

Cysteinyl leukotriene upregulates IL-11 expression in allergic airway disease of mice

Kyung Sun Lee, PhD, So Ri Kim, MD, PhD, Hee Sun Park, MD, PhD, Seoung Ju Park, MD, PhD, Kyung Hoon Min, MD, PhD, Ka Young Lee, MD, Sun Mi Jin, MS, and Yong Chul Lee, MD, PhD Jeonju, South Korea

Background: Chronic airway inflammation and airway remodeling are important features of bronchial asthma. IL-11 is one of the important mediators involved in the process of airway inflammation and remodeling. Cysteinyl leukotrienes (cysLTs) play roles in recruitment of inflammatory cells, airway smooth muscle contraction, vascular leakage, increased mucus secretion, decreased mucociliary clearance, and airway fibrosis. **Objective:** An aim of the present study was to determine the effect of the cysLTs on the regulation of IL-11 expression.

Methods: We used a C57BL/6 mouse model of allergic airway disease and murine tracheal epithelial cells to examine the effects of cysLTs on the regulation of IL-11 expression.

Results: Our present study with an ovalbumin-induced murine model of allergic airway disease revealed that levels of leukotriene C₄ (LTC₄) in bronchoalveolar lavage fluids were increased and that administration of montelukast or pranlukast reduced the increased levels of LTC₄; the increased expression of IL-11 protein and mRNA in lung tissues; airway inflammation, bronchial hyperresponsiveness; the increased levels of TGF- β ₁, IL-4, and IL-13 in bronchoalveolar lavage fluids and lung tissues; and airway fibrosis. In addition, LTC₄ stimulates epithelial cells to produce IL-11. Our results also showed that cysLT type 1 receptor antagonists downregulated the activity of a transcription factor, nuclear factor κ B, and BAY 11-7085 substantially reduced the increased levels of IL-11 after ovalbumin inhalation.

Conclusion: These results suggest that cysLTs regulate the IL-11 expression in allergic airway disease.

Clinical implications: These findings provide one of the molecular mechanisms for the effects of cysLTs on airway inflammation and fibrosis in allergic airway diseases. (J Allergy Clin Immunol 2007;119:141-9.)

Key words: Cysteinyl leukotrienes, IL-11, airway, inflammation, remodeling

Abbreviations used

BAL:	Bronchoalveolar lavage
cysLT:	Cysteinyl leukotriene
cysLT1R:	Cysteinyl leukotriene type 1 receptor
cysLT2R:	Cysteinyl leukotriene type 2 receptor
GAPDH:	Glyceraldehyde-3-phosphate dehydrogenase
LT:	Leukotriene
NF- κ B:	Nuclear factor κ B
OVA:	Ovalbumin
R _L :	Airway resistance

Chronic airway inflammation and airway remodeling are important features of bronchial asthma.¹ The airway obstruction observed in asthma is thought to be caused by airway remodeling. The histopathologic features of this process include epithelial changes, subepithelial fibrosis, infiltration of inflammatory cells, hyperplasia and hypertrophy of the bronchial smooth muscle, and mucus hypersecretion. One of the most prominent features of airway remodeling is subepithelial fibrosis, and this characteristic is reportedly a potential marker for disease severity.² IL-11 is one of the important mediators involved in the process of airway inflammation and remodeling.^{3,4} IL-11 was initially discovered as an IL-6–like plasmacytoma proliferation stimulating activity in supernatants from transformed marrow fibroblasts.⁵ Studies have shown that IL-11 acts as a hematopoietic growth factor, stimulates the acute-phase response, augments Ig production, induces the expression of metalloproteinase inhibitors, regulates neural phenotype, regulates bone metabolism, and protects against the combined effects of radiation and chemotherapy.⁶⁻¹² Previous studies have also demonstrated that human lung fibroblasts and epithelial cells produce IL-11 in response to cytokines (IL-1 and TGF- β), histamine, and viruses that have been epidemiologically associated with asthma exacerbations.¹³⁻¹⁵ In addition, IL-11 has been shown to play an important role in the inflammatory and fibrotic responses in both viral and nonviral human airway disorders.⁴

Leukotrienes (LTs), lipid mediators generated from arachidonic acid by the action of 5-lipoxygenase, play important roles in the pathogenesis of allergic airway inflammation.¹⁶ LTC₄, LTD₄, and LTE₄, known as cysteinyl leukotrienes (cysLTs), are both direct bronchoconstrictors and proinflammatory substances. The cysLTs

From the Department of Internal Medicine, Airway Remodeling Laboratory, Chonbuk National University Medical School, Jeonju.

Supported by a Korean Research Foundation Grant funded by the Korean Government (MOEHRD, Basic Research Promotion Fund; KRF-2005-201-E00014), a grant from the National Research Laboratory Program of the Korean Science and Engineering Foundation, and a grant of the Korea Health 21 R&D Project, Ministry of Health and Welfare (A060169), Republic of Korea.

Disclosure of potential conflict of interest: The authors have declared that they have no conflict of interest.

Received for publication April 3, 2006; revised September 1, 2006; accepted for publication September 1, 2006.

Available online November 2, 2006.

Reprint requests: Yong Chul Lee, MD, PhD, Department of Internal Medicine, Chonbuk National University Medical School, San 2-20, Geumam-dong, Deokjin-gu, Jeonju, 561-180, South Korea. E-mail: leeyc@chonbuk.ac.kr. 0091-6749/\$32.00

© 2007 American Academy of Allergy, Asthma & Immunology

doi:10.1016/j.jaci.2006.09.001

exhibit a variety of physiologic functions involving recruitment of inflammatory cells, airway smooth muscle contraction, vascular leakage, increase of mucus secretion, decrease of mucociliary clearance, and airway fibrosis.¹⁷⁻¹⁹

However, the mechanisms by which cysLTs induce airway inflammation and remodeling, especially airway fibrosis, are not clearly understood.

In the present study we used a murine model of allergic airway disease and murine tracheal epithelial cells to determine the effect of the cysLTs on the regulation of IL-11 expression.

METHODS

More detailed information about the methods used in this study can be found in the Online Repository at www.jacionline.org.

Animals and experimental protocol

Female C57BL/6 mice, 8 to 10 weeks of age and free of murine-specific pathogens, were obtained from the Korean Research Institute of Chemistry Technology (Daejeon, Korea), housed throughout the experiments in a laminar flow cabinet, and maintained on standard laboratory chow *ad libitum*. Mice were sensitized and challenged as previously described,²⁰ with some modifications.

Administration of montelukast, pranlukast, anti-IL-11 antibody, isotype control mAb, or BAY 11-7085

Montelukast sodium (L-706, 631; 5 mg/kg body weight per day; Merck & Co, Inc, Rahway, NJ) dissolved in 1% methyl cellulose or pranlukast (25 mg/kg body weight per day; Ono Pharmaceutical Co, Ltd, Osaka, Japan) dissolved in distilled water was administered by means of oral gavage 11 times at 24-hour intervals on days 19 to 29, beginning 1 day before the first challenge. Anti-IL-11 antibody or isotype control mAb (8 mg/kg body weight per day; R&D systems, Inc, Minneapolis, Minn) was administered intraperitoneally 2 times to each animal, once on day 23 (1 hour after the last airway challenge) and the second time on day 29 (6 days after the last airway challenge). BAY 11-7085 (20 mg/kg body weight per day; BIOMOL International L.P., Plymouth Meeting, Pa) dissolved in dimethyl sulfoxide and diluted with 0.9% NaCl was administered by means of intraperitoneal injection 2 times to each treated animal, once on day 23 (1 hour before the last airway challenge) and the second time on day 29 (6 days after the last airway challenge, see Fig E1 in the Online Repository at www.jacionline.org).^{21,22}

Isolation and primary culture of murine tracheal epithelial cells

Murine tracheal epithelial cells were isolated under sterile conditions, as previously described.²⁰

LTC₄ or LTD₄ stimulation and IL-11 production

Cells were seeded in 6-well culture inserts at a density of 1×10^5 cells/mL and grown in culture medium. At confluence, LTC₄ (Sigma-Aldrich, St Louis, Mo) or LTD₄ (BIOMOL International L.P.) was added to the cells at varying concentrations (1-1000 ng/mL).

Western blot analysis

Protein expression levels were analyzed by means of Western blot analysis, as described previously.²⁰

Cytosolic or nuclear protein extractions for analysis of nuclear factor κ B p65

Cytosolic or nuclear extraction was performed as described previously.^{23,24}

RNA isolation and RT-PCR

Levels of mRNA expression were analyzed by means of RT-PCR assay, with total RNA isolated from lung tissues by using a rapid extraction method (TRI-Reagent), as previously described.²⁵

Quantitative real-time RT-PCR

Quantitative RT-PCR analysis was performed with the Light-Cycler FastStart DNA Master SYBR Green I (Roche Diagnostics, Mannheim, Germany).

Densitometric analysis and statistics

All immunoreactive signals were analyzed by means of densitometric scanning (Gel Doc XR; Bio-Rad, Hercules, Calif). Data were expressed as means $\pm$ SEM. Statistical comparisons were performed by using 1-way ANOVA, followed by the Scheffe test. Significant differences between 2 groups were determined by using the unpaired Student *t* test. Statistical significance was set at a *P* value of less than .05.

RESULTS

IL-11 protein levels and mRNA expression increased in ovalbumin-sensitized and ovalbumin-challenged mice

Western blot analysis revealed that IL-11 protein levels in lung tissues were increased approximately 4.4-, 5.7-, 6.1-, 7.0-, and 7.1-fold at 1/2, 1, 3, 5, and 7 days, respectively, after challenge with ovalbumin (OVA) compared with the levels in the control group (Fig 1, A and B). In contrast, no significant changes in the IL-11 protein level were observed after saline inhalation. Real-time RT-PCR analysis revealed that IL-11 mRNA expression had increased approximately 1.4-, 1.5-, 2.2-, 2.2-, and 2.3-fold at 1/2, 1, 3, 5, and 7 days, respectively, after OVA inhalation compared with the expression in the control group (Fig 1, D). In contrast, no significant changes in the IL-11 mRNA expression were observed after saline inhalation (Fig 1, C and D).

Effect of montelukast or pranlukast on IL-11 protein levels and mRNA expression in lung tissues of OVA-sensitized and OVA-challenged mice

Western blot analysis revealed that levels of IL-11 protein in lung tissues were increased at 7 days after OVA inhalation compared with levels in control mice (Fig 2, A and B). The increased IL-11 levels at 7 days after OVA inhalation were decreased by the administration of montelukast or pranlukast. RT-PCR and real-time RT-PCR analyses showed that IL-11 mRNA expression in lung tissues was increased at 7 days after OVA inhalation compared with the expression after saline inhalation (Fig 2, C and D). The increased IL-11 mRNA expression

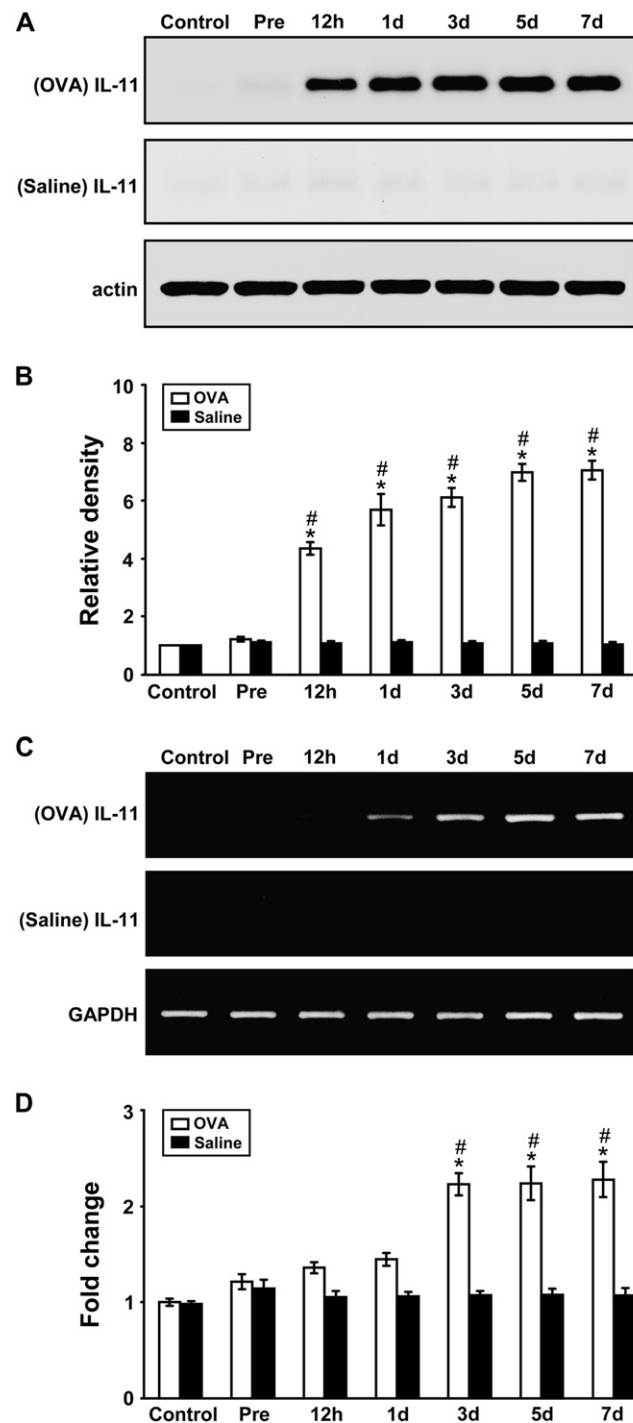


FIG 1. IL-11 protein and mRNA in lung tissues of OVA-sensitized and OVA-challenged mice. Sampling was performed in lung tissues from sensitized mice challenged with OVA or saline. **A**, Western blot analyses of IL-11 protein. **B**, Densitometric analyses are presented as the relative ratio of IL-11 to actin. The relative ratio of IL-11 in the lung tissues of control mice is arbitrarily presented as 1. **C**, Representative RT-PCR analysis of IL-11 mRNA expression. **D**, Quantitative analysis of IL-11 mRNA expression by means of real-time RT-PCR. Data represent means $\pm$ SEM from 8 mice per group. Time periods after the last challenge are indicated as 1/2, 1, 3, 5, and 7 days. *Control*, No treatment; *Pre*, 1 hour before the first challenge. $\#P < .05$ versus *Pre*; $*P < .05$ versus saline inhalation.

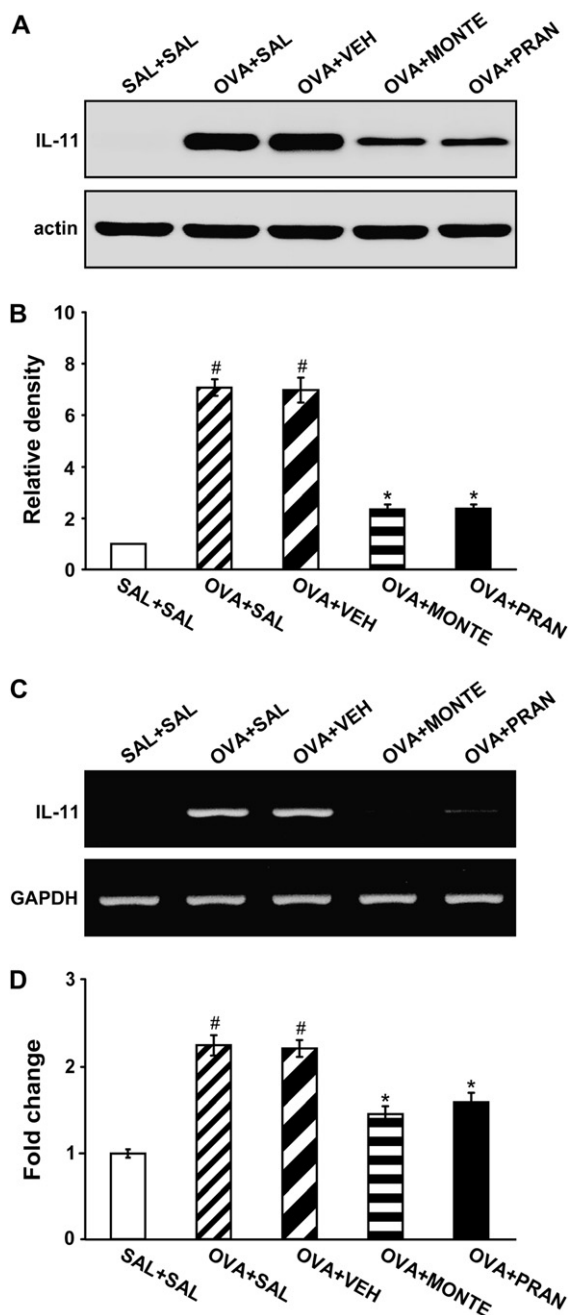


FIG 2. Effect of montelukast or pranlukast on IL-11 protein and mRNA in lung tissues of OVA-sensitized and OVA-challenged mice. **A**, Western blot analysis of IL-11. Sampling was performed at 7 days after the last challenge in saline-challenged mice administered saline (SAL+SAL), OVA-challenged mice administered saline (OVA+SAL), OVA-challenged mice administered drug vehicle (OVA+VEH), OVA-challenged mice administered montelukast (OVA+MONTE), and OVA-challenged mice administered pranlukast (OVA+PRAN). **B**, Densitometric analyses are presented as the relative ratio of IL-11 to actin. The relative ratio of IL-11 in the lung tissues of saline-inhaled mice administered saline is arbitrarily presented as 1. **C**, Representative RT-PCR analysis of IL-11 mRNA expression. **D**, Quantitative analysis of IL-11 mRNA expression by means of real-time RT-PCR. Data represent means $\pm$ SEM from 8 mice per group. [#] $P < .05$ versus SAL+SAL group; ^{*} $P < .05$ versus OVA+SAL group.

was reduced by the administration of montelukast or pranlukast.

Effect of LTC₄ on IL-11 protein and mRNA expression in primary murine tracheal epithelial cells

The effect of LTC₄ on the production of IL-11 from epithelial cells is shown in Fig 3. Western blot analysis revealed that the maximal levels of IL-11 were detected in the presence of LTC₄ (100 ng/mL) at 8 hours of incubation. The release of IL-11 was undetectable at 3 and 5 hours of incubation (data not shown). Epithelial cells were treated with montelukast to suppress the LTC₄-induced IL-11 production. Incubation of montelukast (100 nM) with cells treated with LTC₄ (100 ng/mL) significantly inhibited the production of IL-11 (Fig 3). Montelukast alone had no effect on the IL-11 production of unstimulated cells (data not shown).

RT-PCR analysis was used to detect the mRNA expression of IL-11 in response to stimulation of epithelial cells with LTC₄. At stimulation of epithelial cells with LTC₄, the IL-11 mRNA expression reached a peak at 6 hours (Fig 3, C) and faded at 12 hours and 24 hours (data not shown). Real-time PCR was applied to further confirm the increase of IL-11 mRNA expression. At 6 hours of incubation, the expression of IL-11 mRNA was increased approximately 2.1-fold compared with that seen in unstimulated cells (Fig 3, D). In addition, montelukast inhibited the gene transcription for IL-11 substantially.

Effect of montelukast or pranlukast on nuclear factor κ B p65 protein levels in lung tissues of OVA-sensitized and OVA-challenged mice

Western blot analysis revealed that levels of nuclear factor κ B (NF- κ B) p65 protein in nuclear protein extracts from lung tissues were increased at 7 days after OVA inhalation compared with levels in control mice (Fig 4). The increased NF- κ B p65 levels in nuclear protein extracts were decreased by the administration of montelukast or pranlukast. In contrast, levels of NF- κ B p65 protein in cytosolic protein extracts were decreased compared with levels in control mice. The decreased NF- κ B p65 levels in cytosolic preparations were increased by the administration of montelukast or pranlukast. These results indicate that montelukast and pranlukast inhibit NF- κ B activity by preventing translocation of this transcription factor into the nucleus (Fig 4).

Effect of BAY 11-7085 on IL-11 levels in lung tissues of OVA-sensitized and OVA-challenged mice

Western blot analysis showed that IL-11 protein levels in lung tissues were increased significantly at 7 days after OVA inhalation compared with levels after saline inhalation (Fig 5). The increased IL-11 levels were significantly reduced by the administration of BAY 11-7085.

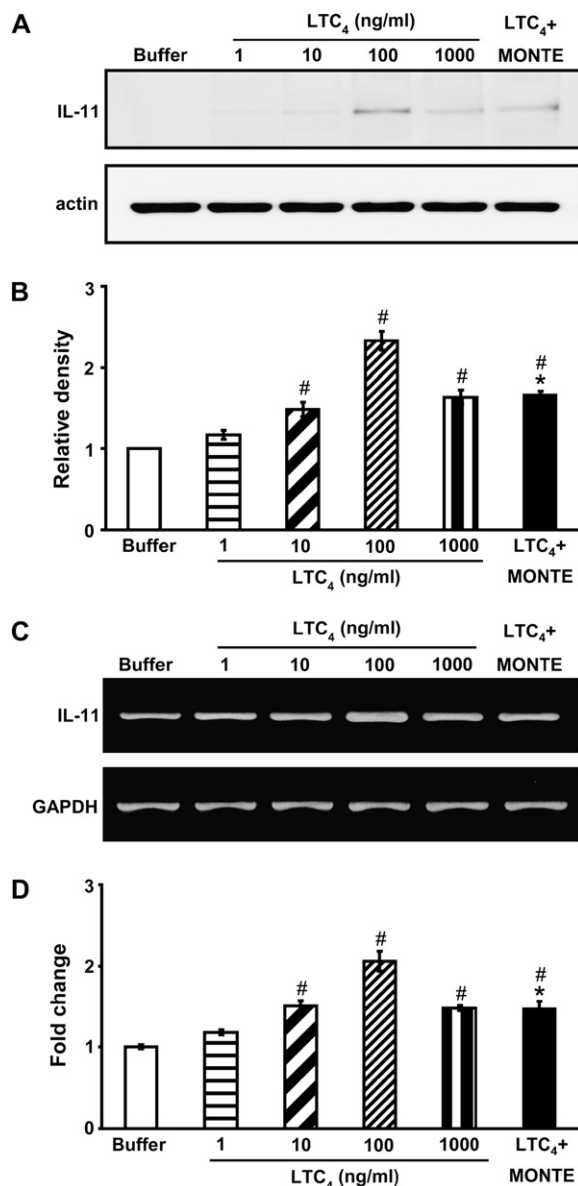


FIG 3. Effect of LTC₄ on IL-11 production by primary tracheal epithelial cells. **A**, Western blot analysis of IL-11. **B**, Densitometric analyses are presented as the relative ratio of IL-11 to actin. The relative ratio of IL-11 in the epithelial cells incubated with buffer alone is arbitrarily presented as 1. **C**, Representative RT-PCR analysis of IL-11 mRNA expression. **D**, Quantitative analysis of IL-11 mRNA expression by means of real-time RT-PCR. Data represent means $\pm$ SEM from 6 independent experiments. MONTE, Montelukast. # $P < .05$ versus buffer alone; * $P < .05$ versus 100 ng/mL LTC₄.

Effect of montelukast or pranlukast on pathologic changes of OVA-sensitized and OVA-challenged mice

Histologic analyses revealed typical pathologic features of allergic airway disease in the OVA-sensitized and OVA-challenged mice. Numerous inflammatory cells, including eosinophils, infiltrated around the bronchioles; the airway wall was thickened; and mucus and debris had

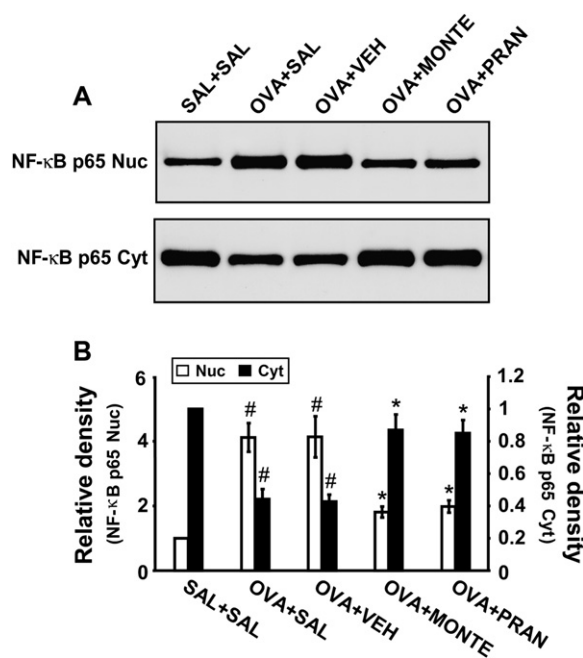


FIG 4. Effect of montelukast or pranlukast on NF-κB p65 protein expression in nuclear and cytosolic protein extracts from lung tissues. **A**, Western blotting of NF-κB p65 in nuclear (Nuc) and cytosolic (Cyt) protein extracts from lung tissues. **B**, Densitometric analyses are presented as the relative ratio of NF-κB p65 levels in the OVA+Sal, OVA+VEH, OVA+MONTE, or OVA+PRAN groups to those in the SAL+Sal group. The NF-κB p65 level in nuclear protein extracts of the SAL+Sal group is arbitrarily presented as 1. Bars represent means $\pm$ SEM from 8 mice per group. For definition of groups, see legend for Fig 3. # $P < .05$ versus SAL+Sal group; * $P < .05$ versus OVA+Sal groups.

accumulated in the lumen of bronchioles. Mice treated with montelukast or pranlukast showed marked reductions in the thickening of the airway wall, the infiltration of inflammatory cells in the peribronchiolar region, and the amount of debris in the airway lumen. These results indicate that montelukast and pranlukast inhibit antigen-induced inflammation in the lungs, including the influx of eosinophils (see Fig E2 in the Online Repository at www.jacionline.org).

Effect of montelukast, pranlukast, or anti-IL-11 antibody on TGF-β₁ levels in lung tissues and bronchoalveolar lavage fluids of OVA-sensitized and OVA-challenged mice

Western blot analysis showed that TGF-β₁ protein levels in lung tissues were significantly increased at 7 days after OVA inhalation compared with levels in control mice (see Fig E3 in the Online Repository at www.jacionline.org). The increased TGF-β₁ levels were significantly reduced by the administration of montelukast, pranlukast, and anti-IL-11 antibody. Consistent with the results obtained from the Western blot analysis, enzyme immunoassays revealed that levels of TGF-β₁ in bronchoalveolar lavage (BAL) fluids were significantly increased at 7 days after OVA inhalation compared with levels in control mice

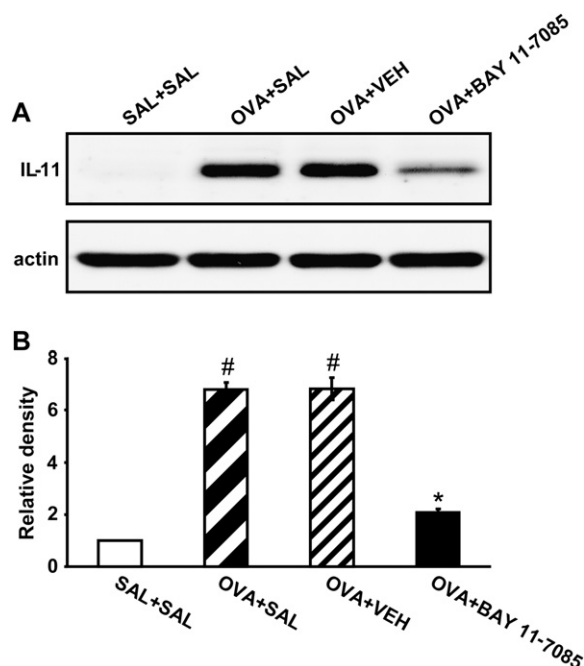


FIG 5. Effects of BAY 11-7085 on IL-11 expression in lung tissues of OVA-sensitized and OVA-challenged mice. **A**, Western blotting of IL-11 in lung tissues. **B**, Densitometric analyses are presented as the relative ratio of IL-11 to actin. The relative ratio of IL-11 in the lung tissues of the SAL+Sal group is arbitrarily presented as 1. Bars represent means $\pm$ SEM from 8 mice per group. For definition of groups, see legend for Fig 3. [#] $P < .05$ versus SAL+Sal group; ^{*} $P < .05$ versus OVA+Sal group.

(see Fig E3 in the Online Repository). The increased TGF- β_1 levels were significantly reduced by the administration of montelukast, pranlukast, and anti-IL-11 antibody. No significant changes were observed in OVA-sensitized and OVA-challenged mice administered isotype control mAb.

Effect of montelukast, pranlukast, or anti-IL-11 antibody on IL-4 and IL-13 levels in lung tissues and BAL fluids of OVA-sensitized and OVA-challenged mice

Western blot analysis showed that IL-4 and IL-13 protein levels in lung tissues were significantly increased at 72 hours after OVA inhalation compared with levels in control mice (see Fig E4 in the Online Repository at www.jacionline.org). The increased IL-4 and IL-13 levels were significantly reduced by the administration of montelukast, pranlukast, and anti-IL-11 antibody. Consistent with the results obtained from the Western blot analysis, enzyme immunoassays revealed that levels of IL-4 and IL-13 in BAL fluids were significantly increased at 72 hours after OVA inhalation compared with levels in control mice (see Fig E4 in the Online Repository). The increased IL-4 and IL-13 levels were significantly reduced by the administration of montelukast, pranlukast, and anti-IL-11 antibody. No significant changes were observed in

OVA-sensitized and OVA-challenged mice administered isotype control mAb.

Effect of montelukast, pranlukast, or anti-IL-11 antibody on peribronchial collagen deposition

OVA-sensitized and OVA-challenged mice had a significant increase in the levels of peribronchial fibrosis compared with levels in control mice, as assessed by means of trichrome staining and determination of total lung collagen content. The administration of montelukast, pranlukast, or anti-IL-11 antibody substantially reduced the increased levels of peribronchial fibrosis after OVA inhalation compared with the levels seen in mice that had no treatment after OVA inhalation (see Fig E5 in the Online Repository at www.jacionline.org).

DISCUSSION

Airway inflammation and fibrosis are prominent features of bronchial asthma and are connected by complex signaling networks. cysLTs and IL-11 are thought to contribute to the pathogenesis of allergic airway disease, but interrelationship between these proteins in airway inflammation and fibrosis has not been clarified. Our present study with an OVA-induced murine model of allergic airway disease has revealed that levels of LTC₄ in BAL fluids were increased (see Fig E6 in Online Repository at www.jacionline.org) and that administration of montelukast or pranlukast reduced the increased levels of LTC₄; the increased expression of IL-11 protein and mRNA in lung tissues; airway inflammation; bronchial hyperresponsiveness (see Fig E7 in Online Repository at www.jacionline.org); the increased levels of TGF- β_1 , IL-4, and IL-13 in BAL fluids and lung tissues; and airway fibrosis. In addition, treatment of epithelial cells with LTC₄ stimulated the expression of IL-11 protein and mRNA. Our results have also indicated that cysLT type 1 receptor (cysLT1R) antagonists downregulated the activity of a transcription factor, NF- κ B, and that an inhibitor of NF- κ B activation, BAY 11-7085, reduced the expression of IL-11 protein. These findings suggest that cysLT1R antagonists inhibit the NF- κ B signal transduction pathway by decreasing NF- κ B binding activity to the promoter region of the IL-11 gene involved in airway inflammation and remodeling in allergic airway disease.

A pleiotropic cytokine, IL-11, is produced by many cell types, including human lung fibroblasts, alveolar and airway epithelial cells, human airway smooth muscle cells, and eosinophils^{13,14,26,27} and is classified as a member of the IL-6 family of cytokines based on functional similarities between IL-11 and IL-6 and the shared use of gp130 molecules in their receptor complexes.^{28,29} Tang et al⁴ have demonstrated that the targeted expression of IL-11 in the mouse airway causes a B and T cell–predominant inflammatory response; airway remodeling with increased type III and I collagen; local accumulation

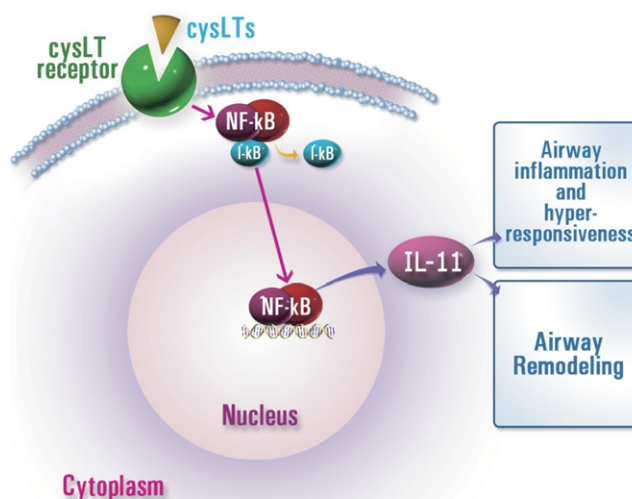


FIG 6. A proposed signaling pathway for cysLT-induced IL-11 expression in allergic airway disease. Our data suggest that cysLT receptor signal is involved in the regulation of IL-11 expression, and the NF-κB pathway could be one of its various regulatory mechanisms. I-κB, Inhibitory kappa B.

of fibroblasts, myofibroblasts, and myocytes; and obstructive physiologic dysregulation. They suggest that IL-11 plays an important role in the inflammatory and fibrotic responses in airway disorders. A previous study has shown that IL-11 expression is found to be increased in the airways of patients with asthma compared with in healthy control subjects and seems to correlate with disease severity and a reduced FEV₁.²⁷ Consistent with these observations, in this study we have found that IL-11 expression was upregulated in OVA-induced allergic airway disease. Interestingly, cysLT1R antagonist blocked LTC₄-stimulated production of IL-11 from epithelial cells, and administration of montelukast or pranlukast reduced the increased IL-11 expression, as well as the increased expression of TGF-β₁, IL-4, and IL-13. In addition, the increased expression of TGF-β₁, IL-4, and IL-13; the increased airway inflammation; and bronchial hyperresponsiveness were decreased substantially by administration of anti-IL-11 antibody. These results suggest that cysLT receptor signaling is associated with the regulation of IL-11 expression and that treatment of the cysLT1R antagonists might improve the features of allergic airway disease through regulation of IL-11 expression.

NF-κB is present in most cell types and is known to play a critical role in immune and inflammatory responses, including asthma.³⁰⁻³⁶ As expected, the NF-κB p65 protein level in nuclear protein extracts of lung tissues was substantially increased in the OVA-induced model of allergic airway disease used for the present study. It is known that activation of this transcription factor induces a variety of inflammatory genes that are abnormally expressed in asthma. These genes include cytokines (eg, IL-4, IL-5, IL-9, IL-11, IL-15, and TNF-α), chemokines (eg, RANTES, eotaxin, and monocyte chemoattractant protein 3), and adhesion molecules (eg, intracellular adhesion

molecule 1 and vascular cell adhesion molecule 1).^{37,38} Previous studies have revealed that the signaling pathways related to IL-11 induction are the H7-sensitive serine/threonine kinase and protein kinase C pathway, activator protein 1-mediated pathway, calmodulin-dependent pathway, and mitogen-activated protein kinase-dependent pathway.^{7,14,15,39-43} However, the regulatory mechanisms of IL-11 expression are not clear at the present time and are complex and cell/tissue specific. In this study expression of IL-11 was increased significantly after allergen challenge in a murine model of allergic airway disease. Administration of montelukast or pranlukast resulted in significant reduction of NF-κB activity, as well as expression of IL-11. In addition, the increased IL-11 protein levels after OVA inhalation were decreased by administration of an inhibitor of NF-κB activation, BAY 11-7085. Moreover, the administration of BAY 11-7085 decreased the increased IL-11 protein levels in LTC₄-stimulated epithelial cells (data not shown). Therefore these observations suggest that the NF-κB-dependent pathway could be one of the various regulatory mechanisms related with induction of IL-11 expression in allergic airway disease.

There are 2 types of cysLT receptors, cysLT1R and cysLT type 2 receptor (cysLT2R), which were originally defined pharmacologically based on their sensitivity to cysLT1R-specific antagonists. cysLT1R and cysLT2R are only loosely homologous (38% amino acid identity)^{44,45} and also share a similar degree of identity (24% to 32%) with the purinergic (P2Y) class of G protein-coupled receptors that mediate cellular responses to extracellular nucleotides.⁴⁶ In the present study we have revealed that the expression of both cysLT1R and cysLT2R in tracheal epithelial cells stimulated with LTC₄ was increased compared with that seen in unstimulated cells (see Fig E8 in

Online Repository at www.jacionline.org). In addition, we have shown that the IL-11 protein levels in epithelial cells were increased significantly after the stimulation of LTD₄, as well as LTC₄, and the increased expression of IL-11 was partially inhibited by montelukast, a cysLT₁R antagonist (see Fig E9 in the Online Repository at www.jacionline.org).

Taken together, these findings suggest that cysLT₂R, as well as cysLT₁R, signaling contributes to the LTC₄- or LTD₄-induced IL-11 expression in allergic airway disease.

In summary, we have examined the effect of the cysLTs on the regulation of IL-11 expression in a murine model of allergic airway disease and murine tracheal epithelial cells. By using montelukast and pranlukast, specific cysLT₁R antagonists, we have shown the important role of cysLTs in OVA-induced airway hyperresponsiveness, inflammation, and fibrosis. Moreover, our results have revealed that LTC₄ stimulated the expression of IL-11 protein and mRNA from epithelial cells, and administration of montelukast and pranlukast reduced IL-11 protein and mRNA expression. On the basis of these observations, we have concluded that cysLT receptor signal is involved in regulation of IL-11 expression (Fig 6). Our findings might also assist a strategy in the treatment of airway fibrosis and inflammation in allergic airway disease.

We thank Professor Mie-Jae Im for critical reading of the manuscript.

REFERENCES

- Bousquet J, Jeffery PK, Busse WW, Johnson M, Vignola AM. Asthma. From bronchoconstriction to airways inflammation and remodeling. *Am J Respir Crit Care Med* 2000;161:1720-45.
- Redington AE, Howarth PH. Airway wall remodelling in asthma. *Thorax* 1997;52:310-2.
- Zhu Z, Lee CG, Zheng T, Chupp G, Wang J, Homer RJ, et al. Airway inflammation and remodeling in asthma. Lessons from interleukin 11 and interleukin 13 transgenic mice. *Am J Respir Crit Care Med* 2001;164(suppl):S67-70.
- Tang W, Geba GP, Zheng T, Ray P, Homer RJ, Kuhn C 3rd, et al. Targeted expression of IL-11 in the murine airway causes lymphocytic inflammation, bronchial remodeling, and airways obstruction. *J Clin Invest* 1996;98:2845-53.
- Paul SR, Bennett F, Calvetti JA, Kelleher K, Wood CR, O'Hara RM Jr, et al. Molecular cloning of a cDNA encoding interleukin 11, a stromal cell-derived lymphopoietic and hematopoietic cytokine. *Proc Natl Acad Sci U S A* 1990;87:7512-6.
- Du XX, Williams DA. Interleukin-11: a multifunctional growth factor derived from the hematopoietic microenvironment. *Blood* 1994;83:2023-30.
- Maier R, Ganu V, Lotz M. Interleukin-11, an inducible cytokine in human articular chondrocytes and synoviocytes, stimulates the production of the tissue inhibitor of metalloproteinases. *J Biol Chem* 1993;268:21527-32.
- Roeb E, Graeve L, Hoffmann R, Decker K, Edwards DR, Heinrich PC. Regulation of tissue inhibitor of metalloproteinases-1 gene expression by cytokines and dexamethasone in rat hepatocyte primary cultures. *Hepatology* 1993;18:1437-42.
- Yin TG, Schendel P, Yang YC. Enhancement of in vitro and in vivo antigen-specific antibody responses by interleukin 11. *J Exp Med* 1992;175:211-6.
- Fann MJ, Patterson PH. Neuropoietic cytokines and activin A differentially regulate the phenotype of cultured sympathetic neurons. *Proc Natl Acad Sci U S A* 1994;91:43-7.
- Girasole G, Passeri G, Jilka RL, Manolagas SC. Interleukin-11: a new cytokine critical for osteoclast development. *J Clin Invest* 1994;93:1516-24.
- Du XX, Doerschuk CM, Orazi A, Williams DA. A bone marrow stromal-derived growth factor, interleukin-11, stimulates recovery of small intestinal mucosal cells after cytoablative therapy. *Blood* 1994;83:33-7.
- Elias JA, Zheng T, Einarsson O, Landry M, Trow T, Rebert N, et al. Epithelial interleukin-11. Regulation by cytokines, respiratory syncytial virus, and retinoic acid. *J Biol Chem* 1994;269:22261-8.
- Elias JA, Zheng T, Whiting NL, Trow TK, Merrill WW, Zitnik R, et al. IL-1 and transforming growth factor-beta regulation of fibroblast-derived IL-11. *J Immunol* 1994;152:2421-9.
- Zheng T, Nathanson MH, Elias JA. Histamine augments cytokine-stimulated IL-11 production by human lung fibroblasts. *J Immunol* 1994;153:4742-52.
- Barnes PJ, Chung KF, Page CP. Inflammatory mediators of asthma: an update. *Pharmacol Rev* 1998;50:515-96.
- Holgate ST, Peters-Golden M, Panettieri RA, Henderson WR Jr. Roles of cysteinyl leukotrienes in airway inflammation, smooth muscle function, and remodeling. *J Allergy Clin Immunol* 2003;111(suppl):S18-34; discussion S34-6.
- Busse WW. Leukotrienes and inflammation. *Am J Respir Crit Care Med* 1998;157(suppl):S210-3; discussion S247-8.
- Lee KS, Kim SR, Park HS, Jin GY, Lee YC. Cysteinyl leukotriene receptor antagonist regulates vascular permeability by reducing VEGF expression. *J Allergy Clin Immunol* 2004;114:1093-9.
- Kwak YG, Song CH, Yi HK, Hwang PH, Kim JS, Lee KS, et al. Involvement of PTEN in airway hyperresponsiveness and inflammation in bronchial asthma. *J Clin Invest* 2003;111:1083-92.
- Pierce JW, Schoenleber R, Jesmok G, Best J, Moore SA, Collins T, et al. Novel inhibitor of cytokine-induced IkappaBalpha phosphorylation and endothelial cell adhesion molecule expression show anti-inflammatory effects in vivo. *J Biol Chem* 1997;272:21096-103.
- Yang G, Abate A, George AG, Weng YH, Dennerly PA. Maturation differences in lung NF-kappaB activation and their role in tolerance to hypoxia. *J Clin Invest* 2004;114:669-78.
- Masmoudi A, Labourdette G, Mersel M, Huang FL, Huang KP, Vincendon G, et al. Protein kinase C located in rat liver nuclei. Partial purification and biochemical and immunochemical characterization. *J Biol Chem* 1989;264:1172-9.
- Cobellis G, Vallarino M, Meccariello R, Pierantoni R, Masini MA, Mathieu M, et al. Fos localization in cytosolic and nuclear compartments in neurons of the frog, *Rana esculenta*, brain: an analysis carried out in parallel with GnRH molecular forms. *J Neuroendocrinol* 1999;11:725-35.
- Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 1987;162:156-9.
- Elias JA, Wu Y, Zheng T, Panettieri R. Cytokine- and virus-stimulated airway smooth muscle cells produce IL-11 and other IL-6-type cytokines. *Am J Physiol Lung Cell Mol Physiol* 1997;273:L648-55.
- Minshall E, Chakir J, Laviolette M, Molet S, Zhu Z, Olivenstein R, et al. IL-11 expression is increased in severe asthma: association with epithelial cells and eosinophils. *J Allergy Clin Immunol* 2000;105:232-8.
- Neben S, Turner K. The biology of interleukin 11. *Stem Cells* 1993;11(suppl 2):156-62.
- Zhang XG, Gu JJ, Lu ZY, Yasukawa K, Yancopoulos GD, Turner K, et al. Ciliary neurotrophic factor, interleukin 11, leukemia inhibitory factor, and oncostatin M are growth factors for human myeloma cell lines using the interleukin 6 signal transducer gp130. *J Exp Med* 1994;179:1337-42.
- Bauerle PA, Baltimore D. NF-kappa B: ten years after. *Cell* 1996;87:13-20.
- Baldwin AS Jr. The NF-kappa B and I kappa B proteins: new discoveries and insights. *Annu Rev Immunol* 1996;14:649-83.
- Barnes PJ, Karin M. Nuclear factor-kappaB: a pivotal transcription factor in chronic inflammatory diseases. *N Engl J Med* 1997;336:1066-71.
- Gilmore TD. The Rel/NF-kappaB signal transduction pathway: introduction. *Oncogene* 1999;18:6842-4.
- Siebenlist U, Franzoso G, Brown K. Structure, regulation and function of NF-kappa B. *Annu Rev Cell Biol* 1994;10:405-55.

35. Barnes PJ, Adcock IM. NF-kappa B: a pivotal role in asthma and a new target for therapy. *Trends Pharmacol Sci* 1997;18:46-50.
36. Kawano T, Matsuse H, Kondo Y, Machida I, Saeki S, Tomari S, et al. Cysteinyl leukotrienes induce nuclear factor kappa b activation and RANTES production in a murine model of asthma. *J Allergy Clin Immunol* 2003;112:369-74.
37. Komura E, Tonetti C, Penard-Lacronique V, Chagraoui H, Lacout C, Lecouedic JP, et al. Role for the nuclear factor kappaB pathway in transforming growth factor-beta1 production in idiopathic myelofibrosis: possible relationship with FK506 binding protein 51 overexpression. *Cancer Res* 2005;65:3281-9.
38. Kumar A, Takada Y, Boriek AM, Aggarwal BB. Nuclear factor-kappaB: its role in health and disease. *J Mol Med* 2004;82:434-48.
39. Yang L, Yang YC. Regulation of interleukin (IL)-11 gene expression in IL-1 induced primate bone marrow stromal cells. *J Biol Chem* 1994;269:32732-9.
40. Yang L, Steussy CN, Fuhrer DK, Hamilton J, Yang YC. Interleukin-11 mRNA stabilization in phorbol ester-stimulated primate bone marrow stromal cells. *Mol Cell Biol* 1996;16:3300-7.
41. Yang L, Yang YC. Heparin inhibits the expression of interleukin-11 and granulocyte-macrophage colony-stimulating factor in primate bone marrow stromal fibroblasts through mRNA destabilization. *Blood* 1995;86:2526-33.
42. Elias JA, Tang W, Horowitz MC. Cytokine and hormonal stimulation of human osteosarcoma interleukin-11 production. *Endocrinology* 1995;136:489-98.
43. Bamba S, Andoh A, Yasui H, Makino J, Kim S, Fujiyama Y. Regulation of IL-11 expression in intestinal myofibroblasts: role of c-Jun AP-1- and MAPK-dependent pathways. *Am J Physiol Gastrointest Liver Physiol* 2003;285:G529-38.
44. Lynch KR, O'Neill GP, Liu Q, Im DS, Sawyer N, Metters KM, et al. Characterization of the human cysteinyl leukotriene CysLT1 receptor. *Nature* 1999;399:789-93.
45. Heise CE, O'Dowd BF, Figueroa DJ, Sawyer N, Nguyen T, Im DS, et al. Characterization of the human cysteinyl leukotriene 2 receptor. *J Biol Chem* 2000;275:30531-6.
46. Di Virgilio F, Chiozzi P, Ferrari D, Falzoni S, Sanz JM, Morelli A, et al. Nucleotide receptors: an emerging family of regulatory molecules in blood cells. *Blood* 2001;97:587-600.

METHODS

Animals and experimental protocol

Female C57BL/6 mice, 8 to 10 weeks of age and free of murine-specific pathogens, were obtained from the Korean Research Institute of Chemistry Technology (Daejeon, Korea), housed throughout the experiments in a laminar flow cabinet, and maintained on standard laboratory chow *ad libitum*. All experimental animals used in this study were under a protocol approved by the Institutional Animal Care and Use Committee of the Chonbuk National University Medical School. Standard guidelines for laboratory animal care were followed.^{E1} Mice were sensitized on days 1 and 14 by means of intraperitoneal injection of 20 μ g of OVA (Sigma-Aldrich) emulsified in 1 mg of aluminum hydroxide (Pierce Chemical Co, Rockford, Ill) in a total volume of 200 μ L, as previously described,^{E2} with some modifications (Fig E1). On days 20, 21, 22, and 23 after the initial sensitization, the mice were challenged for 30 minutes with an aerosol of 3% (wt/vol) OVA in saline (or with saline as a control) by using an ultrasonic nebulizer (NE-U12, Omron, Japan). BAL was performed at 72 hours or 7 days after the last challenge. At the time of lavage, the mice (8 mice in each group) were killed with an overdose of sodium pentobarbitone (pentobarbital sodium, 100 mg/kg of body weight, administered intraperitoneally). The chest cavity was exposed to allow for expansion, after which the trachea was carefully intubated and the catheter was secured with ligatures. Prewarmed 0.9% NaCl solution was slowly infused into the lungs and withdrawn. The aliquots were pooled and then kept at 4°C. Part of each pool was then centrifuged, and the supernatants were kept at -70°C until use. Total cell numbers were counted with a hemocytometer. Smears of BAL cells were prepared with a cytospin (Thermo Electron, Waltham, Mass). The smears were stained with Diff-Quik solution (Dade Diagnostics of Puerto Rico, Inc, Aguada, Puerto Rico) to examine the cell differentials. Two independent blinded investigators counted the cells using a microscope. Approximately 400 cells were counted in each of 4 different random locations. Interinvestigator variation was less than 5%. The mean number from the 2 investigators was used to estimate the cell differentials.

Administration of montelukast, pranlukast, anti-IL-11 antibody, isotype control mAb, or BAY 11-7085

Montelukast sodium (L-706, 631; 5 mg/kg body weight per day; Merck & Co, Inc) dissolved in 1% methyl cellulose or pranlukast (25 mg/kg body weight per day, Ono Pharmaceutical Co, Ltd) dissolved in distilled water was administered by means of oral gavage 11 times at 24-hour intervals on days 19 to 29, beginning 1 day before the first challenge. Anti-IL-11 antibody or isotype control mAb (8 mg/kg body weight per day, R&D systems, Inc) was administered intraperitoneally 2 times to each animal, once on day 23 (1 hour after the last airway challenge) and the second time on day 29 (6 days after the last airway challenge). BAY 11-7085 (20 mg/kg body weight per day, BIOMOL International L.P.) dissolved in dimethyl sulfoxide and diluted with 0.9% NaCl was administered by means of intraperitoneal injection 2 times to each treated animal, once on day 23 (1 hour before the last airway challenge) and the second time on day 29 (6 days after the last airway challenge, Fig E1).^{E2,E3}

Isolation and primary culture of murine tracheal epithelial cells

Murine tracheal epithelial cells were isolated under sterile conditions, as previously described.^{E4} The trachea proximal to the bronchial bifurcation was excised, and adherent adipose tissue was removed. The trachea was opened longitudinally and cut into 3

pieces. The isolated tracheas were incubated in DMEM containing 0.1% protease overnight at 4°C. After tissue digestion, FBS (10% final concentration) was added to the medium to deactivate enzymes, undigested fragments of tissue were removed, and tracheal epithelial cells were harvested by means of centrifugation at 100g for 5 minutes. The epithelial cells were seeded onto 35-mm collagen-coated dishes for submerged culture. The growth medium DMEM/F-12 (Sigma-Aldrich) containing 10% FBS, penicillin, streptomycin, and amphotericin B was supplemented with insulin, transferrin, hydrocortisone, phosphoethanolamine, cholera toxin, ethanolamine, bovine pituitary extract, and BSA. The cells were maintained in a humidified 5% CO₂ incubator at 37°C until they adhered.

LTC₄ or LTD₄ stimulation and IL-11 production

Cells were seeded in 6-well culture inserts at a density of 1×10^5 cells/mL and grown in culture medium. At confluence, LTC₄ (Sigma-Aldrich) or LTD₄ (BIOMOL International L.P.) was added to the cells at varying concentrations (1-1000 ng/mL). Cells were pretreated with montelukast sodium (100 nmol/L) for 2 hours before changing to fresh medium and adding LTC₄ or LTD₄ to the cells to suppress the effect of mediator release induced by LTC₄ or LTD₄. The levels of IL-11 were analyzed by means of Western blotting with antibody against IL-11, as described below. Real-time PCR and RT-PCR were performed as described below to determine IL-11 mRNA expression.

Histology

At 72 hours or 7 days after the last challenge, lungs were removed from the mice after death. Before the lungs were removed, the lungs and trachea were filled intratracheally with a fixative (0.8% formalin and 4% acetic acid) using a ligature around the trachea. Lung tissues were fixed with 10% (vol/vol) neutral buffered formalin. The specimens were dehydrated and embedded in paraffin. For histologic examination, 4- μ m sections of fixed embedded tissues were cut on a Leica model 2165 rotary microtome (Leica, Nussloch, Germany), placed on glass slides, deparaffinized, and stained sequentially with hematoxylin 2 and eosin-Y (Richard-Allan Scientific, Kalamazoo, Mich) or Masson's trichrome stain.

Western blot analysis

Lung tissues were homogenized in the presence of protease inhibitors, and protein concentrations were determined by using the Bradford reagent (Bio-Rad), as described previously.^{E4} Samples (30 μ g of protein per lane) were loaded on a 12% SDS-PAGE gel. After electrophoresis at 120 V for 90 minutes, separated proteins were transferred to polyvinylidene difluoride membranes (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom) by using the wet transfer method (250 mA, 90 minutes). Nonspecific sites were blocked with 5% nonfat dry milk in TBST buffer (25 mmol/L Tris [pH 7.5], 150 mmol/L NaCl, and 0.1% Tween 20) for 1 hour, and the blots were then incubated overnight at 4°C with an anti-IL-11 antibody (R&D Systems, Inc), anti-TGF- β_1 antibody (Santa Cruz Biotechnology, Santa Cruz, Calif), anti-IL-4 antibody (Serotec Ltd, Oxford, United Kingdom), or anti-IL-13 antibody (R&D Systems, Inc). Anti-rabbit or anti-mouse horseradish peroxidase-conjugated IgG was used to detect binding of antibodies. The membranes were stripped and reblotted with an anti-actin antibody (Sigma-Aldrich) to verify equal loading of protein in each lane. The binding of the specific antibodies was visualized by exposing to photographic film after treating with enhanced chemiluminescence system reagents (GE Healthcare).

RNA isolation and RT-PCR

Total RNA from lung tissues and epithelial cells was isolated by using a rapid extraction method (TRI-Reagent), as previously

described.^{E5} RNA was quantified by measuring absorption at 260 nm and stored at -80°C until use. Total RNA (4 μg) was reverse transcribed to cDNA in a buffer containing 20 mmol/L Tris-HCl (pH 8.4), 50 mmol/L KCl, 5 mmol/L MgCl_2 , 10 mmol/L dithiothreitol, 0.5 μg random hexanucleotide primers, 2.5 mmol/L deoxyribonucleoside triphosphate, 40 U of RNase inhibitor, and 50 U/ μL SuperScript II RT (Invitrogen, Carlsbad, Calif) in a final volume of 20 μL . This mixture was incubated for 50 minutes at 42°C and then digested with 2 U/ μL *Escherichia coli* RNase H for 20 minutes at 37°C . The first-strand cDNAs were used for PCR amplification of IL-11 or the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). PCR amplification was performed by mixing 3 μL of the reverse transcription reaction mixture with 47 μL of buffer containing 2.5 U of Taq DNA polymerase (Promega, Madison, Wis) and 30 pmol of specific primer pairs for mouse cDNA of IL-11 or GAPDH, designed from published mouse gene sequences.^{E6} The primers used were as follows: IL-11 (predicted length, 320 bp) sense, 5'-TGCTGACAAGGCTTCGAGTAG-3'; IL-11 antisense, 5'-CAGTCGAGTCTTTAACAACAGC-3'; cysLT1R (predicted length, 162 bp) sense, 5'-CAACGAAGTATCCACCTTCA CC-3'; cysLT1R antisense, 5'-CAACGAAGTATCCACCTTCAACC-3'; cysLT2R (predicted length, 180 bp) sense, 5'-GTCCACGTGC TGCTCATAGG-3'; cysLT2R antisense, 5'-ATTGGCTGCAGCC ATGGTC-3'; GAPDH (predicted length, 609 bp) sense, 5'-GC CATCAACGACCCCTTCATTGAC-3'; and GAPDH antisense, 5'-ACGGAAGGCCATGCCAGTGAGCTT-3'. PCR reactions were performed in a thermocycler (GeneAmp PCR System 2400; Applied Biosystems, Foster City, Calif) by using the following reaction conditions. After an initial incubation for 2 minutes at 95°C , samples were subjected to 35 cycles of 1 minute at 94°C ; 2 minutes at 54°C (GAPDH), 56°C (IL-11), 65°C (cysLT1R), or 68°C (cysLT2R); and 1 minute at 72°C . A final extension step at 72°C for 10 minutes was performed. The RT-PCR products were electrophoretically fractionated on 2% agarose gels stained with ethidium bromide. DNA bands were visualized under UV light.

Quantitative real-time RT-PCR

Quantitative RT-PCR analysis was performed with the LightCycler FastStart DNA Master SYBR Green I (Roche Diagnostics). The sequences of primers used were as follows: IL-11 sense, 5'-TGCTGACAAGGCTTCGAGTAG-3'; IL-11 antisense, 5'-CAGTCGAGTCTTTAACAACAGC-3'; cysLT1R (predicted length, 162 bp) sense, 5'-CAACGAAGTATCCACCTTCA CC-3'; cysLT1R antisense, 5'-CAACGAAGTATCCACCTTCAACC-3'; cysLT2R (predicted length, 180 bp) sense, 5'-GTCCACGTGC TGCTCATAGG-3'; cysLT2R antisense, 5'-ATTGGCTGCAGCC ATGG TC-3'; β -actin sense, 5'-CAGATCATGTTTGAGACCTTC-3'; and β -actin antisense, 5'-ACTTCATGATGGAATTGAATG-3'. Calculation of the relative mRNA levels of each sample was performed according to the manufacturer's protocol. The data have been normalized to the expression of β -actin.

Cytosolic or nuclear protein extractions for analysis of NF- κ B p65

Lungs were removed and homogenized in 2 volumes of buffer A (50 mmol/L Tris-HCl [pH 7.5], 1 mmol/L EDTA, 10% glycerol, 0.5 mmol/L dithiothreitol, 5 mmol/L MgCl_2 , and 1 mmol/L phenylmethylsulfonyl fluoride) containing protease inhibitor cocktails. The homogenates were centrifuged at 1000g for 15 minutes at 4°C . The supernatant fraction was incubated on ice for 10 minutes and centrifuged at 100,000g for 1 hour at 4°C to obtain cytosolic proteins for analysis of NF- κ B p65. The pellets were washed twice in buffer A and resuspended in buffer B (1.3 mol/L sucrose, 1.0 mmol/L MgCl_2 , and 10 mmol/L potassium phosphate buffer [pH 6.8]) and pelleted

at 1000g for 15 minutes. The pellets were suspended in buffer B with a final sucrose concentration of 2.2 mol/L and centrifuged at 100,000g for 1 hour. The resulting pellets were washed once with a solution containing 0.25 mol/L sucrose, 0.5 mmol/L MgCl_2 , and 20 mmol/L Tris-HCl, pH 7.2, and centrifuged at 1000g for 10 minutes. The pellets were solubilized with a solution containing 50 mmol/L Tris-HCl (pH 7.2), 0.3 mol/L sucrose, 150 mmol/L NaCl, 2 mmol/L EDTA, 20% glycerol, 2% Triton X-100, 2 mmol/L phenylmethylsulfonyl fluoride, and protease inhibitor cocktails. The mixture was kept on ice for 1 hour with gentle stirring and centrifuged at 12,000g for 30 minutes. The resulting supernatant was used as soluble nuclear proteins for determination of NF- κ B p65 levels. The levels of NF- κ B p65 were analyzed by means of Western blotting with antibody against NF- κ B p65 (Upstate Biotech, Lake Placid, NY), as described above.

Determination of airway responsiveness

Airway responsiveness was assessed as a change in airway function after challenge with aerosolized methacholine through airways, as described elsewhere.^{E7} Anesthesia was achieved with 80 mg/kg pentobarbital sodium injected intraperitoneally. The trachea was then exposed through a midcervical incision and tracheostomized, and an 18-gauge metal needle was inserted. Mice were connected to a computer-controlled small-animal ventilator (flexiVent; SCIREQ, Montreal, Canada). The mouse was quasinsulinoidally ventilated with a nominal tidal volume of 10 mL/kg at a frequency of 150 breaths/min and a positive end-expiratory pressure of 2 cm H_2O to achieve a mean lung volume close to that during spontaneous breathing. This was achieved by connecting the expiratory port of the ventilator to a water column. Methacholine aerosol was generated with an inline nebulizer and administered directly through the ventilator. Each mouse was challenged with methacholine aerosol in increasing concentrations (2.5–50 mg/mL in saline) to determine the differences in airway response to methacholine. After each methacholine challenge, the data on airway resistance (R_L) were continuously collected. Maximum values of R_L were selected to express changes in airway function, which was represented as a percentage change from baseline after saline aerosol inhalation.

Measurement of LTC₄, TGF- β ₁, IL-4, and IL-13

Levels of LTC₄, TGF- β ₁, IL-4, and IL-13 were quantified in the supernatants of BAL fluids by means of enzyme immunoassay, according to the manufacturer's protocol (LTC₄: Cayman Chemical Co, Ann Arbor, Mich; TGF- β ₁ and IL-13: R&D Systems, Inc; and IL-4: Endogen, Inc, Woburn, Mass). Sensitivity for LTC₄, TGF- β ₁, IL-4, and IL-13 assays were 10, 1.6, 5, 1.5 pg/mL, respectively.

Quantitation of peribronchial fibrosis

Two methods (Masson's trichrome staining and total lung collagen content) were used to quantify peribronchial fibrosis.

Peribronchial trichrome staining. The area of peribronchial trichrome staining in a paraffin-embedded lung was outlined and quantified with a light microscope (Leica DM LB; Leica Mikroskopie & Systeme GmbH, Wetzlar, Germany) attached to an image-analysis system (analySIS Pro version 3.2; Soft Imaging System GmbH, Münster, Germany). Results were expressed as the area of trichrome staining per micrometer length of basement membrane of bronchioles 150 to 200 μm in internal diameter. At least 10 bronchioles were counted in each slide.

Determination of total lung collagen content. The total lung collagen content was determined by using the Sircol Collagen Assay kit (Biocolor Ltd, Belfast, Northern Ireland), according to the manufacturer's protocols.

RESULTS

Effect of montelukast, pranlukast, or anti-IL-11 antibody on cellular changes in BAL fluids

Numbers of total cells, lymphocytes, neutrophils, and eosinophils in BAL fluids were increased significantly at 72 hours after OVA inhalation compared with the numbers after saline inhalation (Fig E2). The increased numbers of total cells, lymphocytes, neutrophils, and eosinophils were significantly reduced by the administration of montelukast, pranlukast, or anti-IL-11 antibody. However, no significant changes were observed in OVA-sensitized and OVA-challenged mice treated with isotype control mAb.

Effect of montelukast or pranlukast on LTC₄ levels in BAL fluids

Levels of LTC₄ in BAL fluids were increased significantly at 72 hours after OVA inhalation compared with levels after saline inhalation (Fig E6). The increased LTC₄ levels were significantly reduced by the administration of montelukast or pranlukast.

cysLT1R antagonists or anti-IL-11 antibody reduced OVA-induced airway hyperresponsiveness

Airway responsiveness was assessed as a percentage increase of R_L in response to increasing doses of methacholine. In OVA-sensitized and OVA-challenged mice, the dose-response curve of R_L shifted to the left compared with that of control mice (Fig E7). In addition, the percentage of R_L produced by methacholine administration (at doses from 25-50 mg/mL) increased significantly in the OVA-sensitized and OVA-challenged mice compared with that in the control animals. OVA-sensitized and OVA-challenged mice treated with montelukast, pranlukast, or anti-IL-11 antibody showed a dose-response curve of percentage R_L that shifted to the right compared with that of untreated mice. These results indicate that administration of montelukast, pranlukast, and anti-IL-11 antibody reduces OVA-induced airway hyperresponsiveness. However, no significant changes of R_L were observed in OVA-sensitized and OVA-challenged mice administered isotype control mAb.

Expression of cysLT1R and cysLT2R mRNA in primary murine tracheal epithelial cells stimulated with LTC₄

We used RT-PCR and quantitative real-time PCR analyses to determine the expression of mRNA for

cysLT1R and cysLT2R in epithelial cells at baseline compared with that seen in epithelial cells stimulated with LTC₄ (Fig E8). These analyses have shown that the expression of mRNA for cysLT1R and cysLT2R was increased approximately 2.5- and 1.7- fold, respectively, compared with that seen in unstimulated cells.

Effect of LTD₄ on IL-11 protein and mRNA expression in primary murine tracheal epithelial cells

Western blot analysis revealed that the levels of IL-11 were increased significantly after incubation with LTD₄ (100 ng/mL) at 8 hours of incubation. Epithelial cells were treated with montelukast to suppress the LTD₄-induced IL-11 production. Incubation of montelukast (100 nM) with cells stimulated with LTD₄ significantly inhibited the production of IL-11 (Fig E9, A and B). Montelukast alone had no effect on the IL-11 production of unstimulated cells (data not shown).

Quantitative real-time PCR was used to measure the mRNA expression of IL-11 in response to stimulation of epithelial cells with LTD₄. At 6 hours of incubation, the expression of IL-11 mRNA was increased approximately 2.2-fold compared with that seen in unstimulated cells (Fig E9, C). In addition, montelukast inhibited the gene transcription for IL-11 substantially.

REFERENCES

1. Institute of Laboratory Animal Research, Commission of Life Sciences, National Research Council. Guide for the care and use of laboratory animals. Washington (DC): National Academy Press; 1996.
2. Pierce JW, Schoenleber R, Jesmok G, Best J, Moore SA, Collins T, et al. Novel inhibitor of cytokine-induced I κ B α phosphorylation and endothelial cell adhesion molecule expression show anti-inflammatory effects in vivo. *J Biol Chem* 1997;272:21096-103.
3. Yang G, Abate A, George AG, Weng YH, Dennery PA. Maturation differences in lung NF- κ B activation and their role in tolerance to hypoxia. *J Clin Invest* 2004;114:669-78.
4. Kwak YG, Song CH, Yi HK, Hwang PH, Kim JS, Lee KS, et al. Involvement of PTEN in airway hyperresponsiveness and inflammation in bronchial asthma. *J Clin Invest* 2003;111:1083-92.
5. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 1987;162:156-9.
6. Lgssiar A, Hassan M, Schott-Ohly P, Friesen N, Nicoletti F, Trepicchio WL, et al. Interleukin-11 inhibits NF- κ B and AP-1 activation in islets and prevents diabetes induced with streptozotocin in mice. *Exp Biol Med* (Maywood) 2004;229:425-36.
7. Lee KS, Park HS, Park SJ, Kim SR, Min KH, Jin SM, et al. A prodrug of cysteine, L-2-oxothiazolidine-4-carboxylic acid, regulates vascular permeability by reducing vascular endothelial growth factor expression in asthma. *Mol Pharmacol* 2005;68:1281-90.

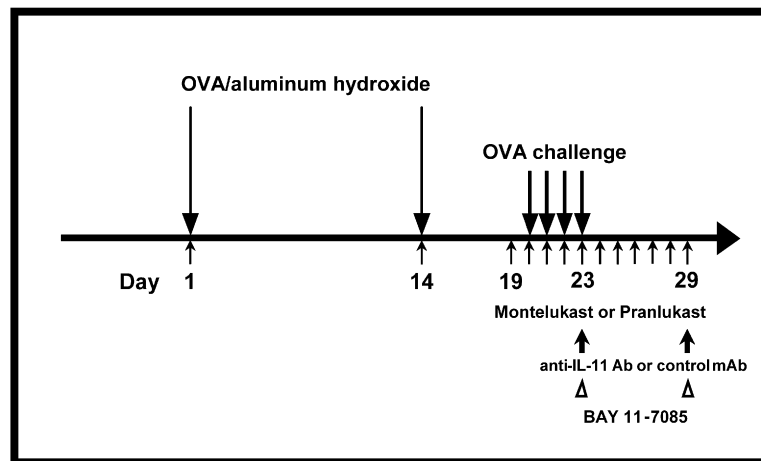


FIG E1. Schematic diagram of the experimental protocol.

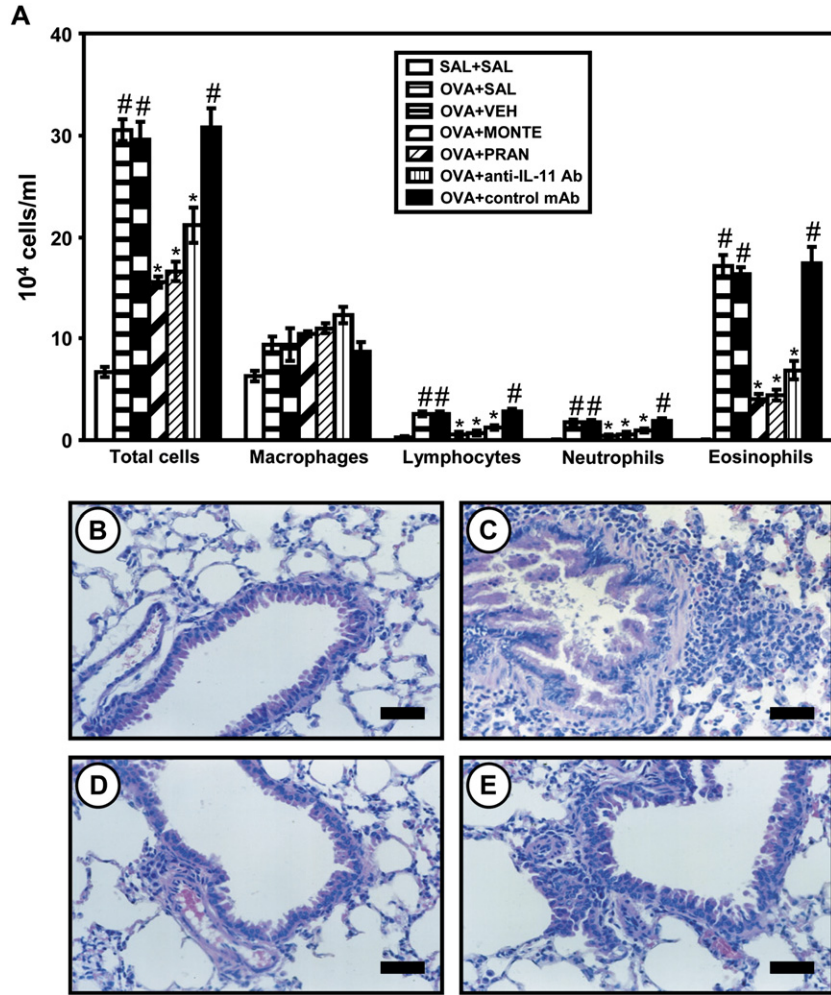


FIG E2. Effect of montelukast, pranlukast, or anti-IL-11 antibody on total and differential cellular components in BAL fluids and pathologic changes in lung tissues of OVA-sensitized and OVA-challenged mice. **A**, Numbers of total and differential cellular components of BAL fluid. Sampling was performed at 72 hours after the last challenge in the SAL+SAL, OVA+SAL, OVA+VEH, OVA+MONTE, OVA+PRAN, OVA+anti-IL-11 Ab, and OVA+control mAb groups. Bars represent means $\pm$ SEM from 8 mice per group. For definition of groups, see legend for Fig 3. $\#P < .05$ versus SAL+SAL group; $*P < .05$ versus OVA+SAL group. **B-E**, Representative hematoxylin 2 and eosin-Y-stained sections of the lungs. Sampling was performed at 72 hours after the last challenge in saline-challenged mice administered saline (Fig E2, **B**), OVA-challenged mice administered saline (Fig E2, **C**), OVA-challenged mice administered montelukast (Fig E2, **D**), and OVA-challenged mice administered pranlukast (Fig E2, **E**). Bars indicate 50 μ m.

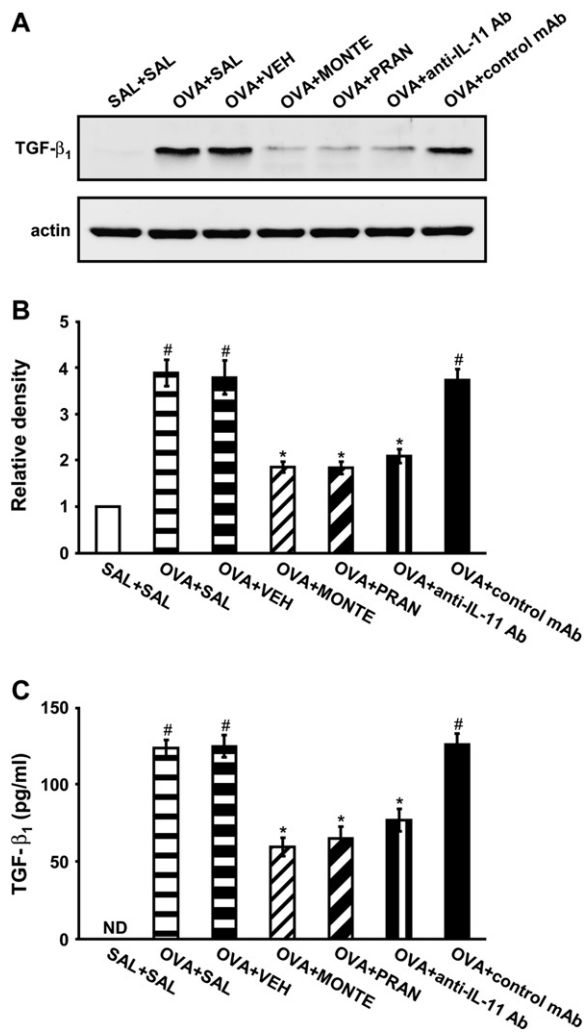


FIG E3. Effect of montelukast, pranlukast, or anti-IL-11 antibody on TGF- β_1 protein expression in lung tissues and BAL fluids of OVA-sensitized and OVA-challenged mice. **A**, Western blot analysis of TGF- β_1 . Sampling was performed at 7 days after the last challenge in the SAL+Sal, OVA+Sal, OVA+VEH, OVA+MONTE, OVA+PRAN, OVA+anti-IL-11 Ab, and OVA+control mAb groups. **B**, Densitometric analyses are presented as the relative ratio of TGF- β_1 to actin. The relative ratio of TGF- β_1 in the lung tissues of control mice is arbitrarily presented as 1. **C**, Enzyme immunoassay of TGF- β_1 in BAL fluids. Bars represent means $\pm$ SEM from 8 mice per group. ND, Not detected. For definition of groups, see legend for Fig 3. # P < .05 versus SAL+Sal group; * P < .05 versus OVA+Sal group.

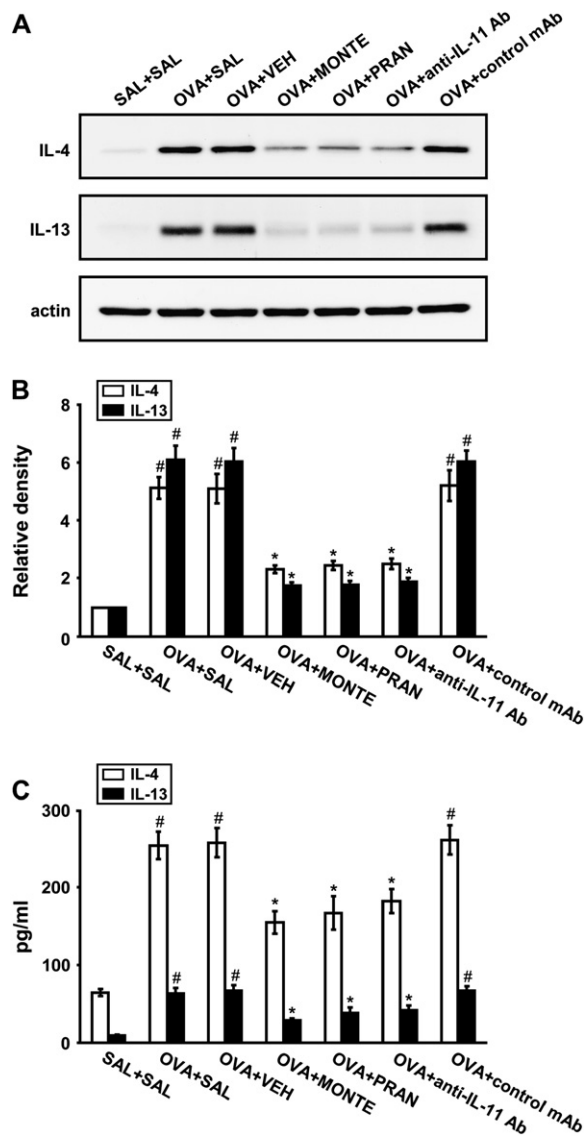


FIG E4. Effect of montelukast, pranlukast, or anti-IL-11 antibody on IL-4 and IL-13 protein levels in lung tissues and BAL fluids of OVA-sensitized and OVA-challenged mice. **A**, Western blot analysis of IL-4 and IL-13. Sampling was performed at 72 hours after the last challenge in the SAL+Sal, OVA+Sal, OVA+VEH, OVA+MONTE, OVA+PRAN, OVA+anti-IL-11 Ab, and OVA+control mAb groups. **B**, Densitometric analyses are presented as the relative ratio of IL-4 and IL-13 to actin. The relative ratio of IL-4 and IL-13 in the lung tissues of control mice is arbitrarily presented as 1. **C**, Enzyme immunoassay of IL-4 and IL-13 in BAL fluids. Bars represent means $\pm$ SEM from 8 mice per group. For definition of groups, see legend for Fig 3. # $P < .05$ versus SAL+Sal group; * $P < .05$ versus OVA+Sal group.

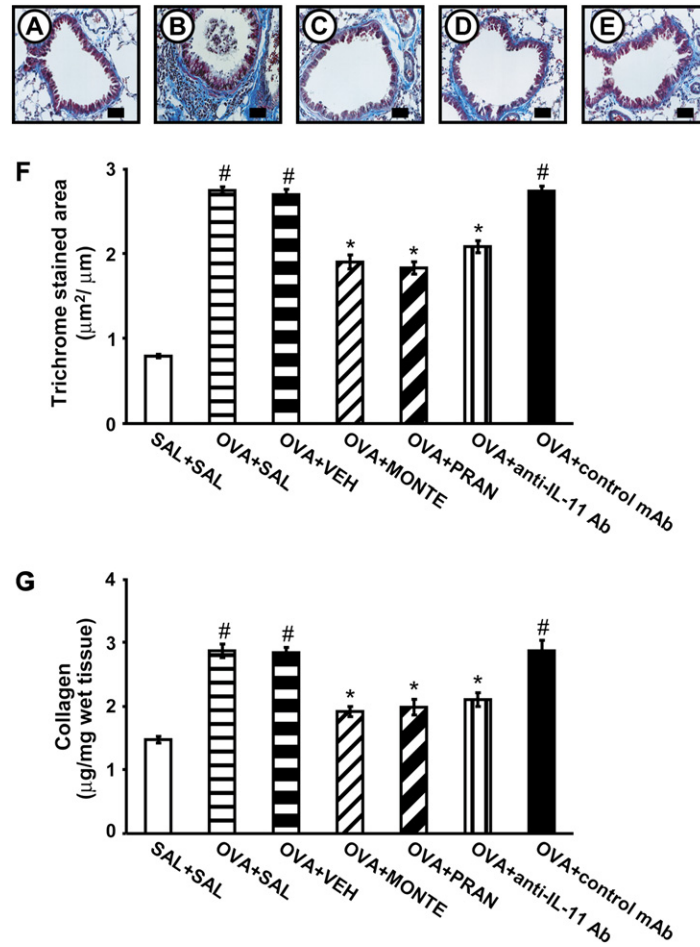


FIG E5. Effect of montelukast, pranlukast or anti-IL-11 antibody on peribronchial fibrosis in lung tissues of OVA-sensitized and OVA-challenged mice. **A-D**, Representative peribronchial trichrome-stained sections of the lungs. Sampling was performed at 7 days after the last challenge in saline-inhaled mice administered saline (Fig E5, A), OVA-challenged mice administered saline (Fig E5, B), OVA-challenged mice administered montelukast (Fig E5, C), OVA-challenged mice administered pranlukast (Fig E5, D), and OVA-challenged mice administered anti-IL-11 antibody (Fig E5, E). Bars indicate 50 μm . **F**, Quantitation of peribronchial fibrosis. Sampling was performed at 7 days after the last challenge in the SAL+Sal, OVA+Sal, OVA+VEH, OVA+MONTE, OVA+PRAN, OVA+anti-IL-11 Ab, and OVA+control mAb groups. **G**, Total lung collagen content. Bars represent means $\pm$ SEM from 8 mice per group. For definition of groups, see legend for Fig 3. # $P < .05$ versus SAL+Sal group; * $P < .05$ versus OVA+Sal group.

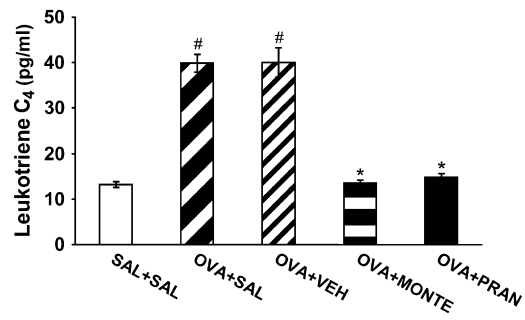


FIG E6. Effect of montelukast or pranlukast on LTC₄ levels in BAL fluids of OVA-sensitized and OVA-challenged mice: enzyme immunoassay of LTC₄. Sampling was performed at 72 hours after the last challenge in the SAL+SAL, OVA+SAL, OVA+VEH, OVA+MONTE, and OVA+PRAN groups. Bars represent means $\pm$ SEM from 8 mice per group. For definition of groups, see legend for Fig 3. # $P < .05$ versus SAL+SAL group; * $P < .05$ versus OVA+SAL group.

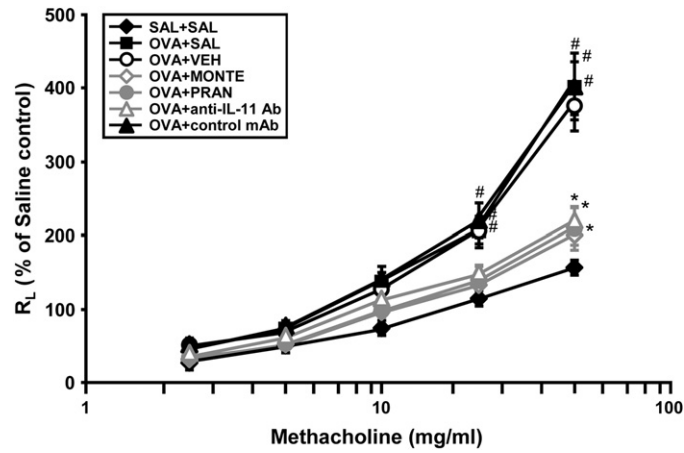


FIG E7. Effect of montelukast, pranlukast, or anti-IL-11 antibody on airway responsiveness in OVA-sensitized and OVA-challenged mice. Airway responsiveness was measured at 72 hours after the last challenge in the SAL+SAL, OVA+SAL, OVA+VEH, OVA+MONTE, OVA+PRAN, OVA+anti-IL-11 Ab, and OVA+control mAb groups. R_L values were obtained in response to increasing doses (2.5-50 mg/mL) of methacholine, as described in the Methods section. Bars represent means $\pm$ SEM from 8 mice per group. For definition of groups, see legend for Fig 3. # $P < .05$ versus SAL+SAL group; * $P < .05$ versus OVA+SAL group.

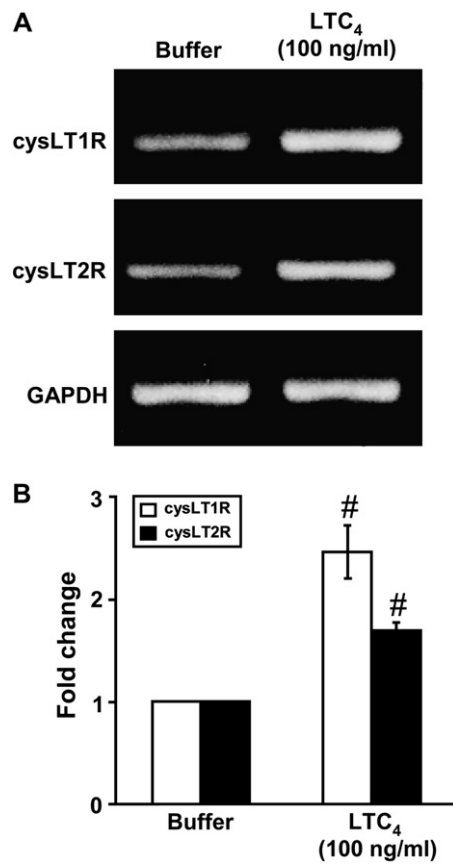


FIG E8. Expression of cysLT1R and cysLT2R mRNA in primary tracheal epithelial cells. **A**, Representative RT-PCR analysis of cysLT1R and cysLT2R mRNA expression after incubation of epithelial cells with LTC₄ at a concentration of 100 ng/mL. **B**, Quantitative analysis of cysLT1R and cysLT2R mRNA expression by means of real-time RT-PCR. Data represent means $\pm$ SEM from 6 independent experiments. #*P* < .05 versus buffer alone.

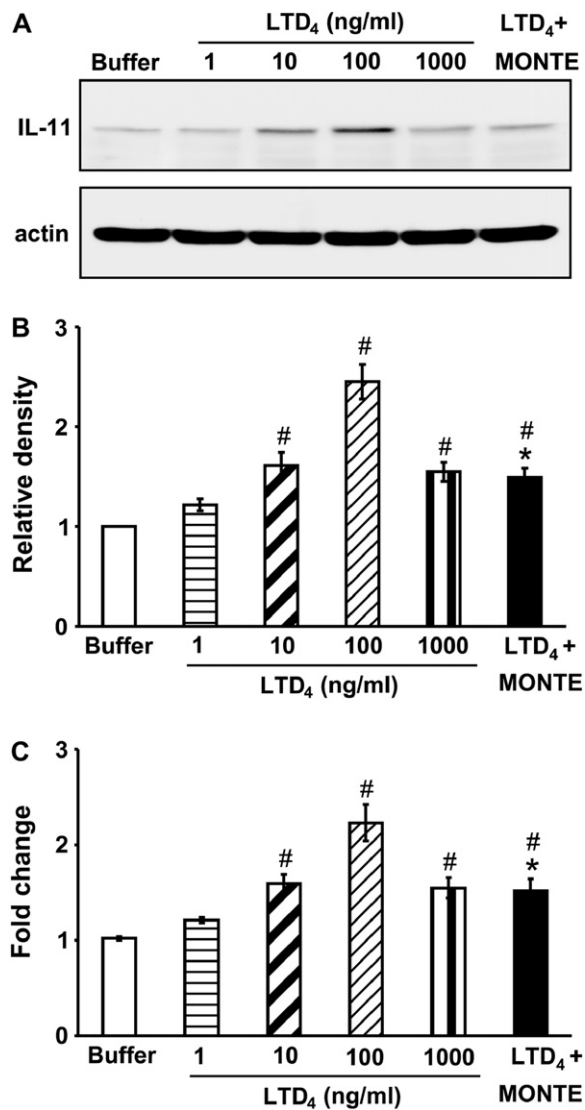


FIG E9. Effect of LTD₄ on IL-11 production by primary tracheal epithelial cells. **A**, Western blot analysis of IL-11. **B**, Densitometric analyses are presented as the relative ratio of IL-11 to actin. The relative ratio of IL-11 in the epithelial cells incubated with buffer alone is arbitrarily presented as 1. **C**, Quantitative analysis of IL-11 mRNA expression by means of real-time RT-PCR. Data represent means $\pm$ SEM from 6 independent experiments. #*P* < .05 versus buffer alone; **P* < .05 versus 100 ng/mL LTD₄.