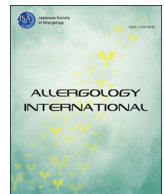




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Original Article

Leukotriene receptor antagonist attenuated airway inflammation and hyperresponsiveness in a double-stranded RNA-induced asthma exacerbation model

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ARTICLE INFO

Article history:

Received 30 January 2017

Received in revised form

24 April 2017

Accepted 1 May 2017

Available online xxx

Keywords:

Asthma

Cysteinyl leukotrienes

Double-stranded RNA

Leukotriene receptor antagonist

Virus

Abbreviations:

Ach, acetylcholine; AHR, airway hyperresponsiveness; ASM, airway smooth muscle; BAL, bronchoalveolar lavage; BALF, bronchoalveolar lavage fluid; CCL, Chemokine (C-C Motif) Ligand; CXCL, Chemokine (C-X-C Motif) Ligand; cysLTs, cysteinyl leukotrienes; cysLT₁R, cysteinyl leukotriene receptor 1; dsRNA, double-stranded RNA; ICS, inhaled corticosteroids; ILC2s, type 2 innate lymphoid cells; i.n., intranasally; i.p., intraperitoneally; KC, Keratinocyte chemoattractant; LTC₄S, leukotriene C₄ synthase; LTD₄, leukotriene D₄; LTRA, leukotriene receptor antagonist; MIP-1α, Macrophage inflammatory protein-1 alpha; OVA, ovalbumin; PBS, phosphate buffered saline; poly I:C, polyinosinic–polycytidylic acid; PSL, prednisolone; Raw, airway resistance; RSV, respiratory syncytial virus; TSLP, thymic stromal lymphopoietin

ABSTRACT

Background: Viral infections are the most common triggers of asthma exacerbation, but the key molecules involved in this process have not been fully identified. Although cysteinyl leukotrienes (cysLTs) have been postulated as the key mediators, their precise roles remain largely unclear. To investigate the roles of cysLTs in virus-induced asthma exacerbation, we developed a murine model using a viral double-stranded RNA analog, polyinosinic–polycytidylic acid (poly I:C), and analyzed the effect of leukotriene receptor antagonist (LTRA) administration.

Methods: A/J mice were immunized with ovalbumin (OVA) + alum (days 0, 28, 42, and 49), followed by intranasal challenge with OVA (phase 1: days 50–52) and poly I:C (phase 2: days 53–55). Montelukast was administered during poly I:C challenge (phase 2) in the reliever model or throughout the OVA and poly I:C challenges (phases 1 and 2) in the controller model. Airway responsiveness to acetylcholine chloride was assessed, and bronchoalveolar lavage (BAL) was performed on day 56.

Results: Administration of poly I:C to OVA-sensitized and -challenged mice increased the number of eosinophils and levels of IL-13, IL-9, CCL3, and CXCL1 in BAL fluid (BALF) and tended to increase airway responsiveness. Montelukast significantly attenuated the poly I:C-induced increase in the number of eosinophils and levels of IL-13, IL-9, and CCL3 in BALF and airway hyperresponsiveness in both the reliever and controller models.

Conclusions: This is the first report showing that LTRA functionally suppressed the pathophysiology of a virus-induced asthma exacerbation model, suggesting the importance of cysLTs as a potential treatment target.

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Peer review under responsibility of Japanese Society of Allergy.

<http://dx.doi.org/10.1016/j.alit.2017.05.004>

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Introduction

Viral infections in the respiratory tract are the most common triggers of an acute asthma exacerbation.¹ The mechanism of virus-induced exacerbations includes multiple interactions between viral components and various inflammatory and structural cells and involves various mediators. However, the precise underlying mechanisms and key molecules involved in the exacerbation have not been completely identified.² Among various mediators, cysteinyl leukotrienes (cysLTs) have been postulated as key mediators of virus-induced exacerbations because their levels in humans are increased in the nasal lavage fluid after viral infections,^{3,4} nasopharyngeal secretions in virus-induced wheezing,⁵ and sputum⁶ or urine⁷ from patients with asthma exacerbation.

In animal experiments, the levels of cysLTs in BALF are also markedly increased by a simple respiratory syncytial virus (RSV) infection,⁸ but the precise roles of cysLTs in virus-induced asthma exacerbation remain largely unclear. Although leukotriene receptor antagonists (LTRAs) are among the main controllers of asthma in clinical practice,⁹ it is yet to be determined if LTRA treatment improves the pathophysiology of virus-induced acute asthma exacerbation.

The aim of the current study was to investigate the roles of cysLTs in a virus-induced asthma exacerbation model. We developed a murine model of asthma exacerbation using a viral double-stranded RNA (dsRNA) analog, polyinosinic–polycytidylic acid (poly I:C), and analyzed the effects of administration of a LTRA, montelukast, on airway inflammation, airway hyperresponsiveness (AHR), and the levels of local cytokines and chemokines. In addition, to determine the appropriate time of administration of LTRA, we administered LTRA in two different time courses resembling the use of LTRA as a controller or reliever.

Methods

Mice

Male A/J mice (6-week old) were purchased from SLC (Shizuoka, Japan) and housed under pathogen-free conditions at the Laboratory Animal Center for Biochemical Research, Teikyo University School of Medicine. A/J mice have a genetic background conferring hypersensitivity to acetylcholine (Ach).¹⁰ The Teikyo University School of Medicine Committee on Animal Research reviewed and approved all experimental procedures.

Experimental protocol

Male A/J mice (6-weeks-old) were prepared as previously described.¹¹ As the negative control group, the mice were intraperitoneally (i.p.) injected with PBS on days 0, 28, 42, and 49, and intranasally (i.n.) and i.p. administered with PBS on days 50–55 [PBS/PBS (PP) group].

Mice were immunized as previously described.¹¹ In brief, the mice were intraperitoneally (i.p.) injected with 10 µg of ovalbumin (OVA) + 2 mg aluminum hydroxide gel on days 0, 28, 42, and 49. Then, on days 50–52, mice were anesthetized and intranasally (i.n.) challenged with OVA (40 µL of 10 mg/mL in normal saline, twice). The simple asthma model was further treated by phosphate buffered saline (PBS) i.n. on days 53–55, and i.p. injections of PBS on days 50–55 [Fig. 1A, OVA/PBS (PP) group].

For the dsRNA-induced asthma exacerbation model, mice were i.n. challenged with poly I:C (40 µL of 15 mg/mL in PBS, Sigma–Aldrich, St. Louis, MO, USA) on days 53–55 (Fig. 1B). The mice were divided into three different treatment groups and PBS or montelukast (30 mg/kg in 500 µL of PBS, provided by Merck & Co.,

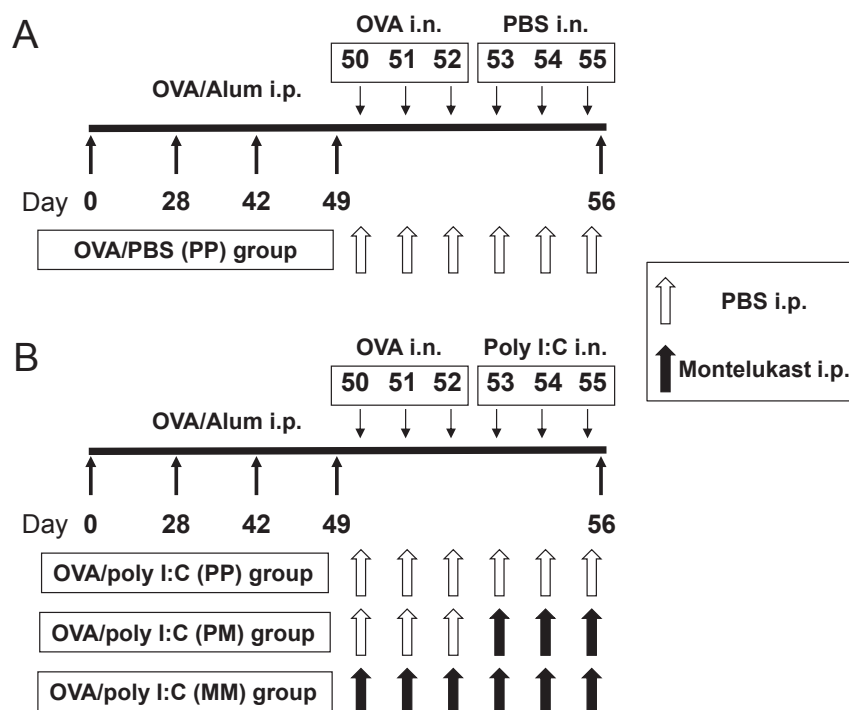


Fig. 1. Study design. A/J mice were immunized with ovalbumin (OVA) + alum (days 0, 28, 42, and 49), followed by intranasal (i.n.) OVA challenge on days 50–52 (phase 1). (A) As simple asthma model [OVA/PBS (PP) group], mice were further treated by phosphate buffered saline (PBS) i.n. on days 53–55 (phase 2), and intraperitoneal (i.p.) injections of PBS on days 50–55 (phase 1–2). (B) For the dsRNA-induced asthma exacerbation model, mice were i.n. challenged with poly I:C (phase 2: days 53–55). Montelukast or PBS was i.p. administered as follows: (1) Control group [OVA/poly I:C (PP): phase 1/2: PBS/PBS], (2) Reliever group [OVA/poly I:C (PM): phase 1/2: PBS/montelukast], and (3) Controller group [OVA/poly I:C (MM) group: phase 1/2: montelukast/montelukast]. Assessment of airway responsiveness and bronchoalveolar lavage was performed on day 56.

Inc., Kenilworth, NJ, USA) was i.p. administered in two phases (phase 1: days 50–52, phase 2: days 53–55). The three groups of mice were treated as follows: (1) Control group [OVA/poly I:C (PP), phase 1: PBS, phase 2: PBS], (2) Reliever model [OVA/poly I:C (PM), phase 1: PBS, phase 2: montelukast], and (3) Controller model [OVA/poly I:C (MM), phase 1: montelukast, phase 2: montelukast].

Airway responsiveness to Ach and bronchoalveolar lavage fluid (BALF) was observed on day 56.

Assessment of airway responsiveness

The assessment of airway responsiveness was undertaken on day 56, as previously described.¹¹ In brief, after the administration of pentobarbital anesthesia, mice were tracheostomized, connected to a Harvard ventilator with 0.3 mL tidal volume and a respiratory frequency of 120/min, followed by injection of pancuronium bromide. Airway resistance (Raw) was measured by whole-body plethysmograph (Buxco Electronics, Troy, NY, USA). Increasing doses of Ach (1.25–5 mg/mL) were administered by ultranebulization for 3 min.

Bronchoalveolar lavage (BAL)

After measurements of pulmonary function, each mouse was exsanguinated. BALF was obtained from selected mice, as previously described.¹² In brief, the lungs were washed with 1 mL of saline until the recovered fluid reached 5 mL. BALF was centrifuged, and the number of cells was counted. BALF was concentrated 10 fold using Amicon Ultra 4 (Millipore, Bedford, MA, USA), and the levels of cytokines and chemokines were determined by Mouse Cytokine 23-Plex Panel Kit (BIO-RAD, Hercules, USA) on a Multiplex ELISA Luminex® system (Hitachi, Tokyo), according to the manufacturer's instructions.

Statistical analysis

The distributions and variances across samples were analyzed using the Shapiro–Wilk and Bartlett's test, respectively. Parametric data were analyzed using the two-way analysis of variance test followed by the Tukey–Kramer test to compare individual groups. Non-parametric data was analyzed using the Wilcoxon signed-rank test, and significance was determined according to the Bonferroni

correction. Data were presented as the means $\pm$ standard error of the mean unless otherwise noted. The analyses were performed using the JMP® software (SAS Institute Japan, Tokyo, Japan).

Results

First, we compared BALF cell numbers in the simple asthma model [OVA/PBS (PP) group] with the negative control group [PBS/PBS (PP) group]. The total number of cells [$6.78 \pm 0.88^{**} \times 10^5$ vs $1.94 \pm 0.30 \times 10^5$ cells; OVA/PBS (PP) group, $n = 12$ vs PBS/PBS (PP) group, $n = 18$, $p < 0.01^{**}$], eosinophils ($4.99 \pm 0.73^{**}$ vs 0.15 ± 0.05), and lymphocytes ($0.25 \pm 0.13^{**}$ vs 0.05 ± 0.01) were significantly upregulated in the simple asthma model, showing that eosinophilic airway inflammation was established in the OVA/PBS (PP) group.

Next, we analyzed the effect of additional administration of poly I:C on the simple asthma model. First, we analyzed the differential cell counts in BALF (Fig. 2). After OVA sensitization and challenge, poly I:C treatment [OVA/poly I:C (PP) group] significantly increased the number of total cells, eosinophils, macrophages, and lymphocytes compared with the number in the simple asthma model [OVA/PBS (PP) group]. In poly I:C challenged groups, although montelukast treatment exerted no significant effect on the number of total cells, macrophages, or lymphocytes in the reliever model [OVA/poly I:C (PM)] or controller model [OVA/poly I:C (MM)] compared with that on the number in the control group [OVA/poly I:C (PP)], montelukast significantly suppressed the number of eosinophils to that in the OVA/PBS (PP) group. The effect on eosinophil counts was observed irrespective of the time of montelukast administration, and there was no significant difference between the OVA/poly I:C (PM) and OVA/poly I:C (MM) groups. In contrast, the number of neutrophils was the same in the OVA/poly I:C (PP) and OVA/poly I:C (PM) groups, whereas slight but significant increase in the number of neutrophils was observed in the OVA/poly I:C (MM) group.

Next, we analyzed the effect of montelukast on airway responsiveness to Ach (Fig. 3). After OVA sensitization and challenge, additional poly I:C treatment tended to increase the response to Ach [OVA/poly I:C (PP) group] compared with that in the OVA/PBS (PP) group. In the poly I:C challenged groups, baseline airway resistance before treatment by Ach was significantly reduced in both OVA/poly I:C (PM) and OVA/poly I:C (MM) groups compared with that in the OVA/poly I:C (PP) group. In addition, airway

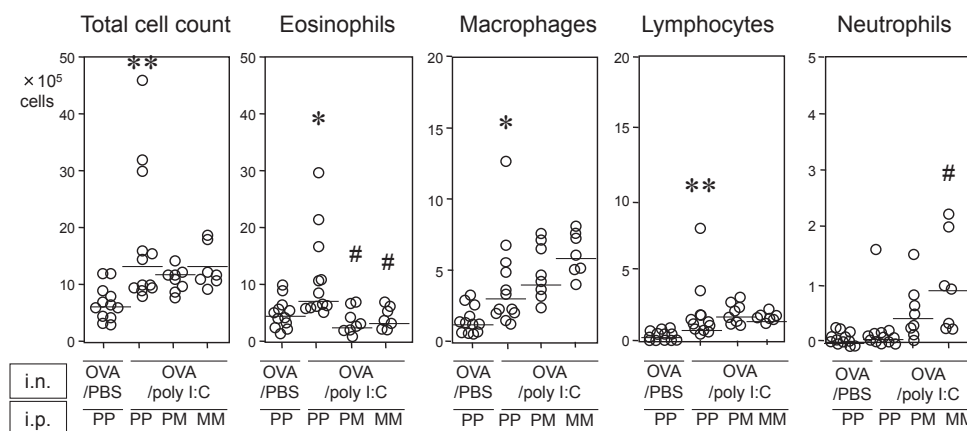


Fig. 2. Bronchoalveolar lavage fluid (BALF) cell analysis. Each left lane shows simple asthma model [OVA/PBS (PP) group, $n = 12$]; OVA intranasal (i.n.) challenge on days 50–52 (phase 1), followed by PBS on days 53–55 (phase 2) with i.p. injections of PBS on days 50–55 (phase 1–2). Other lanes show double-stranded RNA-induced asthma exacerbation model: OVA i.n. challenge in phase 1, followed by poly I:C in phase 2. Montelukast or PBS was i.p. administered as follows: OVA/poly I:C (PP) [Control group ($n = 12$): phase 1/2: PBS/PBS], OVA/poly I:C (PM) [Reliever model ($n = 9$): phase 1/2: PBS/montelukast], OVA/poly I:C (MM) [Controller model ($n = 9$): phase 1/2: montelukast/montelukast]. Data were analyzed by the Wilcoxon signed-rank test followed by Bonferroni correction. Each bar indicates the median values of cell counts. Bonferroni adjusted p value, * $p < 0.0083$, ** $p < 0.00167$ versus OVA/PBS (PP) and # $p < 0.0083$ versus OVA/poly I:C (PP) is statistically significant.

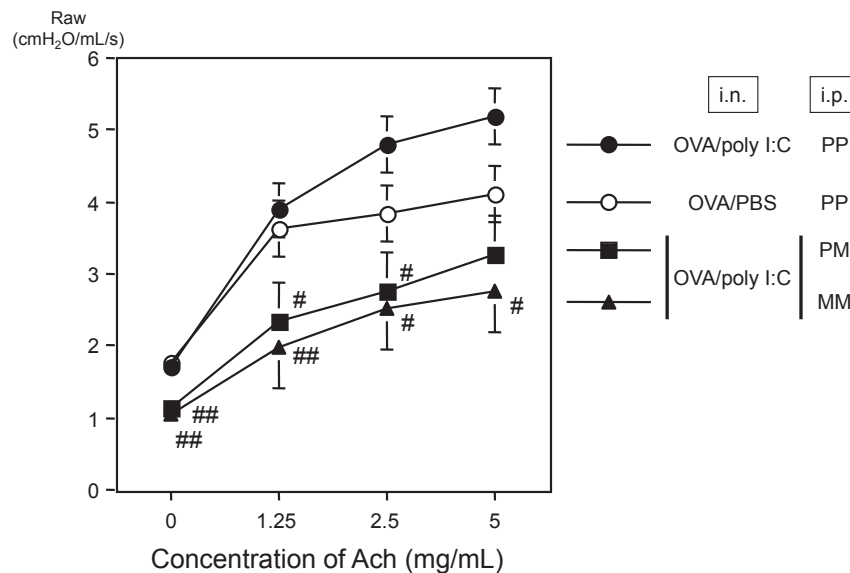


Fig. 3. Airway hyperresponsiveness. Airway responses after treatment with 0, 1.25, 2.5, and 5 mg/mL acetylcholine chloride (Ach) were measured. OVA/PBS (PP) group (simple asthma model, $n = 13$); Ovalbumin (OVA) intranasal challenge (phase 1), followed by phosphate buffered saline (PBS) (phase 2). Other groups were challenged by OVA (phase 1) followed by poly I:C (phase 2). OVA/poly I:C (PP): [Control group ($n = 13$): phase 1/2: PBS/PBS], OVA/poly I:C (PM) [Reliever model ($n = 9$): phase 1/2: PBS/montelukast], OVA/poly I:C (MM): [Controller model ($n = 9$): phase 1/2: montelukast/montelukast]. Data are expressed as mean $\pm$ standard error of the mean values. The airway resistance (Raw) data for the same concentrations of Ach in four groups were analyzed by Wilcoxon signed-rank test followed by Bonferroni correction. Bonferroni adjusted p value, # $p < 0.00167$ and ## $p < 0.0083$ versus OVA/poly I:C (PP) is statistically significant.

responsiveness was also significantly suppressed in both OVA/poly I:C (PM) and OVA/poly I:C (MM) groups after inhalation of 1.25 and 2.5 mg/mL Ach, respectively. The effect was more prominent in the OVA/poly I:C (MM) group, and a significant decrease in airway resistance was observed only in the OVA/poly I:C (MM) group after inhalation of 5.0 mg/mL Ach.

To further analyze the mechanism of decrease in eosinophil and airway responsiveness, we determined the levels of cytokines and chemokines in BALF. The results of molecules detected by the current assay are shown in Figure 4.

After OVA sensitization and challenge, additional poly I:C treatment increased the levels of IL-13, IL-9, Chemokine (C-C Motif) Ligand 3 (CCL3), and Chemokine (C-X-C Motif) Ligand 1 (CXCL1) [OVA/PBS (PP) vs OVA/poly I:C (PP)]. The levels of IL-5 and CCL5 also tended to increase. Th1 cytokine levels, including IFN- γ and IL-2, were below the detectable limit, in all settings.

When montelukast was administered, the levels of IL-13, IL-9, and CCL3 were significantly suppressed in both OVA/poly I:C (PM) and OVA/poly I:C (MM) groups compared with those in the OVA/poly I:C (PP) group and the level of IL-5 was also significantly decreased in the OVA/poly I:C (PM) group. However, these inhibitory effects were not observed for all molecules, and no effect of montelukast on the levels of CCL5 and CXCL1 was observed.

Discussion

In this study, we analyzed the effect of LTRA on a dsRNA-induced asthma exacerbation model. Administration of dsRNA exacerbated allergic airway inflammation and significantly increased the number of eosinophils and the levels of IL-13, IL-9, and CCL3 in BALF, which led to a tendency of increased AHR. Next, we showed that administration of LTRA could suppress the increased airway inflammation induced by dsRNA and improve AHR. Although, a previous report described the effects of LTRA on BALF eosinophils and IFN- γ and IL-5 production from lung lymph nodes in a virus-induced asthma exacerbation model,¹³ the current study is the first to report the suppressive effect on functional AHR and various mediators in BALF both in the reliever and controller models.

In a murine model of asthma without viral infection, LTRA reduced airway eosinophilia and AHR through the suppression of Th2 cytokines and chemokines.^{14,15} In addition to the effect on baseline pathophysiology of asthma, the specific roles of cysLTs in virus-induced exacerbation of asthma were suggested from the current study.

There have been several reports on virus-induced asthma exacerbation models, and the expression of IL-13,^{16–18} IL-5,^{17,19,20} IL-9,^{17,19,20} macrophage inflammatory protein-1 alpha (MIP-1 α)/CCL3,²¹ RANTES/CCL5,^{19,22} and keratinocyte chemoattractant (KC)/CXCL1²⁰ in lungs was upregulated by an additional viral or dsRNA challenge. Recently, increased levels of IL-25, IL-33, and thymic stromal lymphopoietin (TSLP) were also reported after dsRNA-induced exacerbation in an asthma model.^{22,23} In line with these reports, we observed a significant increase in the levels of IL-13, IL-9, CCL3, and CXCL1 as well as a tendency toward increase in the levels of IL-5 and CCL5 after dsRNA challenge. The levels of these cytokines and chemokines were significantly downregulated by LTRA administration, and potential mechanisms may include the following steps.

After viral infection of airway epithelial cells, virus dsRNA can induce the generation of various chemokines and cytokines including IL-25, IL-33, and TSLP.^{24,25} Viruses also upregulate leukotriene C₄ synthase (LTC₄S) expression and induce the secretion of cysLT²⁶ in epithelial cells. Potential cell types that may be responsible for IL-13 and IL-5 production during viral infections include Th2 and type 2 innate lymphoid cells (ILC2s), a novel innate cell lineage. ILC2s rapidly elicit large amounts of IL-5 and IL-13 in response to IL-25 and IL-33 stimulation.²³ Both ILC2²⁷ and Th2²⁸ functionally express cysteinyl leukotriene receptor 1 (cysLT₁R), and leukotriene D₄ (LTD₄)-induced IL-5 generation from ILC2 was inhibited by LTRA.²⁷ Therefore, cysLTs generated during a viral infection may stimulate ILC2 and Th2 to generate IL-5 and IL-13, and LTRA may downregulate cytokine generation from these cells. In addition, cysLT₁R is expressed in various allergic inflammatory cells including mast cells, eosinophils, and basophils,²⁹ and these cells also possess cysLT synthetic enzymes. Further, LTRA may suppress the amplification of cysLT-induced inflammation by these cells.

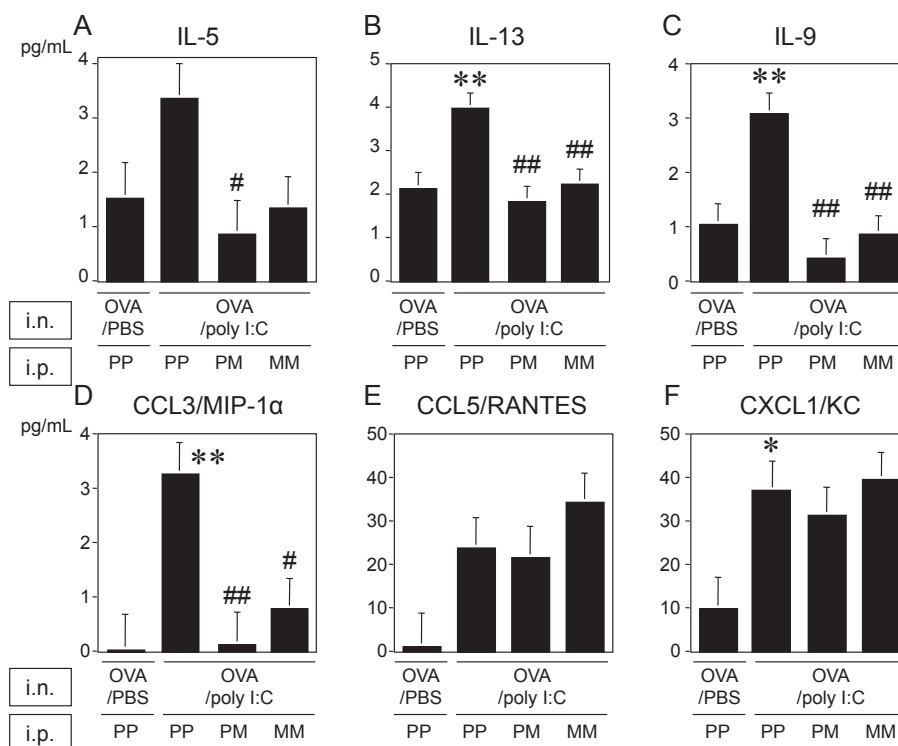


Fig. 4. The levels of cytokines (A–C) and chemokines (D–F) in BALF ($n = 6–8$). Each left lane shows simple asthma model (OVA/PBS (PP) group): Ovalbumin (OVA) intranasal (i.n.) challenge on phase 1, followed by PBS on phase 2 with intraperitoneal (i.p.) injections of PBS during phase 1–2. Other lanes show double-stranded RNA-induced asthma exacerbation model: OVA i.n. challenge in phase 1, followed by poly I:C in phase 2. Montelukast or PBS was i.p. administered as follows: OVA/poly I:C (PP) [Control group: phase 1/2: PBS/PBS], OVA/poly I:C (PM) [Reliever model: phase 1/2: PBS/montelukast], OVA/poly I:C (MM) [Controller model: phase 1/2: montelukast/montelukast]. Data are expressed as means $\pm$ standard error of the mean value. Data were analyzed using the two-way analysis of variance test followed by the Tukey–Kramer test to compare individual groups. ** $p < 0.01$, * $p < 0.05$ versus OVA/PBS (PP) and ## $p < 0.01$, # $p < 0.05$ versus OVA/poly I:C (PP).

Another mechanism may include the inhibitory effect of LTRA on epithelial cells. Although cysLT₁R expression has not been confirmed in bronchial epithelial cells,²⁹ these cells can generate chemokines in response to LTD₄.³⁰ A mechanism of virus-induced increase in chemokine levels may involve autocrine or paracrine effect of the epithelial cell-derived cysLTs,²⁶ and this effect may be inhibited by LTRA. The effect of LTRA was chemokine specific in this study. Although a significant decrease in murine eosinophil-directed chemokine CCL3³¹ was observed, significant effects on CCL5 and CXCL1 were lacking. These findings might partly explain the different effects of LTRA on BALF eosinophil, macrophage, and neutrophil numbers, but further investigation is required to clarify the precise mechanisms.

Concerning AHR, poly I:C upregulates the functional expression of M3 muscarinic receptors (M3R) in airway smooth muscle (ASM),³² supporting the current finding that AHR to Ach was tended to be upregulated by poly I:C. In this study, LTRA tended to downregulate AHR in the OVA/poly I:C group to levels lower than those in the simple asthma model (Fig. 3). If M3R expression in the OVA/poly I:C group is mainly under the control of cysLTs, M3R function may be strongly reduced by LTRA. The precise effect of cysLTs on M3R function in ASM is still unknown, and further investigation is required to clarify this point.

Most asthma patients in the real-world setting are already prescribed inhaled corticosteroids (ICS) before exacerbation. However, it has been reported that there is a corticosteroid insensitive component in the mechanism of virus-induced asthma exacerbation. *In vitro* experiments infecting bronchial epithelial cells with RSV resulted in increased LTC₄S mRNA expression and cysLT generation, but corticosteroid could not significantly alter

LTC₄S mRNA expression,²⁶ suggesting a requirement for further blockade of the cysLT pathway. TSLP induced by viral infection of epithelial cells led to steroid resistance in ILC2 cells.³³ Also, in the dsRNA-induced exacerbation model, chemokine generation could not be completely suppressed by oral corticosteroids.²⁰ These findings suggest a potential role of LTRA in virus-induced exacerbation in patients who were already treated by ICS.

In this study, treating with montelukast effectively augmented the pathophysiology of exacerbation. In addition to the controller model, the reliever model, where montelukast was administered only during the dsRNA challenge, showed downregulation of the number of eosinophil in BALF, AHR, and cytokine/chemokine generation. These findings suggest the effectiveness of adding LTRA, even after the symptoms of viral infection emerge. In fact, a small human study reported that adding LTRA to prednisolone (PSL) after infection-induced asthma exacerbation significantly reduced the cumulative dose of PSL.³⁴ These findings merit an additional large clinical investigation in future.

In the current study, we clearly showed that the pathophysiology of dsRNA-induced exacerbation was downstream of cysLTs. However, to clarify the role of cysLTs in virus-induced exacerbation, further investigations are required concerning the effect of viral components on the level of local generation of cysLTs, the number of cysLT₁R expressing cells, including ILC2, and the level of functional expression of cysLT₁R in various cells.

In conclusion, LTRA suppressed multiple aspects of pathophysiology in a dsRNA-induced asthma exacerbation model, suggesting the importance of cysLTs as potential treatment targets for virus-induced asthma exacerbation. Because LTRAs are already available in clinical practice, further precise positioning of LTRA in

asthma exacerbation is critical to reduce severe exacerbations leading to the asthma deaths.

Acknowledgments

The authors thank Ms. Manami Okabe, Ms. Chise Tamura, and Ms. Rika Kawamura for their excellent technical help and Ms. Asako Tsukamoto for her outstanding secretarial assistance. We are also grateful to Dr. Yusuke Tanaka, Dr. Michio Kuramochi, Dr. Hidenori Arai, Dr. Maho Suzukawa, and Dr. Masao Yamaguchi for helpful discussions. The authors would like to thank Enago (www.enago.jp) for the English language review.

This study was funded by Grants-in-aid for Scientific Research by the Ministry of Education, Culture, Sports, Science and Technology of Japan to HN (20590934).

Conflict of interest

The authors have no conflict of interest to declare.

Authors' contributions

MU, NS and HN designed the study, conducted experiments, interpreted the results, and wrote manuscripts. YuK, SR, YaK, KHA conducted experiments. NY and KO developed A/J mouse asthma model. KO designed the study and interpreted the results. All authors approved the final version of the manuscript.

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