Resolvin E3 attenuates allergic airway inflammation *via* the interleukin-23–interleukin-17A pathway

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ABSTRACT: We investigated the effects of resolvin E (RvE) 1, RvE2, and RvE3 on IL-4– and IL-33–stimulated bone marrow–derived dendritic cells (BMDCs) from house dust mite (HDM)-sensitized mice. We also investigated the role of RvE3 in a murine model of HDM-induced airway inflammation. *In vitro*, BMDCs from HDM-sensitized mice were stimulated with IL-4 and IL-33 and then treated with RvE1, RvE2, RvE3, or vehicle. RvE1, RvE2, and RvE3 suppressed IL-23 release from BMDCs. *In vivo*, RvE3 administrated to HDM-sensitized and challenged mice in the resolution phase promoted a decline in total numbers of inflammatory cells and eosinophils, reduced levels of IL-23 and IL-17 in lavage fluid, and suppressed *IL-23* and *IL-17A* mRNA expression in lung and peribronchial lymph nodes. RvE3 also reduced resistance in the lungs of HDM-sensitized mice. A NanoBiT β-arrestin recruitment assay using human embryonic kidney 293 cells revealed that pretreatment with RvE3 suppressed the leukotriene B4 (LTB4)–induced β-arrestin 2 binding to LTB4 receptor 1 (BLT1R), indicating that RvE3 antagonistically interacts with BLT1R. Collectively, these findings indicate that RvE3 facilitates the resolution of allergic airway inflammation, partly by regulating BLT1R activity and selective cytokine release by dendritic cells. Our results accordingly identify RvE3 as a potential therapeutic target for the management of asthma.—Sato, M., Aoki-Saito, H., Fukuda, H., Ikeda, H., Koga, Y., Yatomi, M., Tsurumaki, H., Maeno, T., Saito, T., Nakakura, T., Mori, T., Yanagawa, M., Abe, M., Sako, Y., Dobashi, K., Ishizuka, T., Yamada, M., Shuto, S., Hisada, T. Resolvin E3 attenuates allergic airway inflammation *via* the interleukin-23–interleukin-17A pathway. FASEB J. 33, 000–000 (2019). www.fasebj.org

KEY WORDS: house dust mite · ω-3 fatty acid · IL-17A

Asthma is a chronic airway inflammatory disease characterized by airway hyperresponsiveness and infiltration of leukocytes, including eosinophils and T lymphocytes, with increased levels of prophlogistic cytokines and lipid mediators (1). Although the majority of patients with asthma have symptoms that respond to standard therapies, such as inhaled corticosteroids, some 5–10% of asthmatic patients have symptoms that are refractory to current therapy.

The ω-3 fatty acids, namely, eicosapentaenoic acid (EPA) and docosahexaenoic acid, are abundant in fish oil, and epidemiologic studies have shown that dietary supplementation with fish oil or ω-3 fatty acids has beneficial effects on asthma (2–5). Resolvins are products of ω-3 fatty acids with potent anti-inflammatory properties (6–9). EPA-derived resolvin E (RvE)1 has previously been observed to show anti-inflammatory and proresolving effects on eosinophilic airway inflammation in a murine asthma model (10–13). Haworth *et al.* reported that RvE1 attenuates allergic airway inflammation through regulating IL-23 and IL-17A production. RvE1 has also been shown to

ABBREVIATIONS: AHR, airway hyperreactivity; BAL, bronchoalveolar lavage; BLT1R, LTB4 receptor 1; BMDC, bone marrow–derived dendritic cell; EGFR, epidermal growth factor receptor; EPA, eicosapentaenoic acid; GFP, green fluorescent protein; H&E, hematoxylin and eosin; HDM, house dust mite; HEK, human embryonic kidney; IC_{50}, half maximal inhibitory concentration; LTB4, leukotriene B4; P2A, peptide sequence from porcine teschovirus-1 2A; PAS, periodic acid–Schiff; PBLN, peribronchial lymph node; RvE, resolvin E; T_h, T helper

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inhibit IL-23 production *in vivo* and suppresses IL-23 release from LPS-stimulated bone marrow–derived dendritic cells (BMDCs) (12). RvE3 [(5*Z*,8*Z*,11*Z*,13*E*,15*E*,17*R*,18*R*)-17,18-dihydroxyicosa-5,8,11,13,15-pentaenoic acid], a novel member of the E series of resolvins, was recently identified in EPA-derived metabolites from eosinophils and has been found to exhibit potent anti-inflammatory action by inhibiting polymorphonuclear leukocyte infiltration in a zymosan-induced peritonitis model (14). However, the role of RvE3 in allergic inflammation is currently unknown.

In this study, we investigated the effect of RvE1, RvE2, and RvE3 on BMDCs and the effects of RvE3 on a murine model of asthma in order to gain an insight on the role of RvE3 in allergic airway inflammation. We also conducted a NanoBiT β-arrestin recruitment assay using human embryonic kidney (HEK)293 cells to determine whether RvE3 interacts with leukotriene B4 (LTB4) receptor 1 (BLT1R).

MATERIALS AND METHODS

Animals

Female BALB/c mice (5–8 wk old) were obtained from Charles River Laboratories (Wilmington, MA, USA) and housed under specific pathogen-free conditions in the animal facility of the Gunma University Standing Committee on Animals. Animal experiments were approved by the Institutional Animal Care and Use Committee of Gunma University Graduate School of Medicine (17-007).

A murine model of airway inflammation

House dust mite (HDM; *Dermatophagoides pteronyssinus*) extract was obtained from Greer Laboratories (Lenoir, NC; containing 0.0809 μg of Der p 1 and 1.7 μg of total protein). RvE1, RvE2, and RvE3 were obtained from the Faculty of Pharmaceutical Science, Hokkaido University (Hokkaido, Japan). Isofluorane-anesthetized mice were intranasally administered 65 μg of HDM extract (total protein) in 50 μl of PBS or 50 μl of PBS alone (control) on d 0, 1, 2, 14, 15, 16, and 17. RvE1 (2 μg) in 100 μl of PBS, RvE3 (2 μg) in 100 μl of PBS, or vehicle (100 μl of PBS) was intraperitoneally injected on d 17 and 18. The protocol used for sensitization and challenge was partly based on the method described by Gold *et al.* (15). The doses of RvE1 and RvE3 and the protocol used for injecting resolvins were selected based on a previous study, Aoki *et al.* (11), conducted on RvE1. On protocol d 19, 48 h after the final HDM challenge, airways were lavaged 3 times with PBS (0.1 ml), and the lung tissues were harvested for histopathological and mRNA expression analyses. Peribronchial lymph node (PBLN) and spleen tissues were also harvested for the analysis of mRNA expression. Differential cell counts (200 cells/slide) were determined in cytospin preparations obtained by centrifuging at 500 rpm for 5 min and staining with May-Grünwald-Giemsa stain.

Histopathological studies

Lung samples were fixed in 10% neutral buffered formalin, dehydrated through a graded ethanol series, and embedded in paraffin. The deparaffinized sections (4 μm thick) were stained with hematoxylin and eosin (H&E), and periodic acid–Schiff (PAS) for identifying mucus-secreting cells (goblet cells) in the airways. We enumerated the stained goblet cells in the large-caliber preterminal bronchi of at least 3 lung sections obtained from each animal. The length of the basal lamina of the corresponding bronchus was measured using ImageJ (National Institutes of Health, Bethesda, MD, USA). Goblet cell scores were calculated as previously described in Grünig *et al.* (16). Briefly, 40–80 consecutive airways from mice were categorized according to the abundance of PAS-positive goblet cells and assigned numerical scores (0: <5% goblet cells; 1: 5–25%; 2: 25–50%; 3: 50–75%; 4: >75%) in a blind manner based on methods previously described in Aoki *et al.* (11). The sum of the airway scores for each lung was divided by the number of airways examined to yield a histologic goblet cell score.

Measurement of cytokines

IL-4, IL-5, IL-13, IL-17, and IL-23 were measured using DuoSet ELISA Development Kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

Culture of BMDCs

Female BALB/c mice were intranasally administered 65 μg of HDM extract (total protein) in 50 μl of PBS on d 0, 1, and 2. On d 14, bone marrow cells obtained from the femurs and tibias of mice were placed in Roswell Park Memorial Institute (RPMI) 1640 medium containing 10% fetal bovine serum and recombinant mouse GM-CSF (10 ng/ml; R&D Systems) and recombinant mouse IL-4 (10 ng/ml; R&D Systems). The culture medium was replaced with the same medium containing these cytokines at 2-d intervals. On d 7, the cells were pulsed with IL-4 (10 ng/ml) and IL-33 (20 ng/ml) and treated with RvE1 (10 nM), RvE2 (10 nM), RvE3 (10 nM), or vehicle (PBS). After 24 h, the culture supernatants were analyzed for IL-23 using ELISA.

Measurement of mRNA

Total RNA was isolated from the lungs, PBLNs, and spleen of mice using RNAiso Plus (Takara, Kyoto, Japan; MilliporeSigma, Burlington, MA, USA), according to the manufacturer's instructions. After treatment with DNaseI (Promega, Madison, WI, USA) to remove traces of contaminant genomic DNA, 2 μg of the purified RNA was reverse-transcribed according to the manufacturer's recommendations (Thermo Fisher Scientific, Waltham, MA, USA). The expression of mRNA was measured by real-time quantitative TaqMan PCR using an Mx3000P Quantitative PCR system (Agilent Technologies, Santa Clara, CA, USA) as previously described in Mogi *et al.* (17), We used the following probes purchased from Thermo Fisher Scientific: murine IL-4 (Mm 00445259_m1), IL-5 (Mm00439646_m1), IL-13 (Mm00434204_m1), IL-17A (Mm00439618_m1), IL-23 (Mm00518984_m1), and glyceraldehyde-3-phosphate dehydrogenase (Mm99999915_g1).

Airway hyperreactivity to methacholine

Airway hyperreactivity (AHR) is one of the major hallmarks of asthma. In the present study, we measured lung resistance 48 h after the final HDM challenge. Anesthetized mice were mechanically ventilated using a Buxco BioSystem XA Resistance and Compliance Analyzer [Data Sciences International (DSI), St. Paul, MN, USA], and aerosolized methacholine (0, 1.25, 2.5, 5, and 10 mg/ml) was delivered in-line through the inhalation port for 2 min. Lung resistance was measured as a percentage of the baseline value with PBS nebulization using the mean of 10 readings taken for each concentration of methacholine.

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DNA synthesis

The coding sequence of rat β-arrestin 2 harbored within a β-arrestin 2–pEGFP-N1 plasmid [a gift from Robert Lefkowitz (Department of Medicine, Duke University Medical Center) (plasmid 35411; Addgene, Watertown, MA, USA)] (18) was inserted with a GS linker downstream of the SmBiT coding region in a pBiT2.1-*N* vector (Promega). In order to generate an HEK293 cell line that stably expresses the SmBiT-fused β-arrestin 2, we subsequently introduced a peptide sequence from porcine teschovirus-1 2A (P2A) (19) downstream of epidermal growth factor receptor (EGFR) in an EGFR–green fluorescent protein (GFP)–pEGFP-C1 plasmid (20). Thereafter, the EGFR coding region was replaced with the SmBiT–β-arrestin 2 coding sequence (SmBiT–β-arrestin 2–P2A-GFP–pEGFP-C1). The human BLT1R coding sequence in a Presto-Tango GPCR Kit [a gift from Bryan Roth (Department of Pharmacology, University of North Carolina, Durham, NC, USA) (1000000068; Addgene)] (21) was inserted with a GS linker upstream of the LgBiT coding region in a pBiT1.1-*C* vector (Promega) (BLT1R-LgBiT-pBiT).

NanoBiT β-arrestin recruitment assay

A NanoBiT β-arrestin recruitment assay was performed according to a method previously described in ref. 22 with slight modifications. HEK293S cells [a gift from Yoshinori Shichida (Research Organization of Science and Technology, Ritsumeikan University, Kusatsu Shiga, Japan)] were transfected with SmBiT–β-arrestin 2–P2A-GFP–pEGFP-C1 and grown in the presence of 400 μg/ml G418 for 2 wk. Colonies expressing GFP (SmBiT–β-arrestin 2 HEK293S cells) were picked up under a fluorescence microscope. These SmBiT–β-arrestin 2 HEK293S cells were transfected with BLT1R-LgBiT-pBiT (4 μg/plate) using Lipofectamine 3000 (Thermo Fisher Scientific) in a white collagen I–coated 96-well plate (Thermo Fisher Scientific) as described in ref. 23. After an overnight incubation at 37°C in 5% CO_2, the medium in each well was exchanged for 80 μl of 0.01% bovine serum albumin–HBSS containing resolvins or vehicle (ethanol) 1 h prior to measurement. The time course of the change in luminescence intensity was detected at room temperature in a microplate reader (FlexStation 3; Molecular Devices, Sunnyvale, CA, USA) using the following protocol: mode, luminescence, flex mode; wavelength, all; integration time, 500 ms; total run time 12 min; interval, 8 s; compound transfer 1, 20 μl 5× NanoGlo Live Cell Substrate (Promega) at 20 s; compound transfer 2, 25 μl 5× concentration ligands or vehicle diluted in 0.01% bovine serum albuin–HBSS at 200 s. This protocol enabled us to measure all lanes with the same equilibration time (3 min) after substrate addition. The data were normalized at the maximum intensity of wells stimulated with 1 μM LTB4 (Cayman Chemical, Ann Arbor, MI, USA) for the dose-response measurement of LTB4 and resolvins, or the mean intensity of vehicle-pretreated wells 240 s after 1 nM LTB4 stimulation for the competitive assay. The data were fitted using Eq. 1 or 2, where the half maximal inhibitory concentration (IC_{50}) is: the 50% inhibitory concentration:

$$f(x) = \text{bottom} + \frac{\text{top} - \text{bottom}}{1 + \frac{EC_{50}}{x}} \quad (1)$$

$$f(x) = \text{top1} + \frac{\text{bottom1} - \text{top1}}{1 + \frac{IC_{50}}{x}} + \text{bottom2} + \frac{\text{top2} - \text{bottom2}}{1 + \frac{EC_{50}}{x}} \quad (2)$$

The curve fittings and the illustrations in the figures were obtained using Igor Pro 8.03 (WaveMetrics, Lake Oswego, OR, USA).

Statistical analysis

Results are presented as means ± SEM. An ANOVA or Welch's *t* test was used to assess the statistical significance of differences. A value of $P < 0.05$ was considered significant.

RESULTS

RvE3 regulates IL-23 release from BMDCs

Given that RvE1 attenuates asthmatic inflammation by regulating IL-23 and IL-17A (12), we investigated the effect of RvE1, RvE2, and RvE3 on BMDCs. BMDCs prepared from HDM-sensitized mice were stimulated with IL-33 and IL-4 and treated with RvE1, RvE2, RvE3, or vehicle. We found that RvE1-, RvE2-, and RvE3-treated BMDCs showed significantly lower levels of IL-23 than the vehicle-treated BMDCs ($P < 0.05$) and that RvE3-treated BMDCs displayed lower IL-23 secretion than either RvE1- or RvE2-treated BMDCs (**Fig. 1**).

RvE3 attenuates HDM-induced airway inflammation and airway hyperresponsiveness

Airway inflammation is one of the characteristic features of asthma, and we further investigated the effect of RvE3 in an HDM-induced asthma model in mice. The mice were sensitized and challenged intranasally with HDM, and RvE3 or vehicle was injected intraperitoneally during the resolution phase. A schematic representation of the experimental protocol is shown in **Fig. 2*A***.

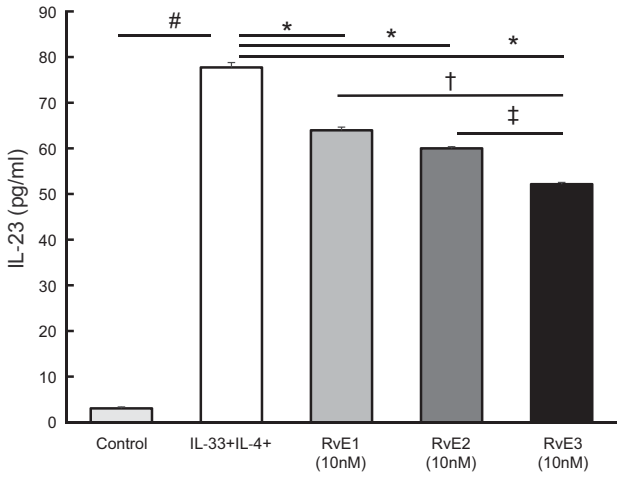

Figure 1. RvE3 regulates IL-23 release from IL-4– and IL-33–stimulated BMDCs obtained from HDM-sensitized BALB/c mice. IL-23 levels were significantly higher in IL-4– and IL-33–stimulated BMDCs compared with control (nonstimulated) BMDCs. RvE1, RvE2, and RvE3 treatments significantly reduced IL-23 levels. Values are expressed as means ± SEM. $^{\#}P < 0.05$, significantly different from the control BMDCs; $^{*}P < 0.05$, significantly different from the IL-4– and IL-33–stimulated and vehicle-treated BMDCs. $^{\dagger}P < 0.05$, significantly different from the RvE1-treated BMDCs; $^{\ddagger}P < 0.05$, significantly different from the RvE2-treated BMDCs.

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On protocol d 19, 48 h after the final HDM challenge, lung resistance was measured to evaluate AHR. Mice treated with RvE3 showed a reduction in maximum lung resistance (Fig. 2*B*). On protocol d 19, bronchoalveolar lavage (BAL) was performed. Compared with the vehicle-treated mice, RvE3-treated mice displayed a significant decrease in the number of total leukocytes and eosinophils in BAL fluid (Fig. 2*C*). These results indicate that RvE3 promoted resolution of both inflammation and AHR.

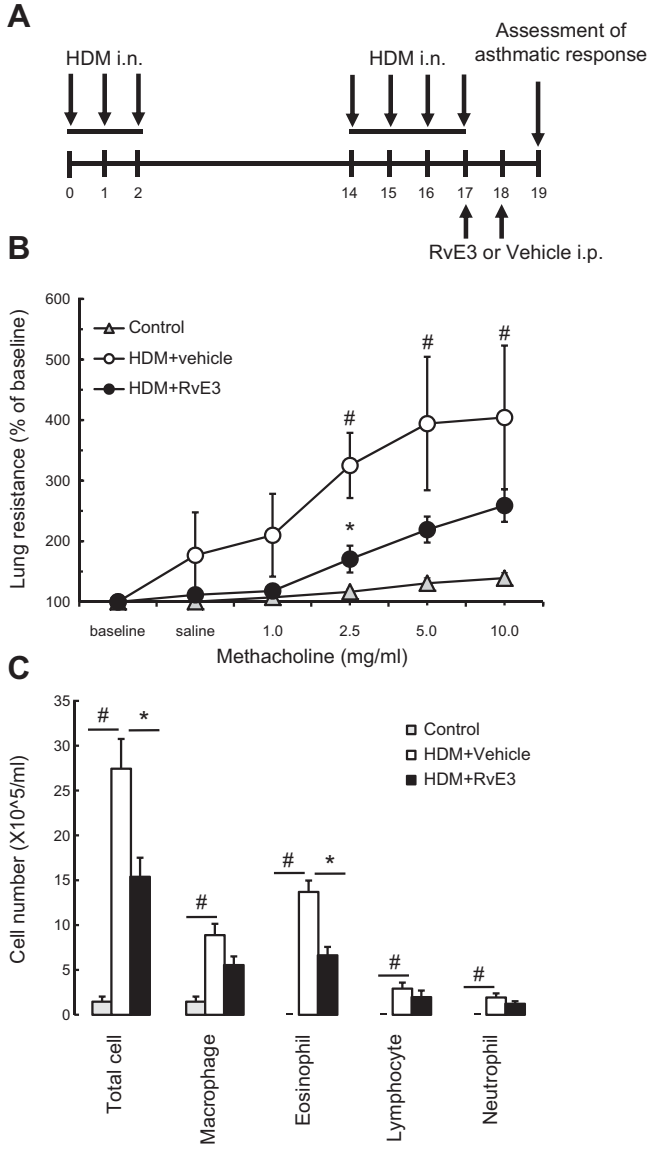

Figure 2. RvE3 promotes resolution of airway inflammation and airway hyperresponsiveness. *A*) Protocol for determining the effect of RvE3 on the resolution phase of HDM-induced airway inflammation. i.n., intranasal. *B*) RvE3-treated mice (filled circles) showed significantly lower levels of lung resistance than vehicle-treated mice (open circles). *C*) RvE3 administration significantly reduced the number of total cells and eosinophils in BAL fluid. Values are expressed as means ± SEM; $n = 8$–10/group. $^{\#}P < 0.05$, significantly different from the control group; $^{*}P < 0.05$ significantly different from the HDM-challenged and vehicle-treated group.

The extent of leukocyte infiltration within anatomic location was determined in H&E- and PAS-stained sections of lung tissue collected from mice on d 19 (**Fig. 3**). Sensitization and challenge with HDM induced widespread peribronchiolar and perivascular inflammation, which was primarily eosinophilic (Fig. 3*B*) compared with nonsensitized (control) mice (Fig. 3*A*). RvE3-treated mice had substantially fewer leukocytes in the peribronchial regions than vehicle-treated mice (Fig. 3*C*). Compared to control mice (Fig. 3*D*), PAS-stained goblet cells are increased in HDM-challenged and vehicle-treated mice (Fig. 3*E*). RvE3 treatment also decreased airway epithelial mucus production (Fig. 3*F*). We also observed that the goblet cell score, which was based on the number of PAS-stained goblet cells, was significantly lower in RvE3-treated mice than in vehicle-treated mice (Fig. 3*G*).

We also investigated the effect of RvE1 in the mouse model of HDM-induced asthma using the same protocol as used for RvE3 treatment. We found that the total cell counts in BAL fluid of mice treated with RvE1 (2 μg, i.p.) on d 19 were similarly reduced, which is consistent with observations previously reported in ref. 11. Furthermore, the total number of cells did not differ significantly from that in RvE3-treated mice (Supplemental Fig. S1), which suggests that, in terms of alleviating allergic airway inflammation *in vivo*, RvE3 might have a potency comparable to that of RvE1.

RvE3 regulates cytokines

To elucidate the proresolving mechanisms of RvE3, we measured the levels of cytokine mRNA expression in the lungs, PBLNs, and spleen of control and vehicle- or RvE3-treated mice on protocol d 19. We accordingly observed that RvE3 treatment significantly reduced *IL-5*, *IL-4*, and *IL-13* mRNA levels in lung tissue on d 19 (**Fig. 4*A*–*C***) and significantly suppressed *IL-23* and *IL-17A* mRNA expression (Fig. 4*D*, *E*). Administration of RvE3 also resulted in significantly lower concentrations of IL-5, IL-4, IL-13, IL-23, and IL-17 in BAL fluid (**Fig. 5**). These results indicate that RvE3 contributes to the regulation of cytokines that play pivotal roles in allergic airway inflammation.

We also observed that RvE3 significantly suppressed IL-23 and IL-17A mRNA expression in PBLNs (**Fig. 6**). The expression levels of IL-23 and IL-17A in the spleens of RvE3-treated and vehicle-treated mice were relatively low and showed no significant differences between treatments (unpublished results).

RvE3 suppresses the LTB4-induced activity of BLT1R in HEK293 cells

Previous studies have demonstrated that RvE1 and RvE2 are ligands for BLT1R (24, 25), and therefore we next compared the effects of RvE1, RvE2, and RvE3 on BLT1R in HEK293 cells using a NanoBiT arrestin recruitment assay (22). We accordingly observed that whereas LTB4 and RvE1 stimulation increased the binding of β-arrestin 2 to BLT1R in a dose-dependent manner (EC_{50}: 18 ± 4.5 nM

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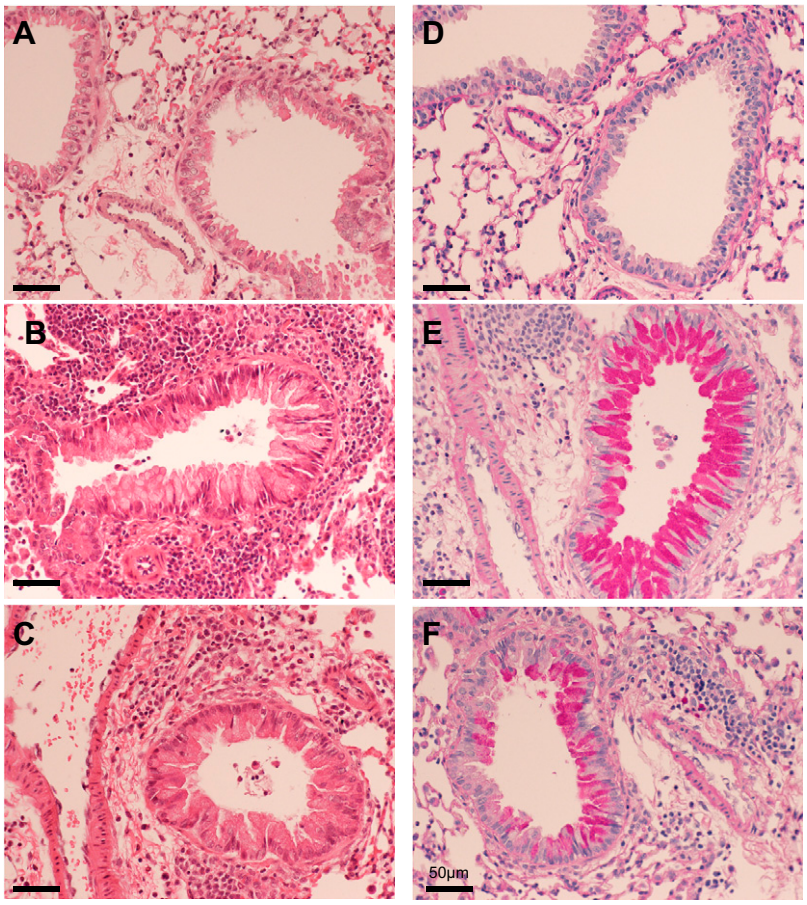

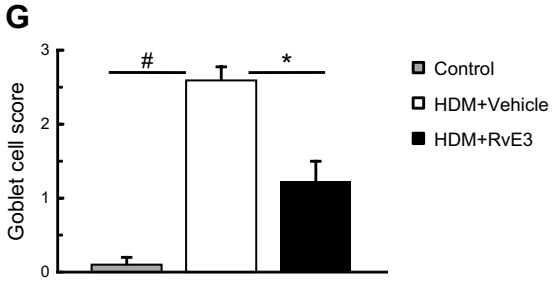

Figure 3. RvE3 reduces airway inflammation in lung tissue. *A–C*) Representative lung tissue sections from nonsensitized (control) mice (*A*), HDM-challenged and vehicle-treated mice (*B*), and HDM-challenged and RvE3-treated (2 μg) mice (*C*) were stained with H&E. Scale bars, 50 μm. *D–F*) Representative lung tissue sections from nonsensitized (control) mice (*D*), HDM-challenged and vehicle-treated mice (*E*), and HDM-challenged and RvE3-treated (2 μg) mice (*F*) were stained with PAS. Scale bars, 50 μm. *G*) Goblet cell scores based on the number of PAS-stained goblet cells were significantly lower in RvE3-treated mice than in vehicle-treated mice. Values are expressed as means ± SEM. $^{\#}P < 0.05$, significantly different from the control group; $^{*}P < 0.05$, significantly different from the HDM-challenged and vehicle-treated group.

and 2.2 ± 0.86 μM, respectively), RvE2 and RvE3 showed no agonist activity (**Fig. 7**). We also examined the antagonistic effect of RvE1, RvE2, and RvE3 on BLT1R by monitoring the suppression of 1 nM LTB4–induced β-arrestin 2 binding and found that pretreatment with more than 32 nM of RvE1, RvE2, or RvE3 significantly suppressed the activation of BLT1R by 1 nM LTB4 (**Fig. 8**). Pretreatment with higher concentrations of RvE1 increased the basal level of β-arrestin 2 binding to BLT1R (Fig. 8*A, B*) and thereby resulted in the bell-shaped dose-response depicted in Fig. 8*D* (IC_{50}: 58 ± 16 nM, EC_{50}: 3.2 ± 2.5 nM). Although a similar bell-shaped dose-response (IC_{50}: 29 ± 12 nM, EC_{50}: 3.5 ± 1.8 μM) was obtained following pretreatment with RvE2, the basal level of β-arrestin 2 binding remained unaltered (Fig. 8*A, E*). In contrast, the antagonistic effect of RvE3 showed a monophasic dose-response curve (IC_{50}: 25 ± 8.7 nM) (Fig. 8*F*).

DISCUSSION

In this study, we investigated the effects of RvE1, RvE2, and RvE3 on IL-4– and IL-33–stimulated BMDCs derived from HDM-sensitized mice and the effect of RvE3 on an experimental mouse model of asthma. We found that RvE3 has an anti-inflammatory effect on allergic airway inflammation. Following HDM sensitization and challenge, RvE3 administration in the resolution phase led to a significant reduction in airway eosinophil recruitment and mucus cell hyperplasia in the lung. In addition to a reduction in inflammation, we observed that AHR was also reduced in the murine asthma model.

Resolvins are lipid mediators generated from ω-3 fatty acids and have been recognized as potent proresolving lipid mediators. RvE1 and RvE2 are formed from 18-hydroxy-5*Z*,8*Z*,11*Z*,14*Z*,16*E*-EPA, and unlike RvE1 and RvE2, which are synthesized in neutrophils *via*

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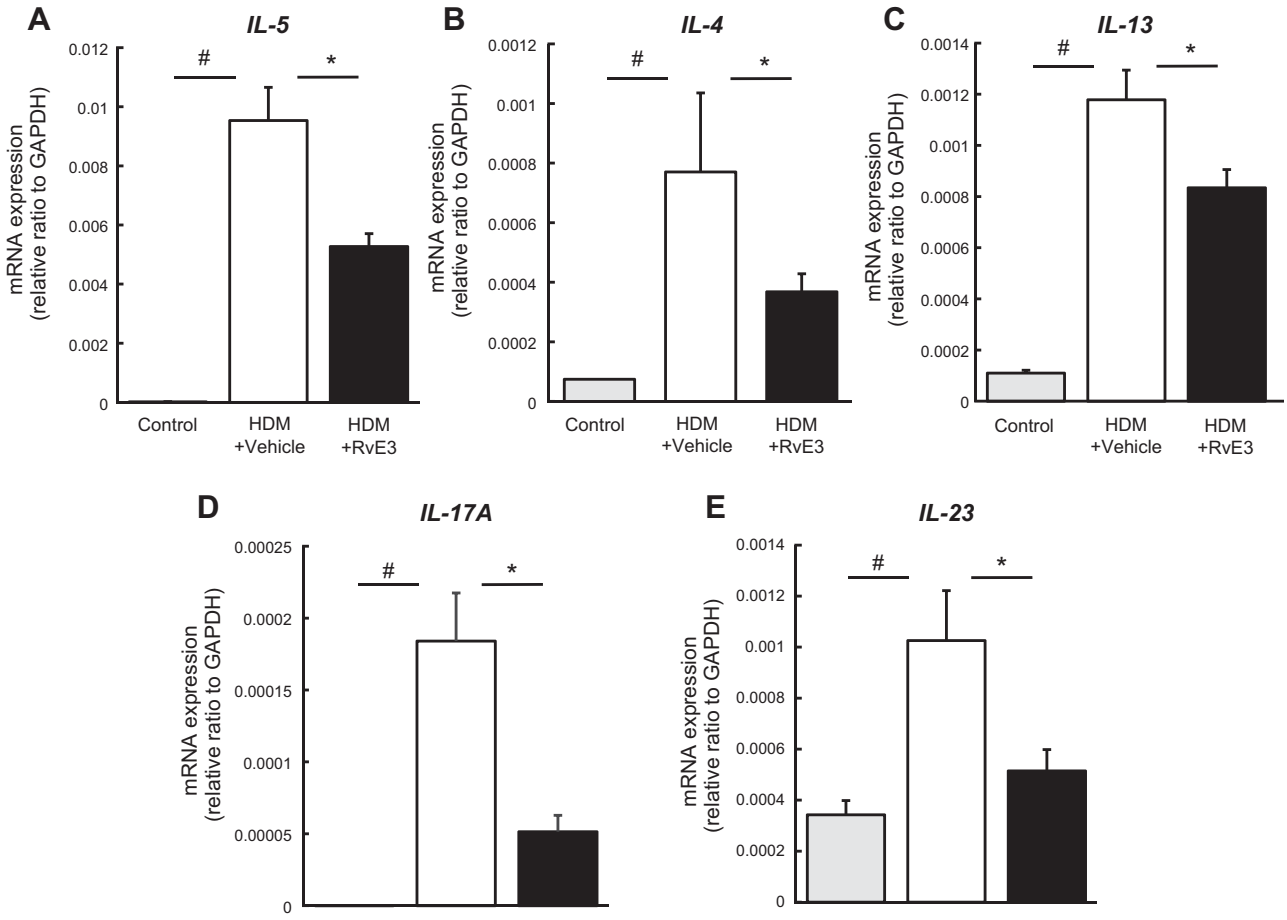

Figure 4. RvE3 regulates the mRNA expression of cytokines in the lung. RvE3 treatment significantly suppressed mRNA expression of *IL-5* (*A*), *IL-4* (*B*), *IL-13* (*C*), *IL-17A* (*D*), and *IL-23* (*E*). GAPDH, glyceraldehyde-3-phosphate dehydrogenase. Values are expressed as means ± SEM; $n > 3$/group in at least 3 independent experiments. $^{\#}P < 0.05$, significantly different from the control group; $^{*}P < 0.05$, significantly different from the HDM-challenged and vehicle-treated group.

the 5-lipoxygenase pathway, RvE3 is biosynthesized primarily by eosinophils *via* the 12- and 15-lipoxygenase pathway (14). 12- and 15-lipoxygenase are expressed in macrophages, eosinophils, dendritic cells, mast cells, and airway epithelial cells and are up-regulated in various cell types in response to induction by T helper (T_h)2 cytokines, including IL-4 and IL-13 (26).

RvE3 has previously been reported to be a potent inhibitor of polymorphonuclear leukocyte chemotaxis *in vitro* (14); to the best of our knowledge, however, the present study is the first to demonstrate that RvE3 has an anti-inflammatory effect with respect to allergic inflammation.

IL-23 is a member of the IL-12 family of cytokines and has been shown to be important in various diseases, including autoimmune diseases and cancers (27–30). In asthma, dendritic cells and macrophages have been reported to be the main sources of IL-23 (31), which displays proinflammatory features *via* the proliferation and maintenance of T_h17 cells. T_h17 cells release IL-17A, which plays a pivotal role in responses to allergens and regulation of airway inflammation (32–34). IL-17A is expressed in the airways of patients with asthma, and the levels of expression are correlated with the severity of asthma (35), whereas neutralization of IL-17A has been shown to attenuate airway inflammation in an asthma model (12, 36).

Haworth *et al.* (12) showed that RvE1 attenuates allergic airway inflammation *via* the regulation of IL-23 and IL-17A production, and in the present study, we found that not only RvE1 but also RvE2 and RvE3 suppressed IL-23 secretion from BMDCs *in vitro*, with IL-23 levels being lowest in the RvE3-treated BMDCs. However, given that, *in vivo*, we detected no significant difference between RvE3-treated and vehicle-treated mice, the clinical implications of these observation are at present unknown. Nevertheless, we did observe that the reduction in total cell counts in BAL fluid attributable to RvE3 was comparable to that induced by RvE1. RvE3 treatment also led to decreases in the expression of *IL-23* and *IL-17A* mRNAs in lung and PBLNs and to lower concentrations of IL-23 and IL-17 in BAL fluid *in vivo*, thereby indicating that RvE3 attenuates allergic airway inflammation *via* the regulation of IL-23 secretion from DCs.

IL-23 plays a pivotal role in eosinophilic airway inflammation, and IL-23 and T_h17 cells are known to be involved in both neutrophil recruitment in the airways and enhancement of T_h2 cell–mediated eosinophilic airway inflammation (36). Recently, Nakajima *et al.* have

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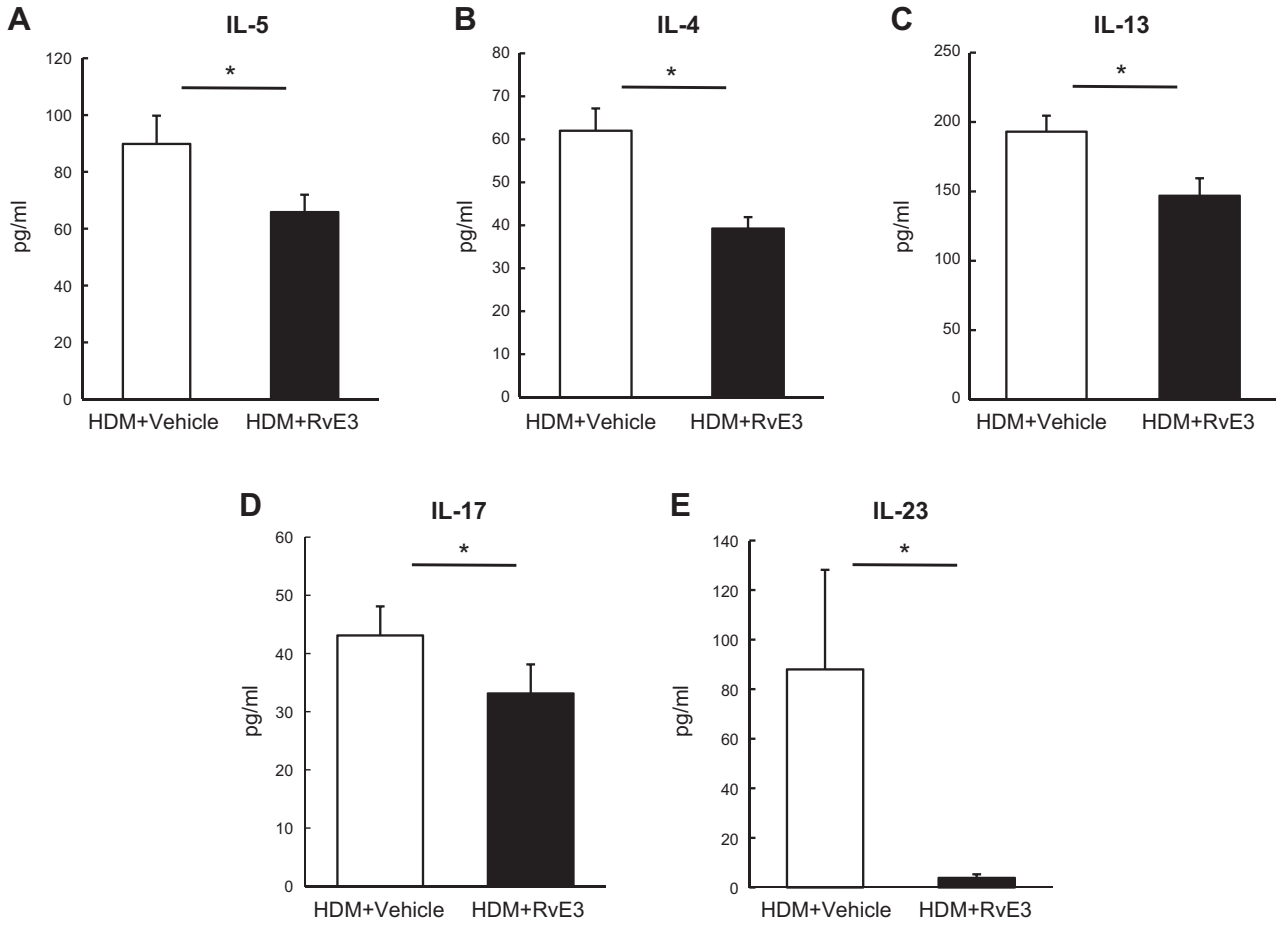

Figure 5. RvE3 reduced concentrations of the cytokines IL-5 (*A*), IL-4 (*B*), IL-13 (*C*), IL-17 (*D*), and IL-23 (*E*) in BAL fluid. Values are expressed as means ± SEM; $n > 3$/group. $^*P < 0.05$, significantly different from the HDM-challenged and vehicle-treated group.

reported that IL-23 neutralization attenuates not only antigen-induced neutrophil recruitment but also antigen-induced eosinophil recruitment to the airways and T_h2 cytokine production in the airways (37). It has also been demonstrated that enforced expression of IL-23 in the lungs enhances not only antigen-induced IL-17A production but also T_h2 cytokine production and eosinophil recruitment in the airways (36).

In the present study, we found that RvE3 markedly decreased mRNA levels of *IL-23* and *IL-17A* in lung and PBLNs and released the levels of IL-23 and IL-17, as well as T_h2 cytokines, in BAL fluid, which is consistent with the findings of previous studies (36–38). Our findings thus indicate that IL-23, IL-17A, and T_h2 cytokines are involved in the regulatory pathways whereby RvE3 attenuates the allergic airway inflammation.

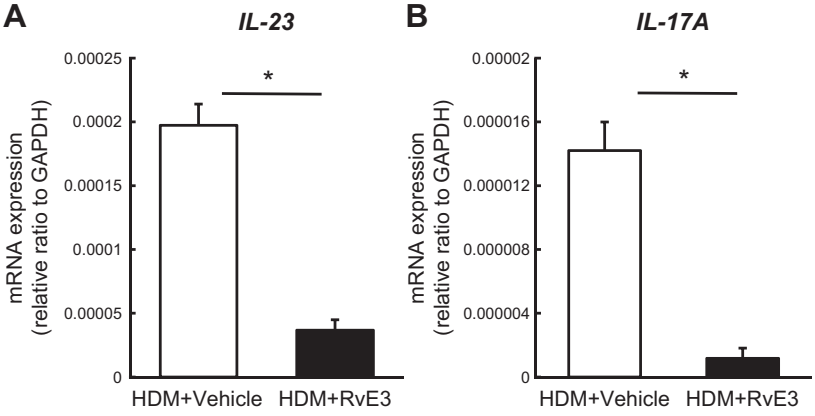

Figure 6. RvE3 treatment significantly regulates the mRNA expression of IL-23 (*A*) and IL-17A (*B*) in the PBLNs. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. Values are expressed as means ± SEM; $n > 3$/group in at least 3 independent experiments. $^*P < 0.05$, significantly different from the HDM-challenged and vehicle-treated group.

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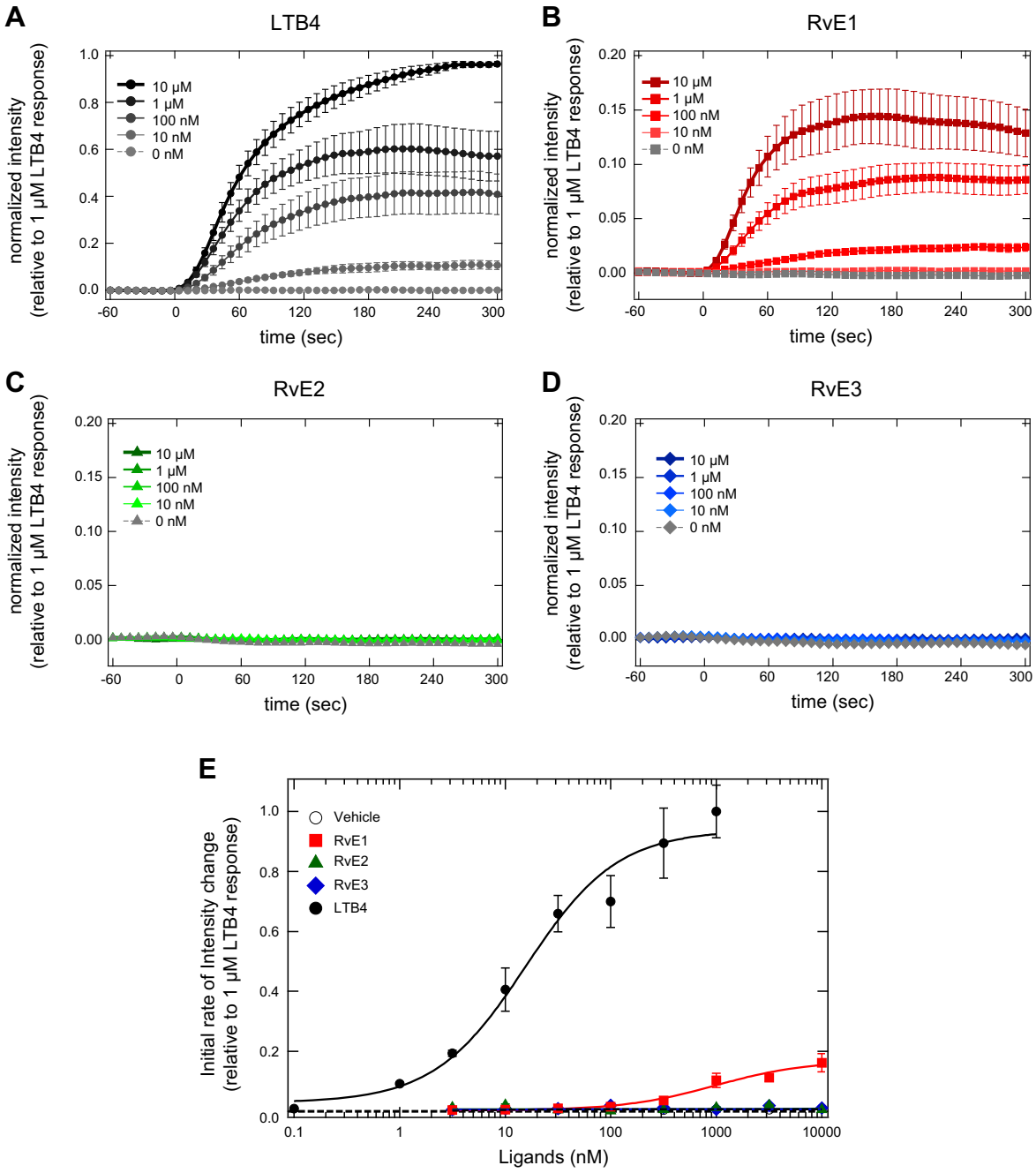

Figure 7. Dose-dependent changes in the binding of β-arrestin 2 to BLT1R. *A–D*) Time courses of normalized intensity changes reflecting the specific interaction between SmBiT–β-arrestin 2 and BLT1R-LgBiT in HEK293S cells upon LTB4 (*A*), RvE1 (*B*), RvE2 (*C*), and RvE3 (*D*) stimulation. *E*) Various concentrations of ligands were added at 0 s. Dose-response curves of the initial rates of the intensity changes. Values are expressed as means ± SEM; n = 4–6 independent experiments.

We also investigated whether RvE3 interacts with BLT1R, which is a GPCR that is widely expressed in immune cells. The activation of BLT1R by LTB4 triggers the chemotaxis of neutrophils and lymphocytes and is also related to various inflammatory diseases, including asthma (39). Here, we showed that the specific interaction between BLT1R and β-arrestin 2 triggered by 1 nM LTB4 was significantly suppressed by RvE1, RvE2, and RvE3 and that the IC_{50} values obtained for RvE1 (58 nM) and RvE2 (29 nM) in the present study are consistent with the K_d values (45 and 25 nM, respectively) that have previously been reported in refs. 24 and 25. In contrast, however, the EC_{50} values of RvE1 (3.2 μM) and RvE2 (3.5 μM) were found to be 2 orders of magnitude higher than the previously reported K_d values. We suspect that the bell-shaped response of RvE1 and RvE2 could be related to the high and low affinity states of BLT1R. In this regard, previous studies have demonstrated that the bimodal response of BLT1R to LTB4 is regulated by multiple phosphorylations of serine and threonine residues in BLT1R (40, 41). Similar to LTB4, stimulation with higher concentrations of RvE1 and RvE2 would increase the low affinity state that is related to the phosphorylation-dependent β-arrestin recruitment. In contrast, RvE3 significantly

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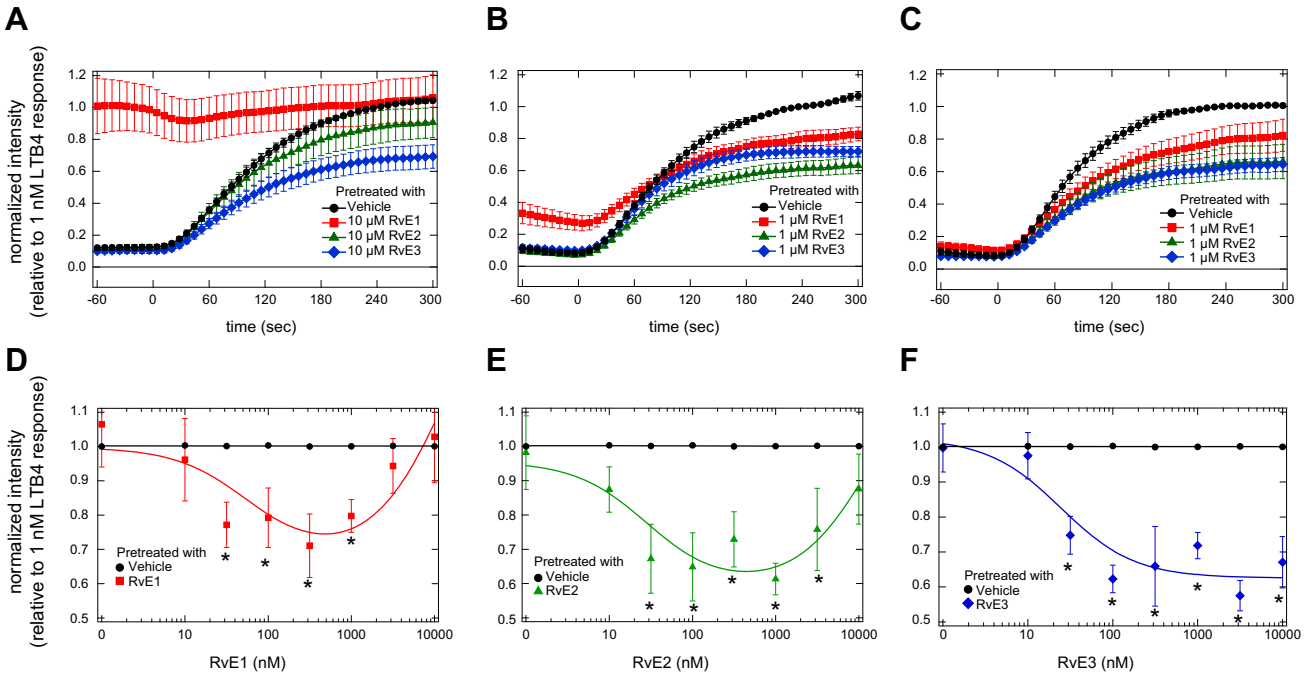

Figure 8. Competitive β-arrestin 2 recruitment assay to assess the effects of resolvins against 1 nM LTB4. Time courses of the normalized intensity changes reflecting specific interaction between SmBiT–β-arrestin 2 and BLT1R-LgBiT in HEK293S cells after pretreatment with 10 μM (*A*), 1 μM (*B*), and 100 nM (*C*) of RvE1 (red squares), RvE2 (green triangles), RvE3 (blue diamonds), or vehicle (black circles) followed by 1 nM LTB4 stimulation at 0 s. RvE1 (*D*), RvE2 (*E*), and RvE3 (*F*) pretreatment dose-response curves of normalized intensity at 240 s after stimulation with 1 nM LTB4. Values are expressed as means ± SEM; $n = 6$ independent experiments performed in duplicate. $^*P < 0.05$ (1-tailed Welch's *t* test), significantly different from vehicle-pretreated cells.

suppressed the LTB4-induced binding of β-arrestin to BLT1R, even following pretreatment with 10 μM RvE3. RvE3 thus acts as a neutral antagonist of LTB4, without any positive effects on BLT1R with respect to β-arrestin recruitment. Further studies will, however, be required to elucidate the detailed mechanisms underlying the action of RvE3.

In summary, our results provide insights on the protective role of RvE3 in the resolution phase of allergic airway inflammation. RvE3 selectively regulates the production of cytokines, including IL-23, IL-17A, and T_h2, thereby indicating the possibility that RvE3 attenuates allergic airway inflammation *via* multiple pathways. RvE3 could thus represent a novel therapeutic agent for the treatment of asthma. FJ

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AUTHOR CONTRIBUTIONS

H. Aoki-Saito, T. Ishizuka, M. Yamada, and T. Hisada designed the research; M. Sato, H. Aoki-Saito, and T. Hisada analyzed the data and wrote the manuscript; M. Sato, H. Aoki-Saito, Y. Koga, M. Yatomi, H. Tsurumaki, and T. Saito conducted the research; H. Fukuda, H. Ikeda, T. Maeno, T. Nakakura, T. Mori, K. Dobashi, and S. Shuto contributed new reagents or analytic tools; and M. Yanagawa, M. Abe, and Y. Sako performed the β-arrestin 2 recruitment assay and analyzed the related data.

REFERENCES

1. Busse, W. W., and Lemanske, R. F., Jr. (2001) Asthma. *N. Engl. J. Med.* **344**, 350–362
2. Mickleborough, T. D., Lindley, M. R., Ionescu, A. A., and Fly, A. D. (2006) Protective effect of fish oil supplementation on exercise-induced bronchoconstriction in asthma. *Chest* **129**, 39–49
3. Li, J., Xun, P., Zamora, D., Sood, A., Liu, K., Daviglus, M., Iribarren, C., Jacobs, D., Jr., Shikany, J. M., and He, K. (2013) Intakes of long-chain omega-3 (n-3) PUFAs and fish in relation to incidence of asthma among American young adults: the CARDIA study. *Am. J. Clin. Nutr.* **97**, 173–178
4. Horrobin, D. F. (1987) Low prevalences of coronary heart disease (CHD), psoriasis, asthma and rheumatoid arthritis in Eskimos: are they caused by high dietary intake of eicosapentaenoic acid (EPA), a genetic variation of essential fatty acid (EFA) metabolism or a combination of both? *Med. Hypotheses* **22**, 421–428
5. Barros, R., Moreira, A., Fonseca, J., Delgado, L., Castel-Branco, M. G., Haahtela, T., Lopes, C., and Moreira, P. (2011) Dietary intake of α-linolenic acid and low ratio of n-6:n-3 PUFA are associated with decreased exhaled NO and improved asthma control. *Br. J. Nutr.* **106**, 441–450

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6. Serhan, C. N., Clish, C. B., Brannon, J., Colgan, S. P., Chiang, N., and Gronert, K. (2000) Novel functional sets of lipid-derived mediators with antiinflammatory actions generated from omega-3 fatty acids via cyclooxygenase 2-nonsteroidal antiinflammatory drugs and transcellular processing. *J. Exp. Med.* **192**, 1197–1204
7. Serhan, C. N., Hong, S., Gronert, K., Colgan, S. P., Devchand, P. R., Mirick, G., and Moussignac, R. L. (2002) Resolvins: a family of bioactive products of omega-3 fatty acid transformation circuits initiated by aspirin treatment that counter proinflammation signals. *J. Exp. Med.* **196**, 1025–1037
8. Gilroy, D. W., Lawrence, T., Perretti, M., and Rossi, A. G. (2004) Inflammatory resolution: new opportunities for drug discovery. *Nat. Rev. Drug Discov.* **3**, 401–416
9. Schwab, J. M., Chiang, N., Arita, M., and Serhan, C. N. (2007) Resolvin E1 and protectin D1 activate inflammation-resolution programmes. *Nature* **447**, 869–874
10. Aoki, H., Hisada, T., Ishizuka, T., Utsugi, M., Kawata, T., Shimizu, Y., Okajima, F., Dobashi, K., and Mori, M. (2008) Resolvin E1 dampens airway inflammation and hyperresponsiveness in a murine model of asthma. *Biochem. Biophys. Res. Commun.* **367**, 509–515
11. Aoki, H., Hisada, T., Ishizuka, T., Utsugi, M., Ono, A., Koga, Y., Sunaga, N., Nakakura, T., Okajima, F., Dobashi, K., and Mori, M. (2010) Protective effect of resolvin E1 on the development of asthmatic airway inflammation. *Biochem. Biophys. Res. Commun.* **400**, 128–133
12. Haworth, O., Cernadas, M., Yang, R., Serhan, C. N., and Levy, B. D. (2008) Resolvin E1 regulates interleukin 23, interferon-γ and lipoxin A4 to promote the resolution of allergic airway inflammation. *Nat. Immunol.* **9**, 873–879
13. Haworth, O., Cernadas, M., and Levy, B. D. (2011) NK cells are effectors for resolvin E1 in the timely resolution of allergic airway inflammation. *J. Immunol.* **186**, 6129–6135
14. Isobe, Y., Arita, M., Matsueda, S., Iwamoto, R., Fujihara, T., Nakanishi, H., Taguchi, R., Masuda, K., Sasaki, K., Urabe, D., Inoue, M., and Arai, H. (2012) Identification and structure determination of novel anti-inflammatory mediator resolvin E3, 17,18-dihydroxyeicosapentaenoic acid. *J. Biol. Chem.* **287**, 10525–10534
15. Gold, M. J., Antignano, F., Halim, T. Y., Hirota, J. A., Blanchet, M. R., Zaph, C., Takei, F., and McNagny, K. M. (2014) Group 2 innate lymphoid cells facilitate sensitization to local, but not systemic, TH2-inducing allergen exposures. *J. Allergy Clin. Immunol.* **133**, 1142–1148
16. Grünig, G., Warnock, M., Wakil, A. E., Venkayya, R., Brombacher, F., Rennick, D. M., Sheppard, D., Mohrs, M., Donaldson, D. D., Locksley, R. M., and Corry, D. B. (1998) Requirement for IL-13 independently of IL-4 in experimental asthma. *Science* **282**, 2261–2263
17. Mogi, C., Tobo, M., Tomura, H., Murata, N., He, X. D., Sato, K., Kimura, T., Ishizuka, T., Sasaki, T., Sato, T., Kihara, Y., Ishii, S., Harada, A., and Okajima, F. (2009) Involvement of proton-sensing TDAG8 in extracellular acidification-induced inhibition of proinflammatory cytokine production in peritoneal macrophages. *J. Immunol.* **182**, 3243–3251
18. Shenoy, S. K., and Lefkowitz, R. J. (2005) Receptor-specific ubiquitination of β-arrestin directs assembly and targeting of seven-transmembrane receptor signalosomes. *J. Biol. Chem.* **280**, 15315–15324
19. Kim, J. H., Lee, S. R., Li, L. H., Park, H. J., Park, J. H., Lee, K. Y., Kim, M. K., Shin, B. A., and Choi, S. Y. (2011) High cleavage efficiency of a 2A peptide derived from porcine teschovirus-1 in human cell lines, zebrafish and mice. *PLoS One* **6**, e18556
20. Hiroshima, M., Pack, C. G., Kaizu, K., Takahashi, K., Ueda, M., and Sako, Y. (2018) Transient acceleration of epidermal growth factor receptor dynamics produces higher-order signaling clusters. *J. Mol. Biol.* **430**, 1386–1401
21. Kroeze, W. K., Sassano, M. F., Huang, X. P., Lansu, K., McCorvy, J. D., Giguère, P. M., Sciaky, N., and Roth, B. L. (2015) PRESTO-Tango as an open-source resource for interrogation of the druggable human GPCRome. *Nat. Struct. Mol. Biol.* **22**, 362–369
22. Dixon, A. S., Schwinn, M. K., Hall, M. P., Zimmerman, K., Otto, P., Lubben, T. H., Butler, B. L., Binkowski, B. F., Machleidt, T., Kirkland, T. A., Wood, M. G., Eggers, C. T., Encell, L. P., and Wood, K. V. (2016) NanoLuc complementation reporter optimized for accurate measurement of protein interactions in cells. *ACS Chem. Biol.* **11**, 400–408
23. Yanagawa, M., Hiroshima, M., Togashi, Y., Abe, M., Yamashita, T., Shichida, Y., Murata, M., Ueda, M., and Sako, Y. (2018) Single-molecule diffusion-based estimation of ligand effects on G protein-coupled receptors. *Sci. Signal.* **11**, eaao1917
24. Arita, M., Ohira, T., Sun, Y. P., Elangovan, S., Chiang, N., and Serhan, C. N. (2007) Resolvin E1 selectively interacts with leukotriene B4 receptor BLT1 and ChemR23 to regulate inflammation. *J. Immunol.* **178**, 3912–3917
25. Oh, S. F., Dona, M., Fredman, G., Krishnamoorthy, S., Irimia, D., and Serhan, C. N. (2012) Resolvin E2 formation and impact in inflammation resolution. *J. Immunol.* **188**, 4527–4534
26. Kühn, H., and O'Donnell, V. B. (2006) Inflammation and immune regulation by 12/15-lipoxygenases. *Prog. Lipid Res.* **45**, 334–356
27. Langrish, C. L., Chen, Y., Blumenschein, W. M., Mattson, J., Basham, B., Sedgwick, J. D., McClanahan, T., Kastelein, R. A., and Cua, D. J. (2005) IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. *J. Exp. Med.* **201**, 233–240
28. Langowski, J. L., Zhang, X., Wu, L., Mattson, J. D., Chen, T., Smith, K., Basham, B., McClanahan, T., Kastelein, R. A., and Oft, M. (2006) IL-23 promotes tumour incidence and growth. *Nature* **442**, 461–465
29. Chan, J. R., Blumenschein, W., Murphy, E., Diveu, C., Wiekowski, M., Abbondanzo, S., Lucian, L., Geissler, R., Brodie, S., Kimball, A. B., Gorman, D. M., Smith, K., de Waal Malefyt, R., Kastelein, R. A., McClanahan, T. K., and Bowman, E. P. (2006) IL-23 stimulates epidermal hyperplasia via TNF and IL-20R2-dependent mechanisms with implications for psoriasis pathogenesis. *J. Exp. Med.* **203**, 2577–2587
30. Yago, T., Nanke, Y., Kawamoto, M., Furuya, T., Kobashigawa, T., Kamatani, N., and Kotake, S. (2007) IL-23 induces human osteoclastogenesis via IL-17 in vitro, and anti-IL-23 antibody attenuates collagen-induced arthritis in rats. *Arthritis Res. Ther.* **9**, R96
31. Lewkowich, I. P., Lajoie, S., Clark, J. R., Herman, N. S., Sproles, A. A., and Wills-Karp, M. (2008) Allergen uptake, activation, and IL-23 production by pulmonary myeloid DCs drives airway hyperresponsiveness in asthma-susceptible mice. *PLoS One* **3**, e3879
32. Vroman, H., van den Blink, B., and Kool, M. (2015) Mode of dendritic cell activation: the decisive hand in Th2/Th17 cell differentiation. Implications in asthma severity? *Immunobiology* **220**, 254–261
33. Martinez, G. J., Nurieva, R. I., Yang, X. O., and Dong, C. (2008) Regulation and function of proinflammatory TH17 cells. *Ann. N. Y. Acad. Sci.* **1143**, 188–211
34. Pichavant, M., Goya, S., Meyer, E. H., Johnston, R. A., Kim, H. Y., Matangkasombut, P., Zhu, M., Iwakura, Y., Savage, P. B., DeKruyff, R. H., Shore, S. A., and Umetsu, D. T. (2008) Ozone exposure in a mouse model induces airway hyperreactivity that requires the presence of natural killer T cells and IL-17. *J. Exp. Med.* **205**, 385–393
35. Molet, S., Hamid, Q., Davoine, F., Nutku, E., Taha, R., Pagé, N., Olivenstein, R., Elias, J., and Chakir, J. (2001) IL-17 is increased in asthmatic airways and induces human bronchial fibroblasts to produce cytokines. *J. Allergy Clin. Immunol.* **108**, 430–438
36. Wakashin, H., Hirose, K., Maezawa, Y., Kagami, S., Suto, A., Watanabe, N., Saito, Y., Hatano, M., Tokuhisa, T., Iwakura, Y., Puccetti, P., Iwamoto, I., and Nakajima, H. (2008) IL-23 and Th17 cells enhance Th2-cell-mediated eosinophilic airway inflammation in mice. *Am. J. Respir. Crit. Care Med.* **178**, 1023–1032
37. Peng, J., Yang, X. O., Chang, S. H., Yang, J., and Dong, C. (2010) IL-23 signaling enhances Th2 polarization and regulates allergic airway inflammation. *Cell Res.* **20**, 62–71
38. Nakajima, H., and Hirose, K. (2010) Role of IL-23 and Th17 cells in airway inflammation in asthma. *Immune Netw.* **10**, 1–4
39. Nakamura, M., and Shimizu, T. (2011) Leukotriene receptors. *Chem. Rev.* **111**, 6231–6298
40. Okuno, T., Ago, H., Terawaki, K., Miyano, M., Shimizu, T., and Yokomizo, T. (2003) Helix 8 of the leukotriene B4 receptor is required for the conformational change to the low affinity state after G-protein activation. *J. Biol. Chem.* **278**, 41500–41509
41. Nakanishi, Y., Tan, M., Ichiki, T., Inoue, A., Yoshihara, J. I., Maekawa, N., Takenoshita, I., Yanagida, K., Yamahira, S., Yamaguchi, S., Aoki, J., Nagamune, T., Yokomizo, T., Shimizu, T., and Nakamura, M. (2018) Stepwise phosphorylation of leukotriene B4 receptor 1 defines cellular responses to leukotriene B4. *Sci. Signal.* **11**, eaao5390

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