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ORIGINAL ARTICLE

Interleukin-1α induced release of interleukin-8 by human bronchial epithelial cells *in vitro*: assessing mechanisms and possible treatment options

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SUMMARY

Survival after lung transplantation is hampered by chronic lung allograft dysfunction (CLAD). Persistently elevated BAL-neutrophilia is observed in some patients despite treatment with azithromycin, which may be induced by IL-1α. Our aim is to establish an *in vitro* model, assess mechanistic pathways and test different therapeutic strategies of IL-1α-induced release of IL-8 by human bronchial epithelial cells. Bronchial epithelial cells (16HBE) were stimulated with IL-1α with or without azithromycin or dexamethasone. IL-8 protein was analyzed in cell supernatant. Different MAP kinases (p38, JNK, ERK$^{1/2}$, Iκβ) and targets known to be involved in tumor formation (PI3K, Akt) were investigated. Finally, different treatment options were tested for their potential inhibitory effect. IL-1α induced IL-8 in bronchial epithelial cells, which was dose-dependently inhibited by dexamethasone but not by azithromycin. IL-1α induced p38 and Akt phosphorylation, but activation of these MAPK was not inhibited by dexamethasone. JNK, ERK$^{1/2}$, Iκβ and PI3K were not activated. None of the tested drugs reduced the IL-1α induced IL-8 production. We established an *in vitro* model wherein steroids inhibit the IL-1α-induced IL-8 production, while azithromycin was ineffective. Despite using this simple *in vitro* model, we could not identify a new treatment option for azithromycin-resistant airway neutrophilia.

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Introduction

For selected patients with end-stage lung disease, lung transplantation is the ultimate treatment option. However, outcome after lung transplantation remains poor, mainly due to high morbidity and mortality associated with chronic lung allograft dysfunction (CLAD) with a five-year prevalence of 50% [1]. CLAD is defined as an irreversible decline in forced expiratory volume in one-second (FEV1) or/and in forced vital capacity (FVC) of at least 20% compared with the mean of the two best postoperative values [2].

In 2008, it became clear that there are different manifestations of CLAD and that the term bronchiolitis obliterans syndrome (BOS) was not sufficient to cover all forms [3]. This was based upon the observation that

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a cohort of LTx patients who experienced a decline in FEV_1 accompanied with an elevated BAL-neutrophilia and IL-8 levels experienced an improvement in their FEV_1 with at least 10% after azithromycin therapy, while others did not show a response [3]. These responders, initially denominated as neutrophilic reversible allograft dysfunction and later on renamed to azithromycin responsive allograft dysfunction (ARAD), typically display an IL-17-mediated airway neutrophilia, which is downregulated after azithromycin treatment [4,5]. IL-17 is shown to indirectly attract neutrophils, via the induction of IL-8 (CXCL8), a known neutrophilic chemo-attractant [6]. As ARAD can be adequately treated with azithromycin, it can no longer be classified as CLAD but as a known and treatable form of graft function decline [2]. As a consequence, azithromycin is now recommended as first-line therapy whenever a persistent FEV_1 decline is observed [7].

Recently, another CLAD phenotype has been described besides BOS: restrictive allograft syndrome (RAS). BOS is typically characterized by an obstructive pulmonary function defect, accompanied with scattered obliterative bronchiolitis (OB) lesions and air trapping on CT [2]. RAS, on the other hand, represents a restrictive defect in pulmonary function, mostly accompanied by persistent infiltrates on chest CT scan [2].

Recently, we identified lung transplant recipients experiencing a persistent, obstructive decline in FEV_1, who despite treatment with azithromycin demonstrated persistently elevated airway neutrophilia (≥ 15%), which we denominate "azithromycin-resistant neutrophilia". Although neutrophilia and IL-8 levels were increased in BAL of these patients, despite azithromycin therapy, we could not demonstrate an upregulation of IL-17-positive lymphocytes in the airway submucosa [8], which alludes to non-IL-17-mediated mechanisms of neutrophil recruitment. This is corroborated by Suwara *et al.,* who demonstrated elevated levels of IL-1α, IL-1Rα, IL-1β, IL-6, IL-8, and tumor necrosis factor-alpha (TNF-α) in BAL of these patients [9,10]. It has been suggested that damaged epithelial cells (considered to be one of the major risk factors for obliterative bronchiolitis) may act as a potential source of IL-1α, serving as an alternative neutrophil chemo-attractant in azithromycin-resistant neutrophilia [9].

Twenty-six percentage of our lung transplant recipients developed elevated (≥ 15%) BAL neutrophilia despite azithromycin treatment and these patients showed an inferior survival compared with patients without elevated BAL-neutrophilia under azithromycin treatment [11]. Up to now, no specific treatment is available and consequently survival remains poor after onset of azithromycin-resistant neutrophilia (<3 years). In our own experience, steroid pulses might bring some temporary improvement in FEV_1. However, due to their detrimental side effects, high doses of steroids can only be given in acute situations but cannot be maintained for longer periods.

We hypothesize that the pro-inflammatory interleukin, IL-1α, acts as key neutrophil chemo-attractant in azithromycin-resistant neutrophilia by stimulating the production of IL-8 from bronchial epithelial cells. Firstly, we aimed to establish an *in vitro* model of bronchial epithelial cells that responds to steroids but not to azithromycin. Secondly, we aim to investigate the involvement of various transcription factors. Finally, we investigate the possibility of drugs already routinely used in other chronic neutrophilic diseases to treat azithromycin-resistant neutrophilia.

Materials and methods

Culture of 16HBE cells

Epithelial cells, 16HBE14o- cells (16HBE), were kindly provided by Dr. Gruenert (university of California, San Francisco, USA) and were cultured according to the standard cell culture protocol as previously described [16]. The cells were used for experiments between passages 3 and 15.

Cytotoxicity measurements

16HBE cells were seeded in a 96-well plate. When at least 80% confluent, cells were rinsed with serum-free DMEM/F12 medium and exposed to 0, 0.01, 0.1, 1, 10, and 100 ng/mL IL-1α (R&D Systems, Oxon, United Kingdom) for 24 h. After incubation, cells were washed with DPBS and freshly diluted WST-1 cell proliferation reagent (1/20) (Roche Diagnostics, Vilvoorde, Belgium) was added for 30 min. Absorbance of cell supernatant was measured using a spectrophotometer (Bio-Rad Model 680XR microplate reader) at 450 nm.

IL-1α induced IL-8 production

Confluent 16HBE cells were stimulated with 0.01, 0.1, 1, and 10 ng/mL IL-1α for 24 h. IL-8 production was measured in cell supernatant in a 1/5 dilution via sandwich ELISA (Thermo Fisher Scientific). Ten ng/mL IL-1α was used for further stimulation experiments, for which cells were incubated with azithromycin (0.01 nM- 1000 nM) or dexamethasone (0.0001 μM-100 μM) 30 min prior to stimulation with IL-1α. After 24 h, cell supernatant was

removed and stored at −80 °C. IL-8 production from 16HBE cells stimulated only with 10 ng/mL IL-1α was considered as reference (100%). All other data were expressed relative to this concentration. Negative control cells were stimulated with an equivalent amount of the vehicle.

Potential of IL-1α to induce IL-8 production was also tested in primary human bronchial epithelial cells ($n = 5$). Two were collected in our own center (unused donor lungs) and three derived from tumor resections. A piece of large, cartilaginous bronchus was selected to derive human broncho-epithelial cells (HBEC) that were allowed to redifferentiate into a pseudo-stratified, mucociliary airway epithelium by an air–liquid interface (ALI) [12]. At day 15 after initiation of the ALI, cells were exposed to 10 ng/mL IL-1α at the apical side (100 μL) as well as the basolateral side (600 μL) to avoid concentration gradients; 24 h after exposure, supernatant of both compartments was collected and stored in −80 °C until IL-8 sandwich ELISA was performed. Per donor, two replicates were tested.

Western Blot analysis of MAP kinases after IL-1α stimulation

Confluent 16HBE cells in T25 culture flasks were either unstimulated or stimulated with 10 ng/mL IL-1α for 15, 20, 30, 45, 60, and 90 min. Afterward, cells were rinsed twice with ice-cold PBS and scraped in cell extraction buffer (Thermo Fisher Scientific) supplemented with phenylmethylsulfonyl fluoride (PMSF) and PhosStop™ (Roche, Brussels, Belgium). Samples were stored at −80 °C. For the Western blot analysis, samples were first separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) in Bolt™ 8% Bis-Tris Plus gels and were afterward transferred to a polyvinylidene difluoride (PVDF) membrane via dry blotting with an iBlot according to the manufacturer's instructions (Thermo Fisher Scientific). Western blotting was performed as described by Wuyts *et al.* [13]. Protein detection was carried out using ECL Prime Western Blotting Detection Reagent (GE HealthCare) using a ProXima 500 apparatus (Isogen Life Sciences, Temse, Belgium). To quantify the band intensity of the Western blots, 1D (Totallab, Newcastle upon Tyne, United Kingdom) was used including background correction.

New treatment options: N-acetylcysteine, pirfenidone, montelukast, dapsone, moxifloxacin, theophylline, and roflumilast

To investigate potential therapeutic strategies, 16HBE cells were exposed to different concentrations of N-acetylcysteine (NAC) (Sigma-Aldrich, Diegem, Belgium), pirfenidone (PFD) (0.001–5 mM) (Genentech, San Francisco, USA), montelukast (0.0001–1 μM) (Cayman, Michigan, USA), dapsone (0.0001–50 μM) (Sigma-Aldrich), moxifloxacin hydrochloride (0.0001 μM–0.1 mM) (Sigma-Aldrich), theophylline (0.0001–10 μM) (Sigma-Aldrich), or roflumilast (0.001–100 μM) (Sigma-Aldrich) 30 min prior to stimulation with 10 ng/mL IL-1α in serum-free DMEM/F-12 medium. The NAC solution was first buffered to reach normal pH (7.2) before addition to the cells. NAC was dissolved in sterile water, pirfenidone in serum-free DMEM/F-12 medium, montelukast in ethanol, dapsone in acetone, moxifloxacin hydrochloride in serum-free DMEM/F12-medium, theophylline in 0.1 M NaOH, and roflumilast in dimethyl sulfoxide (DMSO). Negative control cells were stimulated with an equal amount of vehicle. In all experiments, ethanol, acetone, NaOH, or DMSO never exceeded 0.1% as a final concentration in the culture media. After 24 h, the supernatant was removed and stored at −80 °C and IL-8 was measured via ELISA.

Statistical analysis

Graph prism 4.0 software (San Diego, CA, USA) was used for statistical analysis. Results are presented as mean ± SEM and analyzed using the Mann–Whitney U-test. A $P < 0.05$ was considered statistically significant. IL-8 production from 16HBE cells stimulated with 10 ng/mL IL-1α only was considered the reference (100%). All other data were expressed relative to this concentration.

Results

Establishment of the *in vitro* model of IL-1α-induced IL-8 production

IL-1α was not toxic for the epithelial cells for all tested IL-1α concentrations (Fig. 1a). In supernatant of non-stimulated 16HBE cells, the IL-8 concentration was 398.3 pg/mL ± 73.66 pg/mL ($n = 4$). Higher concentrations of IL-1α (from 0.01 to 10 ng/mL) increased IL-8 production dose-dependently, reaching a concentration of 1399 ± 228.1 pg/mL with 1 ng/mL IL-1α and 1643 pg/mL ± 168.7 pg/mL ($n = 12$) with 10 ng/mL IL-1α (Kruskal–Wallis one-way ANOVA, Dunn's *post hoc* test, $P < 0.001$ vs unstimulated cells), a 4.1-fold increase (Fig. 1b).

In primary bronchial epithelial cells, 10 ng/mL IL-1α also induced significantly upregulated IL-8 production ($P < 0.001$) (Fig. 1c).

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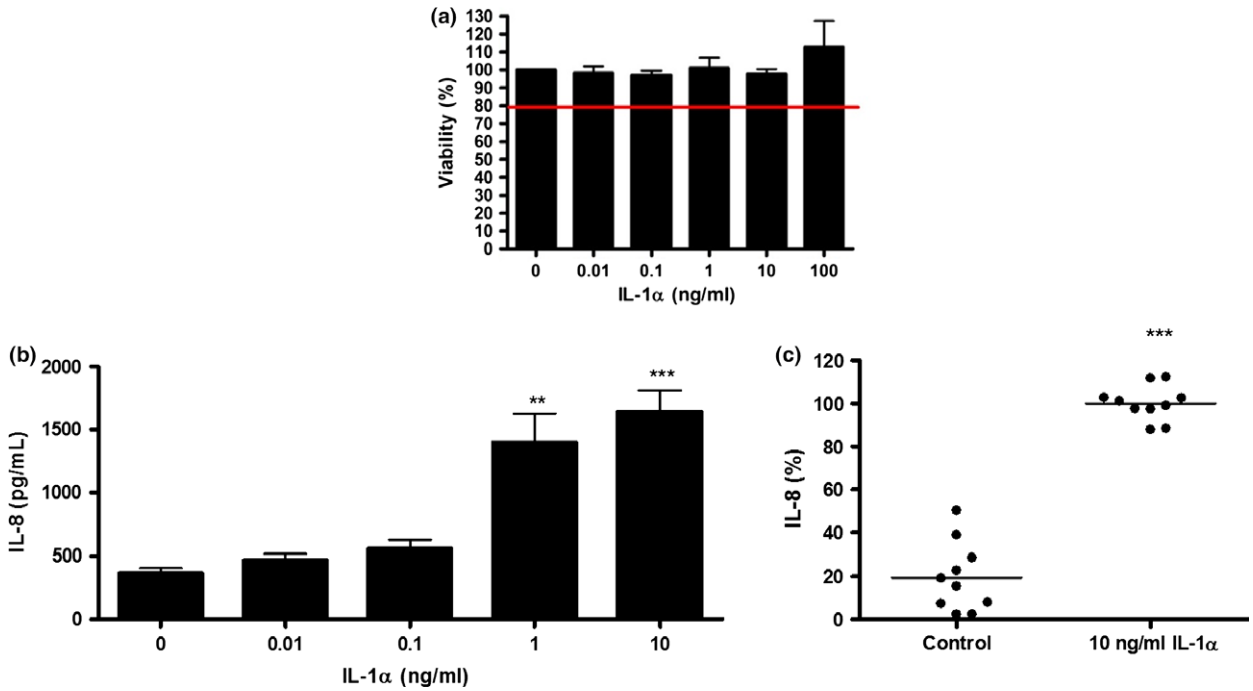

Figure 1 (a) Cytotoxicity assay of IL-1α on 16HBE epithelial cells. Viability of the cells that were not exposed to IL-1α is set at 100%. Viability of the exposed cells is expressed as a percentage of these controls. N = 4. (b) IL-1α induced IL-8 production in 16HBE epithelial cells. **: $P < 0.01$; ***: $P < 0.001$; Kruskal–Wallis one-way ANOVA, Dunn's *post hoc* test. 10 ng/mL IL-1α was chosen for further experiments. N = 12. (c) IL-1α induced IL-8 production in primary bronchial epithelial cells (HBEC). ***: $P < 0.001$; Kruskal–Wallis one-way ANOVA, Dunn's *post hoc* test. Five donors were used to culture HBEC cells. Two replicates per donor were tested.

Neither dexamethasone, nor azithromycin decreased the viability of 16HBE cells in all tested concentrations (highest concentration of 100 μM and 1 μM respectively) after 24-h exposure (data not shown).

Dexamethasone significantly and dose-dependently attenuated the IL-1α induced IL-8 release from 16HBE cells (Fig. 2a) ($n = 9$). From a concentration of 0.01 μM on, a significantly lower IL-8 production was found ($P < 0.01$) compared with cells exposed to 10 ng/mL IL-1α only. Using a concentration of 100 μM dexamethasone, a maximum decrease of 62.2% in IL-1α-induced IL-8 production was obtained.

IL-1α-induced IL-8 production of 16HBE cells could not be inhibited by addition of azithromycin up to a concentration of 1 μM (Fig. 2b) ($n = 9$). Consequently, we can conclude that this model reflects the *in vivo* situation and serves as a good model for azithromycin-resistant neutrophilia.

Signal transduction of 16HBE cells after stimulation with 10 ng/mL IL-1α

IL-1α induced phosphorylation of threonine 180 and tyrosine 182 of p38 MAPK, from 15 min to 60 min after stimulation. Peak phosphorylation levels occurred after 20–30 min. P38 MAPK phosphorylation returned to baseline between 60 and 90 min following stimulation (Fig. 3a) ($n = 5$). IL-1α also induced phosphorylation of threonine 308 of Akt MAPK, from 20 min until 45 min, with peak phosphorylation at 20 min (Fig. 3b) ($n = 5$).

IL-1α did not induce phosphorylation of tyrosine 467 and tyrosine 199 of the MAPK PI3-kinase, threonine 202, and tyrosine 204 of the MAPK p42/44 ERK and threonine 183 and tyrosine 185 of the MAPK SAP/JNK (data not shown, $n = 5$). We also tested the possible involvement of NFκβ in the IL-1α-induced IL-8 production via the inhibitor of NFκβ, Iκβ. IL-1α did not induce phosphorylation of serine 32 of Iκβ and total Iκβ was not changed over time. If NFκβ would be involved in the mechanism, the inhibitor of NFκβ, Iκβ, should be phosphorylated and degraded over time, which was not observed (data not shown).

Effect of dexamethasone on p38 and Akt MAPK

As dexamethasone was able to inhibit IL-1α-induced IL-8 production, we tested whether a simultaneous incubation of 0.1 μM dexamethasone and IL-1α could inhibit the IL-1α-induced p38 and/or Akt phosphorylation.

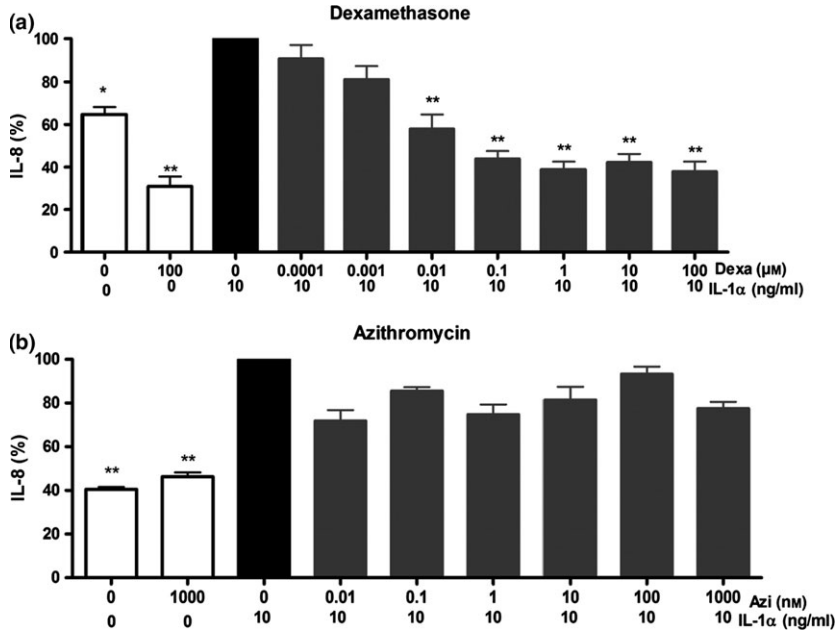

Figure 2 IL-8 production of 16HBE epithelial cells after stimulation with IL-1α and different concentrations of dexamethasone (a) or azithromycin (b). Cells only exposed to 10 ng/mL IL-1α and not to dexamethasone or azithromycin are set at an IL-8 production of 100%. The IL-8 production of the cells exposed to dexamethasone/azithromycin is expressed as a percentage of this. *: $P < 0.05$, **: $P < 0.01$. N = 9 (3 different passages of cells, three replicates per experiment).

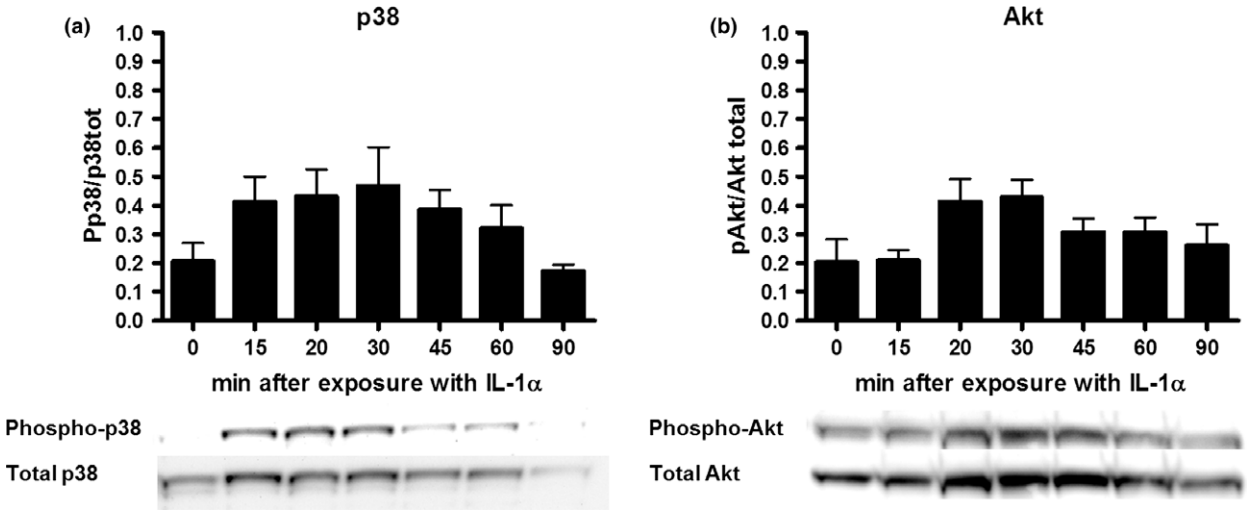

Figure 3 Western blot analysis was performed on nonstimulated cells or cells stimulated with 10 ng/mL IL-1α for 15, 20, 30, 45, 60, and 90 min. (a) Ratio of density measured for Phospho-p38/density for total p38 MAPK. (b) Ratio of density measured for phospho-Akt/density for total Akt. N = 5, 5 different passages of cells.

Therefore, we performed two sets of experiments wherein we added 0.1 μM dexamethasone 30 min or 24 h prior to IL-1α stimulation. After an incubation period of 20 min (as the previous experiment showed a peak phosphorylation after 20 min, Fig. 3), cell extraction and Western blotting were performed. Ten ng/mL IL-1α induced phosphorylation of both p38 and Akt MAPK, confirming our previous observations, but simultaneous exposure with 0.1 μM dexamethasone was not able to inhibit or downregulate phosphorylation of neither these MAPK (data not shown).

In vitro testing of other treatment options for azithromycin-resistant neutrophilia

Neither NAC, nor pirfenidone were toxic for the 16HBE cells up to a concentration of 10 mM and 5 mM, respectively. Montelukast was not toxic for the cells up

to a concentration of 1 μM, while concentrations of 10 μM montelukast and higher were toxic for 16HBE cells (i.e., cell viability below 10% compared with control). Dapsone, moxifloxacin hydrochloride, theophylline, and roflumilast were not toxic for the cells up to a concentration of 50 μM, 100 μM, 10 μM, and 100 μM, respectively (data not shown).

IL-1α-induced IL-8 production of 16HBE cells was not modulated by addition of NAC up to 10 mM (Fig. 4a), pirfenidone up to 0.5 mM (Fig. 4b), dapsone up to 50 μM (Fig. 4d), moxifloxacin hydrochloride up to 100 μM (Fig. 4e), theophylline up to 10 μM (Fig. 4f), or roflumilast up to 100 μM (Fig. 4g). At a pirfenidone concentration of 1 mM and 5 mM, a significantly lower IL-8 production was seen ($P < 0.05$ and $P < 0.01$ respectively) with a decrease of 10% and 25%, respectively, compared with IL-1α-exposed cells without pirfenidone. However, the control cells that received 5 mM pirfenidone without IL-1α stimulation showed a similar decreased IL-8 production. Montelukast had a small impact on IL-1α-induced IL-8 production. At a concentration of 0.01 μM and higher the IL-1α induced IL-8 production was decreased with 10% ($P = 0.028$) IL-8 production of the cells only stimulated with 0.1 μM montelukast did not differ from unstimulated cells (no IL-1α and no montelukast) Fig. 4c. N = 6 for all the tested drugs.

Discussion

To our knowledge, this is the first evidence that IL-1α induces IL-8 production in 16HBE human bronchial epithelial cells and in primary bronchial epithelial cells, which can be inhibited by dexamethasone (up to more than 60%) but not by azithromycin. We showed that p38 and Akt MAPK were activated after stimulation of the cells with IL-1α. However, simultaneous exposure of the cells with steroids did not suppress this effect. Lastly, we showed that NAC, pirfenidone, dapsone, moxifloxacin, theophylline, and roflumilast did not modulate and montelukast did only mildly modulate this IL-1α-induced IL-8 release.

We established an *in vitro* model for azithromycin-resistant neutrophilia. The finding that IL-1α stimulates IL-8 release from bronchial epithelial cells suggests that

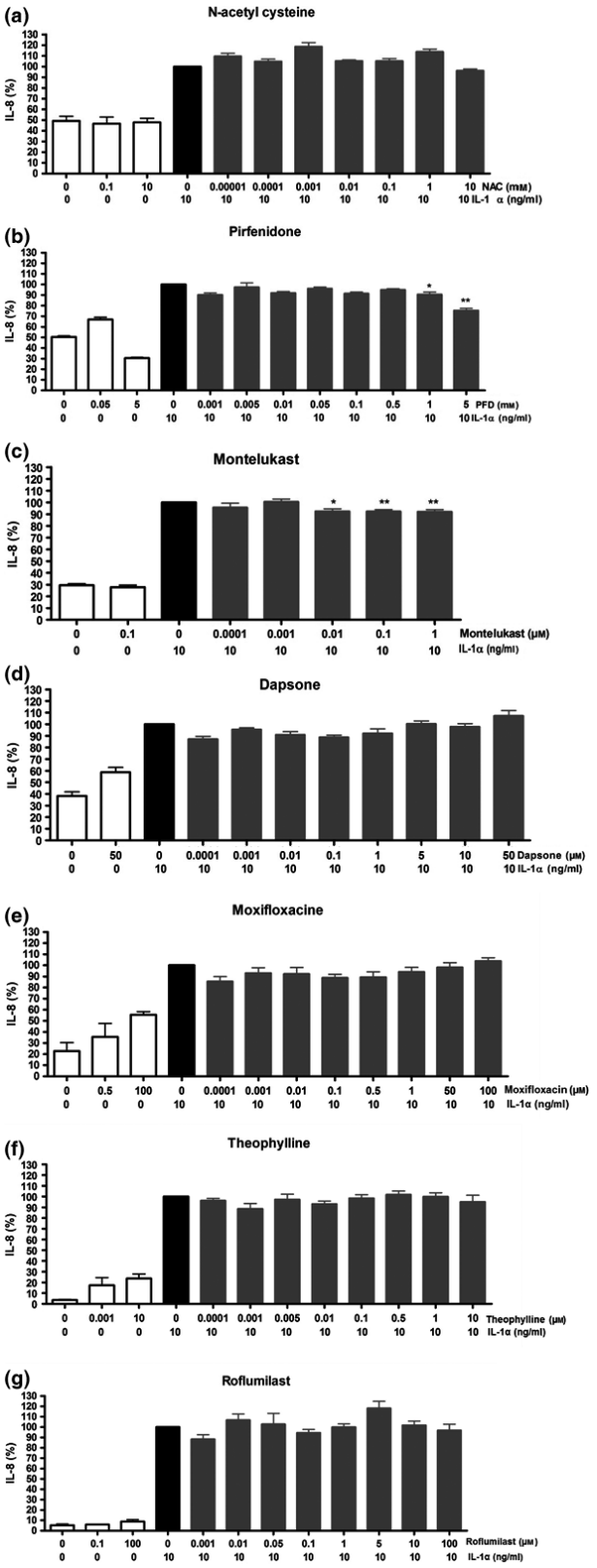

Figure 4 IL-8 production of 16HBE epithelial cells after stimulation with IL-1α and different concentrations of N-acetylcysteine (NAC) (a), pirfenidone (b), montelukast (c), dapsone (d), moxifloxacin (e), theophylline (f), or roflumilast (g). The cells only exposed to 10 ng/mL IL-1α and not to NAC, pirfenidone, montelukast, dapsone, or moxifloxacin are set at an IL-8 production of 100%. The IL-8 production of the cells exposed to NAC, pirfenidone, or montelukast is expressed as a percentage of this. *: $P < 0.05$ and **: $P < 0.01$. N = 8 for each experiment/drug.

the airway epithelium might play a prominent role in the ongoing neutrophilic airway inflammation in some patients with CLAD. Our results are consistent with the clinical situation of our patients with azithromycin-resistant neutrophilia: Their FEV_1 does not improve after azithromycin therapy but responds to a steroid pulse. Similarly, we found *in vitro* no response to azithromycin but a decrease in IL-1α-induced IL-8 production with steroids. Therefore, we suggest using this simple *in vitro* model to investigate mechanistic pathways and possible effects of new treatment options to inhibit BAL-neutrophilia in azithromycin-resistant neutrophilia.

BAL-neutrophilia is considered to be a major risk factor for the development of BOS. With the observation that BAL-neutrophilia was correlated with response to azithromycin, interest in neutrophils decreased. However, neutrophilia may (re)develop in some patients despite azithromycin therapy for an unknown reason. As these patients suffer from a worse survival, neutrophils seem to be carrying an important prognostic role and might be involved in the pathophysiological mechanism [11]. It is well known that activated neutrophils have the potential to cause damage to lung tissue due to their ability to generate reactive oxygen species and to release toxic proteases. IL-8 is the predominant neutrophil chemotactic factor in the lung [6]. As local release of IL-8 by bronchial epithelial cells contributes to the attraction and activation of neutrophils in the transplanted lung, upregulation of this cytokine by 16HBE cells was our main interest, although we acknowledge that other neutrophil chemo-attraction molecules exist, such as other alarmins like IL-33 and IL-18, which we have not examined.

One explanation of the persistently increased neutrophilia could be the presence of microorganisms, which are considered to be major risk factors for CLAD. Indeed, patients with high BAL-neutrophilia under azithromycin showed higher abundance of *Pseudomonas aeruginosa* in BAL fluid [11]. Several studies have already demonstrated a link between bacterial infections/colonization and an increased risk of the development of CLAD after lung transplantation [14–16]. Bacterial infections after lung transplantation can cause injury to the airway epithelium, which in turn can release alarmins that further activate the immune system [17]. A recent study of Borthwick *et al.* demonstrated an increase in IL-1α in BAL of *Pseudomonas Aeruginosa*-infected lung transplant patients, which correlated with both BAL IL-8 and neutrophilia. Furthermore, they showed that *Pseudomonas aeruginosa*-infected primary epithelial cells release IL-1α and IL-8. Lastly, a partly inhibition by dexamethasone of the IL-1α-induced IL-8 secretion in fibroblasts was shown [18]. With our study, we provided evidence of a direct effect of IL-1α on IL-8 production in bronchial epithelial cells, which is probably the place where the first injury takes place.

To unravel the mechanism behind the IL-1α-induced IL-8 production, we investigated the involvement of well-known MAP kinases (p38, JNK, $ERK^{1/2}$, Iκβ) as well as kinases that have been found to be involved in the process leading to tumor cell proliferation and survival (Akt and PI3 kinase) [19,20]. Although we were not able to establish the mechanism behind the IL-1α-induced IL-8 production, we could confirm that JNK, Iκβ, and ERK were not involved, which suggests another mechanism for airway neutrophilia in azithromycin-resistant neutrophilia compared with ARAD observed by Wuyts *et al.* [13]. Additionally, PI3 kinase is also not involved in the mechanism of the IL-1α-induced IL-8 production. We, however, observed that IL-1α induces p38 and Akt MAPK phosphorylation/activation, but these are not the MAP kinases via which dexamethasone blocks the IL-1α-induced IL-8 production and therefore other yet unknown mechanisms are probably causing the presence of high BAL-neutrophilia in azithromycin-resistant neutrophilia. As none of the tested drugs was able to inhibit this IL-1α-induced IL-8 production, we did not look further into the possibility of these drugs to inhibit the activation of both MAP kinases (p38 and Akt).

Concerning the role of therapeutics in the inhibition of azithromycin-resistant neutrophilia, we tested different drugs already in use for the treatment of other persistent neutrophilic or chronic respiratory diseases. We tested whether NAC influenced the IL-1α-induced IL-8 production as NAC already proved to reduce chemokine release via inhibition of p38 MAPK in human airway smooth muscle cells [21]. However, NAC was not able to inhibit the IL-1α induced IL-8 production, which further suggests that p38 MAPK is not involved in the signaling cascade.

Pirfenidone, a small synthetic nonpeptide molecule approved for the treatment of idiopathic pulmonary fibrosis (IPF), possesses anti-fibrotic and anti-inflammatory properties. Liu *et al.* were able to show in a rat lung transplant model a decreased neutrophil recruitment in pirfenidone-treated animals [22]; however, only at a concentration of 1 mM and 5 mM, a small decrease in IL-8 could be detected. As cells stimulated with 5 mM pirfenidone alone and not with IL-1α produced significant lower amounts of IL-8, this suggests that

pirfenidone is likely to inhibit the spontaneous IL-8 production rather than the IL-1α-induced IL-8 production. Nakanishi *et al.* reported that pirfenidone can inhibit the activation of Akt MAPK in cultured hepatocytes [23]. The fact that pirfenidone was proven to inhibit Akt MAPK activation but was not able to inhibit the IL-1α-induced IL-8 production in our *in vitro* model is another clue that Akt MAPK is probably not involved in the IL-1α-induced IL-8 production.

Montelukast is an antagonist of type I cysteinyl leukotriene receptors. Montelukast was able to cause a small decrease in the IL-1α-induced IL-8 production in our *in vitro* model. We were only able to test concentrations up to 1 μM as concentrations above 10 μM montelukast were toxic for the 16HBE cells. There is evidence that montelukast possesses a range of secondary anti-inflammatory effects [24–27] that may be particularly effective in controlling the corticosteroid-insensitive neutrophil production [28].

Dapsone, a synthetic sulfone widely used for the treatment of chronic neutrophil dermatoses, has been shown to inhibit IL-8 secretion from human bronchial epithelial cells stimulated with LPS [29]. However, dapsone seemed unable to inhibit the IL-1α-induced IL-8 production in our *in vitro* model. Kanoh *et al.* suggest that the inhibitory effect of dapsone on IL-8 could be due to inhibition of NF-κβ phosphorylation [29]. We found that NF-κβ is not involved in the IL-1α-induced IL-8 production, which may explain why dapsone is not able to downregulate the IL-1α induced IL-8 production.

Moxifloxacin is a fluoroquinolone which is effective against both gram-positive and gram-negative bacteria. *In vitro* studies on human monocytes suggested inhibitory and stimulatory effects of moxifloxacin on the immune system [30–32]. Araujo *et al.* demonstrated that moxifloxacin was able to inhibit the secretion of IL-1α and TNF-α [31]. However, moxifloxacin was not able to downregulate the IL-1α-induced IL-8 production *in vitro*.

Theophylline, a phosphodiesterase inhibitor that has been shown to demonstrate anti-inflammatory effects in concentrations below the concentration at which it targets phosphodiesterases [33–35] seemed a promising drug to test in low concentrations in our *in vitro* model but was not able to inhibit the IL-1α induced IL-8 production.

Roflumilast, an anti-inflammatory drug, has in preclinical models been shown to a broad spectrum of inflammatory cytokines and reactive oxygen species [36]. Low-dose roflumilast (1 nM and 1 μM) reduced the release of IL-8 in alveolar type II cells [37]. However, the production of IL-8 in these cells after addition of LPS and cigarette smoke appears to be dependent of ERK1/2 [37]. In the IL-1α-induced IL-8 production in our *in vitro* model, ERK1/2 was not involved. This might explain why roflumilast was not able to inhibit the IL-1α induced IL-8 production.

A limitation of our study is that the majority of experiments it is performed with a cell line instead of primary cells. However, we specifically chose to use a cell line for this study instead of primary cells because our aim was to establish a simple and reproducible *in vitro* model for azithromycin-resistant neutrophilia that could simply be used to test and predict the possibility of drugs in downregulating the IL-1α-induced BAL-neutrophilia seen in azithromycin-resistant neutrophilia. It has been described that the risk of infections and contamination for cell lines is much lower than for primary cells, variability is lower when using cell lines so that reproducibility is higher and more standardized and controlled results can be achieved. Furthermore, cell lines can be kept for a longer period of time (3-20 passages), which made an extensive research toward the mechanism of IL-1α-induced IL-8 production possible in a simple manner. However, we also demonstrated that 10 ng/mL IL-1α has the same effect on IL-8 production in primary bronchial epithelial cells. As upregulation of IL-8 production after exposure to 10 ng/mL IL-1α in the primary epithelial cells is comparable with the cell line of human bronchial epithelial cells (16HBE), we expect the same reactions of the cells toward the different treatment options. Recently, a study by Borthwick *et al.* using primary epithelial cells was able to demonstrate the possibility of the primary cells to release IL-1α and IL-8 after infection of the cells with *Pseudomonas aeruginosa* [18].

Another limitation of our study is that we only included one cell type, the bronchial epithelial cell (16HBE), in our *in vitro* model, and that we did not include immune cells. It is possible that in the transplanted lung other cells than the epithelial cells may act as a source of IL-1α, as shown by Borthwick *et al.* [18]. However, the major pathological manifestation of BOS is OB, wherein the disturbance of the epithelial layer is thought to be the "primum movens" for subsequent accumulation of extracellular matrix in the bronchiolar lumen, further indicating the importance of the epithelium.

To our knowledge, there is currently no satisfactory lung transplantation animal model to further test our

hypothesis. The mouse orthotopic lung transplant model seems to reflect the restrictive phenotype of CLAD, while the mouse heterotopic trachea transplant model is a model of fibroproliferation and not chronic rejection [38]. A good *in vivo* model to validate our findings is therefore lacking. This is the reason why we investigated the mechanism in an *in vitro* model.

Whatever the source of IL-1α is, we could demonstrate that bronchial epithelial cells responded with an IL-8 increase. More importantly, this IL-1α-induced IL-8 production could be inhibited by dexamethasone, but not by azithromycin. IL-1α induced p38 and Akt MAPK phosphorylation, but these transcription factors are not likely to be involved in the IL-1α-induced IL-8 production. Although we could not find the exact mechanism that is responsible for the IL-1α-induced IL-8 production, p38, Akt, ERK, PI3K, JNK, and Iκβ MAPK were shown not to be directly involved, alluding to other mechanisms responsible for the neutrophil recruitment in BOS patients with azithromycin-resistant neutrophilia. With this *in vitro* model, we observed no significant effect of NAC, pirfenidone, montelukast, dapsone, moxifloxacin in decreasing the IL-8 production. Further investigational drugs will be tested to hopefully find a treatment for patients with this condition to improve long-term survival.

Authorship

[1]Hannelore Bellon: data acquisition, writing of the paper, data analysis, 1Elly Vandermeulen: critical appraisal of the manuscript, [1]Carolien Mathyssen and Annelore Sacreas: isolation and culturing of the primary bronchial epithelial cells before the ALI interface. [1]Stijn E. Verleden: Participated in research design, participated in the writing of the paper, critical appraisal of the manuscript, 1Tobias Heigl: critical appraisal of the manuscript, [2]Hanne Vriens: data acquisition, 1Elise Lammertyn: critical appraisal of the manuscript, [3]Charles Pilette: provided primary bronchial epithelial cells and the expertise about how to culture them. [2]Peter Hoet: critical appraisal of the manuscript, 1Robin Vos: Participated in research design, participated in the writing of the paper, critical appraisal of the manuscript, 1Bart Vanaudenaerde: Participated in research design, participated in the writing of the paper, critical appraisal of the manuscript, [1]Geert M. Verleden: Participated in researchdesign, participated in the writing of the paper, critical appraisal of the manuscript.

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Conflicts of interest

None of the authors has anything to disclose in relation to this study.

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