



Original research article

Increased expression of CysLT₂ receptors in the lung of asthmatic mice and role in allergic responses

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ARTICLE INFO

Keywords:

Cysteinyl leukotrienes
 CysLT₂ receptors
 CysLT₁ receptors
 Airway remodeling
 Eosinophil

ABSTRACT

Compared with CysLT₁ receptors, the functional role of CysLT₂ receptors in asthma has not been clarified. The purpose of this study was to determine 1) whether CysLT₂ receptors are expressed in the lung of mice and if expression increases in asthmatic mice, and 2) whether CysLT₂ receptors are involved in allergic leukocyte infiltration into the lung and in the development of airway remodeling in asthmatic mice. BALB/c mice were sensitized with ovalbumin (OVA) + Al(OH)₃, and intratracheally challenged with OVA 4 times. Lung tissue was isolated before and after the 4th OVA challenge for detection of CysLT₂ receptors by immunohistochemistry and flow cytometry. The effect of a CysLT₂ receptor antagonist BayCysLT₂RA on multiple antigen challenge-induced leukocyte infiltration into the lung and the development of airway remodeling was evaluated. Even in non-challenged mice, CysLT₂ receptors were expressed in bronchial smooth muscle. After multiple challenges, expression was also observed in leukocytes infiltrating into alveolar spaces. CysLT₂R⁺ leukocytes included alveolar macrophages, conventional dendritic cells, and eosinophils. BayCysLT₂RA significantly inhibited multiple antigen challenge-induced increases in eosinophils and mononuclear cells in the lung. The development of airway remodeling was tended to be suppressed by CysLT₂ receptor antagonist. In conclusion, CysLT₂ receptors were constitutively expressed in the lung, and expression was strengthened in asthmatic mice. Activation of CysLT₂ receptors was functionally involved in allergic leukocyte infiltration into the lung. The CysLT₂ receptor can be a molecular target for the development of new pharmacotherapies for asthma.

1. Introduction

Cysteinyl leukotrienes (CysLTs), LTC₄, LTD₄ and LTE₄, are 5-lipoxygenase pathway metabolites of arachidonic acid and deeply involved in bronchial asthma via activation of CysLT₁ receptors [1,2]. Specific CysLT₁ receptor antagonists, including pranlukast [3,4] and montelukast [5], have widely been used as pharmacotherapy for bronchial asthma. Another receptor subtype of CysLTs, termed the CysLT₂ receptor [2,6–8], is a G-protein-coupled receptor with an amino acid sequence 38% identical to that of the CysLT₁ receptor [6]. CysLT₂ receptor mRNA has been detected in lung macrophages, airway smooth muscle, and peripheral blood leukocytes [6]. We have also demonstrated that CysLT₂ receptors were expressed in lung specimens isolated from asthma subjects, and suggested that activation of CysLT₂ receptors contribute to antigen-induced bronchoconstriction in certain asthma population [9]. However, it is difficult to analyze the expression and functional roles of CysLT₂ receptors in asthma using human lung tissue.

Guinea pig has been extensively used to assess the functional roles of CysLTs because the bronchial smooth muscle responds well to LTD₄ and is constricted through activation of CysLT₁ receptors [10]. We developed a CysLT₁/CysLT₂ dual antagonist [11], and analyzed the role of CysLT₂ receptors in guinea pig models of asthma by focusing on bronchoconstriction [12–15]. However, it is difficult to analyze molecular mechanisms using guinea pig models because various tools for molecular biological experiments are not available. For example, histological existence of CysLT₂ receptors in normal and asthmatic lung tissues of guinea pigs is unclear.

Murine models have also been used to elucidate the cellular and molecular mechanisms underlying asthma pathogenesis. In particular, BALB/c mice have been utilized as an appropriate model for Th2-type airway inflammation. CysLT₁ receptor antagonists suppress eosinophilic airway inflammation and airway remodeling induced by antigen challenges [16–20]. The role of CysLT₂ receptors in murine asthma has been analyzed using CysLT₂ receptor-deficient mice, which revealed

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that CysLT₂ receptors negatively regulate the development of CysLT₁ receptor-dependent Th2-type airway inflammation [21]. Further analysis is needed to elucidate the roles of CysLT₂ receptors using specific receptor antagonists, to examine if CysLT₂ receptors are histologically expressed in murine lung, and to ascertain whether expression is altered in asthmatic lungs.

The purpose of this study was to determine whether the CysLT₂ receptor can be a molecular target for pharmacotherapy of asthma. Thus, murine experiments were designed to elucidate 1) whether CysLT₂ receptors were expressed in the lung and if expression increased in asthmatic mice, and 2) whether CysLT₂ receptors were functionally involved in allergic leukocyte infiltration into the lung and in the development of airway remodeling in asthmatic mice.

2. Materials and methods

2.1. Sensitization and challenge with antigen

The murine asthma model was developed as previously reported [22–26]. Briefly, 5-week-old female BALB/c mice (Japan SLC, Hamamatsu) were sensitized by i.p. injections with OVA + Al(OH)₃ at a dose of 50 µg OVA/2 mg Al(OH)₃/animal on days 0, 14, and 28. Next, sensitized mice were intratracheally challenged with 2% OVA on day 35, 36, 37, and 40 under inhalation anesthesia with isoflurane at a volume of 25 µl/animal.

This animal study was approved by the Experimental Animal Research Committee at Setsunan University.

2.2. Immunohistological detection of CysLT₂ and CysLT₁ receptors in lung tissue

Before the 1st OVA challenge and 4 h after the 4th challenge, the left lobe from the lung tissue was isolated under anesthesia with pentobarbital and xylazine. The tissues were fixed in 10% formaldehyde, embedded in paraffin wax, and cut into 4 µm thick sections. As reported elsewhere [15], after antigenicity activation and treatment with hydrogen peroxide solution, sections were washed, and blocked with 1% bovine serum albumin or normal rabbit serum. Sections were stained with goat polyclonal anti-mouse CysLT₂ receptor antibody (sc-27097, Santa Cruz Biotechnology, Dallas, TX, USA, at a dilution of 1:200) overnight at 4 °C or stained with rabbit polyclonal anti-mouse CysLT₁ receptor antibody (Aviva Systems Biology, San Diego, CA, USA, at a dilution of 1:300) for 1 h at room temperature.

After washing, anti-CysLT₂ receptor-stained sections were treated with anti-goat IgG biotinylated secondary antibody for 30 min at 37 °C and washed followed by staining with streptavidin-horseradish peroxidase for 30 min at 37 °C. The anti-CysLT₁ receptor-stained sections were washed and stained with biotinylated anti-rabbit IgG secondary antibody attached to VECTASTAIN Elite ABC Rabbit IgG Kit (Vector Lab., Burlingame, CA, USA) followed by treatment with ABC reagent from the kit.

Color was developed by soaking the sections in 3,3'-diaminobenzidine solution followed by counterstaining with a hematoxylin solution.

2.3. Flow cytometric detection of CysLT₂R⁺ and CysLT₁R⁺ leukocytes in lung tissue

Lung tissues were isolated under anesthesia with pentobarbital and xylazine before the 1st challenge and 4 h after the 4th challenge. The tissue was cut into approximately 1 × 1 × 1 mm pieces followed by incubation with 3 mg/ml collagenase type-I (Thermo Fisher Scientific, Waltham, MA, USA) at 37 °C for 1 h. Cells were dispersed using a syringe and filtered through 70 µm nylon mesh. After centrifugation, contaminating erythrocytes were lysed with ACK lysing buffer.

Lung cells were stained with Fixable Viability Dye eFluor 780 (Thermo Fisher Scientific) to exclude dead cells for FACS analysis. Cells

were treated with an anti-CD16/32 (FcγRII/III) antibody (2.4G2) to prevent non-specific binding of subsequently used antibodies. After washing, cells were stained with goat polyclonal anti-mouse CysLT₂ receptor antibody (Santa Cruz Biotechnology) or rabbit polyclonal anti-mouse CysLT₁ receptor antibody (Aviva Systems Biology, San Diego, CA, USA) for 30 min at 4 °C followed by staining with secondary antibody, Alexa Fluor 488-labeled AffiniPure donkey anti-goat IgG antibody or Alexa Fluor 488-labeled AffiniPure donkey anti-rabbit IgG antibody (Jackson Immuno Research, West Grove, PA, USA), respectively, for 30 min at 4 °C. After washing, cells were further stained with PerCP-Cy5.5-labeled anti-mouse CD45 antibody (clone 30-F11, BioLegend), and PE-labeled anti-mouse Siglec-F antibody (clone E50-2440), PE-labeled anti-mouse CD11c antibody (clone HL3), PE-labeled anti-mouse Gr-1 antibody (clone RB6-8C5), PE-labeled anti-mouse F4/80 antibody (clone BM8), eFluor 660-labeled anti-mouse CD11c antibody (clone N418), or APC-labeled anti-mouse I-A/I-E antibody (clone M5/114.15.2) (all from Thermo Fisher Scientific) for 30 min at 4 °C. After washing, stained cells were fixed with 4% paraformaldehyde and analyzed by FACSARIA™ Fusion (Becton Dickinson, San Jose, CA, USA) and FACSDiva™ version 8.0.1 (Becton Dickinson).

Based on previous reports [26–31], alveolar macrophages and dendritic cells in lungs were identified as CD11c⁺ F4/80⁺ CD45⁺ cells and CD11c⁺ I-A/I-E⁺ CD45⁺ cells, respectively. In addition, Siglec-F⁺ CD45⁺ cells and Gr-1⁺ CD45⁺ cells were considered eosinophils and neutrophils, respectively [24,32,33].

2.4. Effects of CysLT₂ receptor antagonist and CysLT₁ receptor antagonist on antigen-induced airway inflammation and remodeling

A CysLT₁ receptor antagonist montelukast and a CysLT₂ receptor antagonist BayCysLT₂RA; 1-(5-carboxy-2-{3-[4-(3-cyclohexylpropoxy)phenyl]propoxy}benzoyl)-4-piperidinecarboxylic acid [34] were synthesized by Ono Pharmaceutical Co., Ltd. (Osaka, Japan). Calcium mobilization assays were performed using Fura-2 AM-loaded CHO-K1 cells stably expressing murine CysLT₁ or murine CysLT₂ receptors. IC₅₀ values of montelukast on LTD₄ (1 nM)-induced responses against CysLT₁ and CysLT₂ receptors were 1.1 and 920 nM, respectively, and those of BayCysLT₂RA were 520 and 0.49 nM, respectively (unpublished data). Montelukast and a positive control dexamethasone 21-phosphate disodium salt (Sigma-Aldrich, St. Louis, MO, USA) were dissolved in saline. BayCysLT₂RA was dissolved in 1 M NaOH followed by diluting with saline and checking pH. These drugs or saline were injected intravenously into sensitized mice once-a-day on days 38, 39, 40, 41, 42, and 43. On the days of intratracheal OVA challenge (day 38, 39, 40, and 43), CysLT receptor antagonists and dexamethasone were administered 5 min and 3 h, respectively, before the 1st–4th challenges. Four hours after the 4th challenge, mice were anesthetized by i.p. injection of pentobarbital and xylazine. Bronchoalveolar lavage fluid (BALF) was obtained from the right lung lobes followed by counting total and differential leukocytes in BALF as previously reported [22–27].

Regarding toxicity of BayCysLT₂RA, no macroscopical change was observed in the lung tissue after i.v. treatment at up to 1 mg/kg.

The left lobe of the lung was isolated for histological studies. The tissue was fixed with 10% buffered formalin and embedded in paraffin. Four micrometer sections were stained with periodic acid–Schiff (PAS) and Masson trichrome for light microscope observation. Quantitative analyses of histological changes were conducted as previously reported [23]. Briefly, the degree of epithelial thickening was quantified as follows: the bronchus at a diameter of more than 150 µm was photographed, and the epithelial area (µm²) and the length of the basement membrane (µm) were measured using Adobe Photoshop CS2 (version 9.0; Adobe Systems, San Jose, CA, USA) and Image J (version 1.49; NIH, Bethesda, MD, USA). The degree of epithelial thickening was expressed by dividing the epithelial area by the length of the basement membrane. Mucus accumulation in the epithelial layer was also

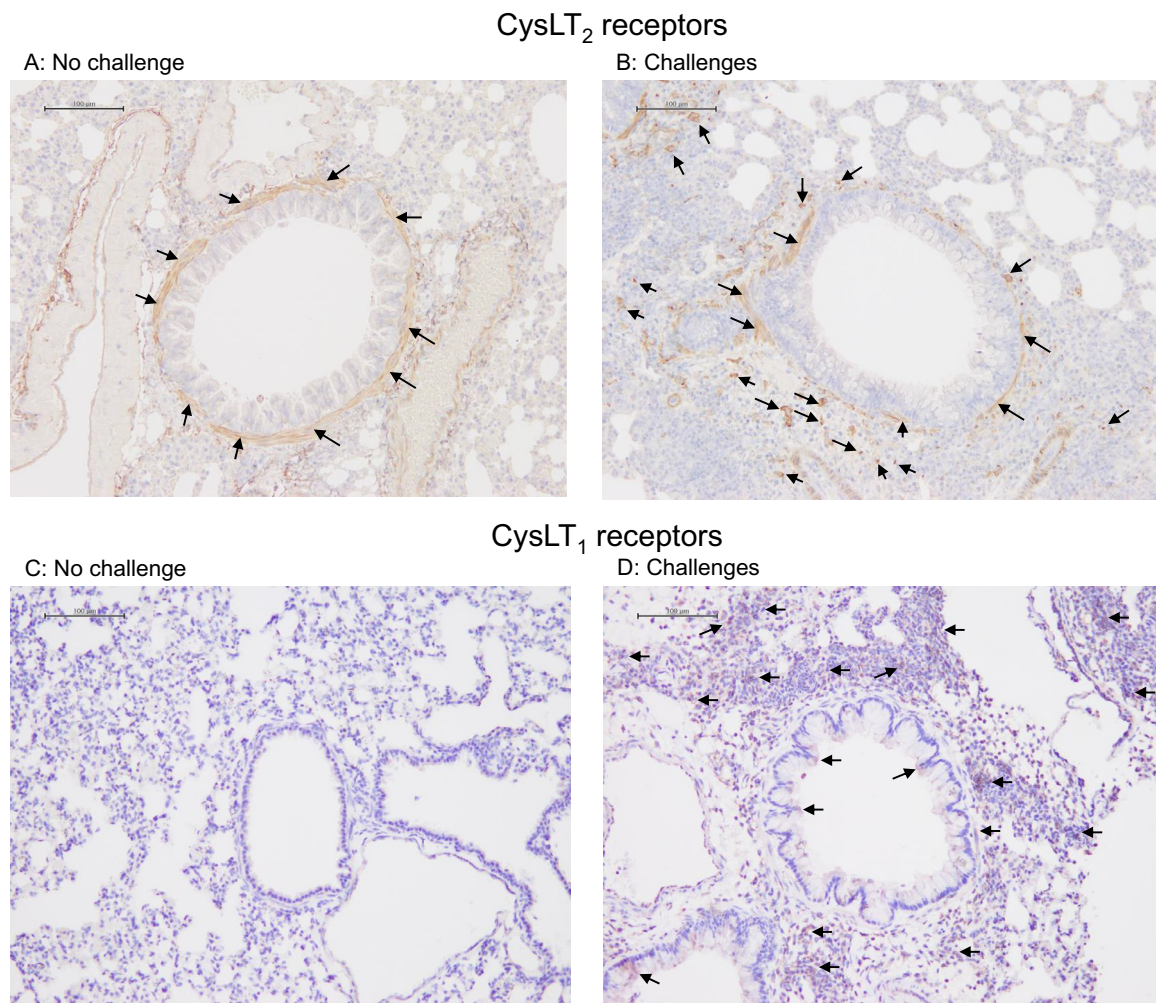


Fig. 1. Expression of CysLT₂ (A and B) and CysLT₁ receptors (C and D) in the lung of non-challenged (A and C) and challenged mice (B and D). Mice were intraperitoneally sensitized with OVA + Al(OH)₃ on days 0, 14, and 28, and then intratracheally challenged with OVA on days 35, 36, 37, and 40. The lung was isolated before multiple challenges on day 35 or 4 h after the 4th challenge on day 40. CysLT₂ and CysLT₁ receptors were immunohistologically detected. Each photograph is a representative of 3 animals. Arrows in each panel show representative parts expressing CysLT₂ or CysLT₁ receptors. The scale bar represents 100 μm.

quantified by dividing the PAS⁺ area in the epithelium (μm²) by the length of the basement membrane (μm).

2.5. Statistical analyses

Two groups were compared by Student's unpaired *t*-test. To compare multiple groups, one-way ANOVA was performed, followed by Dunnett's multiple comparison test. A *P* value less than 0.05 was considered significant. Statistical analyses were performed with Prism (Version 5.0a; GraphPad, La Jolla, CA, USA) or JMP[®] Pro (Version 11.2.0, SAS Institute Japan, Tokyo, Japan).

3. Results

3.1. Expression of CysLT₂ and CysLT₁ receptors in lung tissue

Even in non-challenged mice, CysLT₂ receptors were expressed in bronchial smooth muscle (Fig. 1A). After the 4th challenge, CysLT₂ receptor expression was also detected in leukocytes infiltrating into alveolar spaces (Fig. 1B).

On the other hand, CysLT₁ receptor expression in the lung was relatively weak. CysLT₁ receptor-positive area was hardly observed in lung tissue of non-challenged mice (Fig. 1C). After the 4th challenge, limited parts of the bronchial smooth muscle and epithelium were

positive for CysLT₁ receptors (Fig. 1D). In addition, similar to that of CysLT₂ receptors, leukocytes infiltrating into the alveolar spaces were also positive for CysLT₁ receptors (Fig. 1D).

3.2. Expression of CysLT₂ and CysLT₁ receptors on leukocytes in lung tissue

As described above, both CysLT₂ and CysLT₁ receptors were histologically detected in leukocytes infiltrating into the lungs of asthmatic mice. Thus, CysLT₂R⁺ and CysLT₁R⁺ cells on leukocytes were further analyzed. Expression of CysLT₂ and CysLT₁ receptors on leukocytes (CD45⁺ cells) in single-cell suspensions of enzymatically dispersed lung tissues was analyzed by flow cytometer. Both CysLT₂R⁺ and CysLT₁R⁺ cells from the CD45⁺ cell population were detected even in lung tissue of non-challenged mice (Fig. 2A, B, C, D, E, and F). After the 4th antigen intratracheal challenge, both CysLT₂R⁺ and CysLT₁R⁺ cells were significantly increased (Fig. 2C and F).

Phenotypes of CysLT₂R⁺ CD45⁺ and CysLT₁R⁺ CD45⁺ cells in the lung tissue of challenged mice were further analyzed. Approximately 15% of CysLT₂R⁺ CD45⁺ cells were Siglec-F⁺ cells, which consist mainly of eosinophils [32], whereas the percentage of Gr-1⁺ cells, mainly consisting of neutrophils [24,33], was small (Table 1). The largest population of CysLT₂R⁺ CD45⁺ cells was CD11c⁺F4/80⁺ cells (Table 1), which have been classified as alveolar macrophages [28,30,31]. Approximately half of CysLT₂R⁺ CD45⁺ cells were

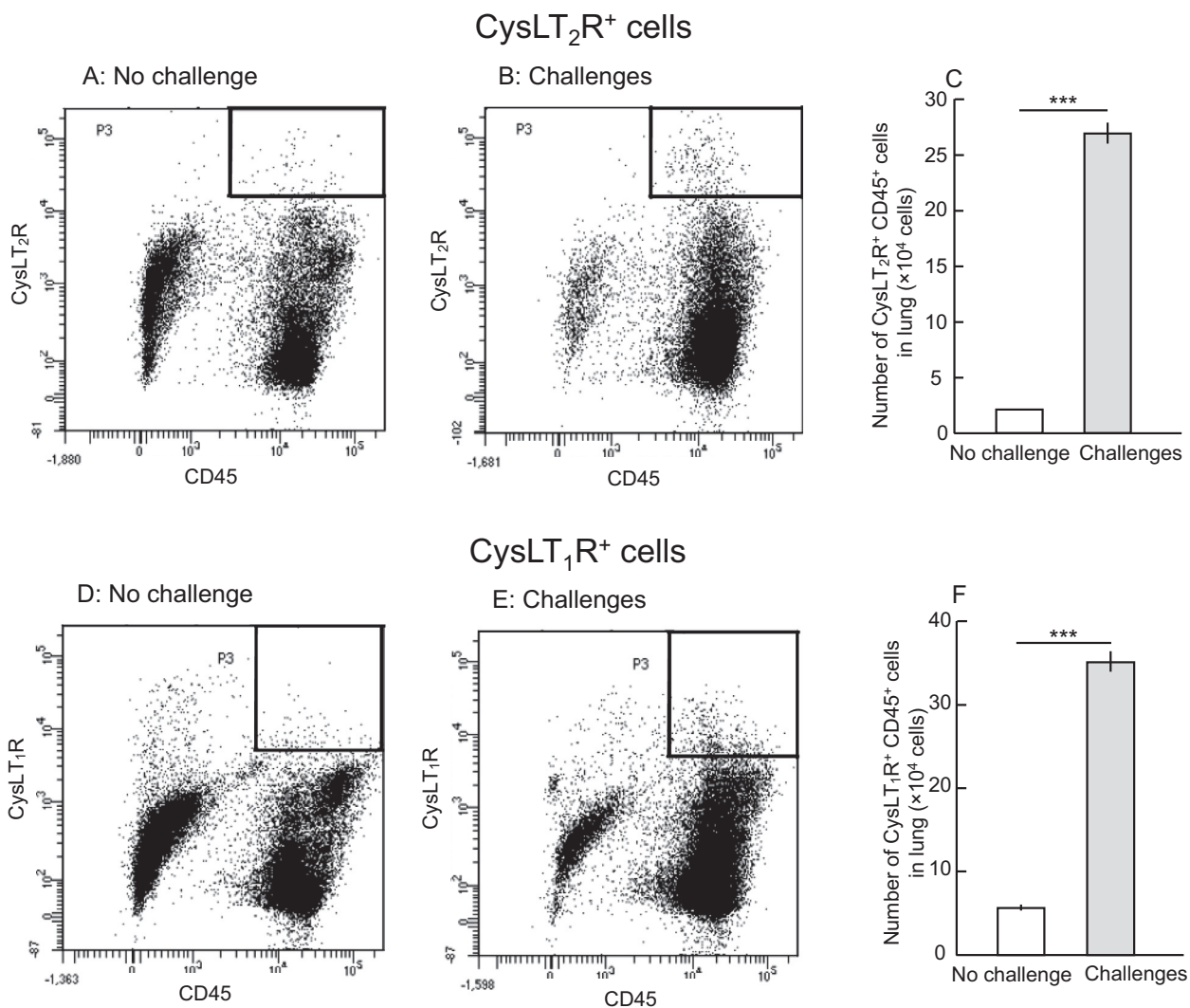


Fig. 2. Expression of CysLT₂ (A, B, and C) and CysLT₁ receptors (D, E, and F) on leukocytes (CD45⁺ cells) in the lung of non-challenged and challenged mice. Mice were intraperitoneally sensitized with OVA + Al(OH)₃ on days 0, 14, and 28, and then intratracheally challenged with OVA on days 35, 36, 37, and 40. The lung was isolated before multiple challenges on day 35 or 4 h after the 4th challenge on day 40, and then enzymatically digested to obtain a single-cell suspension. The single-cell suspension was stained with fluorescently labeled antibodies followed by flow cytometer analyses. Each column represents the mean ± S.E.M. of 3 animals. ****P* < 0.001.

Table 1
Percentage of CD11c⁺ I-A/I-E⁺ cells, CD11c⁺F4/80⁺ cells, Siglec-F⁺ cells and Gr-1⁺ cells in CysLT₂R⁺ CD45⁺ or CysLT₁R⁺ CD45⁺ cells in the lung of sensitized/challenged mice.

	% of CysLT ₂ R ⁺ CD45 ⁺ cells or % of CysLT ₁ R ⁺ CD45 ⁺ cells			
	CD11c ⁺ I-A/I-E ⁺	CD11c ⁺ F4/80 ⁺	Siglec-F ⁺	Gr-1 ⁺
CysLT ₂ R ⁺ CD45 ⁺	51.1 ± 1.9	84.6 ± 5.7	15.5 ± 0.8	2.8 ± 0.8
CysLT ₁ R ⁺ CD45 ⁺	7.1 ± 0.4	32.2 ± 1.8	35.6 ± 4.3	19.0 ± 1.6

Mice were intraperitoneally sensitized with OVA + Al(OH)₃ on days 0, 14 and 28, and then intratracheally challenged with OVA on days 35, 36, 37 and 40. The lung was isolated 4 h after the 4th challenge on day 40, and then enzymatically digested to obtain the single cell suspension. The single cell suspension was stained with fluorescence-labeled antibodies, followed by flow cytometer analyses. Each value represents the mean ± S.E.M. of 3 experiments.

CD11c⁺I-A/I-E⁺ cells (Table 1), which have been classified as conventional dendritic cells [29,31]. CD11c⁺F4/80⁺ cells and CD11c⁺I-A/I-E⁺ cells in the CysLT₂R⁺ cells may be overlapped to each other, calculating the sum of percentages to be more than 100% (Table 1). On the other hand, more than 50% of CysLT₁R⁺ CD45⁺ cells were granulocytes, which consists of Siglec-F⁺ eosinophils (35.6%) and Gr-1⁺ neutrophils (19.0%) (Table 1). CysLT₁R⁺ CD45⁺ cells also contained CD11c⁺F4/80⁺ cells and CD11c⁺ I-A/I-E⁺ cells by 32.2% and 7.1%, respectively (Table 1).

3.3. Effects of a CysLT₂ receptor antagonist BayCysLT₂RA and a CysLT₁ receptor antagonist montelukast on antigen-induced leukocyte infiltration into the lung

Multiple antigen challenges markedly increased the number of leukocytes in BALF (Fig. 3A). Most of the leukocytes consisted of eosinophils, neutrophils, and mononuclear cells, and the percentage of eosinophils from the total leukocyte population was approximately 60% (Fig. 3A and B). Daily i.v. treatment with BayCysLT₂RA partly but significantly suppressed increases in the numbers of eosinophils and mononuclear cells at 0.03 and 0.3 mg/kg, but the effects were not dose-

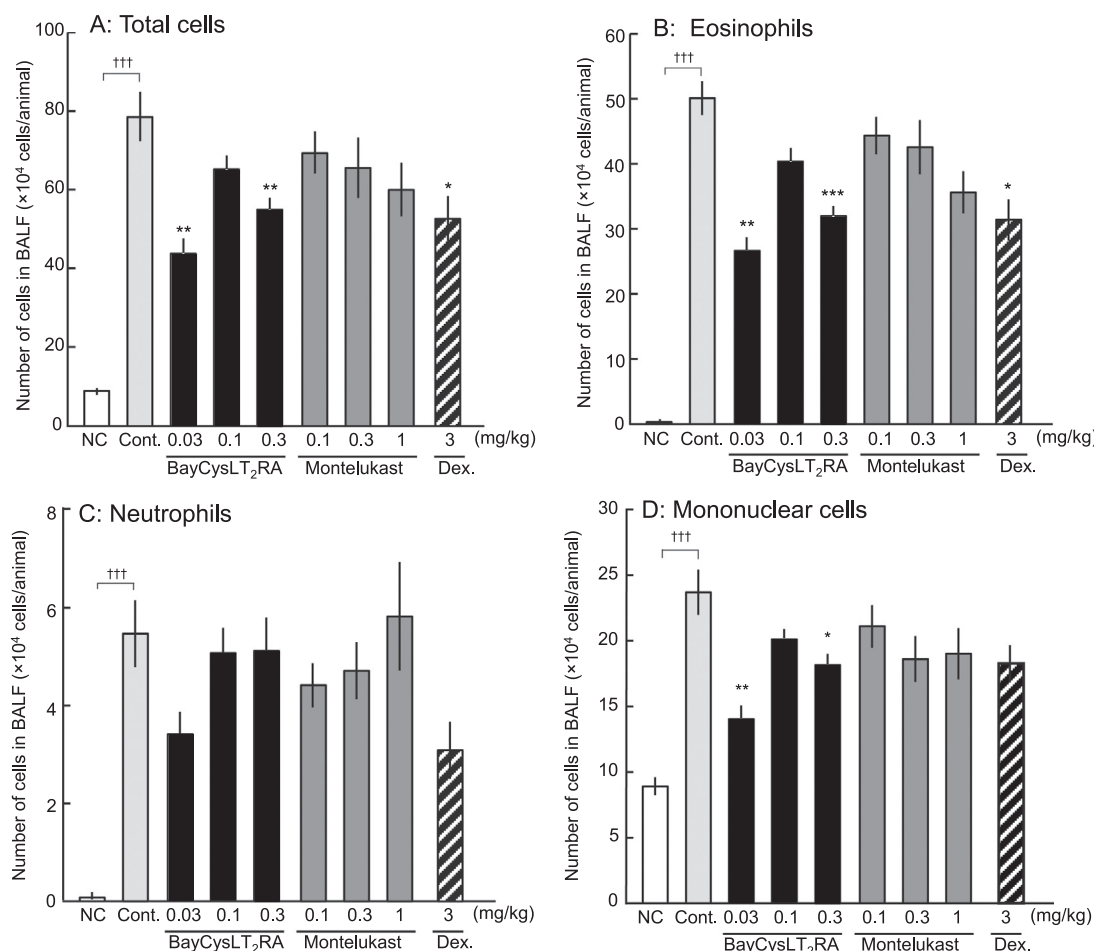


Fig. 3. Effects of a CysLT₂ receptor antagonist BayCysLT₂RA and a CysLT₁ receptor antagonist montelukast on multiple antigen challenge-induced increases in the number of total cells (A), eosinophils (B), neutrophils (C), and mononuclear cells (D) in bronchoalveolar lavage fluid (BALF) of sensitized mice. Mice were intraperitoneally sensitized with OVA + Al(OH)₃ on days 0, 14, and 28, and then intratracheally challenged with OVA on days 35, 36, 37, and 40. BAL was conducted 4 h after the 4th challenge. BayCysLT₂RA (0.03–0.3 mg/kg), montelukast (0.1–1 mg/kg), dexamethasone (Dex., 3 mg/kg), or saline was i.v. administered once-a-day from day 35 to 40. Each column represents the mean $\pm$ S.E.M. of 8–27 animals. *, **, and *** P < 0.05, 0.01, and 0.001, respectively, vs Control (Cont.), ††† P < 0.001. NC: No challenge.

dependent (Fig. 3B and D). Neutrophilia was also slightly attenuated by 0.03 mg/kg BayCysLT₂RA. Daily i.v. treatment with montelukast also decreased the numbers of eosinophils and mononuclear cells at 0.1–1 mg/kg, but the effects were not significant (Fig. 3B and D). When a strong glucocorticoid dexamethasone was administered daily at 3 mg/kg, airway eosinophilia was significantly suppressed but not completely inhibited (Fig. 3B). Increases in the numbers of neutrophils and mononuclear cells were also reduced by dexamethasone, but the effects were not significant (Fig. 3C and D).

3.4. Effects of BayCysLT₂RA and montelukast on the development of airway remodeling induced by antigen challenges

During the 1st–4th antigen challenges, airway remodeling manifested by bronchial epithelial thickening and mucus accumulation in the epithelium developed as previously reported [23]. Both epithelial thickening and mucus accumulation in relatively small bronchi (diameter: 150–400 μ m) were attenuated by BayCysLT₂RA at 0.3 and 1 mg/kg (Fig. 4A, C, and Fig. 5), but inhibition was not significant (Fig. 4A and C). Montelukast significantly suppressed the development of epithelial thickening in relatively small bronchi at 0.1 mg/kg (Fig. 4A), but significant inhibition was not recognized at higher doses of 0.3 and 1 mg/kg (Fig. 4A). Dexamethasone (3 mg/kg) partially inhibited epithelial thickening and mucus accumulation in relatively small bronchi

(Fig. 4A and C). Airway remodeling in larger bronchi (diameter > 400 μ m) were relatively resistant to treatment (Fig. 4B and D).

4. Discussion

We found that CysLT₂ receptors were constitutively expressed in bronchial smooth muscle cells, and that CysLT₂ receptor-expressing leukocytes, mainly consisting of macrophage/dendritic cells and eosinophils, infiltrated into the lung after multiple antigen challenges in sensitized mice. In addition, CysLT₂ receptor activation may be functionally involved in allergic airway infiltration of eosinophils and mononuclear cells. To our knowledge, this is the first report demonstrating the existence of CysLT₂ receptors in the histology of murine lung.

Compared with CysLT₂ receptors, CysLT₁ receptor expression in the lung was not pronounced, even in bronchial smooth muscle. We used two different commercially available primary antibodies against CysLT₁ receptors, but found only a limited number of antibody-positive areas. Low expression of CysLT₁ receptors, even in airway smooth muscle, is consistent with the fact that mouse airway smooth muscle only weakly responds to CysLTs [35]. It is impractical to directly compare expression between CysLT₂ and CysLT₁ receptors because different primary antibodies were used for detection. However, CysLT₂ receptor expression in the murine lung was clearly detected in bronchial smooth

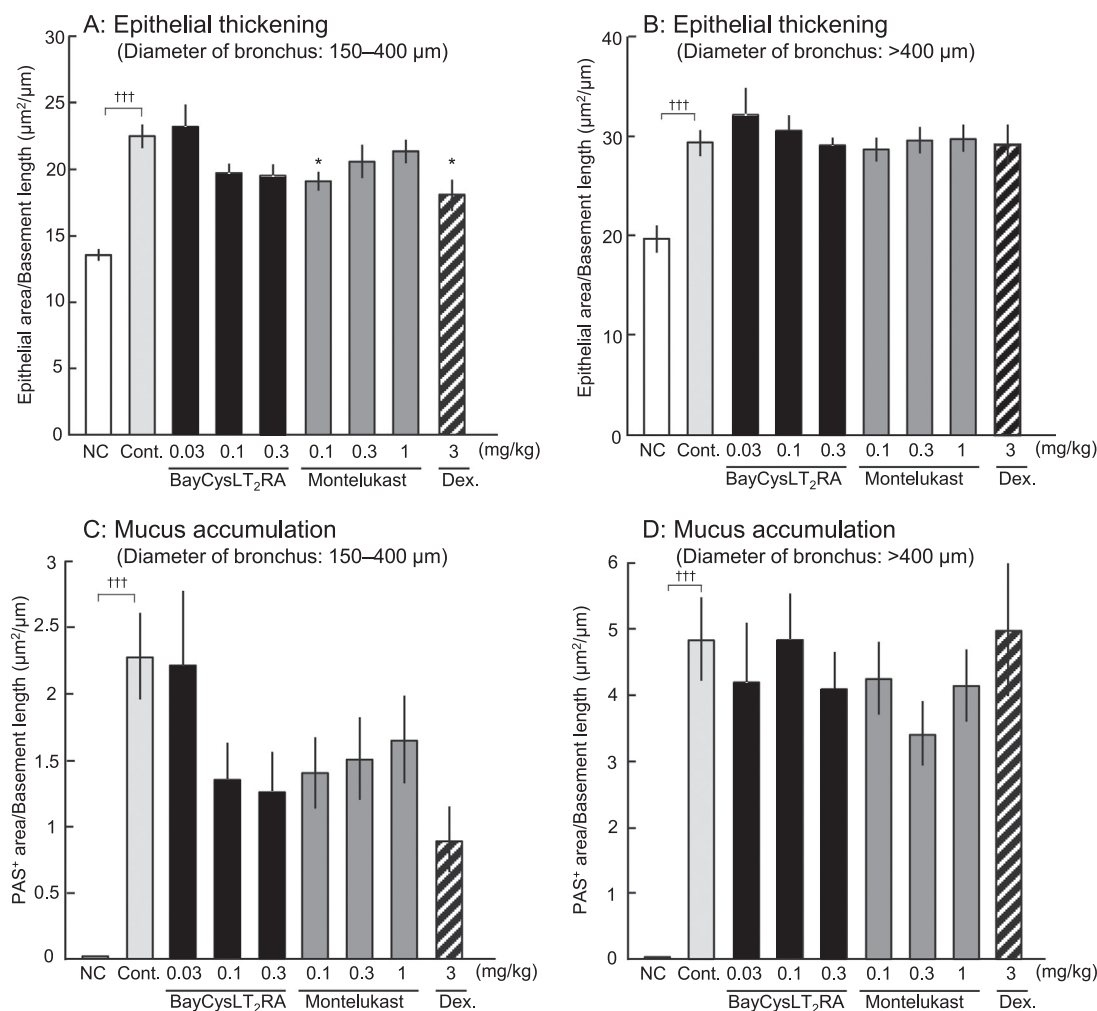


Fig. 4. Effects of a CysLT₂ receptor antagonist BayCysLT₂RA and a CysLT₁ receptor antagonist montelukast on multiple antigen challenge-induced development of airway remodeling in sensitized mice. Mice were intraperitoneally sensitized with OVA + Al(OH)₃ on days 0, 14, and 28, and then intratracheally challenged with OVA on days 35, 36, 37, and 40. BayCysLT₂RA (0.03–0.3 mg/kg), montelukast (0.1–1 mg/kg), dexamethasone (Dex, 3 mg/kg), or saline was i.v. administered once-a-day from day 35 to 40. The lung was isolated before multiple antigen challenges or 4 h after the 4th challenge. Isolated lung tissue was fixed with 10% formalin, embedded in paraffin, and cut into a 4 μm section followed by staining with periodic acid-Schiff (PAS). The degree of epithelial thickening was quantified using PAS-stained sections by dividing epithelial area in the bronchus (diameter: 150–400 μm or >400 μm) by the length of basement membrane (A and B). Degree of mucus deposition was calculated by dividing PAS⁺ area in the bronchus by the length of basement membrane (C and D). Each column represents the mean $\pm$ S.E.M. of 8–22 animals. * P < 0.05 vs Control, $\dagger\dagger\dagger P$ < 0.001. NC: No challenge.

muscle and in leukocytes infiltrating into the lung, implying that CysLT₂ receptors exert functional roles in mouse lung pathophysiology.

The CysLT₂ receptor antagonist BayCysLT₂RA significantly suppressed multiple antigen challenge-induced infiltration of eosinophils and mononuclear cells into the lung, indicating that CysLT₂ receptor activation is involved in leukocyte migration. In contrast to the present finding, Barret et al. [21] reported that antigen-induced eosinophilic pulmonary inflammation was markedly augmented in CysLT₂ receptor-deficient mice. In addition, negative regulation of CysLT₂ receptors in Th2-type pulmonary inflammation was induced by inhibiting both CysLT₁ receptor signaling and expression [21]. An in vitro study suggested that CysLT₂ receptors can negatively regulate CysLT₁ receptor expression and LTD₄-induced mitogenic responses in mast cells [36]. The difference between our results and the literature [21] may be because of different experimental tools, CysLT₂ receptor antagonists, and CysLT₂ receptor-deficient mice. We have confirmed that BayCysLT₂RA lacked effects on other receptors associated with smooth muscle constriction or relaxation, such as adrenergic α_{2A} , α_{2B} and β_2 receptors, histamine H₁ and H₂ receptors, and endothelin ET_A and ET_B receptors (unpublished data). Regarding pharmacokinetics, when BayCysLT₂RA was i.v. administered at 0.3 and 1 mg/kg, $t_{1/2}$ was approximately 0.3 h

(unpublished data). Collectively, the effect of BayCysLT₂RA is specific for CysLT₂ receptors, but not so long-lasting. This situation of lowered function of CysLT₂ receptors under the treatment with BayCysLT₂RA should be different from that in CysLT₂ receptor deficient mice, in which CysLT₂ receptors congenitally defected. In gene knock out mice, however, other compensatory changes due to the gene modification should also be considered. On the other hand, inhibition by CysLT₂ receptor antagonist was not dose-dependent from 0.03–0.3 mg/kg. Increasing the BayCysLT₂RA dose to 1 and 3 mg/kg, did not further inhibit infiltration of eosinophils and mononuclear cells (data not shown). The absence of dose-dependency may be related to the regulatory functions of CysLT₂ receptors as reported by others [21,36], but we have not examined the effect of BayCysLT₂RA on CysLT₁ receptor expression.

Flow cytometric analyses indicated that CysLT₂ receptor-positive leukocytes included Siglec F⁺ cells (eosinophils), CD11c⁺ F4/80⁺ cells (alveolar macrophages), and CD11c⁺ I-A/I-E⁺ cells (conventional dendritic cells). Only a small percentage of Gr-1⁺ cells (neutrophils) was positive for CysLT₂ receptors. Eosinophils, macrophages, and dendritic cells in humans express both CysLT₁ and CysLT₂ receptors [37–39], but whether these receptors are expressed in murine

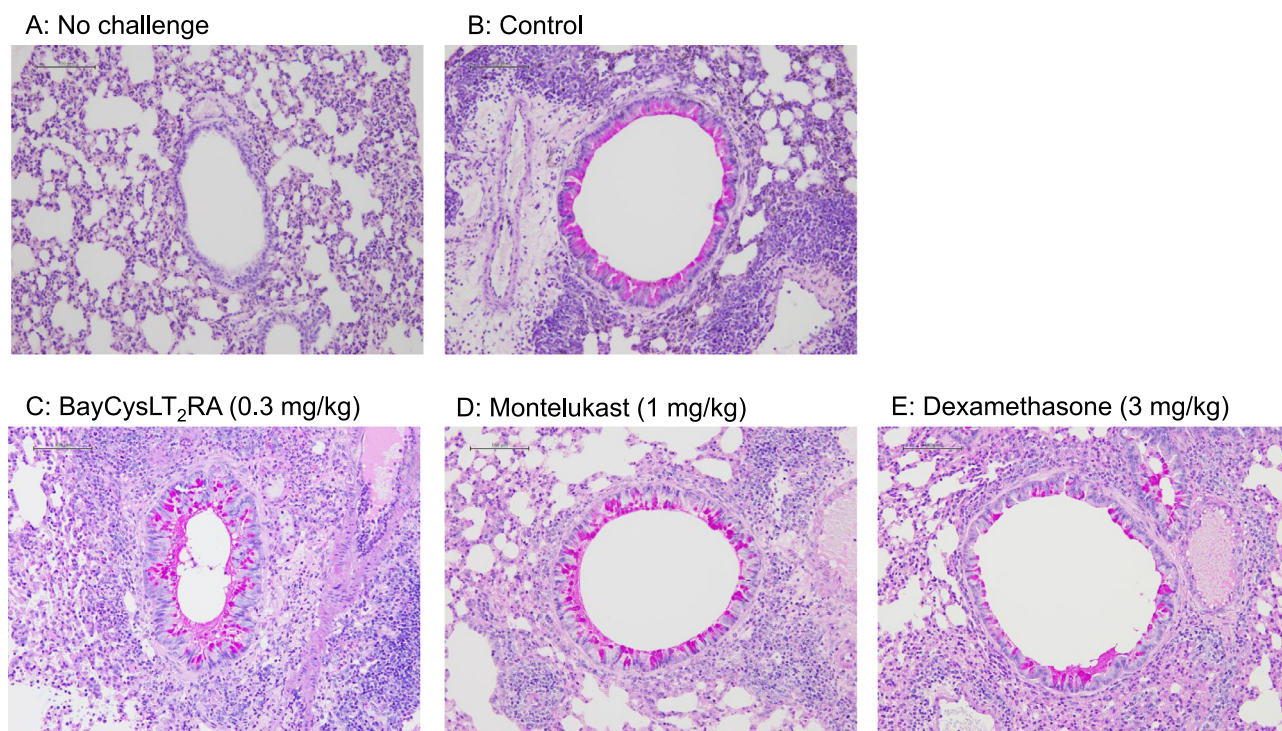


Fig. 5. Representative photographs showing the effects of a CysLT₂ receptor antagonist BayCysLT₂RA (0.3 mg/kg) and a CysLT₁ receptor antagonist montelukast (1 mg/kg) on multiple antigen challenge-induced development of airway remodeling in sensitized mice. Mice were intraperitoneally sensitized with OVA + Al(OH)₃ on days 0, 14, and 28, and then intratracheally challenged with OVA on days 35, 36, 37, and 40. BayCysLT₂RA, montelukast, dexamethasone (3 mg/kg), or saline was i.v. administered once-a-day from day 35 to 40. The lung was isolated before multiple antigen challenges or 4 h after the 4th challenge. Isolated lung tissue was fixed with 10% formalin, embedded in paraffin, and cut into a 4 μm section followed by staining with periodic acid-Schiff (PAS). Black bar in each panel represents 100 μm.

leukocytes has been unclear. Consistent with the expression profiles of CysLT₂ receptors in the present study, CysLT₂ receptor antagonists significantly inhibited both eosinophils and mononuclear cells (macrophages and dendritic cells), but not neutrophils. CysLT₂ receptors expressed in eosinophils, alveolar macrophages, and dendritic cells may be directly related to pulmonary recruitment of these cells to the lung.

The CysLT₁ receptor antagonist montelukast effectively suppresses antigen-induced pulmonary eosinophilia [16–20], but montelukast did not significantly suppress airway eosinophilia even at 1 mg/kg in our murine model. In this study, dose of montelukast was increased to 3 and 10 mg/kg, whereas the inhibitory effect on the eosinophilia was not augmented (data not shown). The difference between the literature [16–21] and the present study may be due to a difference in antigen-challenging protocols. In our study, antigen challenge was conducted by intratracheal administration, but other studies used intranasal administration and inhalation of mist for antigen challenge [16–21]. Intratracheal administration can efficiently deliver antigen to the lung, resulting in lung exposed to a large amount of antigen. In addition, drug resistance to the glucocorticoid dexamethasone was observed in our study. In murine models of pulmonary allergy, dexamethasone markedly suppresses antigen-induced pulmonary eosinophilia at less than 3 mg/kg [20,40]. However, the inhibitory degree of airway eosinophilia in our model by 3 mg/kg dexamethasone was less than 40%. The limited effects of glucocorticoid have been observed elsewhere [23], but the mechanisms underlying glucocorticoid resistance remain unclear. The effectiveness of BayCysLT₂RA implied that blockade of CysLT₂ receptors may be clinically effective for steroid-resistant asthma.

Henderson et al. [20] reported that allergen-induced airway remodeling was blocked by the CysLT₁ receptor antagonist montelukast. The development of epithelial thickening and mucus accumulation at relatively small bronchi was also suppressed by montelukast. The CysLT₂ receptor antagonist BayCysLT₂RA suppressed airway remodeling, but the effect was not significant. The effectiveness of

dexamethasone on airway remodeling was also smaller than that in other reports [20], which suggests that the intratracheal-challenge model is steroid-resistant for the development of airway remodeling.

In conclusion, CysLT₂ receptors were constitutively expressed in bronchial smooth muscle cells of BALB/c mice. Multiple antigen challenges induced infiltration of CysLT₂ receptor-expressing leukocytes (eosinophils and macrophage/dendritic cells) into the lung in sensitized BALB/c mice. CysLT₂ receptor activation may be functionally involved in induction of allergic airway eosinophilia. BALB/c mice are a useful model to examine the mechanisms underlying CysLT₂ receptor-mediated pulmonary allergic responses, and for the development of CysLT₂ receptor antagonists. Finally, CysLT₂ receptors can be a target for the development of new pharmacotherapies for asthma.

Acknowledgment

This study was conducted by funds from Ono Pharmaceutical Co., Ltd., Osaka, Japan.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.plefa.2018.03.007.

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