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Original Article

Azithromycin suppresses Th1- and Th2-related chemokines IP-10/MDC in human monocytic cell line

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KEYWORDS
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Abstract *Background:* Cytokines and chemokines play critical roles in the pathogenesis of asthma. Azithromycin, a macrolides, is frequently used in asthmatic children with lower respiratory tract infection and is reported having anti-inflammatory and immunomodulatory effects. However, the effects of azithromycin on the expression of TNF-α, Th1- and Th2-related chemokines, and neutrophil chemoattractant are unknown. We investigated the *in vitro* effects of azithromycin on the expression of TNF-α, Th1-related chemokine interferon-γ-inducible protein-10 (IP-10/CXCL10), Th2-related chemokine macrophage-derived chemokine (MDC/CCL22) and neutrophil chemoattractant growth-related oncogene-α (GRO-α/CXCL1) in THP-1 cells as a model for human monocytes.

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Methods: THP-1 cells were pretreated with various concentrations of azithromycin before Toll-like receptor 4 (TLR4) agonist lipopolysaccharide (LPS) stimulation. TNF-α, IP-10, MDC and GRO-α were measured by ELISA. Intracellular signaling was investigated by pathway inhibitors and Western blot.
Result: Azithromycin suppressed MDC and IP-10 expression in LPS-stimulated THP-1 cells. However, azithromycin had no effect LPS-induced TNF-α and GRO-α expression. Western blotting revealed that azithromycin suppressed LPS-induced phosphorylation of mitogen-activated protein kinase (MAPK)—JNK and ERK expression, and also suppressed LPS-induced phosphorylation of nuclear factor (NF) κB—p65 expression.
Conclusion: Azithromycin suppressed LPS-induced MDC expression via the MAPK—JNK and the NFκB—p65 pathway. Azithromycin also suppressed LPS-induced IP-10 via the MAPK—JNK/ERK and the NFκB—p65 pathway. Azithromycin may benefit asthmatic patients by suppressing chemokines expression.

Introduction

Asthma is a chronic inflammatory airway disease with increasing prevalence during recent decades.[1] Allergic asthma is characterized with T-helper (Th) cell type 2 (Th2)-mediated immune responses with predominant infiltration of Th2 lymphocytes, eosinophils, basophils and mast cells.[2] Severe refractory asthma is typified with irreversible airway remodeling and the recruitment of neutrophils into the airway.[3] Chemokines recruit inflammatory cells into airway and therefore play important roles in the pathogenesis of asthma. For example, macrophage-derived chemokine (MDC/CCL 22) is a Th2-related chemokine which attracts Th2 cells in response to allergen challenging,[4] and the concentration of MDC is high in both the plasma and the exhaled breath condensate of asthmatic children.[5] Interferon-inducible protein-10 (IP-10/CXCL10) is a Th1-related chemokine contributing to asthmatic airway inflammation and hypersensitivity,[6] and the concentration of IP-10 is increased in the plasma of the asthma children with virus-induced asthma exacerbation.[7] Tumor necrosis factor (TNF)-α is a pleiotropic pro-inflammatory cytokine as well as a chemoattractant to neutrophil,[8] and is regarded as a specific feature in severe refractory asthma.[9] Growth-related oncogene-α (GRO-α/CXCL1), a CXC chemokines, is a neutrophil chemoattractant which recruit neutrophils into asthmatic airways,[10] and the expression of GRO-α is increased during severe exacerbation of asthma.[11] In human allergic disease such as asthma or atopic dermatitis, monocytes and epithelial cells are major producer cytokines and chemokines production in response to microbial challenging, and the expression of chemokines can be modulated by anti-asthmatic or anti-allergic medications.[12,13]

Atypical respiratory tract infections with pathogens such as *Mycoplasma pneumoniae*[14] or *Chlamydia pneumoniae*[15] are important triggers for asthma attack in children, and are possibly positively associated with the development of atopy.[16] The clinical efficacy of macrolides antibiotics in the treatment of atypical upper and lower respiratory tract infections has been established,[17] and azithromycin is a preferred choice of macrolides in children because of its better compliance and less adverse effects in gastrointestinal tract.[18] Macrolides have been observed for additional clinical benefits in patients with chronic airway inflammation by infection due to their anti-inflammatory effect other than antimicrobial activity,[19,20] and the mechanism may be the modulatory effects on immune cell.[21] However, the effects of macrolides on the expression of TNF-α as well as asthma-related chemokines, and the detailed mechanism are unknown. In the present study, we investigated the *in vitro* effects of azithromycin, on the expression of TNF-α, MDC, IP-10 and GRO-α in LPS-stimulated monocytes and also explored the detailed intracellular mechanisms.

Methods

Cell preparation

The human monocytic cell line, THP-1 (American Type Culture Collection, Rockville, MD), was cultured in RPMI 1640 medium (Sigma—Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum, 100 U/mL of penicillin, and 100 μg/mL of streptomycin at 37 °C with 5% CO_2 in a humidified incubator. THP-1 cells were centrifuged and resuspended in fresh media in 24-well round-bottom plates at a concentration of 2×10^5/mL for 24 h before experimental use. According to the pharmacokinetics of azithromycin in human, a single oral dose of 500 mg was bioavailable and produced a peak serum concentration of 0.4 mg/L, and tissue concentrations of azithromycin were much higher than serum concentrations.[22] To investigate the effects of azithromycin, the cells were pretreated with various concentration of azithromycin (Pfizer, New York, NY) 2 h before LPS (0.2 μg/mL) (Escherichia coli-derived; Sigma—Aldrich) stimulation. Cell supernatants were collected 6, 24 and 48 h after LPS stimulation.

Western blotting

After treatment for 2 h with or without azithromycin (1 and 10 μg/mL), the cells were stimulated with LPS (0.2 μg/mL)

for 1 h and were lysed with equal volumes of ice-cold lysis buffer. After centrifugation at 13,000×*g* for 15 min, cell lysates (20 μg) were analyzed by Western blot with anti-MAPK (p38, ERK and JNK), anti-phospho-MAPK (phospho-p38, phospho-ERK and phospho-JNK) antibodies, anti-p65 and anti-phospho-p65 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). Immunoreactive bands were visualized using horseradish peroxidase-conjugated secondary antibody and the enhanced chemiluminescence system (Amersham Pharmacia Biotech, Sunnyvale, CA).

ELISA

The level of TNF-α, MDC, IP-10 and GRO-α in the cell supernatants were measured using commercially available ELISA (R&D system, Minneapolis, MN) following the manufacturer's instruction.

Statistical analysis

All data are presented as mean ± SD. One-way ANOVA followed by Bonferroni's post-hoc comparisons tests were performed in all statistical analyses. *P* value less than 0.05 was considered significant.

Results

Azithromycin suppressed LPS-induced MDC expression in THP-1 cells

MDC is a Th2-related chemokine playing an important role in the pathogenesis of asthma.[5] We firstly investigate the effect of azithromycin on the expression of MDC in LPS-stimulated THP-1 cells. Although at the time 6 h after LPS stimulation azithromycin had no effect on LPS-induced MDC expression (Fig. 1A), azithromycin at the concentration of 5, 10, 20, 40 μg/mL suppressed LPS-induced MDC expression at the time 24 h and 48 h after LPS stimulation (Fig. 1B and C). However, azithromycin alone had no effect on MDC expression (data not shown).

Azithromycin suppressed LPS-induced IP-10 expression in THP-1 cells

We next examined the effect of azithromycin on the expression of IP-10. Interestingly, azithromycin at the concentration of 20 and 40 μg/mL suppressed LPS-induced IP-10 expression at the time 6 h after LPS stimulation (Fig. 2A). However, at the time 24 and 48 h after LPS stimulation, azithromycin had no effect on the expression of IP-10 (Fig. 2B and C), which suggested that the suppressive effect of azithromycin on IP-10 expression was time-dependent. Azithromycin alone in the absence of LPS had no effect on IP-10 expression (data not shown).

Azithromycin had no effect on TNF-α and GRO-α expression in THP-1 cells

Different from allergic asthma which is characterized with Th2-mediated inflammation in the airway, severe refractory asthma is typified with pulmonary inflammation by neutrophils.[3] TNF-α is a pleiotropic proinflammatory cytokine involving in neutrophil recruitment and is an important biomarker in severe refractory asthma.[9] GRO-α recruits neutrophil into airways and is involved in the pathogenesis of severe or refractory asthma.[10] We next investigated the effect of azithromycin on the expression of TNF-α and GRO-α in THP-1 cells. Azithromycin had no effect on LPS-induced TNF-α (Fig. 3) and also had no effect on LPS-induced GRO-α expression (Fig. 4). Azithromycin alone in the absence of LPS had no effect on TNF-α and GRO-α expression (data not shown).

Azithromycin suppressed LPS-induced MDC and IP-10 expression via partly the NFκB—p65 pathway

It has been reported that LPS stimulation of human monocyte activates the IKK—NFκB pathway.[23] We next examined whether the suppressive effect of azithromycin is through the NFκB pathway. As shown in Fig. 5, BAY 117085 (the IKK inhibitor) inhibited LPS-induced MDC expression (Fig. 5A) and also IP-10 expression (Fig. 5B), suggesting that LPS-mediated MDC and IP-10 expression was via the NFκB pathway. Western blot showed that LPS-induced phosphorylation of p65 was suppressed by azithromycin (Fig. 5C). These data suggested that azithromycin may suppress LPS-induced MDC and IP-10 expression via, at least partly, the NFκB—p65 pathway.

Azithromycin suppressed LPS-induced MDC via the MAPK—JNK pathway, and suppressed IP-10 expression via the MAPK—JNK/ERK pathway

MAPK pathway participates in several inflammatory responses. In monocytes, three MAPK pathways can be activated by environmental stimulation such as LPS.[23] We next examined whether azithromycin suppressed LPS-induced MDC and IP-10 expression is through the MAPK pathway. In our previous study we have reported that LPS-induced MDC expression is dependent of the MAPK—p38/JNK pathways without the involvement of the MAPK—ERK pathway, and LPS-induced IP-10 expression is dependent of all the three MAPK pathways.[24] As shown in Fig. 6, azithromycin had no effect on LPS-induced phosphorylation of MAPK—p38 (Fig. 6A), but could suppressed LPS-induced phosphorylation of MAPK—JNK (Fig. 6B) and MAPK—ERK (Fig. 6C). These data suggested azithromycin may enhance LPS-induced MDC expression via, at least partly, the MAPK—JNK pathway, and may suppress LPS-induced IP-10 expression via, at least partly, the MAPK—JNK/ERK pathway.

Discussion

Azithromycin, a macrolide, has been used widely for treatment of Gram-positive and also Gram-negative bacterial infection, as well as used for treatment of *Mycoplasma* or *Chlamydia*. Beyond its antimicrobial activity, azithromycin has been reported having immunomodulatory effects on inflammatory cells[21] and in animal model.[25] Very recent clinical studies implicate that the use of macrolides

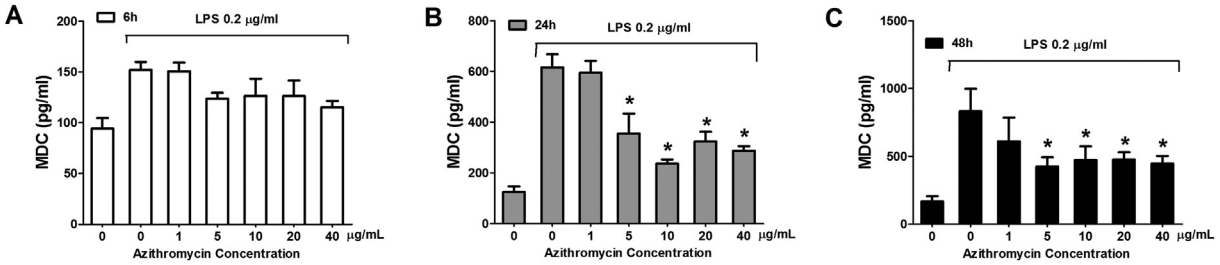

Figure 1. Azithromycin suppressed lipopolysaccharide (LPS)-induced macrophage-derived chemokine (MDC) expression in monocytes. Human monocytic cell line THP-1 cells were pretreated with various concentration of azithromycin for 2 h and were stimulated with LPS. Cell supernatants were collected at indicated time. Azithromycin had no effect on LPS-induced MDC expression at the time (A) 6 h after LPS stimulation, but significantly suppressed LPS-induced MDC expression at the time (B) 24 h and (C) 48 h after LPS stimulation. Results are expressed as mean ± SD of four independent experiments. *$P < 0.05$ compared with the group of LPS-treated cells (B and C).

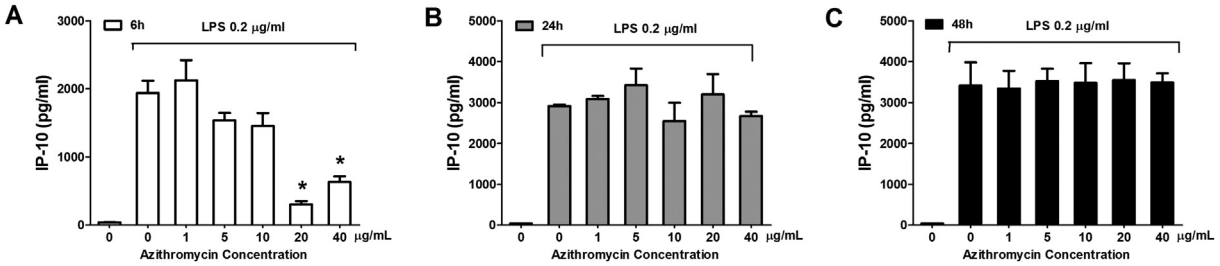

Figure 2. Azithromycin suppressed lipopolysaccharide (LPS)-induced interferon-γ-inducible protein-10 (IP-10) expression in monocytes. THP-1 cells were pretreated with various concentration of azithromycin for 2 h and were stimulated with LPS. Cell supernatants were collected at indicated time. Azithromycin had suppressed LPS-induced IP-10 expression at the time (A) 6 h after LPS stimulation, but had no effect on LPS-induced IP-10 expression at the time (B) 24 h and (C) 48 h after LPS stimulation. Results are expressed as mean ± SD of four independent experiments. *$P < 0.05$ compared with LPS-treated cells (A).

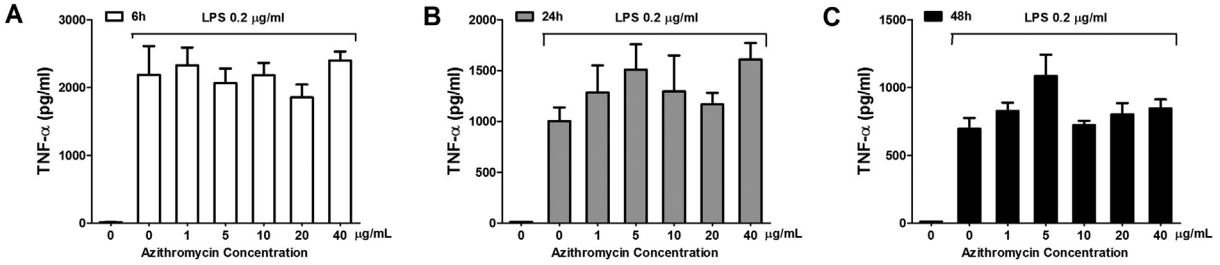

Figure 3. Azithromycin had no effect on lipopolysaccharide (LPS)-induced tumor necrosis factor (TNF)-α expression in monocytes. THP-1 cells were pretreated with various concentration of azithromycin for 2 h and were stimulated with LPS. Cell supernatants were collected at indicated time. Azithromycin had no effects on LPS-induced TNF-α expression at the time (A) 6 h, (B) 24 h, (C) 48 h after LPS stimulation. Results are expressed as mean ± SD of four independent experiments.

can improve symptoms of asthmatic children[26] and benefits patients during acute exacerbation.[27] However, the effect of azithromycin on asthma-related cytokines and chemokines in monocytes, and the mechanisms are unknown. Recently chemokines has been identified their important roles in the pathogenesis of asthma, including recruiting inflammatory cells, activating as well as differentiating T cell responses, and contributing the hyperreactivity and remodeling of asthmatic airway and are therefore become biomarkers and potential targets for the treatment of asthma.[28] In the present study we demonstrated that although azithromycin had no effect on the expression of LPS-induced TNF-α and GRO-α, two neutrophil chemoattractants and the biomarkers of severe refractory asthma, azithromycin significantly suppressed LPS-induced MDC (a Th2-related chemokine) and IP-10 (a Th1-related chemokine) expression in monocytes, and the suppressive effect of azithromycin involves the NFκB and MAPK pathways. Our findings may implicate that azithromycin may benefit asthmatic patients during infection-induced exacerbation by suppressing the expression of asthma-related chemokines.

The immunomodulatory activities of macrolides are found from the fact that prolonged use with low-dose

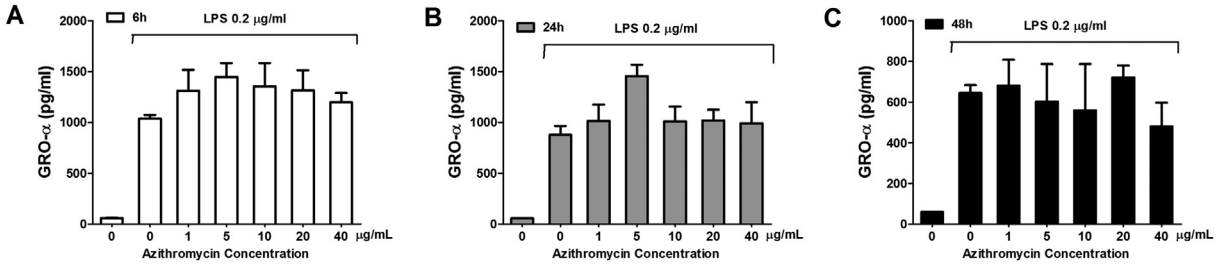

Figure 4. Azithromycin had no effect on lipopolysaccharide (LPS)-induced growth-related oncogene-α (GRO-α) expression in monocytes. THP-1 cells were pretreated with various concentration of azithromycin for 2 h and were stimulated with LPS. Cell supernatants were collected at indicated time. Azithromycin had no effects on LPS-induced GRO-α expression at the time (A) 6 h, (B) 24 h, (C) 48 h after LPS stimulation. Results are expressed as mean $\pm$ SD of four independent experiments.

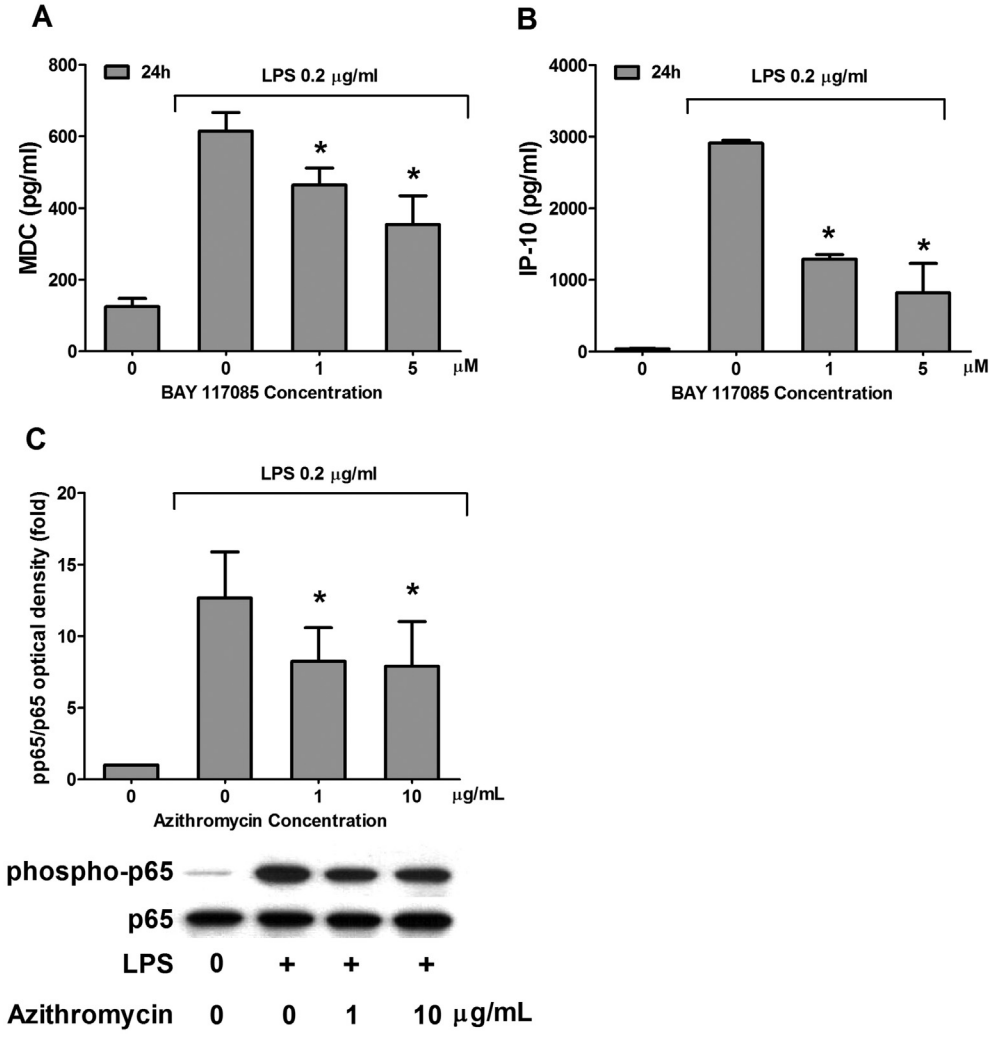

Figure 5. Azithromycin suppressed lipopolysaccharide (LPS)-induced macrophage-derived chemokine (MDC) and interferon-γ-inducible protein-10 (IP-10) expression via partly the nuclear factor (NF)κB—p65 pathway in monocytes. THP-1 cells were pretreated with BAY 117085 (an IKK inhibitor) for 2 h and were stimulated with LPS. Cell supernatants were collected at the time 24 h after LPS treatment. For Western blotting analysis, THP-1 cells were pretreated with azithromycin (1 and 10 μg/mL) for 2 h and were stimulated with LPS for 1 h, and cell lysates were collected for analysis. BAY 117085 suppressed LPS-induced (A) MDC and (B) IP-10 expression. Azithromycin suppressed (C) LPS-induced phospho-p65 expression. Results are expressed as mean $\pm$ SD of four independent experiments. For Western blot analysis, results of optical densitometry are expressed as mean $\pm$ SD from three independent experiments, and one experiment representative of three is shown. *$P < 0.05$ compared with LPS-treated cells (A and B).

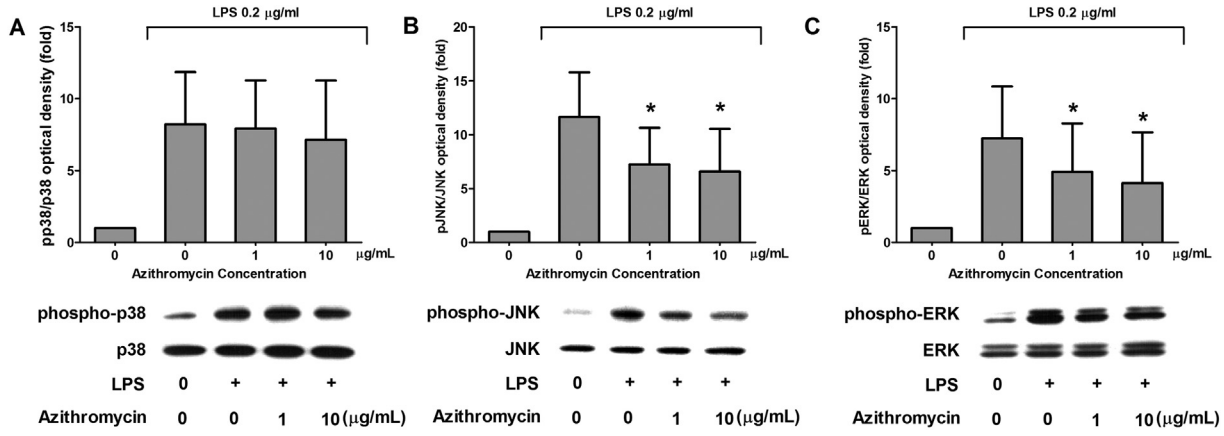

Figure 6. Azithromycin suppressed lipopolysaccharide (LPS)-induced activation of mitogen-activated protein kinase (MAPK) pathway. THP-1 cells were pretreated with azithromycin (1 and 10 μg/mL) for 2 h and were stimulated with LPS for 1 h, and cell lysates were collected for analysis. Azithromycin had no effect on LPS-induced (A) phospho-p38 expression. However, azithromycin suppressed LPS-induced (B) phospho-JNK and (C) phospho-ERK expression. Results of optical densitometry are expressed as mean ± SD from three independent experiments, and one experiment representative of three is shown (A, B and C).

erythromycin can benefit patients with diffuse panbronchiolitis (DPB).[29] The precise mechanisms are not well elucidated, and have been reported to involve the processes of inflammation, including migration as well as cytokines production of inflammatory cells and the oxidative stress in respiratory tract.[30] Macrolides inhibit exhibited pronounced reduction of cytokines/chemokines release from sputum cells isolated from COPD patients with different potency.[31] Macrolides act on the function of immune cells and affect the cytokine production in neutrophils,[21] lymphocytes[32] and monocytes.[33] The present study firstly demonstrated that azithromycin can exert the anti-inflammatory activity via inhibiting LPS-mediated chemokines expression by monocytes.

The present study provided further understanding for intracellular mechanism of action of azithromycin. NFκB activation of inflammatory cells is associated with the expression of inflammatory genes in asthmatic patients,[34] and has been shown to have a specific binding site to the proximal promoter region of MDC in B cells, DCs,[35] and also in human monocytes as reports in our previous work.[36] Interestingly, in interferon-γ activated human blood monocytes, azithromycin suppresses LPS-induced MDC expression, and LPS-signaling pathways are modulated by azithromycin with down-regulation of phosphorylation of STAT1, NFkB—p65, IKK and PI3K.[37] In the present study by IKK inhibitors, we demonstrated that the expression of LPS-induced MDC and IP-10 was dependent of NFκB pathway (Fig. 5A and B), and we also demonstrated that azithromycin suppressed LPS-induced phosphorylation of p65 using Western blotting. These data suggested that azithromycin may suppress LPS-induced MDC and IP-10 expression via, at least partly, the NFκB pathway. The suppressive effect of azithromycin on NFκB is consistent with the report using erythromycin in bronchial epithelial cells[38,39] and a very recent work using azithromycin in murine bone marrow-derived DCs.[40] The suppressive effect of azithromycin on the NFκB—p65 pathway may implicate its potential in modulating NFκB-regulated inflammatory gene expression.

Another important finding of this study is that we provided additional insight into the modulating effect of azithromycin on the MAPK pathway. MAPK pathway is fundamental regulators of most immune cell functions, including chemoattraction and production of inflammatory mediators.[41] The present study revealed that azithromycin suppressed phosphorylation of MAPK—JNK/ERK and subsequently inhibited the expression of MDC and IP-10 in LPS-stimulated monocytes. Interestingly, it is reported that azithromycin inhibits inflammatory cytokine and chemokine expression via suppressing phosphorylation of MAPK—ERK in epithelial cells isolated from women with recurrent Chlamydia trachomatis infection,[42] although the suppressive effect is not consistent in respiratory epithelial cells under different conditions.[43] In asthmatic patients, phosphorylation of ERK and p38 is correlated with disease severity.[44] The suppressive effect of azithromycin on ERK may have potential in modulating the inflammation in asthmatic patients.

In conclusion, azithromycin suppressed asthma-related Th2 chemokine MDC and Th1 chemokine IP-10 in human monocytes through the NFκB—p65 and the MAPK—JNK/ERK pathways. Our findings indicated that with the chemokine-modulating property, azithromycin may have therapeutic potential apart from its anti-microbial activity in treating and suppressing inflammation during infection-related exacerbation of asthma. The present *in vitro* study deserves further clinical *in vivo* studies for potential applications in the feature.

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