In Vitro Effects of 5-Lipoxygenase Pathway Inhibition on Rhinovirus-Associated Bronchial Epithelial Inflammation

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ABSTRACT

Introduction: The leukotriene pathway may be implicated in the induction of virus-induced inflammation. Respiratory epithelial cells may express low levels of 5-lipoxygenase (5-LO) and release leukotrienes (LTs) C4, D4, and E4, upon exposure to viruses or other stimuli. Enhanced expression of 5-LO pathway proteins after rhinovirus (RV) infection has previously been described. We hypothesized that anti-leukotriene treatment of epithelial cells, with or without exposure to RV-infected peripheral blood mononuclear cells (PBMCs)-conditioned media, may inhibit RV-induced up-regulation of inflammatory cytokines.

Methods: PBMCs from a healthy donor were exposed to RV1B and supernatants were harvested at 48 h post infection. BEAS-2B cells were infected with RV, with or without conditioning with the PBMC supernatant. Treatment with anti-LT agents was performed either on both PBMCs and BEAS-2B or at the bronchial epithelial level only, with varying concentrations of montelukast (CysLT receptor antagonist) or MK-886 [FLAP(5-lipoxygenase-activating-protein) inhibitor]. Evaluation of the inflammatory cytokines IL-8, RANTES, IL-11, IL-6, and IP-10 was performed using ELISA.

Results: Our results show that anti-LT treatment of RV-infected bronchial epithelial cells suppresses epithelial RV-mediated cytokine production, independent of conditioning.

Conclusions: This observation may represent an indirect mode of action of the anti-leukotrienes in virus-induced asthma.

Keywords: Anti-inflammatory; Leukotrienes; Rhinovirus; 5-lipoxygenase
Bronchial epithelial cell line is shown to produce LTs (LTB4 and cysLTs) following RV infection.

Anti-LT treated bronchial epithelial cells responded with reduced RV-mediated inflammation.

Inhibition of RV replication is reported after Montelukast (ML) treatment.

5-LO inhibition treatment suppressed the RV-induced inflammation in bronchial epithelial cells.

Inhibition of the 5-LO pathway suppresses the release of RV-induced inflammatory mediators.

DIGITAL FEATURES

This article is published with digital features, including a summary slide, to facilitate understanding of the article. To view digital features for this article go to https://doi.org/10.6084/m9.figshare.14134937.

INTRODUCTION

Viral respiratory infections are the major trigger of asthma exacerbations in adults and children [25, 37]. Rhinoviruses (RV) belong to the Picornaviridae family of positive single-stranded RNA viruses and are implicated in a variety of respiratory disorders ranging from the common cold to pneumonia, sinusitis, and other respiratory conditions [13, 46, 60], most prominently to acute asthma exacerbations [25]. Airway inflammation and remodeling are characteristic features of asthma [9] and it is likely that epithelial infection and the subsequent interaction with the epithelial-mesenchymal trophic unit are critical for RV-mediated pathophysiology contributing to asthma exacerbations [20].

Several studies suggest that virus-induced inflammation may also involve the leukotriene (LT) pathway [5, 52]. There is accumulating evidence that some populations of epithelial cells normally express low levels of 5-lipoxygenase (5-LO) and are thus able to synthesize LTs, despite the fact that LTs are mainly produced by leukocytes [45]. Certain factors, including viral infection, can promote the expression of 5-LO in epithelial cells [34]. Respiratory epithelial cells release LT-C4, D4, and E4, upon exposure to viruses or other stimuli [5].

Montelukast (ML), a potent cysteinyl-leukotriene (CysLT) receptor antagonist, reduces asthma symptoms or cold-induced exacerbations in children and adults [7, 19, 24, 28, 49]. The mechanisms underlying these observations are not fully elucidated, but several explanations have been put forward. RV infection up-regulates nuclear factor kappa B (NF-κB) proteins [41], while ML inhibits tumor necrosis factor-α (TNF-α)-stimulated interleukin IL-8 expression through changes in NF-κB p65-associated histone acetyltransferase (HAT) activity [57]. Both RV epithelial infection and the leukotriene pathway have been implicated in airway remodeling. RV epithelial infection is able to up-regulate vascular endothelial growth factor (VEGF) and mediate angiogenesis [47], but also stimulate fibroblast proliferation through bFGF (basic fibroblast growth factor) [54]. Leukotriene receptor antagonism was able to reduce remodeling in a mouse model [36].

The lower respiratory epithelium, a principal site of RV infection in relation to asthma exacerbations, produced a variety of mediators augmenting the inflammatory response [40]. Respiratory structural cells produce a broad range of mediators upon viral infection, such as IL-1, IL-6, IL-8, IL-11, eotaxins, RANTES, and IP-10 (interferon gamma-induced protein – 10), and most of them are elevated in allergic rhinitis and asthma [15, 16].

We hypothesized that RV-induced inflammation is partly mediated through LTs and that LT pathway inhibitors may reduce or prevent such inflammation. Therefore, we examined the effects of ML and MK-886, a 5-LO inhibitor, on
the inflammatory responses upon RV infection, using two in vitro cell culture systems.

METHODS

Experimental Design (Supplementary data, Fig. S1)

Epithelial Leukotriene Release
In order to study the expression of LTB4 and CysLT mediators following stimulation with several infective doses of RV, BEAS-2B cells were cultured in monolayers on 48-well plates (Corning, Arlington, UK) and exposed to RV at a multiplicity of infection (MOI) of 0.5, 1, 3, and 5 or to control medium (Ohio-HeLa cell lysate, H). Supernatants were removed at 8, 24, 48, 72, and 96 h after infection, clarified by centrifugation (at 3000 \( g \) for 10 min at 4°C), and deep-frozen. Cells were stained with crystal violet to assess RV-induced cytotoxicity. LTB4 and CysLT release was evaluated using commercially available enzyme immunoassay kits according to the manufacturer’s instructions (Cayman Chemical, MI, USA, lower detectable levels were 13 pg/ml).

Inhibition of Leukotriene-Mediated Epithelial Inflammation
BEAS-2B cells were exposed to 1 MOI of RV or to control medium (H) for 1 h. Virus/control solution was then removed; cells were washed twice and replenished with fresh medium. Several concentrations of ML or MK-886 were added to the cultures after RV infection. ML and the FLAP inhibitor MK-886 were kindly supplied by Merck, USA. Both drugs were diluted as stock in dimethyl sulfoxide (DMSO, Sigma-Aldrich, USA) into \( 10^{-1} \) M solutions and after serial dilutions in culture medium, into \( 10^{-1} – 10^{-9} \) M. Although ML is typically used as an asthma control drug [29], the drug is often introduced upon the first signs of an asthma exacerbation and in the case of viral-induced episodes, after the exposure to the virus [49]. Therefore, we exposed the cell cultures to leukotriene pathway inhibition after RV infection. The cell cultures were then incubated at 33°C, 5% CO₂. Supernatants were removed at 48 and 72 h after infection, clarified by centrifugation and deep-frozen. Production of pro-inflammatory and pro-remodeling mediators was evaluated according to the manufacturer’s instructions [CCL5 (RANTES), CXCL8 (IL-8), IL-11, bFGF, (R&D Systems, Europe), IL-6 (eBioscience, San Diego, CA, USA)]. RV-induced cytotoxicity was also evaluated by two different methods: using crystal violet staining [63] and measuring lactate dehydrogenase (LDH) release in cell supernatants, according to manufacturer’s instructions (TAKARA, Japan).

Inhibition of Epithelial Inflammation in the Context of a Systemic Antiviral Immune Response
In order to model the effect of leukotriene pathway inhibition on immune-epithelial interactions, we used a previously described model [63]. Supernatants from RV-infected PBMCs were used instead of purified cytokines, to better represent the concentrations of mediators resulting from the in vivo antiviral immune response [43, 63]. PBMCs were isolated from an adult non-atopic and non-asthmatic donor, as assessed by means of a detailed medical history, skin prick testing, and spirometry. The study was approved by the Medical Ethics Committee of the Panagiots & Aglaia Kyriakou Children’s Hospital. The donor was informed in detail, both written and verbally, about the process and provided written informed consent. Ethics approval in context of European FP7 programme PREDICTA HEALTH-F2-2010–260,895 Protocol no 7790/16-05-2011. Briefly, PBMCs were isolated from whole blood by Ficoll centrifugation [39] and exposed to 5 MOI of RV or control medium, with or without ML/MK-886 treatment. Subsequently, BEAS-2B cells were conditioned with the PBMC supernatants and exposed to RV or control. Culture supernatants were then removed and PBMC supernatant as well as several concentrations of ML or MK-886 were added to the fresh culture medium. BEAS-2B cell cultures were then incubated at 33°C, 5% CO₂, and supernatants were harvested at 48–72 h after exposure to virus or control and inhibition treatment, clarified by centrifugation, and stored at -80°C until further processed. The epithelial inflammatory response was evaluated as described above.
Statistical Analysis

Distribution normality was assessed using the Kolmogorov–Smirnov test. Data are expressed as means ± standard errors of the mean and analyzed using a t test for differences between two groups or using one-way ANOVA with Bonferroni’s multiple comparison test for subsequent between-group effect evaluation. p values < 0.05 were considered significant.

RESULTS

Leukotriene Release (Time and Dose Dependence)

RV-infected BEAS-2B cells produced significantly higher levels of LTB4, 48 and 72 h after infection compared to control (p < 0.05) (Fig. 1a). RV infection at 0.5–5 MOI induced greater release of LTB4 mediators from BEAS-2B cells compared to non-infected cells, control and filtered RV at t = 72 h (p < 0.001) (Fig. 1b). Cell treatment with heat-inactivated or filtered RV did not induce LTB4 release. LTB4 release is higher using 1-MOI RV than using 0.5-MOI, but this response is reduced at higher virus concentrations (3 and 5 MOI) (Fig. 1b). In addition, BEAS-2B cells produced higher levels of CysLTs (LTC4, LTD4, LTE4) at 72 and 96 h after infection with RV (1 MOI), compared to non-infected cells (Fig. 1c). RV infection at 0.5–5 MOI induced greater release of CysLTs at 96 h compared to control, with the highest levels observed at 0.5 and 1 MOI (p < 0.01) (Fig. 1d).
Inhibition of Leukotriene-Mediated Epithelial Inflammation

BEAS-2B cell cultures were next exposed to RV prior to the inhibition of the 5-LO pathway. On the basis of the results of RV-induced LT release, RV at 1 MOI and at the time points of 48 and 72 h were chosen for the inhibition experiments. Release of IL-8, IP-10, IL-6, IL-11, and bFGF was evaluated at 48 and 72 h after infection. Infection with RV induced cytotoxicity at 48 and 72 h after infection, while treatment with ML or MK-886 did not affect the observed cell death, both among infected and non-infected cells (Fig. S2). RV infection induced significant production of IL-8, IP-10, IL-6, IL-11, and bFGF by BEAS-2B cells exposed to RV, as compared to non-infected cells \((p < 0.001)\), while exposure of RV-infected epithelial cells to diluent did not change this effect (data not shown). Treatment with ML significantly inhibited the release of IL-8, IL-6, IP-10, IL-11 from RV-infected BEAS-2B cells after 48 and 72 h, in a dose-dependent manner (Fig. 2a, b, respectively). FGF-2 release was also down-regulated (Fig. 2), but this was significant at higher ML concentrations (Figs. S3 and S4).

Inhibition of Epithelial Inflammation in the Context of a Systemic Antiviral Immune Response

Subsequently, BEAS-2B cells were stimulated by PBMCs supernatants and infected with RV (conditioned cell cultures). The release of inflammatory cytokines in conditioned cell cultures, treated with \(10^{-6}\) M ML or MK-886 or not, was evaluated at 48 h post-infection. RV infection induced significant production of IL-8, IL-6, RANTES, IP-10, and IL-11 in conditioned cell cultures \((p < 0.001)\). Treatment of epithelial cells with RV-infected PBMCs significantly enhanced the virus-induced IL-8, IL-6, RANTES, and IL-11 release. Furthermore, the treatment of conditioned cell cultures with ML \((10^{-6} \text{ M})\) and MK-886 \((10^{-6} \text{ M})\) resulted in significant reduction of cytokine release in supernatants (Fig. 3a). More specifically, the treatment of conditioned cell cultures with ML showed significant reduction of IL-8, IL-6, CCL5, IP-10, and IL-11 \((p < 0.001)\) release. Similar results were shown by the treatment of conditioned cells with MK-886 for IL-8 \((p < 0.001)\), IL-6 \((p < 0.001)\), CCL5 \((p < 0.001)\), IP-10 \((p < 0.001)\), and IL-11 \((p < 0.05)\) release (Fig. 3a). This reduction was sustained in IL-8, CCL5, and IL-11 with a lower dose of ML \((10^{-9} \text{ M})\) and in IL-8 and CCL5 cytokines with a lower dose of MK-866 \((10^{-9} \text{ M})\) (Supplement, Fig. 4).

Similarly, exposure of conditioned cell cultures to drug treatment of both cell types induced significant epithelial production of IL-8, IL-6, RANTES, IP-10, and IL-11, compared to non-infected cells. On the other hand, exposure of RV-infected epithelial cells to supernatants from RV-infected PBMCs treated with diluent did not enhance this effect (data not shown). Prior treatment of PBMCs with ML at a concentration of \(10^{-6} \text{ M}\) significantly inhibited the release of IL-8 and IL-11 by RV-infected BEAS-2B, while PBMCs treated with MK-886 at concentrations of \(10^{-6} \text{ M}\) did not show the same result (Fig. 3b). Treatment of PBMCs with ML or MK-886 significantly inhibited the release of IP10, IL-6, and RANTES by RV-infected BEAS-2B (Fig. 3b). This reduction was sustained in IP10, IL-6, and CCL5 cytokines with higher doses of ML \((10^{-4} \text{ and } 10^{-5} \text{ M})\) and MK-866 \((10^{-4} \text{ and } 10^{-5} \text{ M})\) (Fig. S5).

In addition to the inhibition of inflammatory responses, significantly lower RV replication was observed in BEAS-2B cells treated with ML and exposed to ML-treated, RV-infected PBMC supernatants at 48 h post-infection \((p < 0.05)\) (Fig. 4).

DISCUSSION

Structural cells, such as bronchial epithelial cells, have been considered to synthesize leukotrienes by metabolizing LTA4 produced by a leukocyte donor cell because they lack the 5-LO enzyme [45]. However, although LT synthesis was once thought to be restricted to leukocytes, recent studies suggest that some epithelial cell populations express 5-LO at low concentrations and are thus able to synthesize LTs from arachidonate upon stimulation. More
specifically, it has subsequently been shown that human bronchial epithelial cells [22] and fibroblasts are capable of producing both CysLTs and LTB4 following inflammatory insults [23]. To our knowledge, this is the first time that a bronchial epithelial cell line has been shown to produce LTs (LTB4 and CysLTs) following RV infection. The experiments were performed under serum-free conditions allowing for more precise detection of any differences.

**Fig. 2** Cytokine and chemokine production by BEAS-2B cells exposed to RV1B (1 MOI), as compared to non-infected cells after a 48 h and b 72 h, with and without the addition of montelukast: IL-8, IP10, IL6, FGF-2, IL11 [B2b: BEAS-2B cells exposed to HeLa lysate, B2b + : HRV– (human rhinovirus) infected BEAS-2B cells, D: DMSO 1:10^6, ML: montelukast 10^-6 M] (**p < 0.001, compared to B2b + and B2b + /D)
Fig. 3  a  Cytokine and chemokine production in supernatants of BEAS-2B cells, in PBMCs and a BEAS-2B cell system, in which only BEAS-2B cells were exposed to RV1B (1 MOI) after 48 h, with and without the addition of montelukast: IL-8, IP10, IL6, CCL5 (RANTES), IL11.  

b  Cytokine and chemokine production in supernatants of BEAS-2B cells, in PBMCs and a BEAS-2B cell system, in which both BEAS-2B cells (1 MOI) and PBMCs (5 MOI) were exposed to RV1B after 48 h, with and without the addition of montelukast [control: BEAS-2B cells exposed to HeLa lysate, HRV/D/HRV: RHRV– (human rhinovirus) infected BEAS-2B cells, DMSO 1:10⁶, ML: montelukast 10⁻⁶ M, K: MK-886 10⁻⁶ M] (*p < 0.005, **p < 0.01, ***p < 0.001)
in leukotriene release and better control over physiological responsiveness and evaluation of cellular functions [10, 32].

Bronchial epithelial inflammatory response is a crucial feature of RV infection [53, 62]. In this study, we found significant suppression of the RV-induced inflammation, including the release of IL-8, CCL5, IL-6, IL-11, and IP-10 in bronchial epithelial cells after 5-LO inhibition treatment. Furthermore, we found that anti-LT-treated bronchial epithelial cells exposed to supernatants either of RV-infected PBMCs or anti-LT-treated, RV-infected PBMCs responded with reduced RV-mediated inflammation, which was accompanied by a reduction in virus replication. Interestingly, it is the first time that inhibition of RV replication has been reported after ML treatment, in accordance with clinical studies suggesting improvement of virus-related cytokines in a more direct manner [2]. Furthermore, not only in in vitro models but also in in vivo studies, ML provides clinical benefit to patients with chronic asthma and decreases bronchial hyperresponsiveness. ML caused a statistically significant decrease of serum concentrations in cytokine, ICAM-1, ECP, and peripheral blood eosinophil counts over the 6-week treatment period. This observation raises the possibility that leukotriene receptor antagonists, such as ML, may have effects on parameters of asthmatic inflammation [56].

More specifically, RV-induced expression of IL-8 is greater than that of other respiratory viruses [12] and is shown to be increased in an atopic environment and associated with enhanced neutrophilic inflammation [11, 17]. On the other hand, ML inhibits IL-8 expression in human leukemic monocyte lymphoma and alveolar basal epithelial cell lines [57, 64]. Furthermore, there seems to be an association between IL-8 and LTs through the TLR4 and NF-κB inflammatory pathway, while inhibition of the CysLT receptor represses TNF-α-stimulated IL-8 expression [57]. Besides, 5-LO pathway inhibition may reduce airway inflammation and bronchial hyperresponsiveness, possibly through down-regulation of RV-induced IL-11 and TGF-beta1 expression [33, 44]. Moreover, ML may inhibit RV-associated inflammatory responses through activities not directly related to antagonism of leukotriene receptors [59], such as inhibition of 5-lipoxygenase [48], histone acetyltransferase (HAT) [57], and

However, the relationship between ML and viral infection is not clear. Some data suggest that the antiviral effects of ML may be the result of its anti-inflammatory and anti-allergic properties. The possible relationship between ML and the apoptotic response of the infected cell could also suggest a protective effect of ML against viral infections. Virus-induced genes, such as inflammatory genes, are known to modulate cell death as an antiviral function of the cell [3]. Another study by Andersson et al. [2] showed that montelukast decreased the expression of ICAM-1 (RV1B receptor) in 16HBE cells, which could potentially explain the decrease in viral titer in ML-treated BEAS-2B cells, as well as a reduction in pro-inflammatory cytokines in a more direct manner [2]. Furthermore, not only in in vitro models but also in in vivo studies, ML provides clinical benefit to patients with chronic asthma and decreases bronchial hyperresponsiveness. ML caused a statistically significant decrease of serum concentrations in cytokine, ICAM-1, ECP, and peripheral blood eosinophil counts over the 6-week treatment period. This observation raises the possibility that leukotriene receptor antagonists, such as ML, may have effects on parameters of asthmatic inflammation [56].

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adenosine 3',5'-cyclic monophosphate (cAMP) phosphodiesterase [1], as well as interference with purinergic P2Y receptors [48] and inhibition of eosinophil adhesion to vascular endothelium and migration [50]. This may represent an additional, indirect mode of action of anti-leukotriene medication on virus-induced epithelial inflammation.

We found that treatment with ML did not affect the observed cell death, both among infected and non-infected cells, suggesting that ML does not exert their anti-inflammatory action directly through antiviral effects. Although there is a significant reduction of viral replication at 48 h, this was not significant at 72 h and we found no subsequent effect on the extended in vitro virus-induced epithelial damage.

With the use of a cell-culture system (BEAS-2B and PBMCs stimulated), the levels of cytokine elevation were substantially greater than those from one cell type alone, resulting in more lifelike in vitro experiments [30, 42, 47, 54, 63]. Furthermore, another benefit of using this model is the representation of the natural interplay between immune cells, including neutrophils, eosinophils, NK cells, macrophages from blood, and epithelial cells [35]. The intercellular adhesion molecule 1 (ICAM-1) is the major group rhinovirus receptor, a cell-surface glycoprotein that normally regulates leukocyte trafficking and accumulation at sites of inflammation via engagement of lymphocyte function-associated antigen (LFA)-1 and macrophage-1 antigen (Mac-1) on the cell surface [55]. This may be a potential mechanism by which a virus spreads in the airways and results in the mutual interaction between asthma and rhinitis, for which there is considerable clinical evidence [14, 38].

The main limitation of this study is the use of a bronchial cell line instead of primary bronchial epithelial cells. The difficulty of collecting bronchial cells from donors was the main reason for this experimental drawback. Another limitation of this study was the lack of in vivo experiments, but such investigations are beyond the scope of this paper, as the fact that ML effectively inhibits the inflammatory activities of the 5-LO pathway has already been described and is part of numerous international guidelines for asthma therapy [4, 8]. Another possible disadvantage of our study design is that the level of viremia following a rhinovirus infection is low [61]. However, the percentage of rhinoviremia is higher in asthmatics, suggesting that the direct exposure of the PBMCs significantly contributes to the asthmatic inflammatory response to RV infections.

Our findings support our initial hypothesis that bronchial epithelial cells produce significant amounts of leukotrienes, while inhibition of the 5-LO pathway suppresses the release of RV-induced inflammatory mediators.

**CONCLUSIONS**

In summary, the results of this study support the concept that bronchial epithelial cells are able to synthesize leukotrienes and this production is induced by RV infection. Montelukast and a FLAP inhibitor (MK-866) suppressed RV-induced inflammatory responses. This is the first time that inhibition of rhinovirus replication after montelukast treatment has been reported. These findings identify an important role for CysLT in RV-induced airway responses and provide novel insights into prophylactic approaches for the prevention of RV-mediated sequelae in patients with asthma.

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Compliance with Ethics Guidelines. The study was approved by the Medical Ethics Committee of the Panagiotis & Aglaia Kyriakou Children’s Hospital. The donor was informed in detail, both written and verbally, about the process and provided written informed consent. Ethics approval in context of European FP7 programme PREDICTA HEALTH-F2-2010-260,895 Protocol no 7790/16-05-2011.

Data Availability. All data generated or analyzed during this study are included in this published article/as supplementary information files.

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