Montelukast modulates lung CysLT$_1$ receptor expression and eosinophilic inflammation in asthmatic mice[1]

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ABSTRACT

AIM: To determine the expressions of cysteinyl leukotriene receptors, CysLT$_1$ and CysLT$_2$, in airway eosinophilic inflammation of OVA-induced asthmatic mice and the modulation by montelukast, a CysLT$_1$ receptor antagonist. **METHODS:** Asthma model was induced by chronic exposure to ovalbumin (OVA) in C57BL/6 mice. The eosinophils in bronchoalveolar lavage (BAL) fluid and lung tissues were counted, IL-5 level in BAL fluid was measured, and CysLT$_1$ and CysLT$_2$ receptor mRNA expressions were detected by semi-quantitative RT-PCR. **RESULTS:** Montelukast (6 mg/kg, once per day for 20 d) significantly suppressed the increased eosinophils in BAL fluid and lung tissue, and increased IL-5 level in BAL fluid in OVA challenged mice. OVA challenge increased CysLT$_1$ but decreased CysLT$_2$ receptor mRNA expression. Montelukast inhibited the increased CysLT$_1$ but not the reduced CysLT$_2$ expression after OVA challenge. **CONCLUSION:** CysLT receptors are modulated immunologically, and montelukast inhibits up-regulation of CysLT$_1$ receptor and airway eosinophilic inflammation in asthmatic mice.

INTRODUCTION

Cysteinyl leukotrienes (CysLTs, including LTC$_4$, LTD$_4$, and LTE$_4$) are important inflammatory mediators, which are produced predominantly by eosinophils, mast cells, and macrophages in response to a variety of stimuli activating arachidonate 5-lipoxygenase pathway[1]. CysLT$_1$ selective antagonists, such as montelukast, zafirlukast, and pranlukast, are currently used in the treatment of asthma. Recently, two types of CysLT receptors, CysLT$_1$ and CysLT$_2$, have been cloned, and identified to be G protein-coupled receptors and to mediate CysLT effects[2-7]. CysLT$_1$ receptor mRNA presents in human lung smooth muscle cells, lung macrophages, most peripheral blood eosinophils and pregranulocytic CD34+ cells, and in subsets of monocytes and B lymphocytes[8]. In contrast to the CysLT$_1$ receptor, the strongest expression of the CysLT$_2$ receptor in the lung has been detected in interstitial macrophages, but distinctly weaker expression in smooth muscle cells. CysLT$_2$ receptor expresses abundantly in peripheral blood cell, and especially strong in human eosinophils[9].

The contribution of the CysLT receptors to bronchial asthma has been established by the therapeutic efficacy of CysLT biosynthetic inhibitors[10] and selective CysLT$_1$ receptor antagonists[11]. Ovalbumin sensitization and aerosol challenge in mice elicits LTB$_4$ and

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LTC_4 release into bronchoalveolar lavage (BAL) fluid, eosinophilia in the mucosa and the BAL fluid, and the increased airways reactivity to methacholine[12,13]. Although the involvement of CysLTs in bronchoconstriction is not established in mice[14,15], a $CysLT_1$ receptor selective antagonist, MK-571, recently has been shown to inhibit eosinophilia, bronchial hyperreactivity, and microvascular leakage in a mouse model[16]. Furthermore, in LTA_4 hydrolase gene-disrupted mice subjected to zymosan A-induced peritonitis, neutrophil recruitment was decreased, and protein extravasation because of increased vascular permeability in the peritoneal cavity was substantial[17]. These findings are consistent with the absence of LTB_4 and the increased CysLT generation that attribute to shunting of LTA_4 to LTC_4 synthase. In addition, CysLTs directly increase venular permeability and edema formation at the administration site in mice[18,19].

The roles of CysLTs in asthma have been well investigated. However, no direct evidence elucidates the expressions of $CysLT_1$ and $CysLT_2$ receptors in airway inflammation of asthma. To clarify the implications of CysLT receptors in airway eosinophilic inflammation, we detected the expressions of $CysLT_1$ and $CysLT_2$ receptor mRNAs by RT-PCR and the effect of montelukast, a $CysLT_1$ receptor antagonist, in the lungs of asthmatic mice that were chronically induced by ovalbumin sensitization and aerosol challenge in this study.

MATERIALS AND METHODS

Drugs and reagents Montelukast was a gift from MERCK Research Laboratories (USA); chicken ovalbumin (OVA, grade V) was from Sigma Chemicals, USA; inject alum was from PIERCE Co, USA; recombination mouse IL-5 standard, purified anti-mouse IL-5 antibody, and biotinilated anti-mouse secondary antibody were from Rockland Chemical Co; Trizol for extracting RNA was from Bio Basic Inc, Canada; and chemicals for RT-PCR were from Takara Co, Japan.

Ovalbumin sensitization and challenge[20] Male C57BL/6 mice, weighing 18-22 g, were purchased from Shanghai Experimental Animal Center of Chinese Academy of Sciences. Mice were sensitized by intraperitoneal injections (100 µL) of 20 µg OVA emulsified in 2 mg of Inject Alum [Al $(OH)_3$/Mg$(OH)_2$] on d 0 and d 14. Mice were subsequently challenged with 2 % OVA aerosol in saline or saline alone for 45 min by ultrasonic

nebulization from d 24 to d 41. Montelukast (6 mg/kg) was orally given once daily for 20 d from d 23 to d 42.

Bronchoalveolar lavage fluid (BAL) eosinophil count On d 43, mice were sacrificed and their lungs were lavaged three times with 0.5 mL of PBS containing 2 % FCS. The recovered BAL fluids were pooled and centrifuged, generating a BAL cell pellet and a cell-free supernatant. Total cell counts were determined with a hemacytometer, and eosinophils were counted on Wright stained cytospin slides (Cytospin 3, Shandon Scientific, Pittsburgh, PA) by counting ≥300 cells. Cell-free lavage fluid was frozen on dry ice and stored at -70 ℃ until use.

Lung histology The left lobes of lungs were fixed in 10 % buffered formalin. After embedding in paraffin, the tissues were cut into 5-µm thick sections. Eosinophils were stained with Discombe's solution (0.05 % aqueous eosin and 5 % acetone in distilled water, v/v) for 5 min, rinsed with distilled water, and counterstained with 0.07 % methylene blue.

Interleukin-5 (IL-5) assay IL-5 levels in BAL fluids were measured by ELISA method according to the manufacturer's guideline for users. The limit of this assay was 5 ng/L.

Semi-quantitative RT-PCR Total RNA was prepared from 0.1 mg of the lung tissues with Trizol reagents according to the manufacturer's guidelines. For cDNA synthesis, 20 µL reverse transcription mixture containing total RNA 1 µg, dNTP 1 mmol/L, random primer 0.2 µg, RNasin 20 U, M-MuLV reverse transcriptase 200 U were mixed and incubated at 42 ℃ for 60 min, and then the reverse transcriptase was inactivated by heating the reaction mixture at 70 ℃ for 10 min.

Oligonucleotide primers specific for mouse $CysLT_1$, $CysLT_2$, and G3PDH (an internal standard) were synthesized according to published sequences[8]: $CysLT_1$: (+) CAACGAACTATCCACCTTCACC, (-) AGCCTTCTCCTAAAGTTTCCAC; $CysLT_2$: (+) GTCCACGTGCTGCTCATAGG, (-) ATTGGCTGCA-GCCATGGTC; G3PDH: (+) AGGTTGTCTCCTGCGA-CTTC, (-) CTTGCTCAGTGTCCTTGCTG; with the product sizes 162 bp, 180 bp, and 210 bp respectively. PCR reactions were performed on Eppendorf Master Cycler. The reaction conditions were as follows: 2 µL of cDNA mixture was subjected to amplification in 50 µL of final volume with $MgCl_2$ 1.5 mmol/L, dNTPs 0.2 mmol/L, 20 pmol of each primer, and 2 U of *Taq* DNA polymerase in the reaction buffer. PCR reactions were

as follows: 94 ℃, 5 min; then 94 ℃, 1 min, 65 ℃ for $CysLT_1$, 67 ℃ for G3PDH and 68 ℃ for $CysLT_2$, 1 min; 72 ℃, 45 s, for 30 cycles; and 72 ℃ 10 min to end the reaction. PCR products of 10 µL were separated by 1.8 % agarose gel electrophoresis and visualized using ethidium bromide staining. The density of each band was measured by UVP gel analysis system. This semi-quantitative measure was expressed as ratios compared with G3PDH.

Statistical analysis All values were presented as mean±SD. One-way ANOVA was used for statistical analysis of the differences between groups. $P<0.05$ was considered statistically significant.

RESULTS

Airway eosinophilic inflammation in OVA-sensitized mice After chronically repeated OVA sensitization and challenge, severe airway eosinophilic inflammation appeared in the pulmonary interstitium with numerous eosinophils around the bronchioles and blood vessels as compared to control (Fig 1A-B). Montelukast treatment attenuated eosinophil infiltration (Fig 1C). Eosinophils in both BAL fluid and lung parenchyma significantly increased in chronically OVA-challenged mice, and eosinophils were the predominant inflammatory cells. Montelukast treatment significantly reduced the numbers of total cells and eosinophils in both BAL fluid, and eosinophils in lung parenchyma by 55.9 %, 95.5 %, and 88.5 %, respectively (Tab 1).

Tab 1. Inhibitory effect of montelukast (MK) on eosinophils in BAL fluid and around bronchioles and blood vessels in chronically OVA-challenged mice. *n*=8. Mean±SD. [c]$P<0.01$ *vs* saline control. [f]$P<0.01$ *vs* OVA challenge alone; one-way ANOVA.

Treatment	$10^{-7}×$BALF cells·L^{-1}		$10^{-3}×$Eosinophils around bronchioles and blood vessels/mm^{-2}
	Total	Eosinophils (%)	
Saline	22±7	0.02±0.01 (0.09)	0.006±0.003
OVA	152±57[c]	51±5 (33.7)[c]	1.03±0.07[c]
OVA+MK	67±20[cf]	2.3±0.4 (3.43)[cf]	0.16±0.02[cf]

IL-5 level in BAL fluid IL-5 level in BAL fluid of OVA-challenged mice was 2.15-fold higher than con-
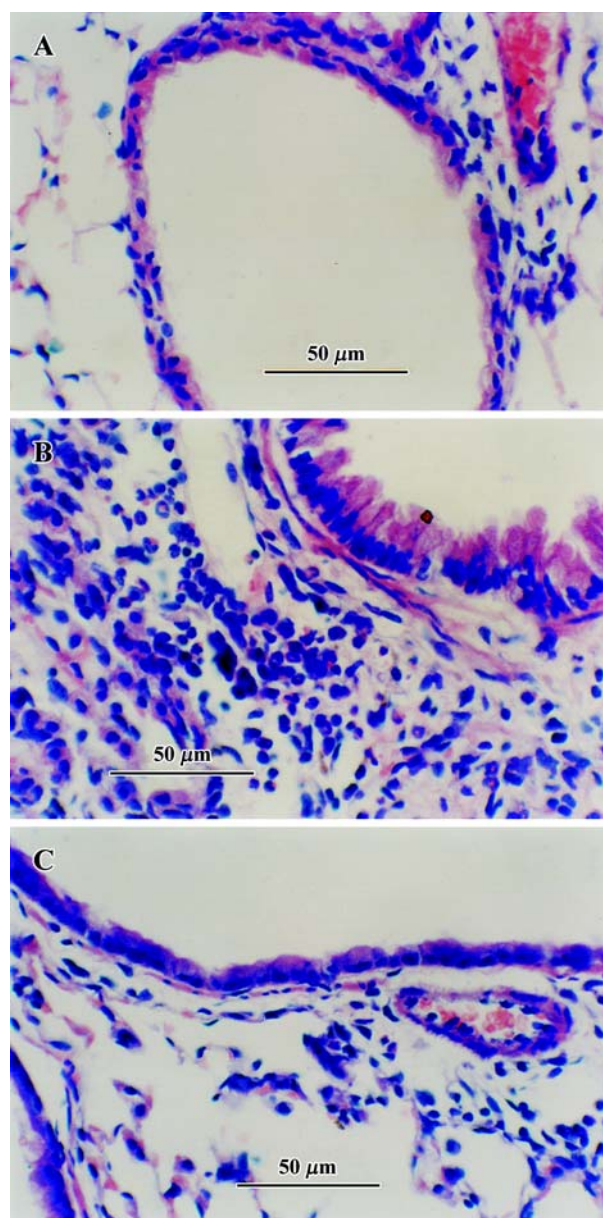

Fig 1. Effect of montelukast on eosinophil infiltration in the pulmonary interstitium. A: control; B: OVA-challenged, there were numerous eosinophils around the bronchioles; C: montelukast- and OVA-treated, there were fewer eosinophils in the peribronchioles tissues.

trol mice, and montelukast treatment decreased IL-5 level by 51.0 % ($P<0.01$, Fig 2).

$CysLT_1$ and $CysLT_2$ mRNA expressions in the lungs of mice $CysLT_1$ and $CysLT_2$ expressions in the lungs of mice were examined by RT-PCR, and only one band of each predicted size for $CysLT_1$ (162 bp) and $CysLT_2$ (180 bp) was found (Fig 3). The expression of $CysLT_1$ mRNA increased 1.3-fold in OVA-challenged mice ($P<0.05$ *vs* control), and montelukast treatment decreased the enhanced expression of $CysLT_1$ mRNA
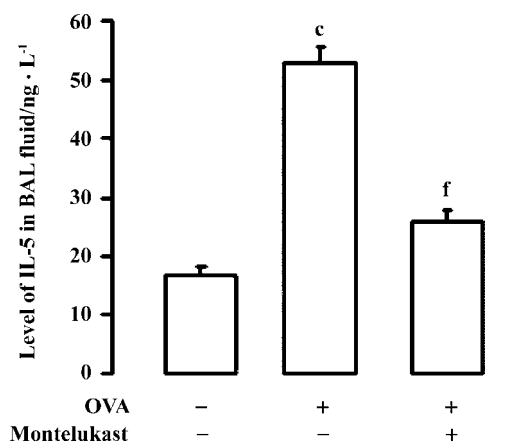
Fig 2. Effect of montelukast on the level of IL-5 in BAL fluid. *n*=5. Mean±SD. [c]*P*<0.01 *vs* control. [f]*P*<0.01 *vs* OVA-challenged mice.
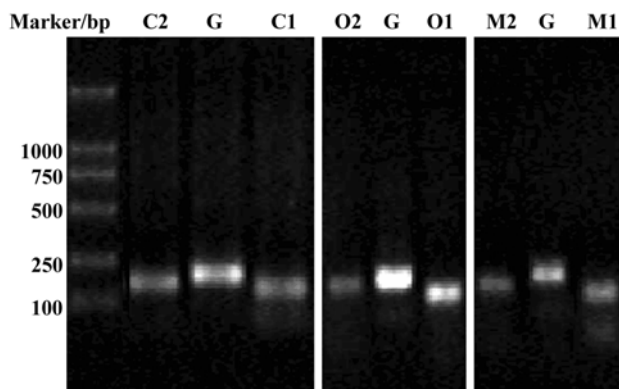

Fig 3. Agarose gel electrophoresis of PCR products. C2 and C1: $CysLT_2$ and $CysLT_1$ of control group; O2 and O1: $CysLT_2$ and $CysLT_1$ of OVA challenged group; M2 and M1: $CysLT_2$ and $CysLT_1$ of montelukast-treated group; G: G3PDH (an internal standard).
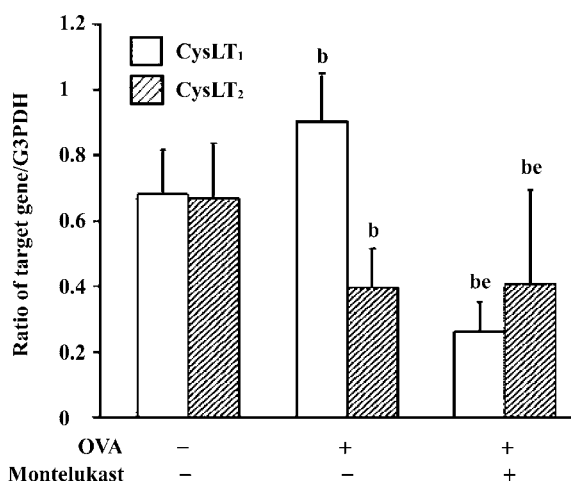

Fig 4. Effect of montelukast on $CysLT_1$ and $CysLT_2$ receptor mRNA expressions in the lungs of mice. *n*=5. Mean±SD. [b]*P*<0.05 *vs* control. [e]*P*<0.05 *vs* OVA.

by 3.4-fold (*P*<0.01, Fig 4). However, the expression of $CysLT_2$ mRNA decreased 1.7-fold in OVA-challenged mice (*P*<0.05), but montelukast treatment had no effect on the decreased expression of $CysLT_2$ mRNA (Fig 4).

DISCUSSION

In this study, a severe airway eosinophilic inflammation and higher level of IL-5 in BAL fluid have been found in a mouse model of asthma, and these alterations can be inhibited effectively by montelukast, a $CysLT_1$ receptor antagonist. Also, up-regulation of $CysLT_1$ and down-regulation of $CysLT_2$ receptor mRNA expression were found in the lungs of asthmatic mice, and montelukast inhibited the enhanced $CysLT_1$ receptor expression, but not $CysLT_2$ receptor. These findings clearly indicate that CysLTs plays an important role in airway eosinophilic inflammation induced by repeated exposure to antigen, and that CsyLT receptors in the lung can be modulated both immunologically and pharmacologically. Evidence for the role of CysLTs in airway eosinophilic inflammation in our study is the results that montelukast greatly suppresses eosinophil infiltration in both pulmonary interstitium and BAL fluid of asthmatic mice. Same effect of montelukast is also reported in acute asthma model of BALB/c mice[21], and another $CysLT_1$ receptor antagonist pranlukast can inhibit human eosinophil activation[22]. These results support the hypothesis that CysLTs induce migration and enhance degranulation of eosinophils via $CysLT_1$ receptor[23]. Furthermore, eosinophils are regulated by a network of cytokines and IL-5 plays a critical role[24], and these are confirmed by the elevation of IL-5 level in BAL fluid in our study.

Interestingly, we found that CysLT receptor expressions in the lungs of asthmatic mice were modulated by both chronic antigen exposure and $CysLT_1$ receptor antagonist. $CysLT_1$ receptor is up-regulated and $CysLT_2$ receptor is down-regulated immunologically. We can not explain the reasons for these changes. But one of the possible reasons for up-regulation of $CysLT_1$ may be related to the elevation of IL-5 level in BAL fluid as we found. IL-5 has been reported to up-regulate $CysLT_1$ receptor expression in HL-60 cells differentiated toward the eosinophils[25]. Also, other cytokines like IL-13, IL-4, IFN-γ and TGF-β increase $CysLT_1$ receptor expression[26-28]. The increased $CysLT_1$ receptors may potentiate the CysLT-mediated actions of eosinophils and pulmonary cells on airway eosinophilic inflammation and

airway hyperresponsiveness as mentioned above. However, the reason why the expression of $CysLT_2$ mRNA decreased in OVA-challenged mice is unclear.

More important finding in this study is that montelukast inhibits the enhanced $CysLT_1$ receptor expression in the lungs of asthmatic mice that related to suppression of eosinophilic inflammation functionally. Since eosinophils, macrophages, and other cells in the lung express $CysLT_1$ receptor, reduced inflammatory cells by montelukast in the lung may result in a lower expression of $CysLT_1$ receptor. Another possible reason is inhibition of IL-5 level in BAL fluid by montelukast that may attenuate one modulator for the up-regulation of $CysLT_1$ receptor expression, but the causal relation is unknown. The mechanism(s) should be further investigated on the basis such as cytokine network, intracellular signal transduction pathways, and receptor-antagonist interactions. But we can not explain why montelukast reduced the expression of $CysLT_1$ receptor even in the lungs of control mice. In contrast, montelukast did not reverse the reduced expression of $CysLT_2$ receptor.

In summary, we found the effect of montelukast on airway eosinophilic inflammation and up-regulation of $CysLT_1$ receptor expression in the lungs of asthmatic mice. Our results provide the evidence of the immunological and pharmacological modulation of CysLT receptor expressions in the lung, and further studies are needed to clarify characteristics and mechanisms like the involved cell types, up- and down-stream events, and time- and dose-dependence.

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