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CHAPTER 3

AZITHROMYCIN DIFFERENTIALLY AFFECTS THE IL-13-INDUCED EXPRESSION PROFILE IN HUMAN BRONCHIAL EPITHELIAL CELLS

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ABSTRACT

The T helper 2 (Th2) cytokine interleukin (IL)-13 is a central regulator in goblet cell metaplasia and induces the recently described Th2 gene signature consisting of periostin (POSTN), chloride channel regulator 1 (CLCA1) and serpin B2 (SERPINB2) in airway epithelial cells. This Th2 gene signature has been proposed as a biomarker to classify asthma into Th2-high and Th2-low phenotypes. Clinical studies have shown that the macrolide antibiotic azithromycin reduced clinical symptoms in neutrophilic asthma, but not in the classical Th2-mediated asthma despite the ability of azithromycin to reduce IL-13-induced mucus production. We therefore hypothesize that azithromycin differentially affects the IL-13-induced expression profile. To investigate this, we focus on IL-13-induced mucin and Th2-signature expression in human bronchial epithelial cells and how this combined expression profile is affected by azithromycin treatment. Primary bronchial epithelial cells were differentiated at air liquid interface in presence of IL-13 with or without azithromycin. Azithromycin inhibited IL-13-induced MUC5AC, which was accompanied by inhibition of IL-13-induced CLCA1 and SERPINB2 expression. In contrast, IL-13-induced expression of POSTN was further increased in cells treated with azithromycin. This indicates that azithromycin has a differential effect on the IL-13-induced Th2 gene signature. Furthermore, the ability of azithromycin to decrease IL-13-induced MUC5AC expression may be mediated by a reduction in CLCA1.
INTRODUCTION

Asthma is a syndrome characterized by airway hyperresponsiveness, chronic inflammation and mucus hypersecretion. Historically asthma has mainly been thought to be driven by a T helper 2 (Th2)-mediated immune response. However, it is now well recognized that asthma consists of multiple phenotypes with different pathophysiological pathways underlying airway inflammation, which may benefit from targeted treatment (1, 2). Different approaches have been taken to develop biomarkers to distinguish these phenotypes to guide clinical treatment. Recently various clinical trials have shown the potential of inhibitors of Th2 inflammation, including IL-13, to modulate clinical outcomes in asthma (3). IL-13 is one of the cytokines produced by Th2 CD4 T cells, and has been shown to have marked effects on the airway epithelium (4).

IL-13 induces goblet cell metaplasia in human bronchial epithelial cells in vitro and in vivo. The main mucins produced by goblet cells, MUC2, MUC5AC and MUC5B, are expressed in healthy human airways and their proportion can vary with health status. MUC2 and MUC5AC expression is increased in bronchial biopsies of Th2-high asthma patients compared to healthy controls. However, MUC5B expression is lower in Th2-high asthma patients compared to healthy controls (5). Whereas a variety of stimuli increase MUC5AC expression, the Th2 cytokine IL-13 appears a central trigger for its production in asthma. Different factors such as chloride channel regulator 1 (CLCA1), SAM pointed domain-containing ETS transcription factor (SPDEF) and forkheadbox A2 (FOXA2) have been implicated in the regulation of IL-13-induced MUC5AC expression (6-8).

In addition to increasing mucin gene expression, IL-13 is a central regulator in the epithelial gene expression of periostin (POSTN) and serpin B2 (SERPINB2), and expression of these genes together with CLCA1, has been used as a signature to classify asthma into Th2-high and Th2-low phenotypes (9). Periostin is of particular interest as a biomarker, as it is detectable in the circulation and therefore may be useful as a blood biomarker for IL-13-activated bronchial epithelial cells. Indeed, there is evidence suggesting that circulating periostin levels may be helpful to identify asthma patients that benefit from anti-IL-13 treatment (1, 10-13).

Several reports indicate that macrolide antibiotics have beneficial effects in the treatment of chronic inflammatory airway diseases such as cystic fibrosis, chronic obstructive pulmonary
disease (COPD) and asthma (14-16). These effects have partly been attributed to immune-modulatory actions, but the mechanisms involved are incompletely understood. Inhibitory effects of macrolides on goblet cell metaplasia and mucin expression induced by various stimuli may contribute to these clinical effects and also IL-13-induced MUC5AC expression has been shown to be inhibited by a macrolide antibiotic (17-23). However, whether macrolides also control the expression of the Th2 signature in epithelial cells is unknown.

Clinical studies have shown that the macrolide antibiotic azithromycin reduces clinical symptoms in neutrophilic asthma, but not in the classical Th2-mediated asthma (16). However, azithromycin has been shown to reduce IL-13-induced mucus expression in various studies. We therefore hypothesize that azithromycin differentially affects the IL-13-induced expression profile. To investigate this, we focus on IL-13-induced mucin and Th2-signature expression in human bronchial epithelial cells and how this combined expression profile is affected by azithromycin treatment.
MATERIAL AND METHODS

Bronchial epithelial cell culture and treatment

Human primary bronchial epithelial cells (PBEC) were isolated from macroscopically normal bronchial tissues obtained from lung cancer patients undergoing lobectomy at the Leiden University Medical Center (Leiden, The Netherlands). Cancer-free trimmed tissues were washed and incubated 2h at 37°C with 0.18% (w/v) proteinase type XIV (Sigma-Aldrich, St. Louis, MO, USA) in Ca²⁺/Mg²⁺-free Hank’s Balanced Salt Solution (Gibco, Bleiswijk, The Netherlands). Epithelial cells were gently scraped off the luminal surface, washed and subsequently cultured in serum-free keratinocyte medium (Gibco) supplemented with 0.2 ng/ml epidermal growth factor (Gibco), 25 µg/ml bovine pituitary extract (Gibco), 1 µM isoproterenol (Sigma-Aldrich), 100 U/ml Penicillin (Lonza, Verviers, Belgium) and 100 µg/ml Streptomycin (Lonza) on coated 6-well plates (coated at 37°C, 5% CO₂ for 2-24h with 30 µg/ml PureCol [Advanced BioMatrix, San Diego, CA, USA], 10 µg/ml Bovine serum albumin [Sigma-Aldrich] and 10 µg/ml fibronectin [isolated from human plasma] diluted in PBS). During the first week of culture following isolation of epithelial cells from lung tissue, 200 µg/ml of the anti-mycoplasma agent ciprofloxacin (Fresenius Kabi, Schelle, Belgium) was added to the medium. After reaching near-confluence, cells were trypsinized (0.03% [w/v] trypsin [Difco, Detroit, USA], 0.01% [w/v] EDTA [BDH, Poole, England], 0.1% glucose [BDH] in PBS) and stored in liquid nitrogen.

These PBEC were used for generation of mucociliary differentiated PBEC cultures by differentiation at the air-liquid interface (ALI) as described previously (24). Briefly, PBEC were cultured submerged on semipermeable transwell inserts with 0.4 µm pore size (Corning Costar, Cambridge, MA) that were coated with a mixture of collagen and fibronectin. Once full confluence was reached, apical medium was removed and PBEC were cultured at ALI during two weeks.

Study design

ALI-PBEC cultures were incubated in presence or absence of recombinant human IL-13 (Peprotech, Rocky Hill, NJ, USA) that was added to the basolateral compartment of the transwell insert during two weeks of ALI differentiation. Azithromycin (Sigma-Aldrich) was also added to the basolateral compartment during ALI differentiation; 0.04% (v/v) DMSO
(Merck, Darmstadt, Germany) was used as vehicle control for azithromycin. Medium with respective treatments was refreshed three times a week. After 14 days exposure, basal medium was collected and stored at -20°C until further use.

**RNA isolation, reverse transcription (RT) and qPCR**

Total RNA was extracted using the Maxwell 16 LEV simplyRNA Tissue Kit (Promega, Leiden, The Netherlands) and quantified using the Nanodrop ND-1000 UV-visible (UV-Vis) spectrophotometer (Nanodrop Technologies, Wilmington, DE). For cDNA synthesis, 1μg of total RNA was reverse transcribed using oligo(dT) primers and Moloney murine leukemia virus (M-MLV) polymerase (Promega) at 37°C. Primer sequences are listed in table 1. RPL13A and ATP5B were used as reference genes. All quantitative PCRs (qPCRs) were carried out in triplicate on a CFX-384 real-time PCR detection system (Bio-Rad Laboratories, Veenendaal, The Netherlands) with the use of SensiFAST™ SYBR green (Bioline, Luckenwalde, Germany). Bio-Rad CFX manager 3.0 software (Bio-Rad) was used to calculate arbitrary gene expression by using the standard curve method.
Azithromycin differentially affects the IL-13-induced expression profile in human bronchial epithelial cells

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence</th>
<th>NCBI Gene ID</th>
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</table>
| POSTN      | F: GAC CGT GTG CTT ACA CAA ATT G  
             R: AAG TGA CCG TCT CTT CCA AGG | 10631        |
| SERPINB2   | F: TCC TGG GTC AAG ACT CAA ACC  
             R: CAT CCT GGT ATC CCC ATC TAC AG | 5055         |
| CLCA1      | F: ATG GCT ATG AAG GCA TTG TCG  
             R: TGG CAC ATT GGA GTC GAT TG | 1179         |
| MUC5AC     | F: CCT TCG ACG GAC AGA GCT AC  
             R: TCT CGG TCA CAC GAA AG | 4586         |
| MUC2       | F: GGA GAT CAC CAA TGA CTG CGA  
             R: GAA TCG TTG TGG TCA CCC TTG | 4583         |
| MUC5B      | F: GGG CTT TGA CAA GAG AGT  
             R: AGG ATG GTC GTG TTG ATG CG | 727897       |
| FOXA2      | F: ACT ACC CCG GCT ACG GTT C  
             R: AGG CCC GTT TTG TTC GTG A | 3170         |
| SPDEF      | F: ATG AAA GAG CGG ACT TCA CCT  
             R: CTG GTC GAG GCA CAG TAG TG | 25803        |
| RPL13A     | F: AAG GTG GTG GTC GTA CGT TGT G  
             R: CCG GAA GGG TTG GTG TTC ATC C | 23521        |
| ATP5B      | F: TCA CCC AGG CTG GTT CAG A  
             R: AGT GGC CAG GGT AGG CTG AT | 506          |

**Mucin analysis**

To determine levels of MUC5AC, MUC5B and MUC2 protein in ALI-PBEC, cell lysates were serially diluted in PBS and 50 µl was spotted on a methanol-preincubated polyvinylidene-difluoride (PVDF)-membrane using a Bio-Dot microfiltration apparatus (Bio-Rad). Non-specific binding sites on the membranes were blocked with PBS/5% (w/v) skim milk (Sigma-Aldrich) overnight at 4°C. Subsequently the membrane was incubated with mouse-anti-MUC5AC (1:100; 45M1; Thermo Fisher Scientific, Breda, The Netherlands), rabbit-anti MUC5B (1:500; H-300; Santa Cruz; Bio-Connect B.V., Huissen, The Netherlands) or rabbit-anti-MUC2 (1:100; H-300; Santa Cruz) in PBS/5% (w/v) skim milk for 1h at room
temperature. HRP-conjugated anti-mouse or anti-rabbit IgG (both 1:10000, Cell signaling Technologies) was used as a secondary antibody and detected using ECL Western Blotting substrate (Thermo Fisher Scientific). Densitometry was performed using Totallab image analysis software (Nonlinear Dynamics, Newcastle upon Tyne, UK).

**SDS-PAGE & Western blot**

Protein lysates were diluted (2:3 [v/v]) in SDS sample buffer, containing 4% (w/v) SDS (Sigma-Aldrich), 20% (v/v) glycerol (Merck), 0.8% (w/v) DL-dithiothreitol (Sigma-Aldrich), 0.5 M Tris pH 6.8 and 0.003% (w/v) bromophenol blue (Sigma-Aldrich), heated for 5min at 100°C, and applied on a 4-15% SDS-PAGE gel (Mini-PROTEAN TGX, Bio-Rad). Next, proteins were blotted on a Trans-Blot Turbo Mini PDVF membrane using the Trans-Blot Turbo Transfer System (Bio-Rad). Nonspecific binding sites were blocked in TBS/0.05% (v/v) Tween-20 containing 5% (w/v) skimmed milk. Membranes were probed with rabbit-anti-CLCA1 (1:2500; EPR12254-88; Abcam, Cambridge, United Kingdom), rabbit-anti-SERPINB2 (1:1000; ab47742; Abcam), rabbit-anti-POSTN (1:2500, ab14041, Abcam) or GAPDH (1:1000; 14C10; Cell Signaling Technologies, Leiden, The Netherlands) in 5% (w/v) BSA TBS/0.05% (v/v) Tween-20 overnight at 4°C. Afterwards, membranes were incubated with anti-rabbit IgG HRP-linked Antibody (1:10000, Cell Signaling Technologies) in blocking buffer for 1h and membranes were subsequently developed with enhanced chemiluminescence substrate (Thermo Fisher Scientific).

**Statistical Analysis**

Graphs were made and statistical analysis was performed in GraphPad PRISM 6.02 (GraphPad Software Inc., La Jolla, Ca). Data are shown as means ± SEM of cultures derived from several donors and differences were considered significant at p-values < 0.05. Differences were explored by one-way ANOVA with Dunnett’s test.
RESULTS

Azithromycin lowers IL-13-induced MUC5AC and MUC2 expression, but does not significantly affect MUC5B expression

We first assessed the ability of IL-13 to induce MUC5AC expression in ALI-PBEC. The concentration of IL-13 was optimized in pilot experiments that showed inconsistent induction of MUC5AC at 1 ng/ml IL-13 whereas consistent induction of MUC5AC was observed for all donors at 5 ng/ml IL-13 (results not shown). We observed that IL-13 not only induced MUC5AC, but also increased MUC2 expression, whereas it lowered MUC5B expression (Fig. 1). Exposure to azithromycin caused a dose-dependent inhibition of IL-13-induced expression of MUC5AC and MUC2. These observations were confirmed at the protein level using dot blot analyses of protein lysates (Fig. 1).

Figure 1. Modulation of mucin expression by azithromycin in IL-13-exposed ALI-PBEC cultures. ALI-PBEC were cultured for 14 days in the presence (black bars) or absence (white bars) of IL-13 (5 ng/ml) and various concentrations of azithromycin (AZM) or its solvent control (CTRL = DMSO; equivalent to the highest concentration of AZM). Mucin gene expression (A) was assessed by qRT-PCR and protein (B) by dot blot for which a representative example is shown. Results are expressed as mean ± SEM fold change compared to IL13 stimulated condition with CTRL (indicated by a horizontal dashed line) (n=4 different donors). *p<0.05.
Azithromycin lowers IL-13-induced SPDEF and CLCA1 expression

To investigate the mechanisms underlying the modulation of IL-13-induced MUC5AC expression by azithromycin, the effect on CLCA1, SPDEF and FOXA2 gene expression was investigated. IL-13 increased SPDEF and CLCA1 expression, while a non-significant decrease in FOXA2 was observed (Fig. 2). Whereas azithromycin caused an increase of FOXA2 expression in control-treated cells which did not reach statistical significance, azithromycin did not prevent the IL-13-induced decrease in FOXA2 expression. In contrast, azithromycin significantly reduced IL-13-induced CLCA1, whereas inhibition of SPDEF expression did not reach statistical significance (p = 0.11). CLCA1 gene expression data was confirmed by measurement of protein levels in basal medium and cell lysates (Fig. 2).

**Figure 2.** Modulation of SPDEF, CLCA1 and FOXA2 expression by azithromycin in IL-13-exposed ALI-PBEC cultures. ALI-PBEC were cultured for 14 days in the presence (black bars) or absence (white bars) of IL-13 (5 ng/ml) and various concentrations of azithromycin (AZM) or its solvent control (CTRL = DMSO; equivalent to the highest concentration of AZM). FOXA2, SPDEF and CLCA1 gene expression (A) was assessed by qRT-PCR and CLCA1 protein (B) by western blot for which a representative example is shown. Results are expressed as mean ± SEM fold change compared to IL-13 stimulated condition with CTRL (indicated by a horizontal dashed line) (n=4 different donors). *p<0.05.
Azithromycin differentially affects the IL-13-induced Th2 gene signature in human bronchial epithelial cells

In addition to CLCA1, POSTN and SERPINB2 are also increased in human airway epithelial cells following IL-13 exposure and have been suggested as biomarkers for Th2-mediated inflammation (5, 9). In the present study, IL-13 exposure significantly increased POSTN and SERPINB2 gene expression in the ALI-PBEC culture system (Fig. 3). Treatment of IL-13-exposed ALI-PBEC with various concentrations of azithromycin resulted in a significant reduction of SERPINB2 expression, similar to what was observed for CLCA1. In contrast, exposure to azithromycin led to a further dose-dependent increase in POSTN expression. Gene expression data was confirmed by measurement of protein levels in basal medium and cell lysate. In line with the gene expression data, azithromycin treatment resulted in lower protein levels of SERPINB2 in cell lysates. SERPINB2 could not be detected in the basal medium. Additionally, azithromycin further increased the IL-13-induced expression of POSTN protein in both cell lysates and basal medium (Fig. 3).
Figure 3. Modulation of the Th2 signature genes by azithromycin in IL-13-exposed ALI-PBEC cultures. ALI-PBEC were cultured for 14 days in the presence (black bars) or absence (white bars) of IL-13 (5 ng/ml) and various concentrations of azithromycin (AZM) or its solvent control (CTRL = DMSO; equivalent to the highest concentration of AZM). POSTN and SERPINB2 gene expression (A) was assessed by qRT-PCR and protein (B) by dot blot for which a representative example is shown. Results are expressed as mean ± SEM fold change compared to IL-13 stimulated condition with CTRL (indicated by a horizontal dashed line) (n=4 different donors). *p<0.05.
DISCUSSION

Results from the present study show that the macrolide azithromycin differentially affects the IL-13-induced expression profile in human bronchial epithelial cells. Azithromycin inhibits IL-13-induced MUC5AC expression, which is accompanied by inhibition of SPDEF, CLCA1 and SERPINB2 expression. In contrast, azithromycin further increases IL-13-induced POSTN expression. CLCA1, SERPINB2 and POSTN were recently described as a biomarker for Th2-high asthma. Our results indicate that azithromycin has a differential effect on this IL-13-induced Th2 gene signature. The modulatory effects of azithromycin on IL-13-induced changes in gene expression is summarized in table 2.

Table 2. Overview effects azithromycin on the IL-13-induced expression pattern. AZM, azithromycin; CTRL, control; IL-13, interleukin-13; ND, not detected

<table>
<thead>
<tr>
<th>Gene</th>
<th>Effect of IL-13</th>
<th>Effect of AZM on IL-13-induced expression</th>
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<tbody>
<tr>
<td></td>
<td>mRNA</td>
<td>protein</td>
</tr>
<tr>
<td>SPDEF</td>
<td>↑</td>
<td>ND</td>
</tr>
<tr>
<td>FOXA2</td>
<td>↓</td>
<td>ND</td>
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<tr>
<td>MUC5AC</td>
<td>↑↑</td>
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<tr>
<td>CLCA1</td>
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<td>↑↑</td>
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<tr>
<td>SERPINB2</td>
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<td>↑</td>
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<tr>
<td>POSTN</td>
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Our study confirms the potential of azithromycin to inhibit mucus production, as shown by its ability to inhibit expression of not only MUC5AC, but also MUC2. Azithromycin has been shown in previous studies to attenuate goblet cell metaplasia and airway inflammation in allergic airway inflammation models in mice (19). Additionally, clarithromycin is able to inhibit IL-13-induced goblet cell metaplasia in human bronchial epithelial cells (17). So far the pathophysiological mechanism is not well described.

The present results show that azithromycin attenuates IL-13-induced CLCA1 expression. Increased CLCA1 expression is found in airway epithelial cells of asthmatics and is linked to airway hyperresponsiveness and goblet cell metaplasia (25-27). A study by Alevy and colleagues indicated an important role for CLCA1 in IL-13-induced MUC5AC expression.
(8). In this study, selective inhibition of CLCA1 and MAPK13 (activated by CLCA1) in IL-13-stimulated human airway epithelial cells significantly reduced MUC5AC expression. Furthermore, niflumic acid, a chloride channel inhibitor, has been shown to suppress IL-13-induced MUC5AC expression in human airway epithelial cells providing indirect evidence for a role of one of these chloride channels, CLCA1 (28). Collectively, these data suggest that IL-13-induced CLCA1 expression mediates MUC5AC expression via MAPK13. Our results show that azithromycin attenuates IL-13-induced CLCA1 expression in addition to MUC5AC expression, and suggest that azithromycin blocks IL-13 induced goblet cell metaplasia by inhibiting CLCA1.

Two other molecules which have been shown to play an important role in IL-13-induced MUC5AC expression are SPDEF and FOXA2. SPDEF expression has been implicated in Th2-mediated goblet cell metaplasia both in vivo and in vitro (43, 44). Increased expression of spdef has been found in the respiratory epithelium of mice challenged with IL-13 (29). Additionally, epithelial overexpression of spdef resulted in goblet cell metaplasia whereas genetic deletion of spdef abrogated goblet cells in the respiratory epithelium after pulmonary allergen exposure (30). In human bronchial epithelial cells treatment with siRNA against SPDEF attenuated IL-13-induced MUC5AC expression (7). In the present study, IL-13 increased SPDEF gene expression, which was non-significantly inhibited by azithromycin. Therefore it can be suggested that azithromycin may reduce goblet cell metaplasia by lowering SPDEF expression although this effect remains to be investigated at the protein level. In contrast to the positive regulators of goblet cell metaplasia SPDEF and CLCA1, FOXA2 is an inhibitory regulator and IL-13 has been shown to reduce FOXA2 expression in human bronchial epithelial cells (6). FOXA2 expression is decreased in lung tissues from patients with a variety of pulmonary diseases (31). In mice, inhibition of Foxa2 causes spontaneous goblet cell metaplasia, whereas conditional expression of Foxa2 inhibits allergen-induced goblet cell differentiation (32). However, the present results show that azithromycin was unable to prevent the IL-13-induced inhibition of FOXA2, making this pathway unlikely to mediate the effects of azithromycin.

The airway epithelial Th2 gene signature can be induced in vitro by IL-13 as demonstrated by the ability of IL-13 to induce POSTN, CLCA1 and SERPINB2 expression in cultured airway epithelial cells (9). Our results in a two weeks exposure model confirm these findings. Importantly, our model also reflects mucin expression patterns found in airway epithelial brushings of Th2-high asthma patients with higher MUC5AC and MUC2 expression and
lower MUC5B expression compared to healthy controls (5). We observed that azithromycin lowered IL-13-induced MUC5AC and MUC2 expression, but did not significantly alter MUC5B expression. The observation that MUC5B expression is maintained in presence of azithromycin is important in view of a recent publication suggesting an important role for MUC5B in airway defense (33).

Azithromycin also inhibited IL-13-induced expression of SERPINB2, another member of the Th2 signature. SERPINB2 is a serine protease inhibitor that can inhibit plasminogen activators thereby preventing plasmin activation. Plasmin is able to degrade the extracellular matrix, either directly by removing glycoproteins or by activating metalloproteinases (34). Therefore, by inhibiting SERPINB2, more plasmin becomes available for extracellular matrix turnover and reducing airway remodeling.

The biological consequences of the increased levels of periostin are so far not clear. POSTN is an extracellular matrix protein and integrin ligand known to be produced by airway epithelial cells and fibroblasts. The functional role of POSTN in asthma remains controversial. Initial studies using POSTN-deficient mice suggested a protective role whereby POSTN was able to prevent allergen-induced goblet cell metaplasia in mouse tracheal epithelial cells (35). Additionally, POSTN has been shown to inhibit allergen-induced IgE production and airway hyperresponsiveness (36). However, a more recent study, also performed in POSTN-deficient mice, suggested that POSTN is required for airway hyperresponsiveness (37). Nonetheless, at present the development of serum POSTN as a biomarker is clinically more important and treatment responses to anti-IL-13 treatments have been linked to baseline levels of POSTN (1, 13). Therefore it is important to identify factors which influence the expression of POSTN and could also affect serum levels in this patient group.

Macrolides are increasingly used for chronic treatment of lung diseases and also in asthma macrolides have been used for prevention and treatment of exacerbations (16, 38-41). Interestingly, the long-term effects of macrolides seem to be dependent on asthma phenotype. Patients with predominantly neutrophilic airway inflammation show beneficial responses to macrolide treatment, whereas macrolides are not effective in patients with eosinophilic airway inflammation (16, 42). Overall, our results indicate that macrolide treatment differentially affects the Th2 signature that has been described to identify Th2-high asthma, which is usually associated with eosinophilic inflammation.
The concentrations of azithromycin used in the present study are likely relevant for understanding the clinical effects of azithromycin treatment. The concentrations used in the present study ranged between 1 and 40 µg/ml, which is within the range reported in the lungs of patients treated with this macrolide: 2.18 µg/ml in the epithelial lining fluid to 3.89 ± 1.2 µg/ml in bronchial mucosal tissue (43). Importantly, concentrations of azithromycin in tissues can be up to 100-fold higher than those in plasma, which range around 0.15 and 0.05 µg/ml (44-46). Additionally, phagocytes accumulate azithromycin which results in 200-fold higher intracellular than extracellular concentrations (47, 48). Since azithromycin accumulation differs in various cell types (47-49), it is likely that both passive and active transport are important for azithromycin accumulation. Overall this suggests that clinically relevant concentrations of azithromycin have been used in this study.

This study has some limitations. We used bronchial epithelial cells derived from macroscopically normal resected tissue obtained during surgery for lung cancer, indicating that cells from smokers were used. Additionally, bronchial epithelial cells from asthmatics were not available to further evaluate the effect of azithromycin on the Th2 signature. We have tried to further elucidate the role of FOXA2 in the effects of azithromycin using siRNA-mediated knockdown of FOXA2 as described previously by Ramachandran and colleagues for the CFTR gene (50). However, whereas transient suppression of FOXA2 gene expression was achieved, we were unable to maintain low levels of FOXA2 during two weeks differentiation. Future studies using alternative approaches using e.g. CRISPR/Cas9 technology are needed, but were outside the scope of the present study.

Our results indicate that azithromycin differentially affects the gene signature that has been described for Th2-high asthma. The observation that azithromycin increases IL-13-induced POSTN expression while decreasing SERPINB2 and CLCA1, is of potential clinical significance because especially POSTN is considered as an emerging, possibly important biomarker for Th2 inflammation in asthma. This study indicates that macrolide treatment may differentially affect biomarkers derived from this gene signature, and that data on modulation of Th2 inflammation based on such biomarkers should be interpreted with caution. Our study further extends previous findings on the ability of macrolides to inhibit goblet cell metaplasia, by demonstrating that azithromycin blocks MUC5AC and MUC2, possibly by inhibiting CLCA1. These observations on macrolide-induced suppression of goblet cell metaplasia secondary to Th2 inflammation, provide a mechanistic basis for the observed beneficial effects of macrolides in asthma and COPD. Clinical results that show that
Azithromycin is not effective in Th2-mediated asthma. Azithromycin inhibits expression of several IL-13-induced genes with the exception of POSTN. Therefore, further investigations should focus on the role of periostin in asthma. Additionally, critical analysis is needed for the effect of various treatments on biomarker expression profiles.
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Azithromycin differentially affects the IL-13-induced expression profile in human bronchial epithelial cells