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RESEARCH PAPER

Azithromycin distinctively modulates classical activation of human monocytes *in vitro*

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azithromycin; dexamethasone; roflumilast; SB203580; human monocyte; human macrophage; classical activation; anti-inflammatory drug; lysosome affecting agent; TLR4 signalling

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BACKGROUND AND PURPOSE
Azithromycin has been reported to modify activation of macrophages towards the M2 phenotype. Here, we have sought to identify the mechanisms underlying this modulatory effect of azithromycin on human monocytes, classically activated in vitro.

EXPERIMENTAL APPROACH
Human blood monocytes were primed with IFN-γ for 24 h and activated with LPS for 24 h. Azithromycin, anti-inflammatory and lysosome-affecting agents were added 2 h before IFN-γ. Cytokine and chemokine expression was determined by quantitative PCR and protein release by ELISA. Signalling molecules were determined by Western blotting and transcription factor activation quantified with a DNA-binding ELISA kit.

KEY RESULTS
Azithromycin (1.5–50 μM) dose-dependently inhibited gene expression and/or release of M1 macrophage markers (CCR7, CXCL 11 and IL-12p70), but enhanced CCL2, without altering TNF-α or IL-6. Azithromycin also enhanced the gene expression and/or release of M2 macrophage markers (IL-10 and CCL18), and the pan-monocyte marker CD163, but inhibited that of CCL22. The Toll-like receptor (TLR) 4 signalling pathway was modulated, down-regulating NF-κB and STAT1 transcription factors. The inhibitory profile of azithromycin differed from that of dexamethasone, the phosphodiesterase-4 inhibitor roflumilast and the p38 kinase inhibitor SB203580 but was similar to that of the lysosomotropic drug chloroquine. Effects of concanamycin and NH4Cl, which also act on lysosomes, differed significantly.

CONCLUSIONS AND IMPLICATIONS
Azithromycin modulated classical activation of human monocytes by inhibition of TLR4-mediated signalling and possible effects on lysosomal function, and generated a mediator expression profile that differs from that of monocyte/macrophage phenotypes so far described.

Abbreviations

AK, adenylate kinase; AP-1, activator protein-1; ELISA, enzyme-linked immunosorbent assay; ERK, extracellular signal-regulated kinase; ICSBP, interferon consensus sequence-binding protein; IKK, IκB kinase; IκB, inhibitor of NF-κB; IRAK, interleukin-1 receptor–associated kinase; M1 macrophage, classically activated macrophage; M2 macrophage, alternatively activated macrophage; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase; NFAT, nuclear factor of activated T cells; NK cells, natural killer cells; PBMC, peripheral blood mononuclear cells; PDE, phosphodiesterase; PDK, 3-phosphoinositide-dependent protein kinase; PLA, phospholipase A;SR, scavenger receptor; STAT, signal transducer and activator of transcription; TLR, Toll-like receptor; TNF, tumour necrosis factor; V-ATPase, vacuolar-type H^+-ATPase

Introduction

Blood monocytes are a crucial source of new mononuclear phagocytes in tissues, where they contribute to both innate and adaptive immune responses. There is increasing awareness of the functional plasticity of the mononuclear phagocyte system. In addition to morphological distinctions between circulating monocytes, tissue macrophages and dendritic cells, it is now clear that they can be committed to a variety of different functions depending on the particular inflammatory or infectious milieu (Gordon and Taylor, 2005; Ueno *et al.*, 2007). Monocyte-derived macrophages, for instance, can be polarized into two broad phenotypic groups. Classically activated macrophages mount crucial innate immune responses to pathogens. They can be induced by IFN-γ alone or together with bacterial LPS or by pro-inflammatory cytokines and are also called M1 cells (Benoit *et al.*, 2008). They characteristically generate high amounts of IL-12 and low amounts of IL-10 and express high levels of the chemokine receptor CCR7. Functionally, M1 cells are highly inflammatory and effective killer cells for microorganisms and tumour cells. Non-classically activated M2 cells, on the other hand, are induced by a variety of stimuli, including IL-4 or IL-13 (M2a), immune complexes (M2b), IL-10 and glucocorticoids (M2c) (Benoit *et al.*, 2008; Gordon and Martinez, 2010). They all exhibit low IL-12 and high IL-10 formation and express high levels of mannose receptors (CD 206) and in most cases, scavenger receptors (CD163). M2 cells are modulators of inflammatory responses. Although the activating stimuli and selective markers for these different macrophage phenotypes have been well studied, much less is known about their pharmacological modulation.

Azithromycin is an antibacterial agent that, together with other members of the macrolide group of antibiotics, has been shown, under a variety of experimental and clinical conditions, to exert anti-inflammatory or immunomodulatory actions (Čulić *et al.*, 2001; Gotfried, 2004; Shinkai *et al.*, 2008; Crosbie and Woodhead, 2009). Recently, it has become apparent that at least some of the immunomodulatory effects of the drug are due to its actions on macrophages. We have shown that the inhibitory effects of azithromycin on LPS-induced lung neutrophilia in mice can be explained by its inhibition of granulocyte macrophage colony-stimulating factor and IL-1β production by macrophages (Bosnar *et al.*, 2009). Like the corticosteroid dexamethasone, azithromycin has recently been shown to clear both neutrophils and macrophages from the zymosan-inflamed mouse peritoneum (Navarro-Xavier *et al.*, 2010). Other groups have reported that azithromycin is able to alter macrophage phenotype from the pro-inflammatory M1 towards the alternatively activated M2 cells (Hodge *et al.*, 2008; Murphy *et al.*, 2008). These studies were carried out with permanent cell lines or cells from chronic obstructive pulmonary disease (COPD) patients.

We have now studied the effects of azithromycin on human peripheral blood monocytes classically activated by IFN-γ and LPS, and we have compared them with those of known anti-inflammatory drugs. Changes in phenotype were assessed by determining the expression profile of a variety of inflammatory markers and by the release of soluble mediators. Previous studies have linked immunomodulatory actions of macrolides with changes in a variety of different molecular targets and signalling processes. These include actions on membrane lipids (Tyteca *et al.*, 2003; Munić *et al.*, 2011), activation of transcription factors (Shinkai *et al.*, 2008; Yamauchi *et al.*, 2009) and on lysosomes in which azithromycin accumulates (Van Bambeke *et al.*, 1996; Tyteca *et al.*, 2002, 2003; Munić *et al.*, 2011). Consequently, we have also investigated the potential involvement of cell signalling processes and lysosomes in the effects of azithromycin on classically activated human monocytes.

Methods

Cells

Blood samples were obtained from healthy donors at the Croatian Institute for Transfusional Medicine, who gave written assurance that they were not taking any medication, in compliance with ethical committee approval (1582/2007, 11.07.2007). Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coat layers obtained from healthy donors. Buffy coat layers were diluted with PBS (Sigma) and centrifuged at 400× *g* on Ficoll-Paque PLUS (GE Healthcare, Bio-Sciences AB, Uppsala, Sweden) for 35 min at room temperature. PBMCs were collected and washed in PBS. Any remaining erythrocytes were lysed in ammonium chloride buffer [0.15 M NH_4Cl (Kemika), 10 mM $NaHCO_3$ (Kemika), 1 mM Na_2EDTA (Sigma), pH 7.4] for 2 min, followed by centrifugation. Human monocytes were isolated from PBMCs on a magnetic separator, VarioMACS (Miltenyi Biotec), by negative selection using the Monocyte Isolation Kit II (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany).

Incubation conditions

Cells were seeded in 48-well plates at a concentration of 2.5×10^5 cells per well in 1 mL of RPMI medium 1640 (Gibco

Invitrogen, Carlsbad, CA, USA), supplemented with 10% FBS (Biowest, Nuaillé, France) and cultured at 37°C in 5% CO_2 and 90% humidity for 48 h. To generate classically activated M1 monocytes/macrophages, monocytes were incubated with IFN-γ (1 ng·mL^{-1}) for 24 h and then activated by the addition of LPS (1 ng·mL^{-1}) for 24 h.

Azithromycin (1.5–50 μM) was added 2 h prior to IFNγ and remained within the well for the whole 48 h incubation period. Other test compounds were added 2 h prior to IFN-γ in the following concentrations: dexamethasone, roflumilast, SB203580, indomethacin, 0.001–10 μM; chloroquine, 3–12 μM; concanamycin, 0.25–1 nM; and NH_4Cl, 5–20 mM. All cell culture experiments were performed on cells from at least two different donors with samples seeded in duplicate. In a preliminary experiment, the potential effects of agents affecting lysosomes were investigated for cytotoxicity and effects on lysosomal pH. The highest concentrations of these agents were not cytotoxic but still affected lysosomal pH in the monocytes.

ELISA

Concentrations of the chemokines CCL2, CCL5, CCL18, CCL22 and the cytokines IL-6, IL-10, IL-12p70 and TNF-α were determined in cell supernatants by sandwich ELISA using capture and detection antibodies according to the manufacturer's instructions. Optical density was measured at 450 nm using a microplate reader (SpectraMax 190; Molecular Devices, Sunnyvale, CA, USA). Concentrations of cytokines were determined by interpolation from standard curves with SoftMax Pro v4.3.1 software (Molecular Devices).

Cell viability

To eliminate the possibility that applied treatments were cytotoxic, release of the cytosolic enzyme, adenylate kinase (AK; ToxiLight; Lonza, Basel, Switzerland), into cell supernatants was used as a measure of necrosis (Benachour and Seralini, 2009; Heinrich *et al.*, 2009). None of the compounds used induced more than a 1.2-fold increase in AK release compared with respective control, unless stated otherwise.

Isolation of total RNA and cDNA synthesis

Total RNA was isolated using RNeasy Plus Mini kit (Qiagen, Valencia, CA, USA). Concentration and quality of isolated total RNA was determined on Agilent Technologies 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). First strand cDNA was synthesized using SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA).

Quantitative RT-PCR

Expression of various genes at the mRNA level was analyzed by quantitative real-time PCR on 7300 Real Time PCR System (Applied Biosystems, Carlsbad, CA, USA) using TaqMan method and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a housekeeping gene. Primers and probes were designed using Primer Express software v3.0 (Applied Biosystems) and used at validated concentrations (Table 1). To quantify expression, the comparative C_T method was applied, using the corresponding control cells [monocytes stimulated with IFNγ and LPS and treated with vehicle (solvent dimethyl sulphoxide)] as comparators. Expression of each gene was normalized to GAPDH expression and calculated relative to the control sample as fold change.

Western blotting of signalling molecules

Monocytes were incubated with IFN-γ (1 ng·mL^{-1}) with or without azithromycin (12.5–50 μM) for 19 h and then activated by the addition of LPS (1 ng·mL^{-1}) for 3 h. A preliminary time course study revealed that at 3 h after LPS, there was broad activation of transcription factors. Cells incubated only with IFN-γ for 22 h were used as negative controls. After 3 h incubation with LPS, cell samples were lysed for 30 min on ice with buffer containing 50 mM HEPES (Sigma), 150 mM NaCl (Kemika), 1% Triton X-100 (GE Healthcare), 1 μg·mL^{-1} leupeptin (Sigma), 2 μg·mL^{-1} aprotinin (Sigma), 1 μg·mL^{-1} pepstatin (Sigma), 17 μg·mL^{-1} PMSF (Sigma), 0.2 mM sodium orthovanadate (Sigma), 2 mM sodium fluoride (Sigma), 4 mM β-glycerophosphate (Sigma), 0.4 mM sodium pyrophosphate (Sigma), pH 7.4. The lysates were centrifuged at 14 000× *g* for 10 min at 4°C and supernatants were stored at –20°C

Lysates prepared in sample buffer for electrophoresis (Invitrogen) were separated by SDS-PAGE (NuPAGE; Invitrogen) for 50 min at 170 V. Proteins were transferred from gels to nitrocellulose membrane using SemiDry (Bio-Rad, Hercules, CA, USA) at 12.5 mA·cm^{-2} in transfer buffer (48 mM Tris, 38 mM glycine, 0.075 % SDS and 20 % methanol). Membranes were incubated in blocking buffer (2% non-fat milk in PBS with 0.02% Tween-20) for 60 min. Primary antibodies [to phospho-Akt (p-Akt), p-Jun, p-ERK 1/2, p-JNK, p-signal transducer and activator of transcription (STAT)1, p-STAT3, p-NF-κB-p65, p-PI3K), p-3-phosphoinositide-dependent protein kinase (PDK)1, p-p38, p-inhibitor of NF-κB (IκB)α, IκBα, p-IκB kinase (IKK) (Cell Signaling) and p-cPLA$_2$, IκBζ and p-NF-κB-p50 (Santa Cruz)] were added to the blocking buffer for 2 h at room temperature. Secondary antibodies labelled with horseradish peroxidase (Cell Signaling) were added and further incubated for 60 min in blocking buffer. Detection was carried out with the substrate for horseradish peroxidase (enhanced chemiluminescence, GE Healthcare) using actin as a control. Densitometric analysis of Western blots was performed using ImageQuant 5.2 software (GE Healthcare, Piscataway, NJ, USA).

Determination of transcription factor activation

Cells were seeded and treated as described earlier for Western blotting. Cells incubated only with IFN-γ for 24 h were used as negative controls. After 3 h incubation with LPS, and according to the manufacturer's instruction, nuclear fractions of human monocytes were prepared and activation of transcription factor NF-κBp65 was determined with a DNA-binding ELISA kit for detection and quantification of transcription factors from nuclear fractions (TransAM Transcription Factor ELISA; Active Motif, Carlsbad, CA, USA). The kit contains a 96-well plate with immobilized oligonucleotides specific for a particular transcription factor. Specifically bound transcription factors are detected using a primary antibody for a particular transcription factor and a secondary antibody conjugated with horseradish peroxidase. Absorbance was measured at 450 nm.

Table 1
Sequences and concentrations of primers and probes used for quantitative RT-PCR

Gene		Sequence	concentration (nM)
GAPDH	F	ACCCACTCCTCCACCTTTGAC	100
	R	CATACCAGGAAATGAGCTTGACAA	100
	P	CTGGCATTGCCCTCAACGACCA	100
CCL2	F	AAGCTGTGATCTTCAAGACCATTG	100
	R	CATGGAATCCTGAACCCACTTC	100
	P	CCAAGGAGATCTGTGCTGACCCCAA	100
CCL5	F	GCATCTGCCTCCCCATATTC	100
	R	AGTGGGCGGGCAATGTAG	100
	P	TCGGACACCACACCCTGCTGCT	100
CXCL11	F	TCCATAATGTACCCAAGTAACAACTGT	200
	R	TTCAACTTTTTTGATTATAAGCCTTGCT	200
	P	CAACGATGCCTAAATCCCAAATCGAAGC	100
IL-10	F	TGAGAACAGCTGCACCCACTT	100
	R	GCTGAAGGCATCTCGGAGAT	100
	P	CAGGCAACCTGCCTAACATGCTTCGA	100
CCR7	F	TTCAGTGGCATGCTCCTACTTC	100
	R	GCTGAGACAGCCTGGACGAT	100
	P	TTGCATCAGCATTGACCGCTACGTG	100
CD206	F	CGCTACTAGGCAATGCCAATG	100
	R	TCGTGCAATCTGCGTACCA	100
	P	AGCAACCTGTGCATTCCCGTTCAAGT	100
CD163	F	GCTGCAGTGAATTGCACAGATAT	100
	R	CGGGATGAGCGACCTGTT	100
	P	CAGTGCAGAAAACCCCACAAAAAGCCA	100
CCL18	F	GGTGTCATCCTCCTAACCAAGAGA	100
	R	GCTGATGTATTTCTGGACCCACTT	100
	P	CCGGCAGATCTGTGCTGACCCC	100
CCL22	F	TGCCGTGATTACGTCCGTTA	100
	R	TCTCCTTATCCCTGAAGGTTAGCA	100
	P	CCCCTGCGCGTGGTGAAACAC	100

Data analysis

Cytokine concentration was determined in pg·mL^{-1}. Owing to high variation between individual donors and to allow comparison with other markers, cytokine formation was calculated as mean fold changes, in comparison with IFN-γ/LPS control samples. All fold changes were $\log_{10}$ transformed, and submitted to ANOVA and Bonferroni post test analysis. Results are presented as mean values ± SEM of $\log_{10}$ transformed fold changes over IFN-γ/LPS-stimulated control values, represented by zero values. Activation of NF-κB was also presented as mean fold changes and analysed by ANOVA and Dunnett's post test analysis. Changes in response to azithromycin observed by Western blot were expressed as mean percentages of inhibition and statistical significance was determined by ANOVA and Dunnett's post test analysis. All changes were considered significant at the $P < 0.05$ level.

For $\log_{10}$-transformed mean fold changes in cytokine concentrations, determined over a range of azithromycin concentrations, regression coefficients for the slopes of the concentration-response curves were determined, and the significance of the difference in slope from zero was calculated.

All statistical tests were performed using GraphPadPrism version 5.03 for Windows (GraphPad Software, San Diego, CA, USA, http://www.graphpad.com).

Materials

Azithromycin dihydrate was purchased from PLIVA Inc. (Zagreb, Croatia), dexamethasone from Biosynth (Staad, Switzerland), SB203580 from Biomol (Lausen, Basel, Switzerland), roflumilast from Altana Pharma (Wessel, Germany), indomethacin, chloroquine and concanamycin A from Sigma-Aldrich (St. Louis, MO, USA) and NH_4Cl from Kemika (Zagreb, Croatia). IFN-γ, as well as antibodies for ELISA were purchased from R&D

Systems (Minneapolis, MN, USA). Bacterial LPS (from *Escherichia coli* 0111:B4) was purchased from Sigma (St. Louis, MO, USA). Antibodies for Western analysis were purchased from Cell Signaling (Danvers, MA, USA). All other reagents, if not indicated otherwise, were from Sigma Chemical Co.

Results

Azithromycin modulation of classically activated monocytes/macrophages

IFN-γ/LPS-treated human blood monocytes showed high expression of CCR7 and CXCL11 and released large amounts of IL-6, TNF-α, CCL2 and CCL5 protein into the culture medium (Table 2). Expression of CD163 and release of IL-10 and CCL18 were low.

Incubation with azithromycin for 48 h reversed, in a concentration-dependent manner, a number of the IFN-γ/LPS-induced changes. It inhibited the release and/or expression of IL-12p70 and CCL22 and decreased the enhanced expression of CXCL11 and CCR7 (Figure 1A and B). Azithromycin only reduced the expression of CD206 at the highest concentration used (Figure 1B). In parallel, azithromycin enhanced the release and expression of IL-10, CCL2 and CCL18, as well as the expression of the surface marker CD163 (Figure 1A and B). There was little or no effect on CCL5, TNF-α and IL-6 secretion (Figure 1A and B).

Effects on signalling pathways and transcription factor activation

Western blotting of classically activated monocytes incubated with azithromycin (12.5–50 μM) showed that after 3 h, phosphorylation of STAT1 (Y701), IKK (S176/180), NF-κBp65 (S536) and PI3K (Y199) were all inhibited by azithromycin (Figure 2A, B). Phosphorylation of NF-κBp65 by Western blot was examined in a single experiment because of technical issues, but direct determination of transcription factor activation revealed statistically significant down-regulation of NF-κBp65 activity (Figure 3). IKK and PI3K were both inhibited equally at all concentrations of azithromycin, whereas STAT1 and NF-κB were only significantly inhibited at 50 μM azithromycin. None of the other signalling molecules investigated showed reproducible changes in response to azithromycin (data not shown).

Comparison with known anti-inflammatory agents

The modulatory effects of azithromycin on selected cytokine and chemokine release by IFN-γ/LPS-activated monocytes were compared with those of other anti-inflammatory agents, namely the corticosteroid dexamethasone, the p38 kinase inhibitor SB203580 and the PDE-4 inhibitor roflumilast (Figure 4). The rationale for their selection was that corticosteroids are the gold standard for anti-inflammatory activity, and SB203580 and roflumilast are novel compounds, which, like azithromycin, have therapeutic potential for inhibiting respiratory inflammation. All tested agents inhibited the release of IL-12p70 in a parallel, concentration-dependent manner, with dexamethasone exhibiting the highest potency (Figure 3). Azithromycin was the only anti-inflammatory compound that did not exert a concentration-dependent inhibitory action on TNF-α release (Figure 4). Like azithromycin, dexamethasone and SB203580 also inhibited CCL22

Table 2

Mean baseline expression of markers and concentrations of cytokines released from unstimulated, negative control and IFN-γ/LPS-activated monocytes

Variable	Concentration ($pg \cdot mL^{-1}$) Negative control	IFN-γ/LPS	Fold change
Cytokines			
TNF-α	17 ± 9	1531 ± 573	131 ± 111
IL-6	150 ± 111	11728 ± 816	104 ± 53
IL-12p70	18 ± 8	434 ± 225	26 ± 16
IL-10	4 ± 4	63 ± 41	32 ± 25
CCL2	190 ± 75	2936 ± 901	18 ± 12
CCL5	67 ± 22	2303 ± 992	34 ± 7
CCL18	45 ± 18	56 ± 11	1 ± 0
CCL22	223 ± 122	867 ± 433	5 ± 3
mRNA expression			
CCR7			91 ± 25
CD163			0.02 ± 0.0
CD206			0.12 ± 0.06
CXCL11			155 ± 10

Fold changes are expressed in relation to unstimulated cells.
Data are means ± SEM from three donors.

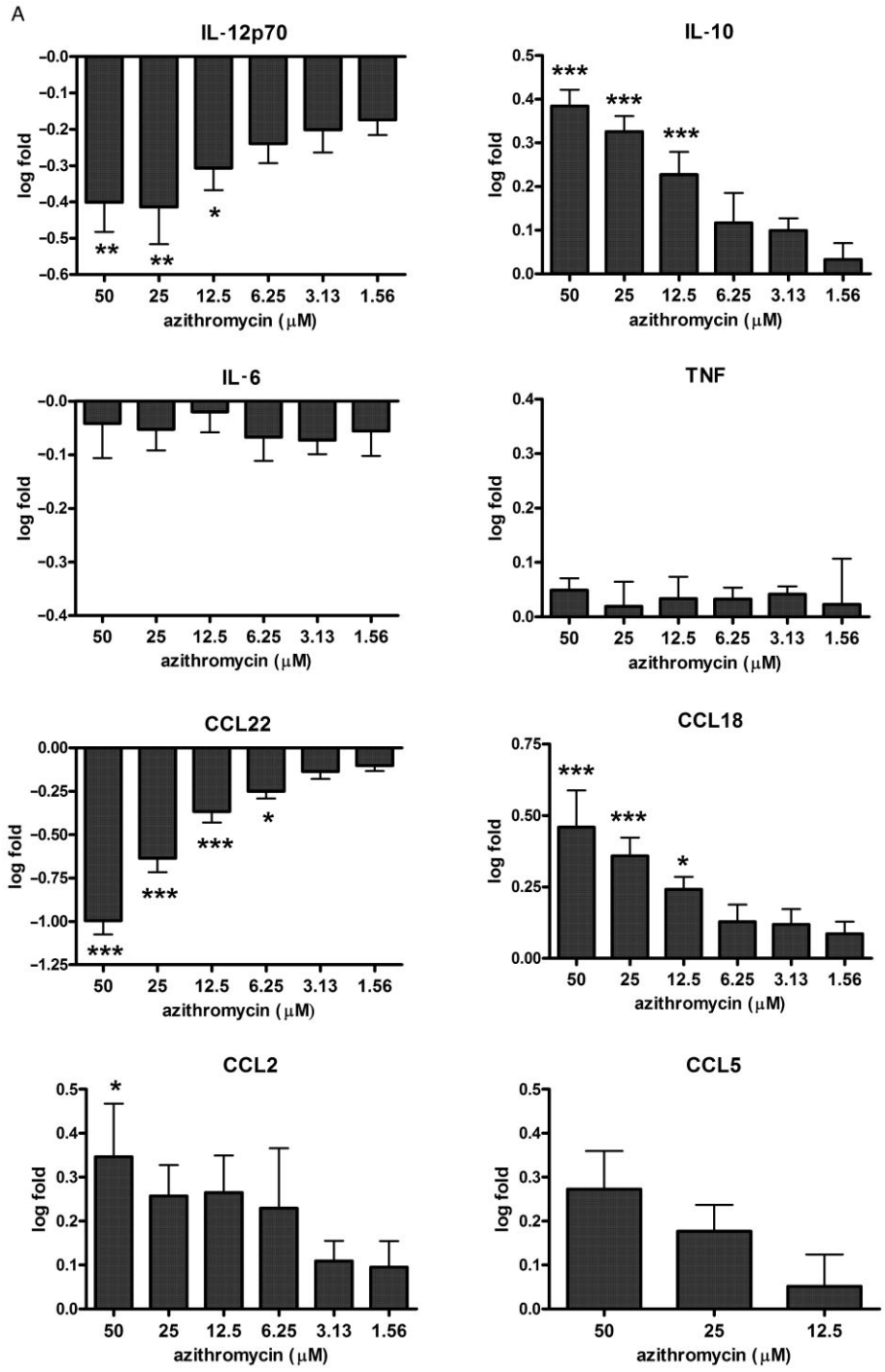

Figure 1

Changes in (A) cytokine production, measured by ELISA (n = 3–5), and (B) mRNA expression of selected markers (n = 3–7) on human monocytes activated with IFN-γ/LPS and treated with azithromycin (AZM;1.56–50 μM). Results are mean values ± SEM of log_{10}-transformed fold changes versus IFN-γ/LPS-activated controls. Note that the IFN-γ/LPS control value is 0. *P < 0.05, **P < 0.01, ***P < 0.001, ANOVA with Bonferroni post test. Regression coefficients for the slopes of the cytokine plots in (A) deviated significantly from zero for CCL2 (P < 0.05), CCL5 (P < 0.01), CCL18 (P < 0.001), CCL22 (P < 0.01), IL-10 (P < 0.01) and for IL-12p70 (P < 0.05, only for the range of concentrations 1.56–25 μM) in contrast to IL-6 and TNF-α for which it was not significantly different.

release, while roflumilast enhanced the production of this chemokine. However, azithromycin enhanced the release of IL-10 and CCL18 with a trend towards an increase in CCL5. In contrast, dexamethasone, SB203580 and roflumilast inhibited or tended to inhibit the production of these mediators. Among these three anti-inflammatory agents, only SB203580 shared an enhancing effect on CCL2 release with azithromycin (Figure 4).

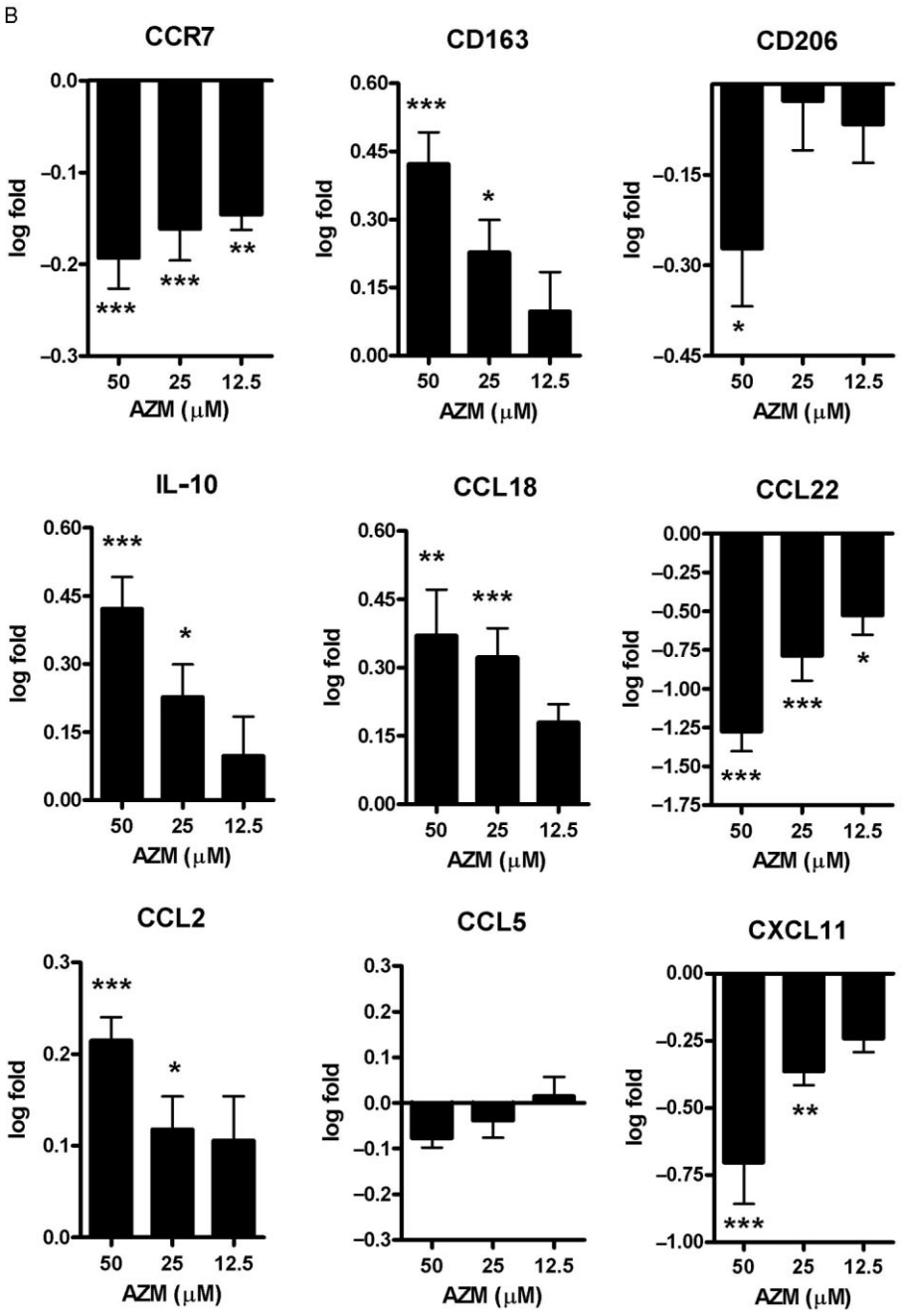

Figure 1
continued.

Comparison with lysosomotropic inhibiting agents

Incubation of IFN-γ/LPS-activated monocytes with the antimalarial/antirheumatic drug chloroquine mirrored exactly the effects of azithromycin by inhibiting IL-12p70 and CCL22 release and enhancing IL-10, CCL2 and CCL18 release (Figure 4). Although trends were observable with azithromycin, chloroquine had no clear effect on CCL5 release. Given that chloroquine, like azithromycin, accumulates in lysosomes (Fox, 1993; Carlier *et al.*, 1994), two other agents that modulate lysosomal function (Sobota *et al.*, 2009), namely, ammonium chloride, which raises the pH in the acidic lysosomes, and concanamycin A, which inhibits pH gradient sustaining vacuolar H^+-ATPase (V-ATPase), were also tested on the release of these four cytokines/chemokines from activated monocytes. The two alkalinizing agents, at non-cytotoxic concentrations, also inhibited IL-12p70 and CCL22, and enhanced CCL18 release, but inhibited, rather than enhanced, IL-10 and CCL2 release (Figure 5).

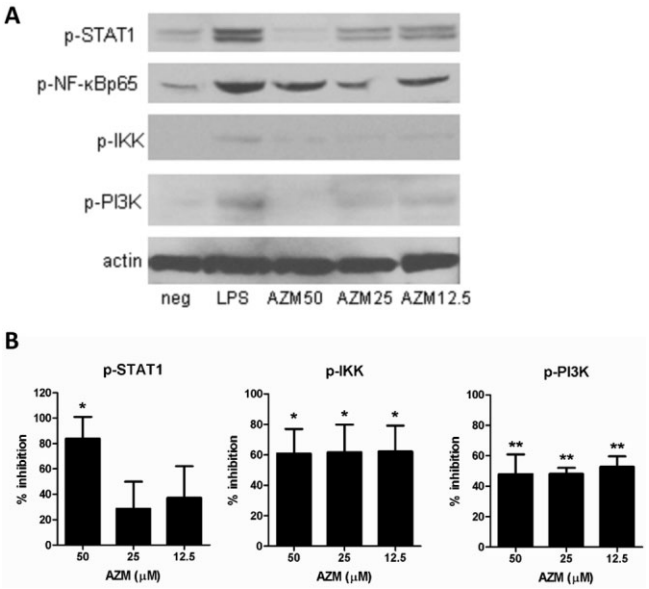

Figure 2

Effects of azithromycin (AZM; 12.5–50 μM) on phosphorylation of signalling molecules in human monocytes activated with IFN-γ/LPS (A) by Western blotting in a single representative experiment; (B) as the mean percent changes in optical density of p-STAT1, p-IKK and p-PI3K from three separate experiments, measured by Western blot. The track labelled neg refers to control cells treated with IFN-γ and DMSO; LPS refers to control cells stimulated with IFN-γ/LPS plus DMSO in the absence of azithromycin. Actin was added as loading control. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ANOVA with Dunnett's post test.

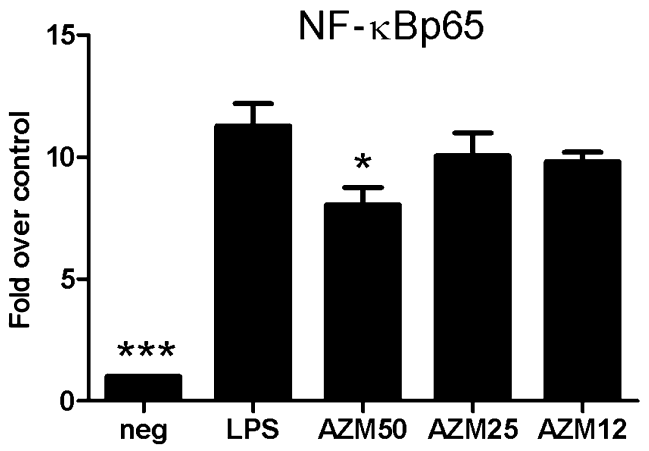

Figure 3

Effects of azithromycin (AZM; 12.5–50 μM) on activation of transcription factor NF-κBp65 in human monocytes activated with IFN-γ/LPS expressed as fold change ($n = 3$). The track labelled neg refers to control cells treated with IFN-γ and DMSO; LPS refers to control cells stimulated with IFN-γ/LPS plus DMSO in the absence of azithromycin. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ANOVA with Dunnett's post test.

Discussion

Azithromycin has been shown to alter the phenotype of permanent macrophage cell lines, as well as phagocytic capacity and CD206 receptor expression in alveolar macrophages from COPD patients (Hodge *et al.*, 2008; Murphy *et al.*, 2008; Yamauchi *et al.*, 2009). We have now demonstrated, in classically activated human blood monocytes, that the azithromycin-conditioned cells have a mediator release profile that differs, in some respect, from those often seen with reported monocyte/macrophage phenotypes. Toll-like receptor (TLR)4-related signalling processes and probably lysosomes appear to be intracellular targets of the macrolide.

Previous studies have shown that, in IFN-γ/LPS-activated permanent murine monocytic cell lines, azithromycin inhibits pro-inflammatory IL-12p40, while enhancing IL-10 expression and release (Murphy *et al.*, 2008; Yamauchi *et al.*, 2009). We have confirmed these effects on human blood monocytes and extended the modulatory effects to dose-dependent inhibition of the expression and/or release of the M1 macrophage markers CCR7, CXCL11 and IL-12p70 (Benoit *et al.*, 2008) but enhancement of CCL2. In contrast to the findings in the murine J774 cell line (Murphy *et al.*, 2008), we did not observe inhibition of IL-6 release, nor enhanced expression of the CD206 mannose receptor. These discrepancies are probably because of species differences and cell differentiation status.

In addition to enhancing the expression and release of IL-10, azithromycin also enhanced the release of another presumed M2 macrophage marker, CCL18 (Benoit *et al.*, 2008). This is not unexpected, as CCL18 production by monocytes is regulated by IL-10 (Van Lieshout *et al.*, 2006). In addition, the expression of the SR CD163, a broad monocyte/macrophage marker, was enhanced, a tendency that has been noted with a variety of anti-inflammatory signals (Van Gorp *et al.*, 2010). However, azithromycin inhibited the release of the declared M2 marker, CCL22. On balance, then, we have confirmed that azithromycin tends to induce an anti-inflammatory profile in human blood monocytes, but only with partial M2 characteristics.

LPS activates mononuclear cells through the trimolecular membrane complex of CD14, TLR4 and MD2, and signalling is initiated by dimerization of the TLR (Carpenter and O'Neill, 2009). The subsequent downstream pathway, leading to expression of pro-inflammatory cytokines and surface molecules, includes a range of signalling molecules and transcription factors such as MAPKs, NF-κB, the STATs and activator protein (AP)-1 (Swantek *et al.*, 1997; Qin *et al.*, 2005; Brikos and O'Neill, 2008).

Macrolide antibacterials, including azithromycin, clarithromycin and erythromycin, have been shown to inhibit ERK1/2 and p38 MAPK in several different cell types and it has been proposed that an initial effect on MAPK leads to modulation of ERK1/2 resulting in later inhibition of NF-κB and AP-1 transcription factors (Shinkai *et al.*, 2008). There is also some evidence that macrolide antibacterials may affect the nuclear translocation and DNA binding of NF-κB (Kanoh and Rubin, 2010). In line with this proposal, we found inhibitory effects of azithromycin on both IKK phosphorylation and NF-κB activation. We saw no effect of azithromycin on the AP-1 constituent c-Jun possibly because

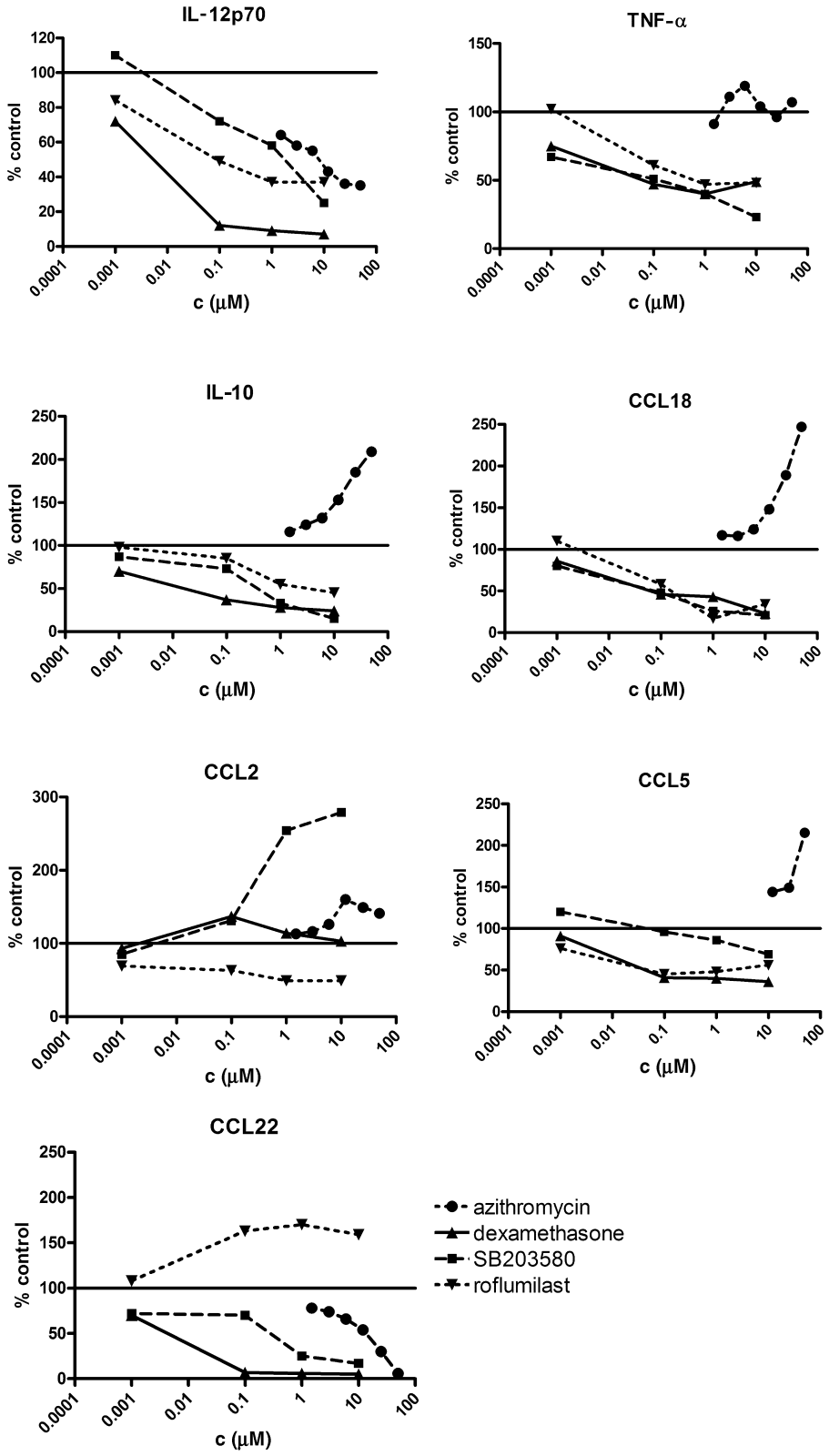

Figure 4

Comparative effects of different concentrations of anti-inflammatory agents, azithromycin, dexamethasone, SB203580 and roflumilast on concentrations of cytokines released from IFN-γ/LPS-stimulated human monocytes. Values are mean percentages of positive control (IFN-γ/LPS-stimulated cells plus DMSO) of observations from at least two separate experiments (n = 2–4). Negative controls (in the absence of IFN-γ/LPS activation) for IL-12p40, TNF, IL-10, CCL18, CCL2, CCL5 and CCL2 were 6, 1, 0, 52, 3, 4 and 16 % of the IFN-γ/LPS-positive control, respectively.

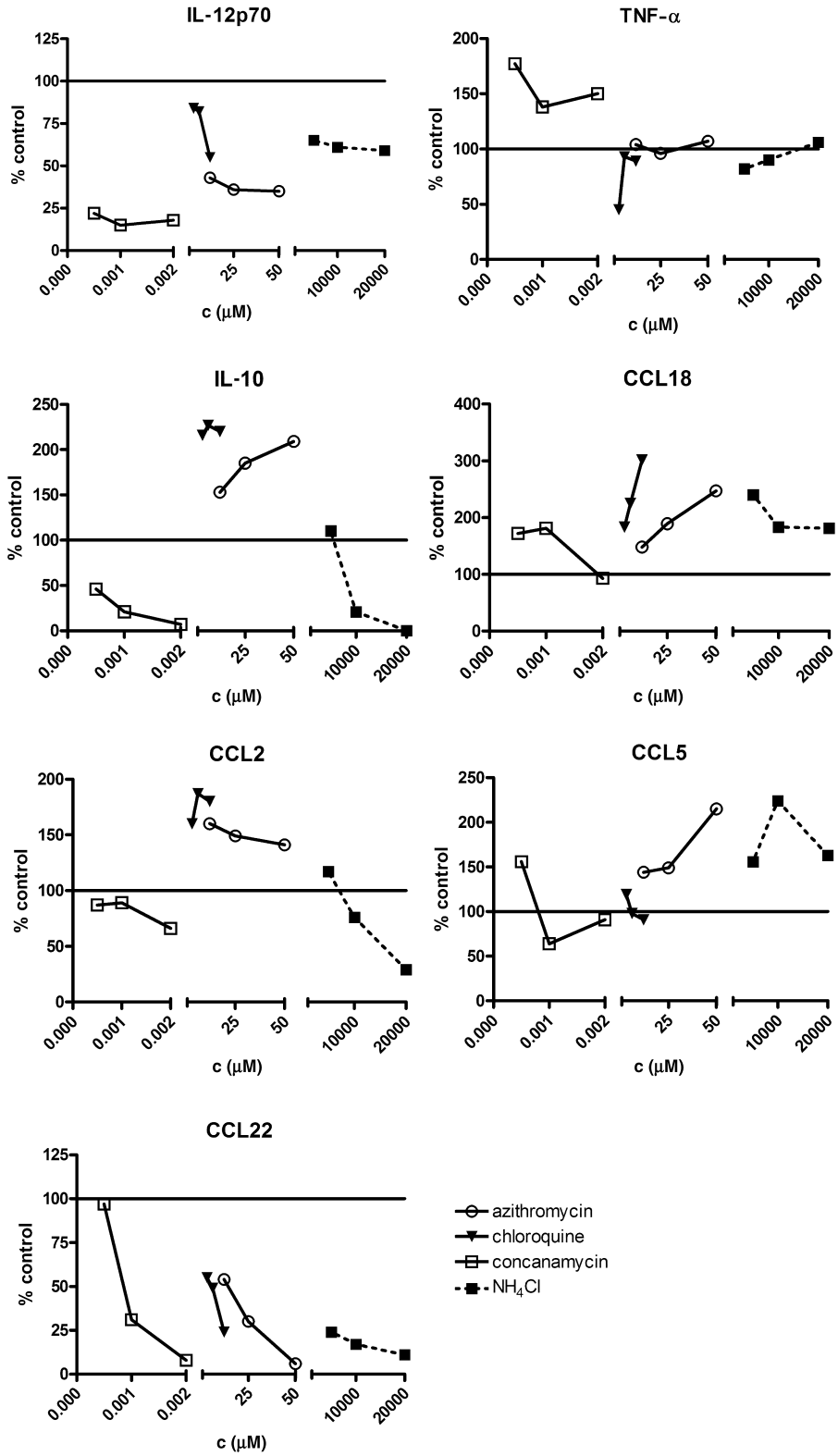

Figure 5

Comparative effects of azithromycin and lysosome-affecting agents (chloroquine, concanamycin A and ammonium chloride) on concentrations of cytokines released from IFN-γ/LPS-stimulated human monocytes. Values are mean percentages of positive control (IFN-γ/LPS-stimulated cells plus DMSO) of observations from at least two separate experiments (n = 2–4).

of the early time point (3 h) at which analyses were carried out. The inhibition of STAT-1 by azithromycin suggests that the MD2-independent pathway was also affected (Prêle *et al.*, 2008).

Stimulation by LPS of the CD14 receptor results in recruitment of Src family kinases and PI3K signalling, which then promotes translocation of nuclear factor of activated T cells (NFAT) and subsequently also NF-κB activation in mononuclear phagocytes (Darieva *et al.*, 2004; Ostuni *et al.*, 2010). Because we observed that azithromycin also inhibits PI3K phosphorylation, over the same concentration range as that at which IKK was inhibited, the macrolide would appear to affect, in parallel, several signalling pathways to monocyte NF-κB activation. Recently, IL-12p40 was shown to be inhibited by azithromycin in RAW264.7 macrophages by a mechanism involving inhibition of the binding of AP-1, NFAT and the interferon consensus sequence-binding protein (ICSBP) to the IL-12p40 promoter site (Yamauchi *et al.*, 2009). However, this was observed after 48 h incubation with azithromycin. A number of time-dependent effects of macrolide antibiotics have been reported, probably involving sequential changes in intracellular signalling processes (Shinkai *et al.*, 2008).

In view of their experimental and clinical anti-inflammatory activities, it is not surprising that all the anti-inflammatory agents tested, including azithromycin, inhibited the production of pro-inflammatory IL-12p70. Azithromycin showed similarity to dexamethasone and the p38 kinase inhibitor SB203580, in contrast to roflumilast, in inhibiting the production of the M2 macrophage marker, CCL22. CCL22 is chemotactic for dendritic and Th2 lymphocytes, playing a role in Th2-related diseases (Yamashita and Kuroda, 2002). Inhibition of this chemokine by azithromycin would tend to extend the potential anti-inflammatory spectrum of the antibiotic. However, azithromycin, in contrast to the other agents, slightly enhanced the production of CCL5, a chemotactic factor for T cells, dendritic cells, eosinophils, natural killer (NK) cells, mast cells and basophils (Levy, 2009), an effect that might, to some extent, counteract the reduction of CCL22. Azithromycin also contrasted with dexamethasone, SB203580 and roflumilast in that it failed to inhibit TNF-α release from classically activated human monocytes. Interestingly, in murine bone marrow-derived dendritic cells stimulated with LPS, azithromycin also failed to inhibit TNF-α release, while significantly enhancing IL-10 release (Sugiyama *et al.*, 2007). Because azithromycin has been shown repeatedly to inhibit TNF-α release from neutrophils and endothelial cells, and to reduce elevated TNF-α levels during chronic inflammatory disorders (Čulić *et al.*, 2001; Gotfried, 2004; Shinkai *et al.*, 2008), the lack of effect we observed may have been because of an alteration of the monocyte phenotype in the presence of azithromycin, rather than its inability to affect TNF-α release.

Azithromycin showed a distinct advantage over the other anti-inflammatory agents, except chloroquine, in that it enhanced the production of IL-10 and IL-10-regulated CCL18. IL-10 has broad anti-inflammatory properties and its up-regulation is crucial to the limitation of host tissue injury by the immune system in response to pathogens and autoimmunity (Saraiva and O'Garra, 2010). The up-regulation of IL-10 by azithromycin not only indicates modulation of the pro-inflammatory characteristics of classically activated monocytes, but also that the macrolide, together with chloroquine, has added anti-inflammatory benefits to those of other drugs.

Chloroquine is used clinically both as an antimalarial and antirheumatic drug. Its antirheumatic activity is thought to be related to its effects on lysosomal function and protein processing (Fox, 1993). Because of their similar cationic and lysosomal accumulating properties, as well as interaction with membrane phospholipids, the actions of chloroquine and azithromycin have been investigated on a number of intracellular processes. Alkalinizing agents affecting lysosomes (including azithromycin, chloroquine and ammonium chloride) inhibit the processing and secretion of TGF-β, in association with decreased Smad2 signalling, in cell lines and mice *in vivo* (Basque *et al.*, 2008). Chloroquine and ammonium chloride also inhibit the expression and release of TNF-α from *Candida*-stimulated human PBMC (Weber and Levitz, 2001). IL-10 production, as in our experiments with LPS, was not inhibited by chloroquine in these fungal-stimulated cells. In contrast, the inhibition by ammonium chloride and concanamycin A of IL-10 production in our study suggests that the effects of azithromycin and chloroquine cannot be attributed entirely to alkalinization of lysosomes. In earlier studies on RAW264.7 macrophage cell lines, chloroquine inhibited LPS-induced TNF-α production by a mechanism different from that of ammonium chloride (Jeong and Jue, 1997). In a recent study on the J774.A1 murine macrophage cell line, we found that chloroquine, azithromycin and several other macrolides inhibited pro-inflammatory cytokine production in correlation with cellular accumulation and phospholipid binding (Munić *et al.*, 2011). Early interaction with cell and/or lysosomal membrane phospholipids (Tyteca *et al.*, 2003) could well have been involved in the effects we observed in human monocytes. Some effects of concanamycin A on LPS-stimulated monocytes could be because of the inhibition of processing and surface expression of TLR4 receptors (Eswarappa *et al.*, 2008).

In conclusion, azithromycin modulates classical activation of human monocytes, generating a mediator expression profile that differs from monocyte/macrophage phenotypes that have been described previously. The mechanism of action of the macrolide appears to involve deregulation of TLR4-mediated signalling through inhibition of NF-κBp65 and STAT1 activation and possible effects on lysosomal function.

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Conflicts of interest

When the studies were performed, all authors were employees of GSK, the originator of the p38 kinase inhibitor,

SB203580, used in the study. Prior to employment by GSK, all authors had been employees of PLIVA Inc., the originator and manufacturer of azithromycin.

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