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Protective effect of resolvin E1 on the development of asthmatic airway inflammation

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ABSTRACT

Resolvin E1 (RvE1) is an anti-inflammatory lipid mediator derived from the omega-3 fatty acid eicosapentaenoic acid (EPA), and strongly acts in the resolution of inflammation. We previously reported that RvE1 dampens airway inflammation and hyperresponsiveness in a murine model of asthma. In the present study, to elucidate the effects of RvE1 on the development of asthmatic airway inflammation, we investigated whether RvE1 acts on different phases of an OVA-sensitized and -challenged mouse model of asthma. RvE1 treatments at the time of either OVA sensitization or at the time of OVA challenge were investigated and compared with RvE1 treatments at the time of both OVA sensitization and challenge. After RvE1 was administered to mice intraperitoneally at the time of both OVA sensitization and challenge, there were decreases in airway eosinophil and lymphocyte recruitment, as well as a reduction in Th2 cytokine and airway hyperresponsiveness. RvE1 treatment at the time of either OVA sensitization or challenge also improved AHR and airway inflammation. Our results suggest that RvE1 acts on several phases of asthmatic inflammation and may have anti-inflammatory effects on various cell types.

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1. Introduction

Resolvins were first identified for their ability to resolve inflammatory exudates and are now recognized as a class of anti-inflammatory, pro-resolving lipid mediators derived from omega-3 polyunsaturated fatty acids [1]. RvE1, which is a member of the E series of resolvins, is derived from eicosapentaenoic acid (EPA) [1,2]. RvE1 is produced by the cyclo-oxygenase (COX)-2 pathway in the presence of aspirin and is regenerated in an aspirin-triggered form. A growing body of evidence indicates that resolvins possess potent anti-inflammatory and immunoregulatory actions that include blocking the production of pro-inflammatory mediators and regulating the trafficking of leukocytes and mediators to sites of inflammation [3]. In a mouse TNBS (2,4,6-trinitrobenzenesulfonic acid)-induced colitis model, RvE1 decreased neutrophil recruitment and pro-inflammatory gene expression, reduced weight loss, and improved survival [4]. In a rabbit *Porphyromonas gingivalis*-induced periodontitis model, RvE1 reduced neutrophil infiltration, prevented connective tissue and bone loss, promoted healing of diseased tissue, and regenerated lost soft tissue and bone [5].

Asthma is a complex syndrome characterized by airway inflammation and airway hyperresponsiveness (AHR). In asthmatic patients, numerous inflammatory cells such as eosinophils and lymphocytes infiltrate peribronchial tissue [6]. In a previous study, we reported that RvE1 dampens airway inflammation and hyperresponsiveness in a mouse model of asthma [7]. Moreover, Haworth et al. demonstrated that RvE1 regulates IL-23, IFN-γ, and LXA_4 to promote the resolution of allergic airway inflammation in a mouse model of asthma [8]. Findings from two laboratories have recently linked RvE1 to the control of allergic airway responses [9]. However, little is known about the mechanism of RvE1 action regarding asthmatic inflammation. To elucidate the mechanism of action of RvE1 in asthmatic inflammation, we investigated the effect of RvE1 on the sensitization phase and/or challenge phase of a mouse experimental model of asthma. RvE1 treatment at the time of either OVA sensitization or challenge improved AHR and airway inflammation. We speculated that RvE1 would act on dendritic cells (DCs) because ChemR23 has been identified as a receptor for RvE1 and is expressed on DCs. Moreover, it has been proposed that airway DCs are not only crucial for sensitization to inhaled antigens, leading to allergies, but also play a crucial role in established inflammation [10]. However, the effect of RvE1 on DCs in asthma *in vivo* is not yet known.

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Our results suggest that RvE1 may play a role in the development of asthmatic airway inflammation by acting on various cell types, including DCs.

2. Materials and methods

Five-week-old female BALB/c mice were obtained from Charles River Laboratories (Tsukuba, Japan). RvE1 was purchased from Cayman Chemical Co. (Ann Arbor, MI), and its physical and biological properties were assessed.

The experimental protocol is shown in Fig. 1A. Balb/c mice were divided into five groups. Group 1 (named N) mice were untreated, i.e., given i.p. injections with PBS and nebulized with PBS. Group 2 (named V) mice were sensitized with i.p. injections of ovalbumin (OVA) (Grade V; Sigma Chemical, St. Louis, MO) (10 μg) plus 1 mg of aluminum hydroxide as an adjuvant in 0.2 ml on days 0 and 14 and challenged with OVA nebulization (1% in PBS, for 20 min) on days 28, 29, and 30. Group 3 (named R) mice were administered i.p. RvE1 (1.0 μg per mouse; 50 μg/kg) on days −8, −1, 6, 13, 20, 27, 28, 29, and 30 before the induction of bronchial asthma. The protocol of i.p. injection of RvE1 was partly based on the method of Arita et al. [4]. Group 4 (named RC) mice were administered i.p. RvE1 (1.0 μg per mouse; 50 μg/kg) on days 28, 29, and 30. Group 5 (named RS) mice were administered i.p. RvE1 (1.0 μg per mouse; 50 μg/kg) on days -8, -1, 6, and 13. Group 3, 4, and 5 mice were sensitized and challenged with OVA using the same protocol with Group 2. On day 32, 48 h after the last aerosol challenge, airway responsiveness to aerosolized methacholine (Mch; 0, 2.5, 5, 10, and 20 mg/ml, for 3 min) was measured, bilateral bronchoalveolar lavage (BAL) was performed, and tissues were harvested for histological analysis. After measurement of airway responsiveness, mice were given a lethal dose of pentobarbital and the lungs were lavaged. The lavage fluid was centrifuged and the BAL fluid supernatant was stored at −20 °C prior to subsequent assay. The cell pellet was resuspended in 1 ml of Hanks' balanced salt solution. For total cell count, stained cells were counted in a Neubauer chamber. Differential cell counts were made on cytospin preparations, and performed by staining with May-Grünwald-

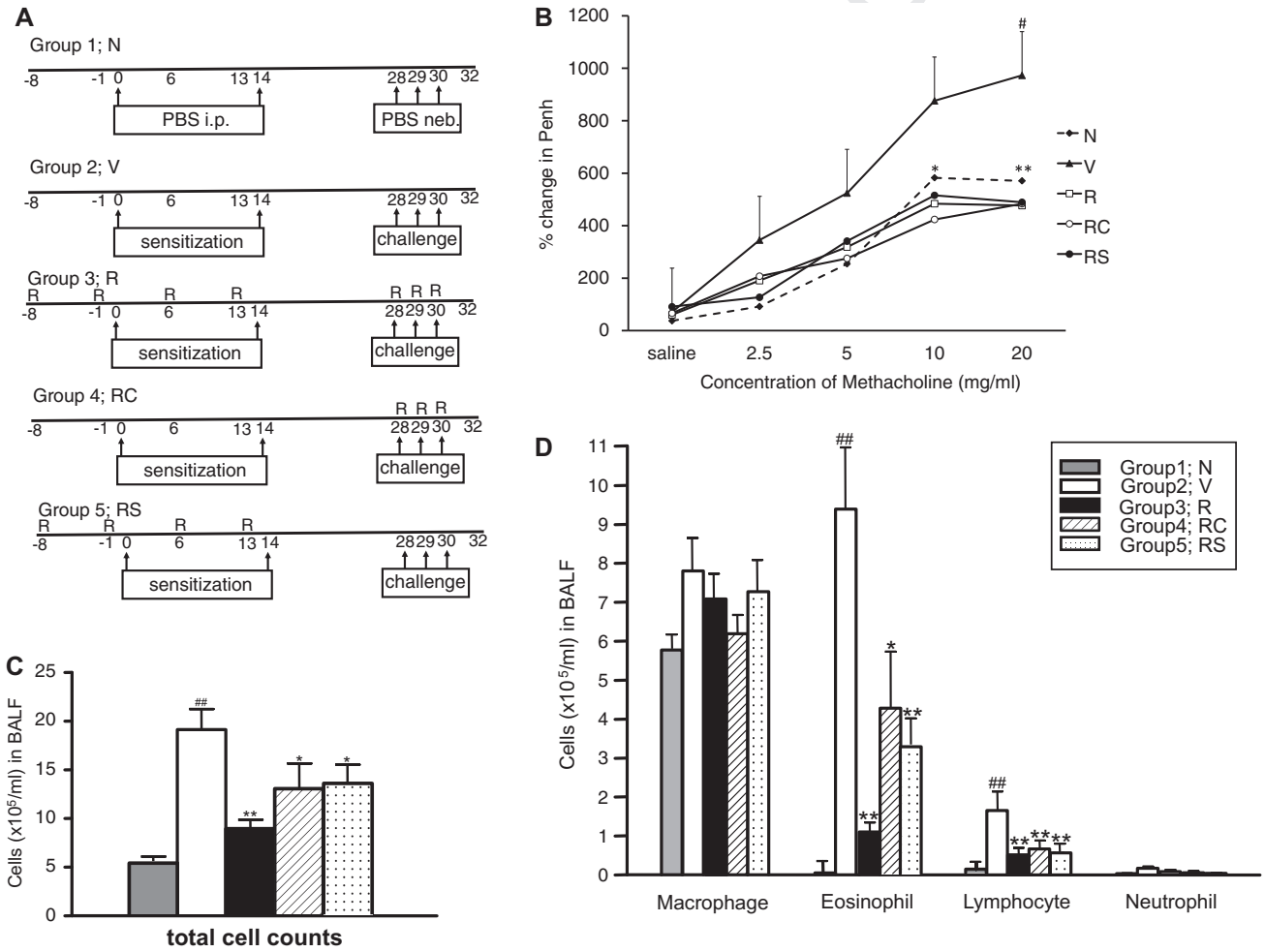

Fig. 1. (A) Schematic summary of experimental protocol. Mice in each Group, 1–5, were treated or untreated as described in Section 2. (B) RvE1 treatments at either the sensitization or challenge phase decreased airway responsiveness to aerosolized methacholine. The degree of bronchoconstriction is expressed as enhanced pause (Penh), as described in Section 2. Relative value of increase to baseline was evaluated as an index of airway responsiveness. RvE1 treatment at both the time of sensitization and challenge significantly decreased AHR to aerosolized Mch. Group 4 (named RC) significantly decreased AHR. Moreover, RvE1 treatment at the sensitization phase significantly suppressed AHR in Group 5 (named RS). Relative values of increase to baseline are expressed as mean ± SEM; $n = 10$ per group. #Significantly different from control group $p < 0.05$. *Significantly different from vehicle group $p < 0.05$. **Significantly different from vehicle group $p < 0.01$. (C and D) RvE1 treatments at either the sensitization or challenge phase prevented airway inflammation in OVA-sensitized and -challenged mice. BAL was isolated as described in Section 2. In sharp contrast, RvE1 administration at both the sensitization and challenge time led to significant decrements in the numbers of total cells (C), eosinophils, and lymphocytes in BAL fluid (D). RvE1 treatment at either the sensitization or challenge phase also led to decrements in the numbers of total cells (C), eosinophils, and lymphocytes in BAL fluid (D). Values are expressed as mean ± SEM; $n = 10$ per group. #Significantly different from control group $p < 0.05$. ##Significantly different from control group $p < 0.01$. *Significantly different from vehicle group $p < 0.05$. **Significantly different from vehicle group $p < 0.01$.

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Giemsa stain. Cells were identified according to standard morphology.

Airway responsiveness was measured in mice 48 h after the final OVA challenge by recording respiratory pressure curves by whole body plethysmography (Buxco Electronics, Inc., Troy, NY) in response to inhaled methacholine according to the method of Hamelmann et al. [11]. In conscious mice, airway responsiveness was measured by recording the enhanced pause (Penh) values using whole body plethysmography obtained in response to inhaled methacholine. Peak and 5-min average Penh values were determined. To compare the increase of Penh, the relative value of the increase to the baseline was evaluated as an index of airway responsiveness. Evaluating airway resistance by Penh has some limitations, for example Penh can be influenced by nasal resistance. This is why we also assessed airway resistance in some settings as described in a previous paper [12]. All experimental animals and protocols used in this study were approved by the institutional animal care and use committee of Gunma University Graduate School of Medicine.

The histological analysis by staining with hematoxylin and eosin (H&E) was performed as reported previously [7]. Goblet cells were evaluated on Periodic Acid-Schiff (PAS)-stained lung sections.

BAL supernatant cytokine levels were determined using commercially available ELISAs according to the manufacturers' instructions. Assays of IL-4, IL-5, IL-13, and RANTES in supernatants were determined using the Bio-Plex Suspension Array System (Nippon Bio-Rad Laboratories, Tokyo, Japan).

Levels of anti-OVA IgE were measured in serum by ELISA using paired antibodies according to the manufacturer's instructions (Dainippon Sumitomo Pharma Co., Osaka, Japan).

All values are expressed as means ± SEM. Nonparametric analysis of variance (Kruskal–Wallis method) was used to determine significance among groups. We used the Mann–Whitney *U* test to analyze for significant differences between individual groups, and a value of $p < 0.05$ was considered significant.

3. Results

3.1. RvE1 prevents OVA-induced airway hyperresponsiveness

In a previous study, we showed that RvE1 administration prevented AHR in OVA-sensitized Balb/c mice [7]. In the present study, mice were divided into five experimental groups and each experiment was performed on each group ($n = 10$ per group). The schematic protocol is shown in Fig. 1A. In OVA-sensitized and -challenged mice (Group 2, named V in Fig. 1A), airway responsiveness to aerosolized Mch was significantly increased when compared with untreated mice (Group 1, named N). When RvE1 was administered to mice intraperitoneally at both the sensitization and challenge phases (Group 3, named R in Fig. 1A), AHR to Mch was markedly reduced as shown in our previous study [7]. Moreover, RvE1 treatment during the challenge but after OVA sensitization (Group 4, named RC in Fig. 1A) significantly decreased AHR to aerosolized Mch in OVA-sensitized and -challenged mice (Fig. 1B). Next, we investigated whether RvE1 treatment at the time of OVA sensitization could modulate AHR in OVA-sensitized and -challenged mice. RvE1 treatment at the sensitization phase significantly suppressed AHR to aerosolized Mch in Group 5 (named RS). These results indicate that Mch-induced bronchoconstriction is significantly reduced by administration of RvE1 at several phases.

3.2. RvE1 inhibits allergic pulmonary inflammation

Persistent airway inflammation and AHR have been observed in patients with asthma as well as in asthma animal models. Leukocytic infiltrates are found within airway lumen, as observed in BAL, and in the lung interstitium, as observed in histological sections. The predominant leukocyte is the eosinophil in conjunction with lymphocytes. We determined the extent of inflammation in both compartments of the lung in sensitized mice after serial OVA challenge (Fig. 1C). Cell recruitment to BAL and lung was comparable between untreated mice (Group 1, named N) and OVA-sensitized and -challenged mice (Group 2, named V). Differential counts (Fig. 3B) revealed that infiltrates in Group 2 mice were composed mainly of eosinophils and lymphocytes. In BAL fluid, total leukocytes, eosinophils, and lymphocytes in Group 3 (named R) mice were sharply reduced. Administration of RvE1 before OVA challenge (Group 4) or sensitization (Group 5) significantly reduced the total cell number and the numbers of eosinophils and lymphocytes in the BAL fluid of OVA-sensitized and -challenged mice.

The extent and anatomical location of leukocyte infiltrates was determined in H&E-stained and PAS-stained sections taken from mice 48 h after the final allergen challenge (Fig. 2A). The challenge with OVA induced widespread peribronchiolar and perivascular inflammation, which was primarily eosinophilic (Fig. 2B). Mice receiving RvE1 before the sensitization and challenge (Fig. 2C) had substantially less eosinophils and lymphocytes in the peribronchial regions and airspaces compared with OVA-sensitized and -challenged mice. RvE1 treatment at the time of challenge with OVA improved airway inflammation in OVA-sensitized and -challenged mice (Fig. 2D). Moreover, RvE1 treatment at the time of sensitization with OVA improved airway inflammation in these mice (Fig. 2E). Although respiratory mucus protects the lower airways from dehydration and damage, excessive secretion from hyperplastic goblet cells contributes to the morbidity and mortality of many respiratory diseases, including asthma. The number of goblet cells stained with PAS was lower in mice that received RvE1 at both phases (Group 3) (Fig. 2H) compared with OVA-sensitized and -challenged mice (Fig. 2G). RvE1 treatment during challenge/OVA sensitization and challenge (Group 4), and RvE1 treatment at sensitization/OVA sensitization and challenge (Group 5) showed lower PAS-stained goblet cells (Fig. 2I and J).

Serum OVA-specific IgE levels in Group 1–5 mice were 20 ng/ml, 8000 ng/ml ($p < 0.05$, Group 1 vs. Group 2), 3000 ng/ml ($p < 0.05$, Group 2 vs. Group 3), 5135 ng/ml ($p < 0.05$, Group 2 vs. Group 4), and 3490 ng/ml ($p < 0.05$, Group 2 vs. Group 5), respectively. Serum OVA-specific IgE levels in Groups 3, 4, and 5 were significantly different from Group 2 (Fig. 3A). These results indicate that administration of RvE1 can significantly inhibit the features of allergic pulmonary inflammation, including leukocyte infiltration and the formation of specific key mediators in airway pathophysiology.

IL-4, IL-5, IL-13, and RANTES are thought to be key Th2 cytokines that affect many of the cell types involved in asthma pathogenesis, such as eosinophil recruitment and activation, goblet cell metaplasia, mucus hypersecretion in epithelial cells, smooth muscle proliferation, and cholinergic-induced smooth muscle contraction [13]. Their levels in BAL fluid in Groups 3, 4, and 5 were significantly reduced between OVA-sensitized and -challenged mice (Fig. 3B–E). Together, these results indicate that RvE1 significantly reduced allergic pulmonary inflammation.

4. Discussion

We previously showed that one of the resolution molecules, EPA-derived RvE1, can play an important suppressive role in the development of allergen-induced AHR [7]. Here we showed that administration of RvE1 during OVA challenge suppressed AHR to inhaled methacholine and airway inflammation in OVA-sensitized and

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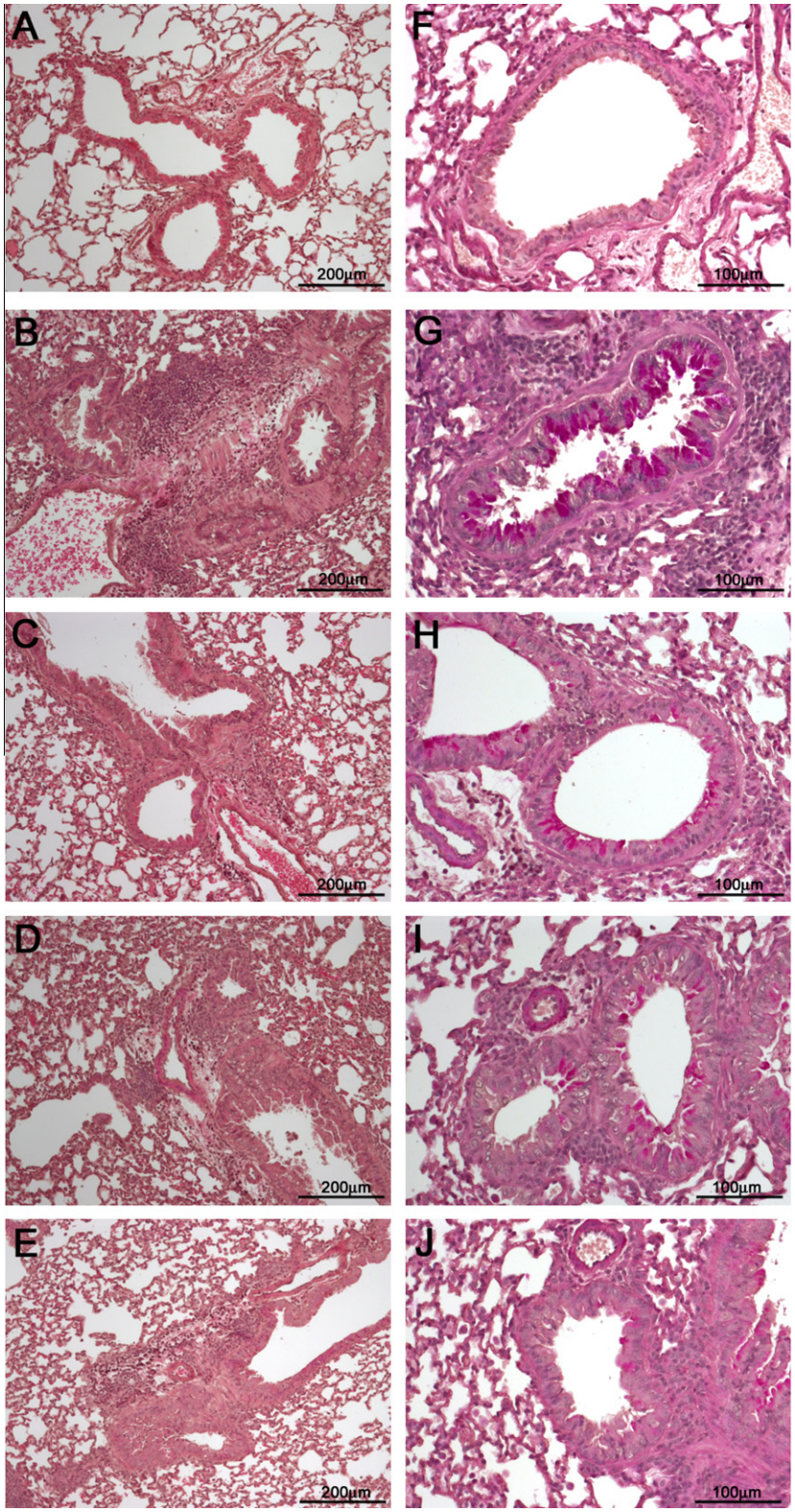

Fig. 2. Protective effect of RvE1 on airway inflammation in lung tissue. Each panel show lung tissues from each corresponding group; panels A and F from Group1, panels B and G from Group 2, panels C and H from Group 3, panels D and I from Group 4, and panels E and J from Group 5. (A–E) shows H&E-stained and (F–J) shows PAS-stained sections. RvE1 treatment (Groups 3–5) improved airway inflammation in OVA-sensitized and -challenged mice (C–E). RvE1 treatment (Groups 3–5) showed fewer PAS-stained goblet cells in OVA-sensitized and -challenged mice (H–J).

-challenged mice (RC group). Moreover, treatment with RvE1 at the time of OVA sensitization also improved AHR and airway inflammation, including Th2 cytokine production, in the BAL fluid in OVA-sensitized and -challenged mice (RS group). Both treatments also prevented OVA-specific IgE production significantly. In our experiments, we showed the effects on airway hyperresponsiveness using

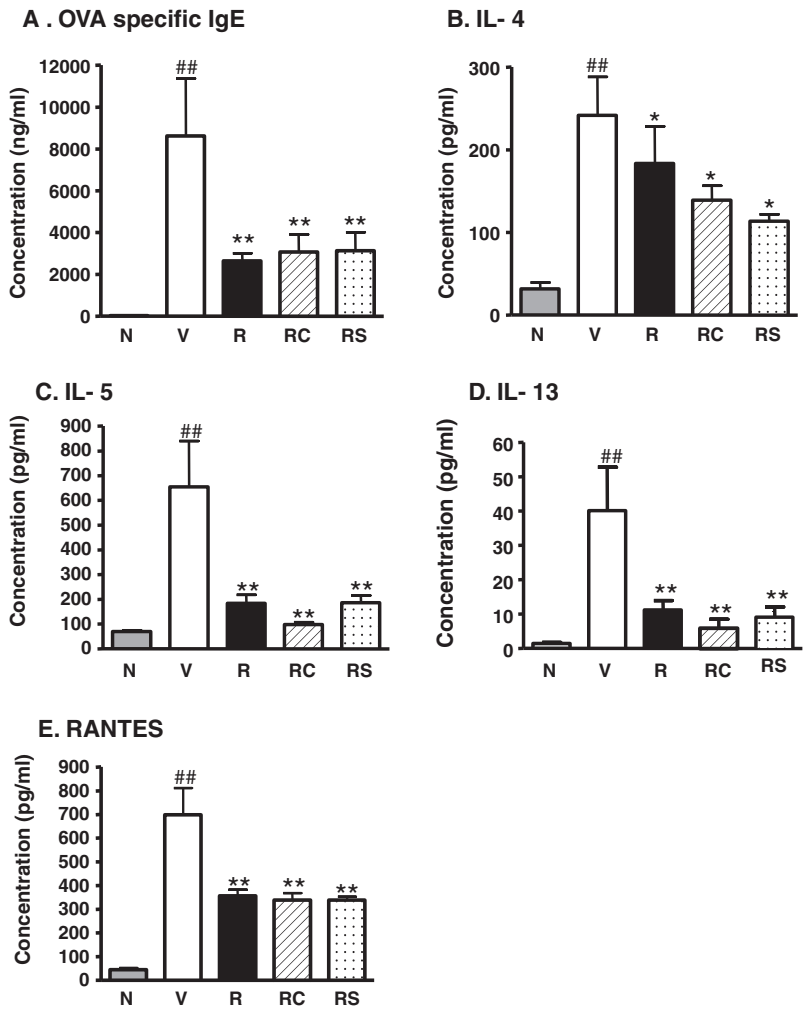

Fig. 3. Effect of RvE1 on OVA-specific IgE (A) and IL-4 (B), IL-5 (C), IL-13 (D), and RANTES (E) production. Serum IgE levels and IL-4, IL-5, IL-13, and RANTES concentrations in BAL fluid were assessed by ELISA. RvE1 treatment at both the sensitization and challenge phase (Group 3) decreased both serum OVA-specific IgE (A) and IL-4, IL-5, and RANTES (B–E). RvE1 treatment at the challenge phase (Group 4) and at the sensitization phase (Group 5) also decreased both serum OVA-specific IgE (A) and IL-4, IL-5, and RANTES in BAL fluid (B–E). These results are expressed as the mean ± SEM of 10 animals. ##Significantly different from control group $p < 0.01$. *Significantly different from vehicle group $p < 0.05$. **Significantly different from vehicle group $p < 0.01$.

body plethysmography. Evaluating airway resistance by Penh sometimes has limitations, such as being influenced by nasal resistance. This is why we also assessed airway resistance as described in our previous paper [12]. The results from airway resistance measurement showed the same tendency as those from Penh measurement (data not shown). Our results suggest that the administration of RvE1 at the time of either sensitization or challenge can prevent allergen-specific Th2 cell development and IgE production in an experimental mouse model of asthma.

In a previous study of ours, we speculated that the inhibitory effect of RvE1 on the LTB_4 and BLT1 axis could be important [7]. RvE1 interacts with the LTB_4 receptor, BLT1, on human PMN as a partial antagonist resulting in the inhibition of the LTB_4-induced calcium response in human peripheral blood mononuclear cells [14]. Recent studies in allergen-induced AHR and inflammation using mice lacking BLT1 have shown crucial roles for LTB_4 and BLT1 in Th2 cytokine production from lung T cells and recruitment of antigen-specific effector $CD8^+$ T cells [15–18]. The LTB_4-BLT1 interaction leads to the recruitment/activation of allergen-specific $CD4^+$Th2 and allergen-specific effector $CD8^+$ T cells. This is why the LTB_4-BLT1 pathway is thought to be an important target for the treatment of bronchial asthma. When RvE1 is administered before both OVA sensitization and OVA challenge, BLT1 might be blocked by RvE1, and the LTB_4-BLT1 interaction leading to IL-4, IL-5, IL-13, and IgE production can be prevented. Pulmonary eosinophilia was also sharply reduced in mice given RvE1. Eosinophil recruitment to the lung in asthma is primarily a consequence of Th2 lymphocyte activation, which was reduced by RvE1, as shown by the lower levels of Th2 cytokine in BAL fluid and the decreased number of lymphocytes in both BAL fluid and lung tissue. Although it has not yet been demonstrated, RvE1 might act on eosinophils directly through the receptor BLT1 leading to the apoptosis of eosinophils and the inhibition of eosinophil migration. So it might be that one of the mechanisms by which the administration of RvE1 during OVA challenge suppressed AHR to inhaled methacholine and airway inflammation in OVA-sensitized and -challenged mice is the inhibition of LTB_4 and BLT1.

In the present study, even in the experimental group during the sensitization phase treatment of RvE1, AHR and airway inflammation were also reduced. It could be that DCs are also affected by RvE1 in this mouse model of asthma. ChemR23 has been identified as a receptor for RvE1 [19]. ChemR23 is expressed on monocytes, macrophages, and DCs. Among the human peripheral blood leukocytes, ChemR23 was abundantly expressed in monocytes, with lower amounts in neutrophils and T lymphocytes, findings consistent with the observation that this receptor is expressed in APCs such as macrophages and DCs. RvE1 transmits signals as a selective ago-

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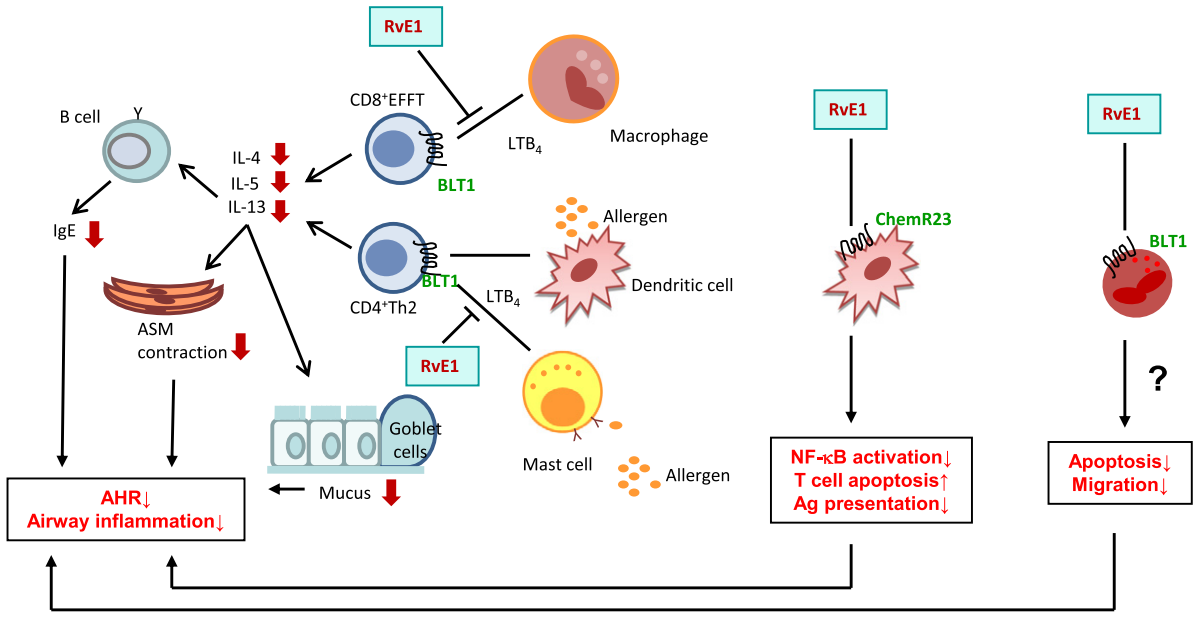

Fig. 4. Presumed mechanisms of the protective effect of RvE1 on allergic airway disease in sensitized hosts following allergen exposure. When RvE1 is administered, BLT1 might be blocked by RvE1, and the LTB_4-BLT1 interaction leading to IL-4, IL-5, IL-13, and IgE production can be prevented. Moreover, RvE1 might act on DCs via an RvE1 and ChemR23 interaction and prevent antigen presentation, and induce T cell apoptosis and inhibition of NF-κB activation. APC, antigen-presenting cell; $CD8^+_{EFFT}$, effector memory $CD8^+$ T cell; ASM, airway smooth muscle; AHR, airway hyperresponsiveness.

nist via ChemR23 and counter regulates TNF-α-stimulated NF-κB activation [19]. RvE1 may serve as an autocrine and/or paracrine signal during resolution to terminate further NF-κB activation and cytokine production in a temporal and spatially regulated fashion. NF-κB plays a critical role in host defence and in chronic inflammatory diseases such as asthma. Many extracellular stimuli, including viruses, oxidants, inflammatory cytokines, and immune stimuli, activate NF-κB. Glucocorticoids also inhibit activated NF-κB and this is likely to be important in the anti-inflammatory action of steroids. Recently, Vassiliou et al. reported the effects of RvE1 on DCs *in vitro*. Bone marrow-derived DCs (BMDC) exposed to RvE1, especially during differentiation, have been shown to acquire the capacity to induce apoptosis of activated T cells through the induction of indoleamine 2,3-dioxygenase [20]. Under the effect of RvE1, these BMDCs do not switch their chemokine receptor profile from CCR5 to CCR7, even following LPS stimulation. RvE1-exposed DCs maintain an immature chemokine receptor expression pattern even following TLR stimulation, with high CCR5 and no CCR7 expression. This effect implies that DCs exposed to RvE1 and pathogens remain at the inflammatory site instead of migrating to the lymph nodes, and induce apoptosis in effector T cells infiltrating the inflammatory site. As it has been proposed that airway DCs are not only crucial for sensitization to inhaled antigens, leading to allergies, but also play a crucial role in established inflammation, DCs represent a therapeutic target to prevent the development of airway diseases [21]. Taken together, antagonist activity for pro-inflammatory BLT1 receptors and agonist activity for anti-inflammatory ChemR23 receptors could provide a molecular basis for the various anti-inflammatory effects of RvE1.

Haworth et al. demonstrated that RvE1 regulates IL-23, IFN-γ, and lipoxin A_4 (LXA_4) to promote the resolution of allergic airway inflammation in a mouse model of asthma [8]. Both our results and those of Haworth et al. show that the anti-inflammatory lipid mediator RvE1 reduces both allergic airway inflammation and hyperresponsiveness, which are characteristic features of asthma. However, there are some differences between these reports. Haworth et al. highlighted the resolution phase to investigate the effect of RvE1. They used a protocol to induce allergic airway inflammation in which mice were sensitized with OVA on days 0 and 7 and challenged with aerosolized OVA on days 14–17. RvE1 was administered for 3 consecutive days after the final allergen challenge. Between protocol days 18 and 25, which was the resolution phase, the endogenous resolution interval was confirmed by counting BAL fluid leukocytes. According to the results from Haworth and colleagues, the mechanism by which RvE1 promotes the resolution of inflammation is as follows [8]: RvE1 decreases IL-17A, IL-23, IL-6, and LTB_4, all of which are involved in the maintenance of airway inflammation, and increases IFN-γ and LXA_4, which are required for the resolution of inflammation. The mechanism of RvE1 in the regulation of Th17 cells, through the suppression of IL-23 and IL-6, is especially emphasized. In this way, RvE1 may play important roles in the resolution phase of the mouse model of asthma. Taken together with our data, RvE1 may act on various cells including lymphocytes, PMNs, and DCs to prevent the development of asthmatic inflammation in the mouse experimental model. We speculate that RvE1 plays important roles by different anti-inflammatory mechanisms depending on the inflammation phase (Fig. 4).

In conclusion, our results demonstrate broad anti-inflammatory effects and protective and regulatory roles for RvE1 in airway hyperresponsiveness and asthmatic inflammation. RvE1 may promote the resolution of inflammation via multiple mechanisms. In light of its ability to inhibit both of these key asthma phenotypes *in vivo*, RvE1 may represent a new treatment and therapeutic approach for asthma. Further studies are needed to understand the precise mechanism of anti-inflammatory action of RvE1, and to realize useful treatments for asthma.

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