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Montelukast suppresses epithelial to mesenchymal transition of bronchial epithelial cells induced by eosinophils

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ABSTRACT

Epithelial to mesenchymal transition (EMT) is a mechanism by which eosinophils can induce airway remodeling. Montelukast, an antagonist of the cysteinyl leukotriene receptor, can suppress airway remodeling in asthma. The purpose of this study was to evaluate whether montelukast can ameliorate airway remodeling by blocking EMT induced by eosinophils. EMT induced was assessed using a co-culture system of human bronchial epithelial cells and human eosinophils or the eosinophilic leukemia cell lines, Eol-1. Montelukast inhibited co-culture associated morphological changes of BEAS-2b cells, decreased the expression of vimentin and collagen I, and increased the expression of E-cadherin. Montelukast mitigated the rise of TGF-β1 production and Smad3 phosphorylation. Co-culture of human eosinophils with BEAS-2B cells significantly enhanced the production of CysLTs compared with BEAS-2B cells or eosinophils alone. The increase of CysLTs was abolished by montelukast pre-treatment. Montelukast had similar effects when co-culture system of Eol-1 and BEAS-2B was used. This study showed that montelukast suppresses eosinophils-induced EMT of airway epithelial cells. This finding may explain the mechanism of montelukast-mediated amelioration of airway remodeling in bronchial asthma.

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1. Introduction

Bronchial asthma is a chronic airway inflammatory diseases with a high prevalence worldwide [1]. Cysteinyl leukotrienes (CysLTs) are important inflammatory lipid mediators that can contribute to the pathogenesis of acute asthma by causing smooth muscle constriction, vascular permeability, and mucus production [2–4]. Allergen challenge in patients with asthma increases the level of CysLTs in the exhaled breath condensate [5,6]. The level of CysLTs in nasopharyngeal secretions in patients with virus-induced wheezing is higher than in children with acute virus infection without wheezing [7]. In chronic asthma, CysLTs play a key role in airway remodeling, which is characterized by subepithelial fibrosis, myofibroblast hyperplasia, thickening of the lamina reticularis, and increased smooth muscle mass [8,9]. CysLTs in the exhaled breath condensate are correlated with reticular basement membrane thickness in childhood asthma [10]. Montelukast, which is a potent and selective antagonist of the cysteinyl leukotriene receptor used for the treatment of asthma, has been shown to inhibit or reverse airway remodeling in both human asthma and animal models of the disease [11–15].

Airway eosinophilic inflammation is a characteristic pathological feature of asthma [16]. Eosinophils are major source of CysLTs [17]. Eosinophils secrete CysLTs after stimulation by cytokines including Interleukin (IL)-5, IL-3, and granulocyte–macrophage colony-stimulating factor (GM-CSF) [18,19]. Interestingly, eosinophils isolated from asthmatic patients can produce higher CysLTs than those from healthy subjects [20,21]. CysLTs can attract and activate eosinophils, because eosinophils express cysteinyl leukotriene receptors (CysLTs1R and CysLTs2R) [22]. Inhalation of CysLTs causes eosinophil recruitment into airways [23,24]. CysLTs induce eosinophil migration and ROS generation, degranulation, and cytokine production from eosinophils [25,26]. CysLTs can also stimulate the production and secretion of transforming growth factor (TGF)-β1 from eosinophils [27]. The secretion of TGF-β1 from eosinophils contributes to airway remodeling in chronic asthma [28–30]. TGF-β1 is also known as a strong inducer of epithelial to

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mesenchymal transition (EMT) in bronchial epithelial cells [31,32]. EMT can explain the increase number of myofibroblasts in cancer and fibrotic diseases [33]. A recent study has demonstrated that EMT may also contribute to airway remodeling in asthma [34,35]. Previously, we demonstrated that eosinophils infiltrating the airway increase the expression of TGF-β1 leading to EMT and airway remodeling [36]. In the present study, we hypothesized that montelukast suppresses EMT of airway epithelial cells induced by eosinophils.

2. Materials and methods

2.1. Reagent

L-glutamine, penicillin/streptomycin, donkey anti-mouse IgG-Alexa Fluor 488, chicken anti-rabbit IgG-Alexa Fluor 594, Texas Red-X phalloidin, Laemmli sample buffer and Trizol Reagent were purchased from Invitrogen (Carlsbad, CA), RPMI-1640 and bovine serum albumin (BSA) from Sigma (St Louis, MO), and fetal bovine serum (FBS) from BioWhittaker (Walkersville, MD, USA). Goat anti-human E-cadherin antibody (HECD-1) was purchased from Abcam (Cambridge, MA), mouse anti-human E-cadherin antibody from BD Biosciences (Mississauga, ON, Canada), anti-TGF-β1 monoclonal antibody (mAb) (1D11) from R&D Systems (Minneapolis, MN), anti-human Smad3 antibody and rabbit anti-human phosphorylation-Smad3 (p-Smad-3) polyclonal antibody (Ser423/425) were from Cell Signaling Technology (Beverly, MA). Mouse anti-human α-tubulin monoclonal antibody (mAb) (B-7) and radioimmunoprecipitation (RIPA) lysis buffer were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase-conjugated goat anti-rabbit IgG and horseradish peroxidase-conjugated goat anti-mouse IgG were from BIO-RAD (Hercules, CA). Anti-CD16 and anti-CD14 bound micromagnetic beads were obtained from Miltenyi Biotec (Auburn, CA).

2.2. Cell lines

BEAS-2B, which is an adenovirus 12-SV40 virus hybrid (Ad12SV40) transformed human epithelial cells, was obtained from the Riken Cell Bank (Tsukuba, Japan), grown in DMEM supplemented with 10% (v/v) heat-inactivated FBS, 0.03% (w/v) L-glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin. EoL-1 cells were obtained from the Riken Cell Bank, maintained in suspension culture at 37 °C and 5% CO_2 in humidified atmosphere in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated FBS, 0.03% (w/v) L-glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin. For differentiation, EoL-1 cells were diluted to 5×10^5 cells/ml and 0.5 mM sodium n-butyrate (BA) was added. EoL-1 cells were incubated with 0.5 mM BA for 5 days.

2.3. Preparation of human eosinophils

Eosinophils from healthy human volunteers were purified by negative selection using anti-CD16 and anti-CD14 bound micromagnetic beads as previously described [37]. The purity of eosinophils was more than 97% as measured by the Randolph's stain [37]. Written informed consent was obtained from all healthy volunteers before blood sampling. The protocol of this study was approved by the Institutional Ethic Board for Clinical Investigation (No. 1004; 2008.1.9), and informed consent was obtained from all healthy volunteers before blood sampling.

2.4. Co-culture experiment and morphological analysis

BEAS-2B cells were cultured in 6- or 12-well plates until 60–70% cell confluence, then serum-starved for 24 h. BEAS-2B cells were pre-treated with montelukast (provided by Merck Sharp & Dohme Corp) at 10^{-4} M for 1 h. Human eosinophils or BA-differentiated EoL-1 cells (1×10^6 cells for 12-well plate, 2×10^6 cells for 6-well plate) were added to the culture RPMI culture medium and incubated for further 48 h. After co-culture, human eosinophils or Eol-1 cells were removed from adherent BEAS-2B cells by gentle pipetting. BEAS-2B cells were stained with Diff-Quick solutions and photographed for morphological analysis. Furthermore, nuclei and F-actin cytoskeleton were stained by using immunofluorescence. Briefly, BEAS-2B was fixed with 4% paraformaldehyde for 10 min at room temperature. After washing, BEAS-2B cells were stained with Texas Red-X phalloidin (1:50) for 1 h at room temperature; DAPI was used to stain nuclei for 5 min at room temperature. Fluorescent images were captured using a fluorescence microscope (I × 71 Olympus) after appropriate cell washing.

2.5. RT-PCR

After the co-culture of BEAS-2B cells with eosinophils or Eol-1 cells for 48 h as described above, eosinophils or Eol-1 cells were removed. Total RNA was extracted from BEAS-2B cells by the guanidine isothiocyanate procedure using Trizol Reagent. RNA was reverse-transcribed using oligo-dT primers and then the DNA was amplified by PCR. The sequences of the primers are as follows: for human vimentin, forward 5′-GAGAACTTTGCCGTTGAAGC-3′ and reverse 5′- GCTTCCTGTAGGTGGCAATC-3′; for human collagen, 5′-AACCTGGATGCCATCAAAGTC-3′ and reverse 5′-TCCATGTAGGCCACGCTGTTC-3′, for human E-cadherin forward: 5′- GTATCTTC CCCGCCCTGCCAATCC-3′ and reverse 5′- CCTGGCCGATAGAATGAGACCCTG-3′; for human GAPDH, forward 5′- GTGAAGG TCGGAGTCAACGGA-3′ and reverse 5′-GGTGAAGACGCCAGTGGACTC-3′. PCR was carried for 35 cycles (E-cadherin), 27 cycle (Vimentin), 25 cycle (GAPDH), denaturation at 94 °C for 30 s, annealing at 65 °C for E-cadherin and GAPDH, and 59 °C for vimentin for 30 s, and elongation at 72 °C for 1 min; at the end of these cycles, a further extension was carried out at 72 °C for 5 min. The PCR products were separated on a 2% agarose gel containing 0.01% ethidium bromide. The RNA concentration and purity were determined by UV absorption at 260:280 using an Ultrospec 1100 pro UV/Vis spectrophotometer (Amersham Biosciences, NJ). The amount of mRNA was normalized against the GAPDH mRNA and expressed as percentage of controls.

2.6. ELISA

The immunoassay kit for measuring TGF-β1 (R&D, McKinley Place, MN) and GM-CSF were purchased from BD Biosciences Pharmingen (San Jose, CA), and that for measuring CycLT was obtained from Cayman Chemicals (Ann Arbor, MI); each parameter was measured according to the manufacturer's instructions.

2.7. Immunoprecipitation

For Western blot analysis, BEAS-2B cells were lysed in RIPA lysis buffer. Cell debris was removed by centrifugation at 15000 × *g* for 15 min, and supernatant was warmed in Laemmli sample buffer for 5 min at 70 °C. An equal amount of proteins was subjected to sodium dodecyl sulphate–10% polyacrylamide gel electrophoresis before blotting onto a PVDF (polyvinylidine fluoride) membrane (Amersham Pharmacia Biotech). The membrane was blocked with 5% bovine serum albumin (BSA) in TBS buffer containing 0.1% Tween-20, 25 mM Tris–HCl, 0.15 M NaCl, pH 7.6 at 4 °C overnight and probed with anti-human Smad3 antibody or rabbit anti-human phosphorylation-Smad3 (p-Smad-3) polyclonal antibody (Ser423/425) for 2 h at room temperature. After washing, the membrane was incubated with secondary goat anti-rabbit anti-

body coupled to horseradish peroxidase for 2 h at room temperature. Antibody–antigen complex were then detected using Western lightning plus-ECL according to the manufacturer's instructions (Perkin Elmer). Analysis was carried out by Image Quant LAS-4000mini (GE imagination at work).

2.8. Statistical analysis

All data were expressed as the mean ± SEM. The statistical difference between two variables was calculated by the Mann–Whitney *U* test, and that between three or more variables by analysis of variance with post hoc analysis using the Bonferroni test. The software package GraphPad Prism 6 (GraphPad Software, San Diego, CA) was used for all data statistical analyses and preparation of graphs. All statistical analyses considered data significant at $P < 0.05$.

3. Results

3.1. Montelukast inhibits EMT caused by human eosinophils

BEAS-2B cells co-cultured with human eosinophils exhibited fibroblast-like morphology consistent with EMT [36], but this was inhibited when BEAS-2B cells were pre-treated with montelukast (Fig. 1A). Formation of long stress fibers of F-actin was also evaluated in BEAS-2B cells co-cultured with human eosinophils in the presence or absence of montelukast. Also, montelukast inhibited formation of long stress fibers of F-actin in BEAS-2B cells (Fig. 1A). RT-PCR analysis showed that montelukast significantly inhibited the decrease in the expression of E-cadherin and the increase in the expression of vimentin in BEAS-2B cells co-cultured with human eosinophils (Fig. 1B). The increase in the ratio of vimentin to E-cadherin by the co-culture, was significantly suppressed by montelukast (Fig. 1B). The mRNA expression of collagen I in BEAS-2B cells was also enhanced by co-culture with human eosinophils but montelukast significantly inhibited it (Fig. 1C).

3.2. Montelukast suppresses the increase of TGF-β1 production and phosphorylation of Smad3 in BEAS-2B cells induced by human eosinophils

As we have shown before, co-culture of BEAS-2B cells with human eosinophils enhances the production of TGF-β1 compared with single culture of BEAS-2B cells or eosinophils [36]. Pre-treatment with montelukast significantly inhibited the increase of TGF-β1 in the supernatant obtained during co-culture of BEAS-2B cells with human eosinophils (Fig. 2A). Phosphorylation of the Smad3, an important player of the TGF-β1 receptor pathway, was significantly increased in BEAS-2B cells co-cultured with human eosinophils, but it was also significantly decreased when the cells were co-culture in the presence of montelukast (Fig. 2B). In addition, the production of GM-CSF was remarkably augmented by co-culture of BEAS-2B cells with human eosinophils compared to BEAS-2B cells or eosinophils alone, but the increase of GM-CSF was repressed by pretreatment with montelukast (Fig. 2C).

3.3. Montelukast suppresses the EMT induced by EoL-1 cells

To corroborate the results obtained using primary culture of human eosinophils, similar experiments were performed using the human eosinophilic leukemia cell line, EoL-1 cells, which can

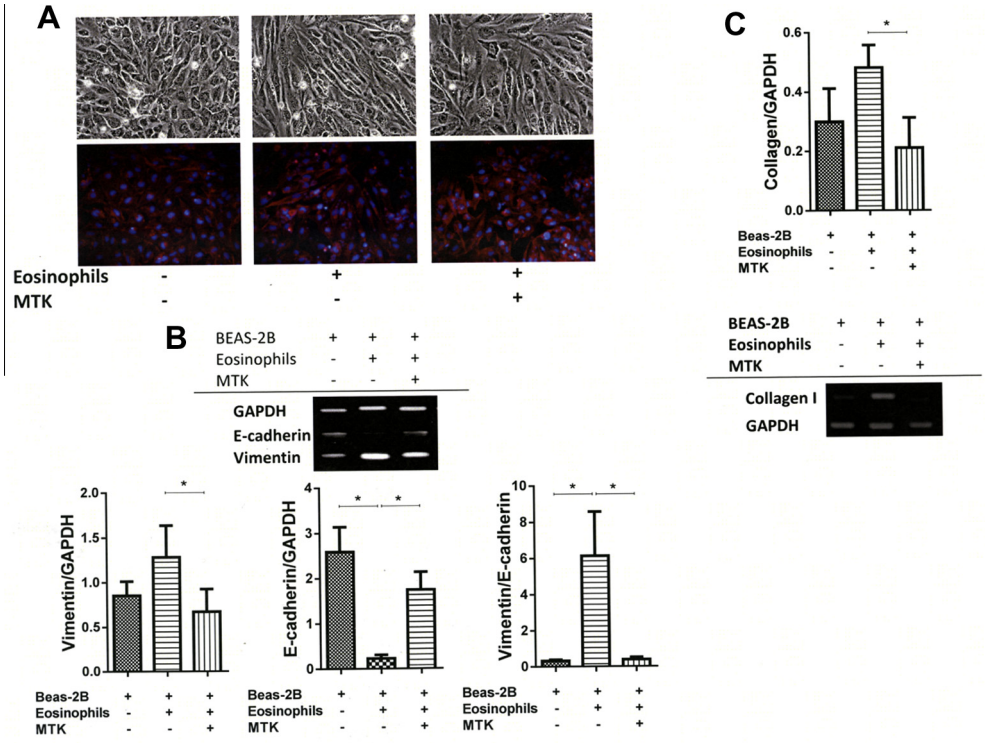

Fig. 1. EMT induced by human eosinophils is inhibited by montelukast. (A) Control and BEAS-2B cells co-cultured with eosinophils in the presence or absence of montelukast; the upper panel shows BEAS-2B cells stained with DIFF-QUICK after washing out eosinophils; the lower panel shows BEAS-2B cells stained with phalloidin (red) and DAPI (blue) after washing out eosinophils. Original magnifications, ×400. (B) Gene expression of vimentin (left panel), E-cadherin (middle panel), and the ratio of vimentin to E-cadherin (right panel) in BEAS-2B cells co-cultured with eosinophils in the presence or absence of montelukast. (C) Gene expression of collagen I in BEAS-2B cells co-cultured with eosinophils in the presence or absence of montelukast. Gene expression was assessed by RT-PCR and densitometry analysis. Data are expressed as mean ± SEM. $^{*}P < 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

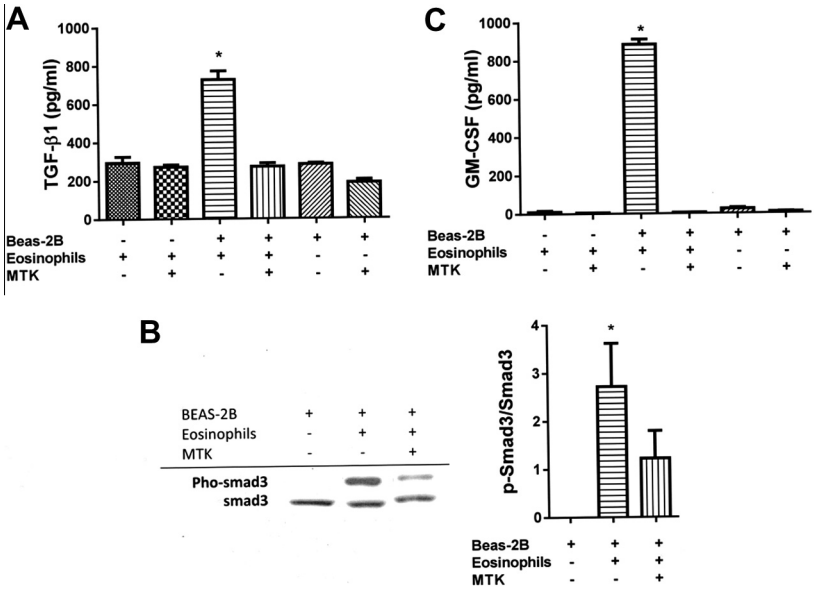

Fig. 2. Montelukast suppresses the TGF-β production and Smad pathway. (A) TGF-β1 levels in the supernatant from each cell condition. (B) Representative Western blot of Smad3 and p-Smad3, and the ratio of p-Smad3 to Smad3 as analyzed by densitometry. (C) GM-CSF levels in the supernatant from each cell condition. Data are expressed as mean ± SEM. *$P < 0.05$ vs. each group.

also induce EMT in BEAS-2B cells [36]. Montelukast significantly suppressed the induction of EMT in BEAS-2B cells and the production of TGF-β1 and GM-CSF compared to untreated cells (Fig. 3A–C).

3.4. Montelukast inhibits increased production of CysLTs caused by eosinophils

Co-culture of BEAS-2B cells with human eosinophils (Fig. 4A) or EoL-1 cells (Fig. 4B) significantly enhances the production of CysLTs compared with single culture of BEAS-2B cells or eosinophils. Pre-treatment of BEAS-2B cells with montelukast abolished the increase of CysLTs in the culture supernatant.

4. Discussion

The results of the present study provides the first evidence that montelukast suppresses EMT of bronchial epithelial cells caused by eosinophils.

The relationship between TGF-β1 and airway remodeling has been well documented. The contribution of CysLTs to the pathogenesis of airway remodeling is not clear. Previous studies have shown that CysLTs can induce TGF-β1 production from human airway epithelial cells and human eosinophils and that airway smooth muscle proliferation caused by LTD4 depends on TGF-β1 [27,38,39]. Here we found that montelukast suppresses EMT of BEAS-2B after co-culture with eosinophils. This inhibition of EMT was associated with decreased expression of TGF-β1 in the co-culture supernatants suggesting that the inhibitory activity of montelukast on EMT depends on the suppression of TGF-β1 secretion. The fact that montelukast also suppressed the activity of the Smad signaling pathway further supports the involvement of the TGF-β1 pathway in the inhibitory activity of montelukast on EMT. In the present study, we have also evaluated the role of GM-CSF in eosinophil regulation [18]. Like TGF-β1, the level of GM-CSF was significantly increased in the co-culture medium of BEAS-2B cells with eosinophils compared with cell cultured alone, and the increase was also abolished by montelukast. A recent report suggested that increased production of GM-CSF by exogenous TGF-β1 can induce EMT in Hertwig's epithelial root sheath/epithelial rests of Malassez cells [40]. Therefore, GM-CSF may further stimulate EMT induced by TGF-β1.

EMT of airway epithelial cells plays a critical role in airway remodeling associated with chronic bronchial asthma [41]; by this process mesenchymal cells that resulted from EMT migrate to the subepithelial connective tissue where they produce extracellular matrix proteins and thus contributing to airway wall fibrosis [41]. A positive relationship between the level of CysLTs in exhaled breath condensate and reticular basement membrane thickness and reduction of collagen deposition by montelukast in ovalbumin sensitized/challenged mouse model were previously reported, suggesting the contribution of CysLTs to extracellular matrix deposition in the airways [12,13,42]. In the present study, we found that montelukast inhibits the expression of collagen I in BEAS-2B cells co-cultured with eosinophils further supporting the inhibitory activity of montelukast on airway collagen deposition.

Secretion of CysLTs from eosinophils can be stimulated by various stimuli [18,19,38]. Consistent with previous studies, here we showed that, co-culture of BEAS-2B cells with human eosinophils increases the production of CysLTs, and that the production was inhibited in the presence of montelukast; these findings suggest the existence of an autocrine/paracrine loop causing enhanced secretion of CysLTs in the co-culture system of BEAS-2B cells and human eosinophils. This finding is consistent with previous studies describing the existence of CysLTs autocrine/paracrine loops in murine lung fibrocytes and human peripheral eosinophils [39,40]. In addition, previous studies have shown that montelukast can also act as inverse agonist that can suppress the spontaneous activity of CysLT receptors, and that it can elicit effects independently of its leukotriene receptor blockade activity [41]; for example, montelukast may alter eosinophil protease activity or suppress the adhesion of eosinophils to VCAM-1 [42,43]. Therefore, further studies are needed to completely elucidate the mechanism of action of montelukast.

The present study has some limitations. The purity of eosinophils was not 100%, and thus EMT could be caused by hematopoietic cells rather than eosinophils. However, in a previous study, we have already demonstrated that eosinophils, purified using the same method, but not the other contaminating cells,

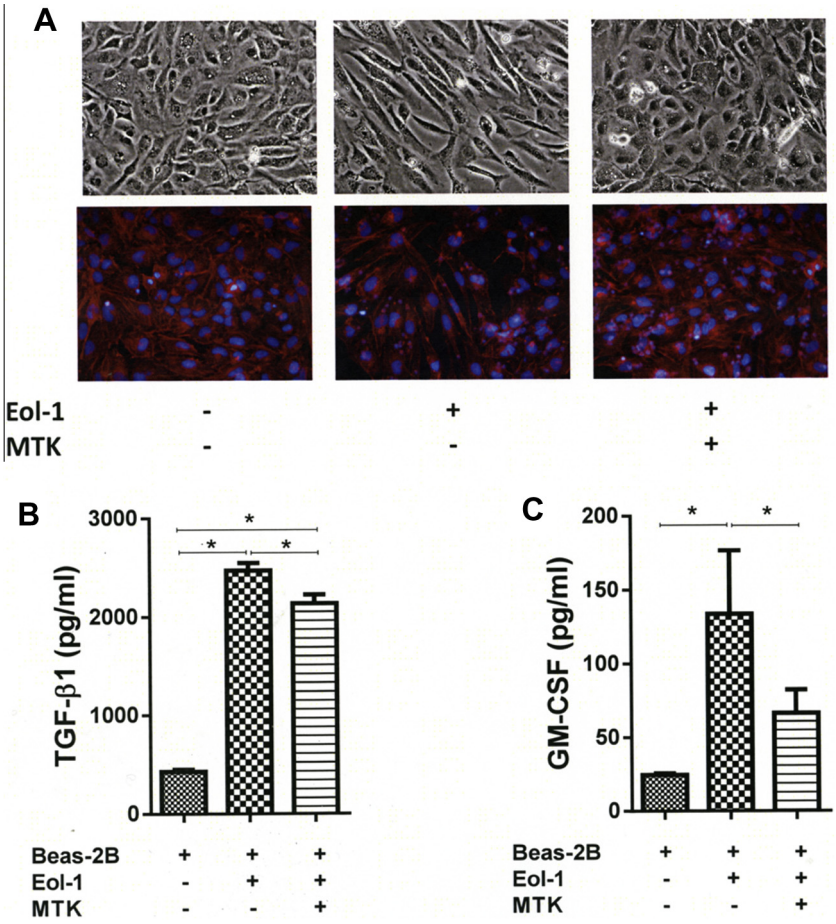

Fig. 3. EMT induced by human Eol-1 cells is inhibited by montelukast. (A) Control and BEAS-2B cells co-cultured with Eol-1 cells in the presence or absence of montelukast; the upper panel shows BEAS-2B cells stained with DIFF-QUICK after washing out eosinophils; the lower panel shows BEAS-2B cells stained with phalloidin (red) and DAPI for nuclei staining (blue) after washing out eosinophils. Original magnification, ×400. (B, C) TGF-β1 and GM-CSF levels in the supernatant from each cell condition. Data are expressed as mean ± SEM. $^{*}P < 0.05$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

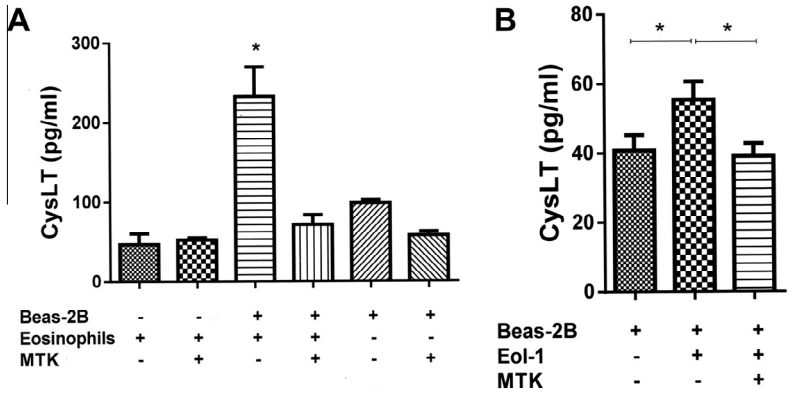

Fig. 4. Production of CysLTs is inhibited by montelukast. CysLTs levels in the supernatant from each cell condition using human eosinophils (A) or EoL-1 cell lines (B). The level of CysLTs was decreased in the presence of montelukast. Data are expressed as mean ± SEM. $^{*}P < 0.05$.

promote EMT in the model [36]. The fact that the EMT induced by a cell line of eosinophil, Eol-1 cells, was inhibited by montelukast also supports the role of human eosinophils in our model of EMT. The lack of an *in vivo* study is another limitation; but it has been previously shown that eosinophils play a critical role in airway remodeling *in vivo* and that montelukast inhibits or reverses airway remodeling in asthma [12–15]. Therefore, it is likely that suppression of eosinophils-induced EMT by montelukast is a relevant mechanism even *in vivo*.

In brief, this study showed that montelukast suppresses eosinophils-induced EMT of airway epithelial cells, and this finding may explain the mechanism by which montelukast ameliorates airway remodeling in bronchial asthma.

Conflict of interest

The other authors declared no conflict of interest.

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