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Azithromycin modulates release of steroid-insensitive cytokines from peripheral blood mononuclear cells of patients with chronic obstructive pulmonary disease

Abstract

Introduction: Oxidative stress reduces responsiveness of peripheral blood mononuclear cells (PBMCs) from patients with chronic obstructive pulmonary disease (COPD) to the anti-inflammatory effects of glucocorticoids (GCs). Azithromycin was shown to modulate corticosteroid resistance mechanisms in COPD patients. However, its ability to enhance anti-inflammatory properties of GCs on the production of cytokines by PBMCs has not well been elucidated.

Material and methods: Heparinised blood was collected from 27 patients with COPD. Phytohaemagglutinin-induced release of pro-inflammatory mediators from PBMCs and production of intracellular cytokines by CD4+ and CD8+ T cells stimulated with phorbol 12-myristate 13-acetate and ionomycin in the presence or absence of 10 μg/mL azithromycin and 10 nM budesonide were determined using enzyme linked immunosorbent assay and flow cytometry.

Results: Azithromycin decreased the secretion of interleukin (IL) 4, IL-5, IL-13, and IL-17A from PBMCs, as well as the production of IL-4 and IL-8 by CD4+ and CD8+ T cells. The combination of azithromycin and budesonide suppressed inflammatory response by inhibition of IL-4, IL-5, IL-8, IL-13, IL-17A, IL-33, thymic stromal lymphopoietin (TSLP), macrophage migration inhibitory factor (MIF) release from PBMCs and by reduction of the percentage of IL-4-, IL-8-, interferon γ- and tumor necrosis factor α-expressing CD4+ and CD8+ T cells. The inhibitory effect of azithromycin combined with budesonide on IL-4, IL-5, IL-8, IL-17A, TSLP production by PBMCs, as well as IL-4 and IL-8 production by T helper cells and cytotoxic T lymphocytes was significantly greater than the effect of budesonide alone.

Conclusions: Azithromycin in combination with budesonide enhances GC properties by inhibiting synthesis of pro-inflammatory cytokines in blood cells of COPD patients.

Key words: azithromycin; corticosteroid resistance; cytokines; peripheral blood mononuclear cells; chronic obstructive pulmonary disease

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Introduction

An important feature of chronic obstructive pulmonary disease (COPD) is the persistent inflammatory response even after elimination of the etiological factor [1]. As a result, lung function is impaired over time and the disease progresses.

Oxidative stress plays a pivotal role in the pathogenesis of COPD. It is caused by reactive oxygen species present in cigarette smoke, air pollution, occupational dusts and smoke from biomass fuel and by oxidants formed by inflammatory and structural cells in the respiratory tract. There are also reduced antioxidant defences, which are unable to counteract the oxidant effects in the airways, with the decreased non-enzymatic antioxidant concentration, repressed antioxidant enzymes, and suppressed expression of the tran-

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scription factor nuclear factor erythroid 2-related factor 2 (Nrf2) that regulates antioxidant and cytoprotective genes [2]. An imbalance between oxidants and antioxidants leads to the activation of transcription factor nuclear factor-κB (NF-κB), which stimulates expression of genes encoding inflammatory proteins such as interleukin (IL) 4 (IL-4), IL-5, IL-8, IL-17A, interferon γ, tumor necrosis factor α, thymic stromal lymphopoietin (TSLP) [3].

Oxidative stress also causes glucocorticoid (GC) insensitivity of cells [4]. Steroid resistance is characterised by the inability or reduced ability of cells to respond to GCs that results in the synthesis and secretion of pro-inflammatory proteins (cytokines, chemokines, enzymes, adhesion molecules) from cells despite adequate corticosteroid therapy. Peripheral blood mononuclear cells (PBMCs) from patients with COPD were shown to be corticosteroid insensitive [5]. These cells are significant producers of cytokines that control inflammatory response in COPD.

Under conditions not accompanied by oxidative stress, GCs act by binding to glucocorticoid receptor (GR), which then translocates from the cytosol into the nucleus and interacts with co-activator molecules to suppress NF-κB-dependent inflammatory genes [4]. Moreover, enzyme histone deacetylase 2 (HDAC2) causes deacetylation of GR enabling it to interact with NF-κB, and thus blocks this transcription factor activity [4]. Oxidative stress decreases HDAC2 activity through activation of phosphoinositide-3-kinase (PI3K) δ. This kinase causes phosphorylation (activation) of downstream kinase Akt, which finally phosphorylates (inactivates) HDAC2 [6]. There is reduced HDAC2 expression in PBMCs of patients with COPD compared with healthy smokers and healthy non-smokers [7].

Azithromycin is a macrolide antibiotic that demonstrates bacteriostatic activity against many gram-positive and gram-negative bacteria as well as atypical agents. It reduces bacterial virulence by inhibition of biofilm formation, bacterial protein synthesis and release of pathogen-associated molecular patterns (PAMPs) from bacteria, and also by enhancement of phagocytosis and intracellular killing of bacteria by monocytes [8]. Additionally, azithromycin possesses immunomodulatory properties through inhibition of several pro-inflammatory cytokines and chemokines, attenuating the migration of effector cells to the airways [9]. Macrolides, including azithromycin, were shown to inhibit Akt phosphorylation, indicating their ability to modulate corticosteroid resistance mechanisms [6]. We hypothesised here that azithromycin can enhance anti-inflammatory properties of GCs and improve steroid responsiveness of PBMCs in COPD patients.

Table 1. Demographics of the study population

Parameter	COPD patients
No. of subjects	27
Male/female	22/5
Age (years)	66.4 ± 1.5
BMI [kg/m^2]	27.6 ± 1.1
Current/ex-smoker	12/15
Smoking history (pack-years)	36.3 ± 2.6
FEV_1 (% predicted)	51.0 ± 3.3
FEV_1/FVC ratio [%]	55.4 ± 2.3

Data are presented as n or mean ± standard *error* of the mean (SEM).
BMI — body mass index; COPD — chronic obstructive pulmonary disease; FEV_1 — forced expiratory volume in 1 s; FVC — forced vital capacity

The aim of this study was to evaluate suppressive efficacy of azithromycin, alone and in combination with GCs, on the production of cytokines by PBMCs of patients with COPD. Results of the current study may reveal the benefits of combined use of azithromycin with GCs to inhibit inflammatory response.

Materials and methods

Subjects

The study was approved by the ethics committee of Belarusian State Medical University (Minsk, Belarus). All patients were informed of the research process and signed informed consent forms. Based on the Global Initiative for Chronic Obstructive Lung Disease (GOLD) criteria, 27 patients with stable COPD were enrolled (clinical characteristics listed in Table 1). Subjects did not have history of asthma or allergy, did not use antibiotics and systemic corticosteroids, and did not report acute infections for 6 weeks preceding the study entry.

Sample collection and PBMC isolation

Peripheral blood samples were collected in heparin-treated tubes. PBMCs were isolated by density gradient centrifugation according to the manufacturer's instructions (Lymphopure, Biolegend, San Diego, CA, USA). Cells were re-suspended at a density of 10^6/mL in RPMI 1640 medium (Gibco, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal calf serum (FCS, Capricorn Scientific, Ebsdorfergrund, Germa-

ny), 2 mM glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin (all from Sigma-Aldrich, St. Louis, MO, USA).

PBMC culture

Freshly isolated PBMCs were seeded onto 96-well plates and subsequently cultured with budesonide (10 nM, Glentham Life Sciences Ltd, Corsham, Wiltshire, UK), azithromycin (10 μg/mL, Glentham Life Sciences Ltd), or their combination for 1 hour followed by phytohaemagglutinin (PHA, Sigma-Aldrich, St. Louis, MO, USA, 10 μg/mL) stimulation for 24 hours. PHA is known as a potent activator of T lymphocytes, which contribute to the most significant proportion of cells among PBMCs. Supernatants were then removed and analysed for IL-4, IL-5, IL-8, IL-13, IL-17A, IL-33, TSLP, macrophage migration inhibitory factor (MIF) by enzyme linked immunosorbent assay (ELISA, Bioassay Technology Laboratory, Shanghai, China; Vector-Best, Novosibirsk, Russian Federation).

It is worth noting that the concentrations of azithromycin and budesonide used in this study are physiologically relevant and concordant with concentrations that were found in the lungs of non-COPD patients treated with a 500 mg/day oral dose of azithromycin or 1.6 mg inhaled budesonide, respectively [10, 11].

Flow cytometry

Intracellular cytokine production by T helper (CD3+CD4+) cells and cytotoxic (CD3+CD8+) T lymphocytes was evaluated using flow cytometry. The staining was performed using anti-human-specific monoclonal antibodies (mAbs) conjugated with the following fluorochromes: fluorescein isothiocyanate (FITC), phycoerythrin (PE), PE-DyLight 594, PE-Cy5, allophycocyanin (APC), and APC-Alexa Fluor 750. These human mAbs included anti-CD45, anti-CD3, anti-CD4, anti-CD8, anti-IL-4, anti-IL-8, anti-IFNγ, and anti-TNFα mAbs, as well as isotype mAbs, which were purchased from Beckman Coulter, Marseille, France, Exbio, Prague, Czech Republic, and R&D systems Europe, Abingdon, UK.

Heparinised whole blood was diluted 1:1 with RPMI 1640 containing 10% FCS and treated in the presence or absence of budesonide (10 nM) and azithromycin (10 μg/mL) at 37°C, 5% CO_2 for 1 hour. Blood cultures were then stimulated with phorbol 12-myristate 13-acetate (PMA, 50 ng/mL) (Cayman Chemical, Ann Arbor, Michigan, USA) and ionomycin (1 μg/mL) (Cayman Chemical, Israel) in the presence of 10 μg/mL of brefeldin A (Cayman Chemical, Israel) and the tubes incubated at 37°C, 5% CO_2 with tube caps loosened to allow entry of CO_2-containing air. At 6 hours 100 μL of 20 mM disodium ethylenediaminetetraacetate dihydrate in phosphate buffered saline was added to the culture tubes to arrest activation and remove adherent cells.

Following washing of cells, mAbs to surface markers were added for 15 minutes at room temperature (RT), and red blood cells were then lysed using VersaLyse (Beckman Coulter). After 10 minutes, tubes were centrifuged at 500 × g for 5 minutes, supernatant removed, and leucocytes fixed and permeabilised using IntraPrep Permeabilization Reagent (Beckman Coulter). Appropriately diluted mAbs to intracellular markers were further added for 15 minutes at RT. Unstimulated and isotype controls were additionally used to enable the correct compensation and confirm antibody specificity. After washing cells in wash buffer, centrifugation and decanting the supernatant, cells were fixed with Fixative Solution (Beckman Coulter) prior to flow cytometric analysis. Samples were acquired on a Navios flow cytometer using Kaluza Analysis software (Beckman Coulter, Brea, CA, USA).

Statistical analysis

GraphPad Prism 7.00 (GraphPad Software Inc., San Diego, CA, USA) was used for data analysis. Results are expressed as mean ± standard error of the mean (SEM) for baseline characteristics of the patients and experimental data. Comparisons of the data between different groups were calculated using one-way analysis of variance (ANOVA). If the difference was significant, the Tukey post hoc test was performed for multiple comparisons. A P value of less than 0.05 was considered statistically significant.

Results

Effect of azithromycin and budesonide on PHA-induced cytokine release from PBMCs

After preincubation of PBMCs with PHA for 24 hours, there was a marked increase in IL-4, IL-5, IL-8, IL-13, IL-17A, IL-33, and TSLP release, but not MIF secretion (Figures 1, 2). This effect of PHA on IL-4, IL-5, IL-8, and IL-13 release from PBMCs was partially or completely inhibited by budesonide. Addition of azithromycin alone to PBMCs cultures resulted in a decrease of IL-4, IL-5, IL-13, and IL-17A production.

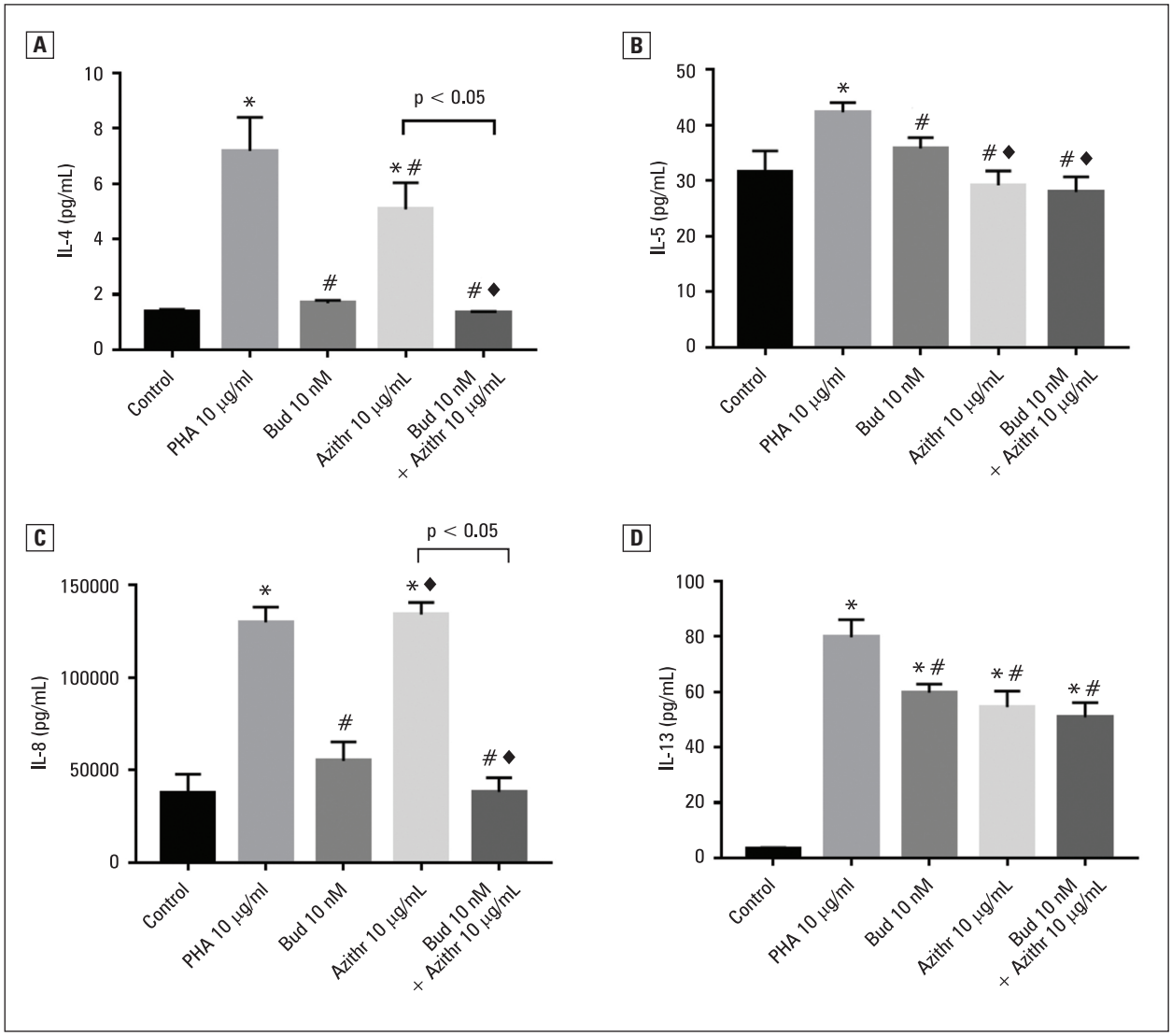

Figure 1. Effect of azithromycin and budesonide alone and in combination on phytohaemagglutinin (PHA)-induced release of interleukin (IL)-4, IL-5, IL-8 and IL-13 from peripheral blood mononuclear cells (PBMCs) of chronic obstructive pulmonary disease (COPD) patients. PBMCs from patients with COPD were treated with azithromycin (Azithr; 10 μg/mL), budesonide (Bud; 10 nM), or their combination for 1 hour, and then stimulated with PHA (10 μg/mL) for 24 hours. Cell-free supernatants were collected and the concentrations of IL-4 (**A**), IL-5 (**B**), IL-8 (**C**) and IL-13 (**D**) were measured by enzyme-linked immunosorbent assay (ELISA). Results are expressed as mean ± standard *error* of the mean (SEM); n = 6. One-way ANOVA followed by post hoc Tukey test: *p < 0.05 versus control; #p < 0.05 versus PHA; ♦p < 0.05 versus Bud

In contrast to single azithromycin or budesonide exposure, the association of these drugs effectively decreased IL-33, TSLP and MIF production induced by PHA. The combination of azithromycin with budesonide also showed greater IL-4, IL-8, and TSLP reduction than either compound alone. In addition, the inhibitory effect of azithromycin combined with budesonide on IL-5 and IL-17A synthesis by PBMCs was significantly greater than the effect of budesonide alone.

Effect of azithromycin and budesonide on PMA/ionomycin-induced cytokine production by T cells

Having ascertained that combination of azithromycin and budesonide could suppress the glucocorticosteroid-insensitive release of IL-17A, IL-33, TSLP, and MIF from PBMCs, we then aimed to determine the effect of these drugs on IL-4, IL-8, IFNγ and TNFα expression by CD4+ and CD8+ T cells. IL-4 and IL-8 production by T helper cells

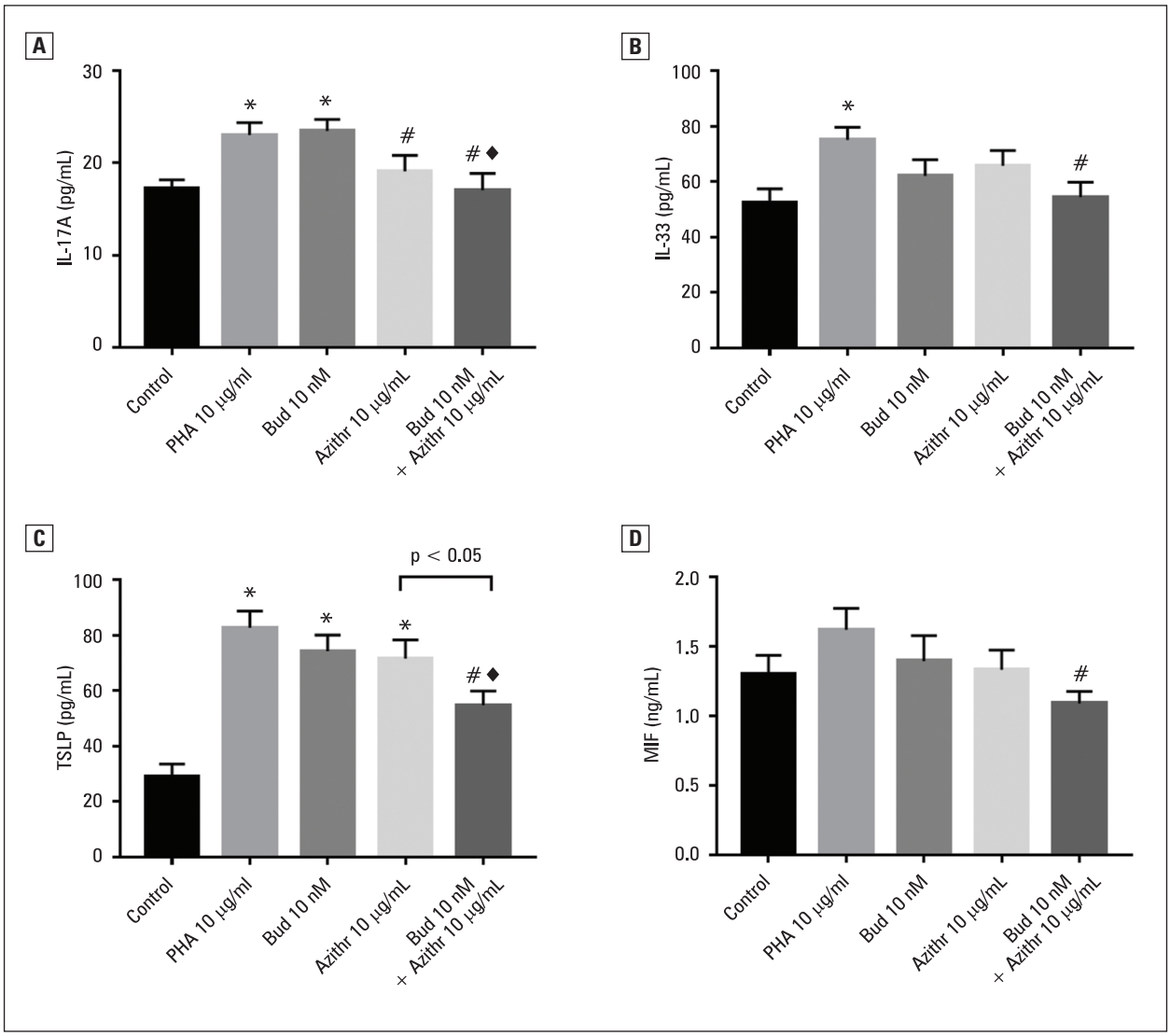

Figure 2. Effect of azithromycin and budesonide alone and in combination on phytohaemagglutinin (PHA)-induced release of interleukin (IL)-17A, IL-33, thymic stromal lymphopoietin (TSLP) and macrophage migration inhibitory factor (MIF) from peripheral blood mononuclear cells (PBMCs) of chronic obstructive pulmonary disease (COPD) patients. PBMCs from patients with COPD were treated with azithromycin (Azithr; 10 μg/mL), budesonide (Bud; 10 nM), or their combination for 1 hour, and then stimulated with PHA (10 μg/mL) for 24 hours. Cell-free supernatants were collected and the concentrations of IL-17A (**A**), IL-33 (**B**), TSLP (**C**) and MIF (**D**) were measured by enzyme-linked immunosorbent assay (ELISA). Results are expressed as mean ± standard error of the mean (SEM); n = 6. One-way ANOVA followed by post hoc Tukey test: *$p < 0.05$ versus control; #$p < 0.05$ versus PHA; ♦$p < 0.05$ versus Bud

and cytotoxic T lymphocytes was significantly decreased in the presence of budesonide or azithromycin alone compared with cultures with no drug (Figure 3). However, neither azithromycin nor budesonide had any effect on IFNγ-expressing CD4+ T cells and TNFα-producing CD8+ T lymphocytes (Figure 4).

The association of budesonide and azithromycin effectively reduced IL-4, IL-8, IFNγ, and TNFα expression by both CD4+ and CD8+ T cells (Figure 5). Moreover, combined treatment with budesonide and azithromycin markedly enhanced suppressive effect seen with budesonide alone on IL-4 and IL-8 expression by CD4+ and CD8+ T lymphocytes.

Discussion

We have shown here the anti-inflammatory effects of azithromycin on cytokine production by PBMCs and in particular by T cells from patients with COPD. Azithromycin in combination with budesonide enhances GC properties by inhibiting synthesis of pro-inflammatory cytokines in blood cells of COPD patients. These results may provide pharmacological approach for combination use of

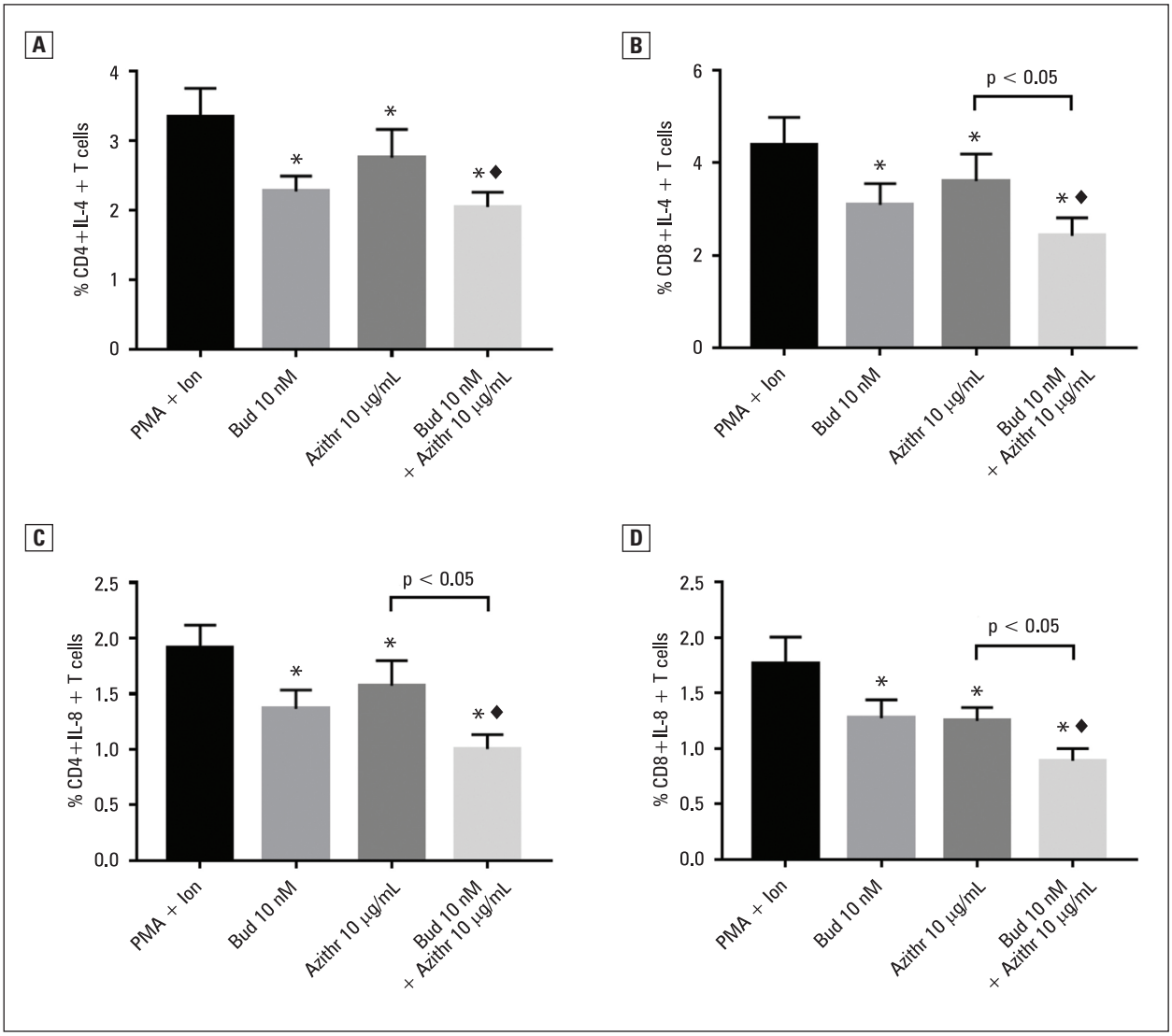

Figure 3. Effect of azithromycin and budesonide alone and in combination on interleukin (IL)-4 and IL-8 expression by CD4+ and CD8+ T cells of chronic obstructive pulmonary disease (COPD) patients. Human peripheral blood cells from patients with COPD were incubated with azithromycin (Azithr; 10 μg/mL), budesonide (Bud, 10 nM), or their combination for 1 hour, and then stimulated with phorbol 12-myristate 13-acetate (PMA, 50 ng/mL) and ionomycin (Ion, 1 μg/mL) for 6 hours. Production of IL-4 (**A** and **B**) and IL-8 (**C** and **D**) by CD4+ and CD8+ T cells was measured by flow cytometry. Results are expressed as mean ± standard *error* of the mean (SEM) of 5–6 independent experiments. One-way ANOVA followed by post hoc Tukey test: *$p < 0.05$ versus PMA + Ion; ♦$P < 0.05$ versus Bud

azithromycin and GCs in the treatment of COPD patients.

PBMCs from patients with COPD secrete a broad range of cytokines and chemokines. Several of the intracellular signalling pathways regulating the production of these mediators are activated by oxidative stress as they include NF-κB, which activity is under control of oxidants [2]. NF-κB expression has been shown to be increased in PBMCs of patients with COPD [7]. In the current article, in order to stimulate cells we used PHA and PMA, which are known to induce generation of reactive oxygen species and to increase NF-κB activity [12, 13].

Inflammatory response in COPD is mainly orchestrated by Th1 cells. However, recent evidence suggests that Th2 and Th17 cells are also involved in initiation and maintenance of inflammation in COPD. Th1 cells predominantly secrete IFNγ and TNFα, Th2 cells produce IL-4, IL-5, and IL-13, while Th17 cells synthesize IL-17A [14].

In this study, azithromycin reduced TNFα production by T helper cells. However, azithromycin showed no effect on the percentage of TNFα-positive cytotoxic T lymphocytes as well as IFNγ-expressing CD4+ and CD8+ T cells, whereas the combined azithromycin/budesonide

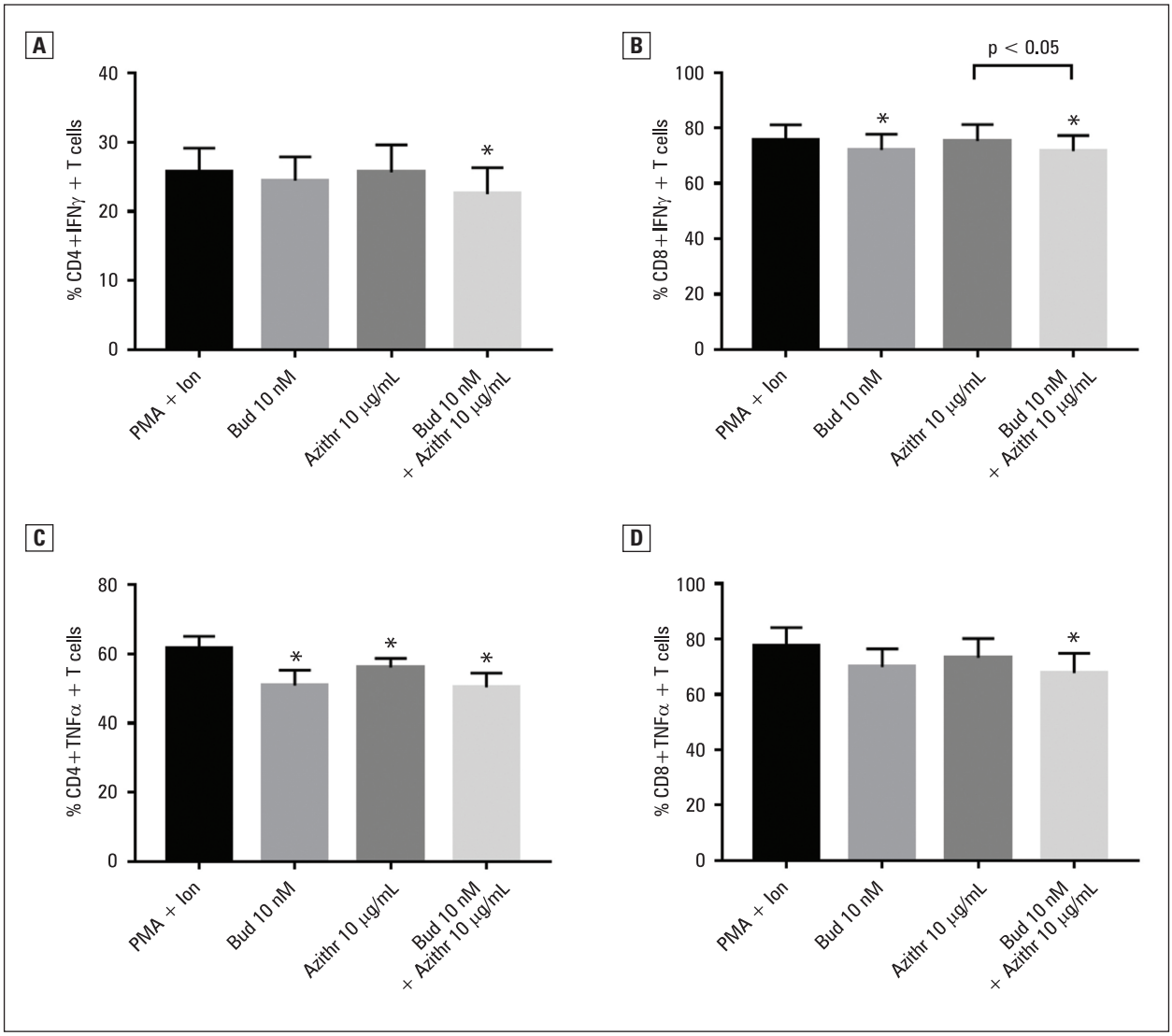

Figure 4. Effect of azithromycin and budesonide alone and in combination on interferon γ (IFNγ) and tumor necrosis factor α (TNFα) expression by CD4+ and CD8+ T cells of chronic obstructive pulmonary disease (COPD) patients. Human peripheral blood cells from patients with COPD were incubated with azithromycin (Azithr; 10 μg/mL), budesonide (Bud, 10 nM), or their combination for 1 hour, and then stimulated with PMA (50 ng/mL) and ionomycin (Ion, 1 μg/mL) for 6 hours. Production of IFNγ (**A** and **B**) and TNFα (**C** and **D**) by CD4+ and CD8+ T cells was measured by flow cytometry. Results are expressed as mean ± standard *error* of the mean (SEM) of 6 independent experiments. One-way ANOVA followed by post hoc Tukey test: *$p < 0.05$ versus PMA + Ion

treatment significantly reduced production of IFNγ and TNFα by T helper cells and cytotoxic T lymphocytes. Synergistic interactions of TNFα with IFNγ can potentiate the secretion of CXCL9, CXCL10 and CXCL11 from structural cells of the airways [15]. Released chemokines further bind on chemokine receptor CXCR3 to attract circulating T lymphocytes into the bronchial tissue [16]. Thus, taking into account our results, T lymphocyte recruitment in COPD patients is likely to be repressed by combination of azithromycin and budesonide through the inhibition of both TNFα and IFNγ.

IL-4 regulates the differentiation of uncommitted precursors to Th2 cells. Together with IL-13, it shares many functions, such as induction of IgE production by B lymphocytes and recruitment of eosinophils to the airways via the secretion of CCL26 from bronchial epithelial cells. IL-4 and IL-13 can also promote airway fibrosis and mucus hypersecretion [17]. COPD patients had a higher percentage of bronchoalveolar lavage CD8+ T lymphocytes producing IL-4 and IL-13 when compared with healthy non-smokers or smokers with normal lung function [18]. In the current article, azithromycin alone reduced IL-4 and

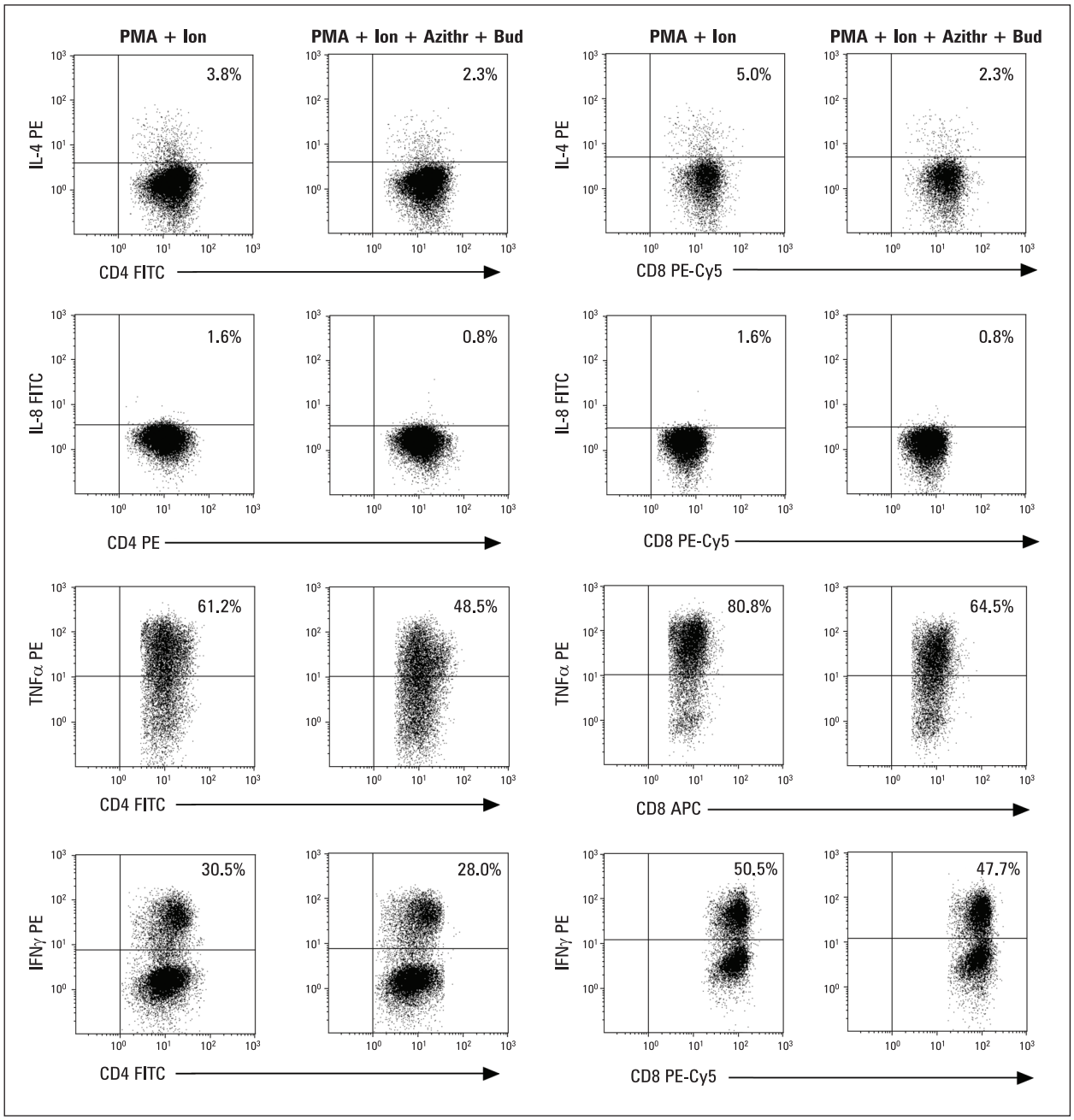

Figure 5. Representative dot plots showing the combined effect of 10 μg/mL azithromycin (Azithr) and 10 nM budesonide (Bud) on the percentage of CD4+ and CD8+ T cells producing interleukin (IL)-4, IL-8, interferon γ (IFNγ), tumor necrosis factor α (TNFα) from a patient with chronic obstructive pulmonary disease

IL-13 release from PBMCs, as well as IL-4 expression by CD4+ and CD8+ T cells. Addition of budesonide to culture tubes also attenuated impact of PHA or PMA/ionomycin stimulation of cells on IL-4 and IL-13 production. More importantly, we observed improvements in the suppressive effects of budesonide by azithromycin for IL-4 production by PBMCs, CD4+ and CD8+ T cells. To our knowledge, this is the first study, which characterizes inhibitory profiles of the combined azithromycin/budesonide therapy on IL-4 and IL-13 release from PBMCs of COPD patients.

IL-5 induces eosinophilic inflammation as it is involved in eosinophil differentiation and maturation in the bone marrow, migration, activation and degranulation of eosinophils in the airways [1]. Agents blocking IL-5 have been shown to reduce exacerbation rates, sputum eosinophil counts, and health-related quality of life

scores in COPD patients with eosinophilia [19]. In the current study, azithromycin suppressed PHA-induced IL-5 secretion from PBMCs of COPD patients. Additionally, the concentration of IL-5 in the supernatants of concanavalin A- or toxic shock syndrome toxin 1 (TSST-1)-stimulated PBMCs from healthy subjects was decreased in the presence of azithromycin, as reported previously [20]. We observed that azithromycin as well as the association of azithromycin with budesonide reduced the production of IL-5 by PBMCs stronger, than single budesonide treatment, indicating that azithromycin has the potential to target eosinophilic inflammation in COPD.

IL-33 drives type 2 immunity and induces production of IL-4, IL-5 and IL-13 by Th2 cells and type 2 innate lymphoid cells (ILC2) [17]. IL-33 has been reported to be upregulated in the airway epithelial cells and peripheral blood lymphocytes from COPD patients, and IL-33 expression in these cells can be affected by factors contributing to COPD pathogenesis, including cigarette smoke exposure and oxidative stress [21, 22]. Moreover, IL-33 is also involved in molecular events, provoked by viral-induced exacerbations of COPD [21]. In this work, both budesonide and azithromycin alone showed a poor inhibitory effect on IL-33 release from PBMCs. However, the association of azithromycin with budesonide reduced IL-33 production by PBMCs, implying that azithromycin has the potential to enhance corticosteroid sensitivity in COPD.

In the current study, azithromycin and budesonide affected TSLP release from PBMCs in a manner similar to that observed for IL-33 production. Recent studies showed that TSLP, apart from Th2-type immune response, has a remarkable effect on non-Th2-type processes by involving immune and structural cells. TSLP also upregulates expression of chemokines CXCL8, CCL1, CCL17, CCL18, CCL22, CCL24 and promote subsequent migration of effector cells to the airways [23]. Furthermore, TSLP mitigates corticosteroid sensitivity of ILC2-cells by activation of STAT5 [24]. In this work, we observed that combination of azithromycin with budesonide was more potent than single medications in the suppression of PHA-induced TSLP release from PBMCs of COPD patients. Thus, combination azithromycin/budesonide therapy may allow to achieve the desired effect of TSLP inhibition in COPD.

IL-17A augments release of IL-8 and granulocyte-macrophage colony-stimulating factor (GM-CSF) from airway epithelial cells [25]. Recent evidence showed that genetic variants in IL-17A are associated with the risk of COPD related to tobacco smoking and biomass burning exposure [26]. Moreover, IL-17A-mediated inflammation in COPD can contribute to steroid resistance as IL-17A may activate PI3K and subsequently suppress HDAC2 activity [27]. Here, we report that corticosteroid-insensitive IL-17A release from PBMCs of patients with COPD was reduced by azithromycin. Another study found that azithromycin at the same concentration of 10 μg/mL decreased IL-17A production by CD4+ T cells of healthy subjects [28]. Furthermore, in our study azithromycin/budesonide combination suppressed PHA-induced IL-17A secretion greater, than budesonide alone. These findings suggest that azithromycin might reverse the corticosteroid insensitivity of IL-17A in PBMCs of COPD patients.

IL-8 acts as a chemokine that stimulates neutrophil migration into the airways. It upregulates mucin gene expression in airway epithelial cells, leading to mucus hypersecretion and heightened rate of COPD exacerbations [29]. As suggested by our experimental findings, azithromycin failed to decrease IL-8 release from PBMCs of COPD patients. In contrast to PBMCs, IL-8 production by CD4+ and CD8+ T-cells was inhibited in the presence of azithromycin compared with cultures with no drug. Moreover, azithromycin had no effect on IL-8 expression by CD14+ monocytes (data not shown), indicating different effect of the drug on blood cell subpopulations for IL-8 production. In agreement with our results, Gualdoni et al. [30] found no alterations of IL-8 production by lipopolysaccharide (LPS)-stimulated monocytes of healthy volunteers co-treated with azithromycin. Other authors have shown no effect of azithromycin on IL-8 secretion from LPS-induced dendritic cells [31], which are presented in PBMC fraction, besides lymphocytes and monocytes. In addition, in the present study azithromycin and budesonide exhibited additive effect, suppressing IL-8 production by PBMCs, CD4+ and CD8+ T cells. A recent study showed that combination of macrolide erythromycin with dexamethasone was more effective at reducing IL-8 production by PBMCs of COPD patients than erythromycin alone [5], supporting our findings with azithromycin.

MIF plays an important role in innate and adaptive immune responses. This cytokine is released by wide range of cell types implicated in the pathogenesis of COPD, including lympho-

cytes, monocytes/macrophages, epithelial, and endothelial cells, upon stimulation with microbial products and oxidative stress [32]. MIF modulates transcription factor Nrf2, which is a crucial regulator of antioxidant genes [32]. Moreover, MIF limits cellular sensitivity to GCs via regulation of mitogen-activated protein kinase phosphatase 1 expression, p38 mitogen-activated protein kinase phosphorylation, and NF-κB/IκB signaling cascade [33]. We have previously demonstrated that MIF level in blood plasma is significantly higher in steroid resistant patients with COPD compared with steroid sensitive [34]. In this work, we observed that combination of azithromycin and budesonide decreased the release of MIF from PBMCs. This contrasts with the effect of these drugs alone, which failed to suppress MIF secretion. These findings suggest that azithromycin might regain corticosteroid sensitivity in patients with COPD.

Accumulating evidence suggests that macrolides might also reverse steroid resistance through restoration of HDAC2 expression, inhibition of the PI3Kδ/Akt pathway, and enhancement of GRα expression [5]. Moreover, azithromycin inhibits extracellular signal-regulated kinase (ERK) and c-jun N-terminal kinase (JNK) activity [20]. These kinases can phosphorylate GRα, blocking its nuclear translocation and reducing steroid sensitivity [4].

A clinical trial aimed to identify clinical subgroups of COPD patients most likely to benefit from azithromycin at a dose of 250 mg daily for 1 year in addition to usual care showed that azithromycin may be less effective in current smokers than in ex-smokers [35]. However, we did not take into account smoking status of patients enrolled in the current study, which represents a limitation of our study.

In conclusion, azithromycin alone demonstrates anti-inflammatory effects on blood cells from patients with COPD. This drug reduces the secretion of IL-4, IL-5, IL-13, and IL-17A from PBMCs, as well as the production of IL-4 and IL-8 by CD4+ and CD8+ T cells. Azithromycin combined with budesonide is more potent at suppressing IL-4, IL-5, IL-8, IL-17A, TSLP production by PBMCs, as well as IL-4 and IL-8 production by T helper cells and cytotoxic T lymphocytes when compared with budesonide alone. The data obtained provide an evidence of the effectiveness of azithromycin/budesonide combination in suppressing inflammation in COPD.

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Conflict of interest

None declared.

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