectively controlled by p38 mitogen-activated protein kinase: regulation of prostaglandin H synthase-2, metalloproteinases, and IL-6 at different levels. *J. Immunol.,* **158,** 3165 – 3173.

SALLERFORS, B. & OLOFSSON, T. (1992). Granulocyte-macrophage colony-stimulating factor (GM-CSF) and granulocyte colony-stimulating factor (G-CSF) secretion by adherent monocytes measured by quantitative immunoassays. *Eur. J. Haematol.,* **49,** 199 – 207.

SCHAFER, P.H., WANG, L., WADSWORTH, S.A., DAVIS, J.E. & SIEKIERKA, J.J. (1999). T cell activation signals up-regulate p38 mitogen-activated protein kinase activity and induce TNFα production in a manner distinct from LPS activation of monocytes. *J. Immunol.,* **162,** 659 – 668.

SCHERLE, P.A., JONES, E.A., FAVATA, M.F., DAULERIO, A.J., COVINGTON, M.B., NURNBERG, S.A., MAGOLDA, R.L. & TRZASKOS, J.M. (1998). Inhibition of MAP kinase kinase prevents cytokine and prostaglandin E_2 production in lipopolysaccharide-stimulated monocytes. *J. Immunol.,* **161,** 5681 – 5686.

SCHRECK, R. & BAEUERLE, P.A. (1990). NF-κB as inducible transcriptional activator of the granulocyte/macrophage colony-stimulating factor gene. *Mol. Cell Biol.,* **10,** 1281 – 1286.

SELDON, P.M., STEVENS, D.A., ADCOCK, I.M., O'CONNOR, B.J., BARNES, P.J. & GIEMBYCZ, M.A. (1998). Albuterol does not antagonize the inhibitory effect of dexamethasone on monocyte cytokine release. *Am. J. Respir. Crit. Care Med.,* **157,** 803 – 809.

SHANNON, M.F., COLES, L.S., VADAS, M.A. & COCKERILL, P.N. (1997). Signals for activation of the GM-CSF promoter and enhancer in T cells. *Crit. Rev. Immunol.,* **17,** 301 – 323.

SILBERSTEIN, D.S., OWEN, W.F., GASSON, J.C., DIPERSIO, J.F., GOLDE, D.W., BINA, J.C., SOBERMAN, R., AUSTEN, K.F. & DAVID, J.R. (1986). Enhancement of human eosinophil cytotoxicity and leukotriene synthesis by biosynthetic (recombinant) granulocyte-macrophage colony-stimulating factor. *J. Immunol.,* **137,** 3290 – 3294.

SOLOMON, K.R., KURT-JONES, E.A., SALADINO, R.A., STACK, A.M., DUNN, I.F., FERRETTI, M., GOLENBOCK, D., FLEISHER, G.R. & FINBERG, R.W. (1998). Heterotrimeric G proteins physically associated with the lipopolysaccharide receptor CD14 modulate both *in vivo* and *in vitro* responses to lipopolysaccharide. *J. Clin. Invest.,* **102,** 2019 – 2027.

SOUSA, A.R., POSTON, R.N., LANE, S.J., NAKHOSTEEN, J.A. & LEE, T.H. (1993). Detection of GM-CSF in asthmatic bronchial epithelium and decrease by inhaled corticosteroids. *Am. Rev. Respir. Dis.,* **147,** 1557 – 1561.

THORENS, B., MERMOD, J.J. & VASSALLI, P. (1987). Phagocytosis and inflammatory stimuli induce GM-CSF mRNA in macrophages through post-transcriptional regulation. *Cell.,* **48,** 671 – 679.

TILL, S., LI, B., DURHAM, S., HUMBERT, M., ASSOUFI, B., HUSTON, D., DICKASON, R., JEANNIN, P., KAY, A.B. & CORRIGAN, C. (1995). Secretion of the eosinophil-active cytokines interleukin-5, granulocyte/macrophage colony-stimulating factor and interleukin-3 by bronchoalveolar lavage $CD4^+$ and $CD8^+$ T cell lines in atopic asthmatics, and atopic and non-atopic controls. *Eur. J. Immunol.,* **25,** 2727 – 2731.

TSUBOI, A., SUGIMOTO, K., YODOI, J., MIYATAKE, S., ARAI, K. & ARAI, N. (1991). A nuclear factor NF-GM2 that interacts with a regulatory region of the GM-CSF gene essential for its induction in responses to T- cell activation: purification from human T-cell leukemia line Jurkat cells and similarity to NF-κB. *Int. Immunol.,* **3,** 807 – 817.

VALLEJO, J.G., KNUEFERMANN, P., MANN, D.L. & SIVASUBRAMANIAN, N. (2000). Group B *Streptococcus* induces TNF-α gene expression and activation of the transcription factors NF-κB and activator protein-1 in human cord blood monocytes. *J. Immunol.,* **165,** 419 – 425.

VAN DER BRUGGEN, T., NIJENHUIS, S., VAN RAAIJ, E., VERHOEF, J. & VAN ASBECK, B.S. (1999). Lipopolysaccharide-induced tumor necrosis factor alpha production by human monocytes involves the Raf-1/MEK1-MEK2/ERK1-ERK2 pathway. *Infect. Immun.,* **67,** 3824 – 3829.

VINCENTI, M.P., BURRELL, T.A. & TAFFET, S.M. (1992). Regulation of NF-κB activity in murine macrophages: effect of bacterial lipopolysaccharide and phorbol ester. *J. Cell Physiol.,* **150,** 204 – 213.

WADSWORTH, S.A., CAVENDER, D.E., BEERS, S.A., LALAN, P., SCHAFER, P.H., MALLOY, E.A., WU, W., FAHMY, B., OLINI, G.C., DAVIS, J.E., PELLEGRINO-GENSEY, J.L., WACHTER, M.P. & SIEKIERKA, J.J. (1999). RWJ 67657, a potent, orally-active inhibitor of p38 mitogen-activated protein kinase. *J. Pharmacol. Exp. Ther.,* **291,** 680 – 687.

WANG, P., WU, P., SIEGEL, M.I., EGAN, R.W. & BILLAH, M.M. (1995). Interleukin (IL)-10 inhibits nuclear factor (B (NFκB) activation in human monocytes. IL-10 and IL-4 suppress cytokine synthesis by different mechanisms. *J. Biol. Chem.,* **270,** 9558 – 9563.

WARD, C., CHILVERS, E.R., LAWSON, M.F., PRYDE, J.G., FUJIHARA, S., FARROW, S.N., HASLETT, C. & ROSSI, A.G. (1999). NF-κB activation is a critical regulator of human granulocyte apoptosis in vitro. *J. Biol. Chem.,* **274,** 4309 – 4318.

WESSELBORG, S., BAUER, M.K.A., VOGT, M., SCHMITZ, M.L., SCHULZE-OSTHOFF, K. (1997). Activation of transcription factor NF-κB and p38 mitogen-activated protein kinase is mediated by distinct and separate stress effector pathways. *J. Biol. Chem.,* **272,** 12422 – 12429.

WOOLLEY, K.L., ADELROTH, E., WOOLLEY, M.J., ELLIS, R., JORDANA, M. & O'BYRNE, P.M. (1995). Effects of allergen challenge on eosinophils, eosinophil cationic protein, and granulocyte-macrophage colony-stimulating factor in mild asthma. *Am. J. Respir. Crit. Care Med.,* **151,** 1915 – 1924.

XING, Z., OHKAWARA, Y., JORDANA, M., GRAHAM, F. & GAULDIE, J. (1996). Transfer of granulocyte-macrophage colony-stimulating factor gene to rat lung induces eosinophilia, monocytosis, and fibrotic reactions. *J. Clin. Invest.,* **97,** 1102 – 1110.

XU, J., ZUTTER, M.M., SANTORO, S.A. & CLARK, R.A. (1998). A three-dimensional collagen lattice activates NF-κB in human fibroblasts: role in integrin α_2 gene expression and tissue remodeling. *J. Cell. Biol.,* **140,** 709 – 719.

ZIEGLER-HEITBROCK, H.W., STERNSDORF, T., LIESE, J., BELOHRADSKY, B., WEBER, C., WEDEL, A., SCHRECK, R., BAUERLE, P. & STROBEL, M. (1993). Pyrrolidine dithiocarbamate inhibits NF-κB mobilization and TNF production in human monocytes. *J. Immunol.,* **151,** 6986 – 6993.

(Received March 29, 2000
Revised August 16, 2000
Accepted August 31, 2000)