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Resolvin E1 regulates interleukin-23, interferon- γ and lipoxin A₄ to promote resolution of allergic airway inflammation

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Abstract

Interleukin-23 (IL-23) is integral to the pathogenesis of chronic inflammation. Resolution of acute inflammation is an active process mediated by specific signals and mediators, such as resolvin E1 (RvE1). Here, we provide the first evidence that RvE1, in nanogram quantities, promotes resolution of inflammatory airway responses in part by directly suppressing IL-23 and IL-6 production in the lung. Also contributing to the pro-resolving effects of RvE1 treatment were increased concentrations of interferon- γ in the lungs of RvE1-treated animals. These findings point to a pivotal role of IL-23 and IL-6—which promote survival and differentiation of T_H-17 cells—in maintaining inflammation, and uncover an RvE1-initiated resolution program for allergic airway responses.

INTRODUCTION

Asthma is a chronic inflammatory disease of the airways characterized by infiltration of eosinophils and T lymphocytes that produce cytokines and pro-inflammatory lipid mediators, such as leukotrienes, which contribute to disease severity^{1, 2}. The incidence of asthma is rising in Western countries¹. Approximately 5% of individuals with asthma suffer from severe disease that is refractory to current medical treatment³. Importantly, no therapies are available to actively resolve the aberrant immune responses in asthmatic patients.

Epidemiological studies have indicated that diets rich in omega-3 fatty acids are associated with lower asthma prevalence⁴; however the mechanisms underlying this observation remain unclear. Resolvins are products of omega-3 fatty acids that were named based on their original identification in resolving exudates and their capacity to exert potent anti-inflammatory properties to accelerate the resolution phase of acute peritoneal inflammation^{5–8}. Resolvin E1 (RvE1; 5S, 12R, 18R-tri-hydroxy-6Z, 8E, 10E, 14Z, 16E-eicosapentaenoic acid) is a potent inhibitor of polymorphonuclear leukocyte (PMN) transmigration across endothelial and

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AUTHOR CONTRIBUTIONS

O.H performed experiments, analyzed data, was involved in experimental design and wrote the manuscript. M.C provided reagents, analyzed data and was involved in experimental design. R.Y provided reagents. C.N.S provided reagents, analyzed data, was involved in experimental design and helped write the manuscript. B.D.L conceived the project, performed and designed experiments, analyzed data and helped write the manuscript.

COMPETING INTERESTS STATEMENT

B.D. Levy, C.N. Serhan together with Brigham and Women's Hospital, have a pending patent on the use of RvE1 in the treatment of airway diseases and asthma.

epithelial barriers and promotes clearance of apoptotic PMNs and microbial products by macrophages^{9, 10}. However, the impact of RvE1 on adaptive immunity is not known.

T_H-17 cells, a recently identified subset of CD4⁺ T cells characterized by production of interleukin-17A (IL-17A), have been implicated in the pathogenesis of many inflammatory diseases including arthritis, experimental autoimmune encephalitis, inflammatory bowel disease and airway inflammation¹¹. T_H-17 cell population expansion and survival depends upon IL-23, which like IL-17 has been linked to the pathobiology of chronic inflammatory diseases¹²⁻¹⁵. IL-17 is present in asthmatic airways, can induce lung inflammation and stimulate mucus production¹⁶⁻²³.

Here, we have investigated the impact of RvE1 on allergic airway inflammation in an experimental model of asthma. RvE1 both dampened the development and promoted the resolution of airway inflammation, in part by inhibiting IL-23 release from dendritic cells (DCs).

RESULTS

RvE1 impairs development of airway inflammation

Murine experimental models of asthma involve allergen sensitization and airway challenge²⁴. Little information exists on the time course of natural resolution of allergic airway inflammation after discontinuation of allergen airway challenge. To determine the kinetics of spontaneous resolution of allergic lung inflammation, airway leukocyte numbers were monitored in bronchoalveolar lavage fluids (BALFs) 1, 4 and 8 days after the last of four daily allergen aerosol challenges. These time points corresponded to protocol days 18, 21 and 25 (Fig. 1a). Significant decrements in total BALF cell numbers were evident with a ~27% decrease between days 18 and 21 and a further ~60% decrease between days 21 and 25, (Fig. 1b). There was a distinct temporal regulation for each class of leukocyte. Similar to total leukocytes, eosinophils decreased by ~25% between days 18 and 21 and a further ~70% by day 25. In contrast, macrophages decreased by ~35% between day 18 and day 21, but no significant further decrease was evident by day 25. Of interest, lymphocytes increased significantly between day 18 and day 21 and then markedly decreased by ~85% by day 25. These findings indicate that the natural resolution of inflammatory cell trafficking in the airway is an active and highly regulated process.

Because diets rich in eicosapentaenoic acid (EPA) are linked to lower asthma prevalence⁴ and the EPA-derived RvE1 regulates acute inflammatory responses¹⁰, we next determined the impact of RvE1 on inflammatory cell trafficking in allergic airway inflammation. Administration of RvE1 (200ng/day, i.v.) 30 minutes prior to OVA challenge on protocol days 14-17 (Fig. 1a) significantly decreased BALF leukocyte numbers at day 18 by ~80% compared to vehicle (0.1% ethanol) exposed animals (Fig. 1d). The numbers of BALF eosinophils, macrophages and lymphocytes were all markedly reduced by RvE1 treatment (Fig. 1e). These findings indicate that RvE1 can prevent the development of allergic airway inflammation. Of note, endogenous concentrations of RvE1 in normal and inflamed murine lung were below the limits of detection by liquid chromatography tandem mass spectrometry (LC-MS-MS).

RvE1 promotes resolution of airway inflammation

As these findings revealed potent immunomodulatory actions of RvE1, and in view of the current absence of pro-resolving therapies for asthma, we next determined the potential influence of RvE1 on the resolution of established airway inflammation. RvE1 (100ng/day, i.v.) or vehicle (0.1% ethanol) was administered for 3 consecutive days (protocol days 18-20) after the last allergen challenge and BALF leukocyte numbers were counted on days 21 and

25 (Fig. 2a). On day 21, RvE1-treated animals contained significantly decreased numbers of total BALF leukocytes, including eosinophils, macrophages and lymphocytes, compared to vehicle-treated animals (Fig. 2b,c). The resolution interval (R_i) is defined as the time required for cell numbers to reach 50% of the numbers observed at the peak of inflammation²⁵. In vehicle-treated animals, between protocol days 18 and 25, the endogenous R_i for BALF leukocytes was ~5 days (Fig. 2d). Of interest, the R_i for RvE1 treated mice was markedly shortened to ~2.5 days. Together, these findings indicate that RvE1 exerts both anti-inflammatory and pro-resolving effects in this model of airway inflammation.

At day 21 in the resolution protocol, histopathological analyses revealed marked decrements in leukocyte infiltration and adherence to vascular endothelial surfaces, and less reactive bronchial epithelial cells in the lungs of RvE1-treated animals (Fig. 3a,b and Supplementary Fig. 1, online). In addition, Periodic Acid Schiff (PAS) staining revealed that RvE1 also decreased airway epithelial mucus production (Fig. 3c,d). Thus, in the absence of continued allergen exposure RvE1 rapidly resolved airway leukocyte infiltration and lung morphological changes associated with allergic inflammation.

Airway hyper-responsiveness (AHR) is a functional hallmark of asthma³. At protocol day 21, AHR was measured in anesthetized mice that were mechanically ventilated in the presence of escalating doses of inhaled methacholine (MCh). Animals treated with RvE1 displayed significant protection from MCh-induced increases in mean lung resistance (Fig. 3e). The effective dose of MCh required to increase mean lung resistance 200% from baseline (ED_{200}) was markedly higher for RvE1-treated mice than vehicle-treated counterparts (Fig. 3f). These results indicate that in addition to inflammation, RvE1 also accelerated resolution of allergic airway pathology and AHR.

RvE1 regulates cytokine and lipid mediators

To uncover mechanisms through which RvE1 regulates airway responses, select cytokines and lipid mediators that modulate T cell population expansion and differentiation were measured by cytokine bead array in protocol day 21 BALF from vehicle or RvE1-treated mice. Notably, RvE1 significantly decreased IL-17A concentrations by ~70% (Fig. 4a). In control animals that were sensitized to OVA, but challenged with aerosol saline, IL-17A concentrations were undetectable at day 21. T_H -17 T cell survival and differentiation is promoted by the cytokines IL-23 and IL-6, respectively²⁶. Administration of RvE1 significantly decreased BALF IL-23 and IL-6 concentrations by ~65% and ~70%, respectively (Fig. 4b,c). Indicative of its agonist properties, RvE1 markedly increased interferon (IFN)- γ (<http://www.signaling-gateway.org/molecule/query?afcsid=A001238>) Fig. 4d). In sharp contrast to the marked RvE1-mediated regulation of IL-23, IL-6 and IFN- γ , RvE1 had no apparent effect at protocol day 21 on IL-10 or the T_H 2 cytokines IL-4, 5 and 13 (Fig. 4e-h). In addition, there was no significant difference in serum IgE concentrations (25.3 ± 2.7 ng/ml (vehicle) versus 26.5 ± 3.4 ng/ml (RvE1)). Because IL-27 can block the development of both T_H 2 and T_H -17 inflammatory responses²⁷⁻²⁹, we also measured BALF IL-27 concentrations. RvE1 decreased IL-27 by ~60% (Fig. 4i), indicating that RvE1 suppressed T_H -17 responses in an IL-27-independent manner. Together, these results indicate that RvE1 is a potent and selective regulator of cytokines that are pivotal for airway inflammation.

Given their important roles in inflammation and asthma, the pro-inflammatory lipid mediators leukotriene B₄ (LTB₄) and cysteinyl leukotrienes (CysLTs) were also measured in the BALF of vehicle- and RvE1-treated mice³. RvE1 treatment led to a significant decrease in LTB₄ (Fig. 4j). Of interest, endogenous concentrations of the counter-regulatory eicosanoid mediator lipoxin A₄ (LXA₄) increased from peak inflammation (day 18; 64.1 ± 12.9 pg/ml) to resolution (day 21; 183.7 ± 31.3 pg/ml). RvE1 administration led to further significant increases in LXA₄ concentrations on protocol day 21 (330.00 ± 55.5 pg/ml (Fig. 4k). No significant

differences in concentrations of the lipid mediator prostaglandin D₂ (954.1 ± 34.0 (vehicle) versus 943.5 ± 24.5 pg/ml (RvE1)) were noted. Together, these findings uncover a “fingerprint” of selective mediator regulation by RvE1, and suggest that T_H-17 cells and LTB₄ may promote maintenance of inflammation in a manner distinct from the pathogenic roles played by T_H2 cells and CysLTs in the initiation of allergic inflammatory responses.

RvE1 regulates T cell IL-17 and IFN- γ production

To investigate if changes in T cell phenotype were responsible for changes in IL-17A and IFN- γ production, lung, lung-draining mediastinal lymph nodes (MLNs) and spleen were excised during protocol day 21 and CD4⁺ T cell production of IL-17A and IFN- γ was measured by flow cytometry (Fig. 5a). Administration of RvE1 (days 18-20) significantly increased the ratio of CD4⁺ T cells expressing IFN- γ to those expressing IL-17A in all tissues (Fig. 5b). To determine the effect of IL-17A on the resolution of allergic airway inflammation, animals were given IL-17A-neutralizing antibody (10 μ g/day), isotype control antibody, or recombinant murine IL-17 (1 μ g/day) by intranasal administration each day on protocol days 18-20. Treatment with IL-17A-neutralizing antibody led to significant decreases in the total numbers of BALF leukocytes, eosinophils and macrophages relative to both vehicle and isotype control-treated animals (Fig. 5c and Supplementary Fig. 2a, online). BALF leukocyte numbers at protocol day 21 after treatment with IL-17A-neutralizing antibody were similar to those seen after RvE1 treatment (Fig. 5c). Administration of recombinant murine IL-17A had no significant effect on total BALF leukocytes, but administration of IL-17A in combination with RvE1 blocked the ability of RvE1 to induce resolution of leukocyte trafficking, as measured by BALF leukocyte numbers (Fig. 5c and Supplementary Fig. 2a, online). Administration of recombinant murine IL-23 together with RvE1 also blocked the pro-resolution effects of RvE1 (Fig. 5c and Supplementary Fig. 2b, online). As IL-6 promotes T_H-17 cell differentiation³⁰ and RvE1 decreased BALF IL-6 concentrations (Fig. 4c), we examined the effects of exposure to recombinant murine IL-6 on RvE1-mediated resolution. Of interest, like IL-23 and IL-17, IL-6 blocked RvE1-mediated decrements in BALF leukocyte numbers (Fig. 5d). Together, these findings reveal pivotal roles for IL-23 and T_H-17 cells in the persistence of airway inflammation.

To determine if the RvE1-mediated increase in IFN- γ (Fig. 4d) contributed to the pro-resolving actions of RvE1, mice were given an IFN- γ -neutralizing antibody. Compared to treatment with RvE1, anti-IFN- γ led to significant increases in BALF total leukocytes (Fig. 5d) that were primarily the result of increased numbers of eosinophils (Supplementary Fig. 2c, online). Mice receiving anti-IFN- γ together with RvE1 contained fewer BALF leukocytes compared to mice treated with anti-IFN- γ and vehicle, but more BALF leukocytes than mice treated with RvE1 alone (Fig. 5d). However, anti-IFN- γ did not influence BALF IL-23 concentrations, and BALF IL-23 concentrations were similar in animals treated with anti-IFN- γ plus RvE1 and those treated with RvE1 alone (Supplementary Fig. 3). These findings indicate that IFN- γ does not regulate IL-23 in this setting, but does contribute to RvE1-mediated inflammation resolution.

Examination of lung histopathology revealed similar trends as mice treated with anti-IL-17A exhibited decreased leukocyte infiltration and hypertrophic changes to the airway epithelium, similar to mice treated with RvE1, and treatment with anti-IFN- γ reversed the ameliorative effects of RvE1 (Fig. 5e). Together, these findings implicate IL-23, IL-17 and IL-6 in the maintenance of airway inflammation and IFN- γ in its resolution after discontinuation of allergen challenge.

Overlapping yet distinct effects of RvE1 and LX

Because LXA₄ concentrations increased during spontaneous resolution (day 21) and RvE1 treatment further increased LXA₄ concentrations (Fig. 4k), we next investigated the impact of

an LXA₄ analog, 15-epi-lipoxin analog (ATLa), alone or in combination with RvE1 on airway inflammation. This LX stable analog prevents the development of allergic airway responses²⁴, but its influence on resolution in this model has not been determined. ATLa significantly decreased BALF leukocyte numbers, measured at protocol day 21, by a similar magnitude as RvE1 (100ng/day) (Fig. 6a). Of interest, we noted no synergistic effect between RvE1 (50ng) and ATLa (100ng) (Fig. 6a). Quantification of BALF IL-17, IL-23 and IFN- γ concentrations revealed distinct regulation of these cytokines by RvE1 and ATLa. Both RvE1 and ATLa significantly decreased IL-17 concentrations (Fig. 6b), but RvE1 and not ATLa decreased IL-23 (Fig. 6c). In sharp contrast to the RvE1-induced increase in IFN- γ production, ATLa alone and together with RvE1 significantly decreased IFN- γ concentrations (Fig. 6d).

RvE1 inhibited IL-23 production *in vivo* and this cytokine can be released by activated DCs^{31, 32}. In addition, lung DCs removed from mice at protocol day 21 and bone marrow-derived DCs (BMDCs) expressed RNA transcripts encoding receptors for RvE1 (ChemR23 and BLT-1) and ATLa (ALX) (data not shown). Therefore, we measured the impact of RvE1 on IL-23 release from lipopolysaccharide-stimulated BMDCs. RvE1 potently inhibited IL-23 release in a concentration-dependent manner (Fig. 6e). Addition of BOC-2 peptide, an LXA₄ receptor (ALX) antagonist³³, did not block inhibition of IL-23 release by RvE1. Of note, RvE1 (1ng/ml) also inhibited release of IL-6 and tumor necrosis factor from these activated BMDCs (Fig. 6f). Together, these findings show that LXA₄ and RvE1 both inhibit IL-17 *in vivo*, yet RvE1 alone regulates IL-23 *in vivo* and *in vitro* in a LXA₄-independent manner.

DISCUSSION

Here, we provided evidence that RvE1 acts as an anti-inflammatory and pro-resolving lipid mediator for allergic airway inflammation. After antigen sensitization and aerosol challenge, there was a natural and spontaneous resolution of inflammation in the airway that was accelerated by administration of nanogram quantities of RvE1. In addition to inflammation, RvE1 also dampened airway mucus and hyper-reactivity, responses typical of asthma. RvE1 decreased IL-23, IL-6 and IL-17 and increased IFN- γ concentrations; these cytokine alterations were reflected in shifting percentages of T_H-17 and IFN- γ -producing CD4⁺ T cells in lung, lung-draining MLNs and spleen. Antibody neutralization experiments implicated IL-17A as an essential driver of airway inflammation *in vivo*. Moreover, exogenous addition of recombinant IL-23, IL-6 or a neutralizing antibody against IFN- γ prevented RvE1-mediated resolution. RvE1 treatment also increased LXA₄, and a stable LXA₄ analog shared the pro-resolving and IL-17 counter-regulating actions of RvE1. Together, these findings point to IL-23 and T_H-17 cells as essential for the maintenance of airway inflammation, and provide the first evidence for counter-regulatory lipid mediators with the capacity to control these pro-inflammatory cytokines and T cells.

IL-23 is involved in the pathobiology of numerous chronic diseases, including colitis, encephalitis, psoriasis, rheumatoid arthritis and cancer^{12, 14, 15, 34}, and is critical for the survival of T_H-17 cells^{12, 30}. These T cells release IL-17A, a cytokine that increases production of several proinflammatory molecules, including IL-1 and TNF³⁵. RvE1 markedly decreased BALF concentrations of IL-6, IL-23 and IL-17A, indicating that the pathways promoting T_H-17 cell differentiation and survival are regulatory checkpoints countered by RvE1 during resolution of airway inflammation. RvE1 alone regulated IL-23 both *in vivo* and *in vitro*. Administration of IL-23 in combination with RvE1 blocked its ability to promote resolution, indicating that IL-23 was important for prolonging airway inflammation. Of interest, eosinophils express the IL-23 receptor and IL-23 induces the release of pro-inflammatory chemokines from eosinophils²². Highlighting the importance of IL-23 and T_H-17 cells in general inflammation, IL-23 deficient mice are protected in models of colitis¹³. Similar to IL-23, RvE1 can also protect against the development of colitis in model systems^{36, 37}. Thus,

regulation of IL-23 and T_H-17 cells by RvE1 may serve as a common mechanism for its pro-resolving actions in mucosal inflammation.

IL-17 has a pivotal role in regulating airway inflammation and responses to allergen^{20, 21, 38}. Expression of an IL-17 transgene driven by the airway epithelial promoter CC10 leads to spontaneous airway inflammation characterized by infiltration of eosinophils and lymphocytes, hypertrophic changes to the epithelium and increased mucus production¹⁸. In addition, IL-17 receptor knockout mice do not develop allergen-induced airway inflammation²⁰. Moreover, elevated concentrations of IL-17 in human BALF and induced sputum are associated with asthma^{16, 20, 39}. Of interest, IL-17 may have dual regulatory roles in allergic airway inflammation, as it can also inhibit T_H2-driven immune responses²⁰. Concurrent administration of allergen and recombinant IL-17 to sensitized animals dampens airway inflammation by decreasing IL-5, IL-13, CCL11 and CCL17 production in an IL-4 receptor dependent manner²⁰. In contrast, in the experiments performed here, IL-17 BALF concentrations remained elevated in established asthma three days after allergen exposure. In promoting resolution, RvE1 significantly decreased both IL-17 and allergic airway responses. In addition, IL-17-neutralizing antibody significantly decreased in BALF leukocyte numbers and recombinant murine IL-17 blocked the capacity of RvE1 to promote resolution. RvE1 also downregulated IL-6 in BALF *in vivo*, and *in vitro* from LPS-stimulated BMDCs. IL-6 has been found to be increased in the serum of asthmatic subjects compared to nonasthmatic patients⁴⁰ and IL-6 *trans*-signalling via soluble IL-6 receptor supports T_H2 and T_H-17 cell differentiation^{30, 40}. Together these results indicate that RvE1-mediated regulation of IL-17 is important in promoting resolution of airway inflammation and suggest a pathogenic relationship between IL-17 and persistent airway inflammation in this model.

In contrast, RvE1 did not significantly impact concentrations of IL-10 and the T_H2 cytokines IL-4, IL-5 and IL-13 during resolution. Thus, no longer exposed to allergen, the T_H-17 cells and cytokines that maintained the inflammatory response exerted effects distinct from the T_H2 cells and cytokines involved in the initiation of allergic inflammatory responses. Of note, RvE1 facilitated resolution despite decreased concentrations of IL-27, which can regulate both T_H-17 and T_H2 T cells²⁷⁻²⁹.

RvE1 increased IFN- γ production, and T_H-17 immune responses are inhibited by IFN- γ ¹⁸. IFN- γ can contribute to resolution by upregulating Fas (CD95) expression on lymphocytes to increase their rate of apoptosis⁴¹. Mice lacking IFN- γ or Fas exhibit defective resolution of inflammation in experimental models of asthma^{42, 43}. As an IFN- γ -neutralizing antibody partially blocked RvE1-mediated resolution, IFN- γ is important for resolution of airway inflammation in this setting. However, as anti-IFN- γ did not reduce BALF IL-23 concentrations, IFN- γ promoted resolution via a mechanism distinct from that of RvE1.

ChemR23 is a cognate receptor for RvE1 and is expressed on several immune regulatory cell types, including DCs⁴⁴. ChemR23-deficient mice, in a model of zymosan induced peritonitis, exhibit increased inflammation, indicating that this receptor is important for counter-regulatory signalling⁴⁵. The new findings presented here bolster the emerging concept of resolution as an active process with specific counter-regulatory signalling pathways.

The ideal outcome of resolution of inflammation is complete tissue catabasis⁴⁶. RvE1-mediated increases in both IFN- γ and LXA₄ concentrations are indicative of its agonist properties, and provide further evidence that signalling for inflammation resolution is an active process⁸. RvE1 displays autacoid properties as it is rapidly formed, rapidly inactivated and carries potent bioactions at sub-nanomolar concentrations⁸. Difficult to detect *in vivo* here in inflamed lungs, endogenous RvE1 has been detected in several models of spontaneously resolving inflammation^{5, 6}. In addition, quantities of RvE1 can be increased by dietary modification,

administration of aspirin or transgenic expression of the *fat-1* gene^{36, 37, 44}. Moreover, disruption of endogenous formation of RvE1 or LXA₄ by biosynthetic enzyme inhibition disrupts resolution of acute inflammation, resulting in prolonged inflammatory responses^{10, 47}. In addition, severe and uncontrolled asthma are characterized by a decreased capacity to generate these protective mediators^{48, 49}. During resolution of allergic airway inflammation, exogenous RvE1 increased production of LXA₄ and inhibited production of the leukocyte chemoattractant and secretagogue LTB₄. Of interest, both RvE1 and LTB₄ can interact with the LTB₄ receptor BLT-1 with RvE1 serving as a receptor antagonist⁵⁰. In addition to its potent pro-resolving signals for airway inflammation^{24, 47}, LXA₄ can also block LTB₄-mediated responses in leukocytes⁵¹. A stable lipoxin analog (ATLa) significantly decreased BALF leukocyte numbers and IL-17 concentrations, but the mechanism by which ATLa promoted resolution was distinct from RvE1-mediated regulation of IL-23 production, suggesting that IL-17 production is downstream of IL-23. These results show that two classes of pro-resolution lipid mediators target IL-17 via distinct mechanisms to promote resolution.

In summary, RvE1 is a potent regulator of both the initiation and resolution of complex airway inflammation. To promote resolution, RvE1 decreased production of the pro-inflammatory cytokines IL-23, IL-6 and IL-17, and increased production of the counter-regulatory mediators IFN- γ and LXA₄. Regulation of adaptive immune responses by RvE1 points to important pathological roles for IL-23 and T_H-17 cells in the persistent and unrestrained immune responses of asthma and other chronic inflammatory diseases.

METHODS

Sensitization and challenge protocols

Five to seven week old male FVB mice (Charles River Laboratories) were housed in isolation cages under viral antibody-free conditions. Mice were fed a standard diet (Laboratory Rodent Diet 5001, PMI Nutrition International) that contained no less than 4.5% total fat with 0.26% omega-3 fatty acids and <0.01% C20:4. After Harvard Medical Area IRB approval (Protocol #03618), mice were sensitized with intra-peritoneal injections of ovalbumin (OVA) (Grade III; Sigma) (10 μ g) plus 1mg aluminum hydroxide (alum) (Sigma) as adjuvant in 0.2ml saline on days 0 and 7. On days 14, 15, 16, 17 mice received aerosol challenge with 6% OVA for 25 minutes/day. To investigate inflammation development, some animals received RvE1 methyl ester (100ng) or vehicle (0.1% ethanol) in saline by intravenous injection 30 minutes before OVA aerosol challenge on days 14, 15, 16 and 17. This dose of RvE1 was chosen based on its regulation of murine peritoneal inflammation¹⁰. To investigate inflammation resolution, some mice received RvE1 methyl ester (50 ng or 100 ng), a 15 epi-LXA₄ analog (15-epi-16 para-fluoro-phenoxy-LXA₄ methyl ester, ATLa) (100ng) or vehicle by intravenous injection on only days 18, 19 and 20. Some animals were treated with recombinant murine IL-17A (1 μ g/day) (eBiosciences), a neutralizing IL-17A-specific antibody (10 μ g/day) (eBioMM17F3) or an isotype control antibody (eBiosciences), recombinant murine IL-23 (1 μ g/day) (R&D Systems), anti-IFN- γ (10 μ g/day) (XMG1.2, Biolegend), or recombinant murine IL-6 (1 μ g/day) (PeproTech) by intranasal administration. Bronchoalveolar lavage was performed with 2 $\times$ 1 ml aliquots of phosphate buffered saline with 0.6mM EDTA, or tissues were harvested for histological analysis.

Measurement of lung resistance

To measure airway hyper-responsiveness, anesthetized mice were mechanically ventilated using a flexiVent (SciReq) and aerosolized methacholine (0, 1, 3, 10, 30 and 100mg/ml) was delivered in-line via the inhalation port for 10 sec. Lung resistance was measured as a percentage of baseline with PBS nebulization using the mean of 10 readings taken for each

concentration of methacholine. No BALF or histological analysis was performed on those animals undergoing measurement of airway hyper-responsiveness.

Allergen-initiated respiratory inflammation

Murine lungs were fixed in 10% buffered formalin and paraffin embedded for hematoxylin and eosin and Periodic Acid Schiff staining (Sigma). Samples of cell-free BALF (centrifuged at 2000g for 10 minutes) were coded and cytokines were measured in deidentified aliquots by bead array (Pierce Biotechnology, Inc.); lipid mediators were measured by ELISA (LTB₄, CysLTs and PGD₂ Cayman Chemical; LXA₄ Neogen). Cells in BALF were resuspended in PBS, enumerated by a hemocytometer. Cytospins were performed by cytocentrifuge (STATspin) (265×g). Cells were stained with a Wright-Giemsa stain (Sigma) to quantify leukocyte subsets, counting ≥ 200 cells per slide. Total serum IgE was measured by ELISA (BD Biosciences).

Intracellular cytokine flow cytometry

A single-cell suspension of lung, MLNs and spleen was generated by passing tissue through a 70µm filter (BD Pharmingen). Lung lymphocytes were enriched using Ficoll (Sigma). Cells were incubated (4 h, 37°C) *in vitro* with phorbol-12-myristate (50ng/ml; Sigma) and ionomycin (1µg/ml; Sigma) plus Golgi-stop (BD-Pharmingen). Fc receptors were blocked with anti-CD16/32 (Caltag), followed by cell surface staining with CD4 PE-Cy5 (eBiosciences). Cells were then resuspended in Fixation-Permeabilization solution (BD Cytofix/Cytoperm kit; BD Pharmingen) and intracellular staining with anti-IL-17A-FITC (eBio17B7; eBiosciences) and anti-IFN-γ-PE (XMG1.2; Caltag/Invitrogen) was performed at 4°C. A FACSCalibur (Becton Dickinson) was used for flow cytometry and data was analysed using FloJo software (Tree Star).

Generation and purification of BMDCs

The femurs and tibia of FVB mice were opened at both ends and BM cells were extruded with ice cold RPMI-1640 and passed through a 70µm filter. Cells were cultured at a density of 1×10⁶/ml for 7 days with IL-4 (1ng/ml and GM-CSF (10ng/ml) (R&D Systems)) in RPMI-1640 complete media (10% FCS, 2% Pen-Strep), changing the medium every other day. On day 7, the CD11c⁺ BMDCs were further purified from the non-adherent cells using anti-CD11c—coated magnetic beads according to the manufacturer's instructions (Miltenyi Biotech). The purity of the cells was assessed by flow cytometry (>93%). BMDCs were plated at a density of 1×10⁶/ml and incubated with vehicle or RvE1 free acid (1, 10 or 100ng) in 1ml final volume for 24 h before the addition of LPS (Sigma) (1µg/ml) for a further 24 h (37°C, 5% CO₂). IL-23 (p19/p40) release was determined by ELISA (eBiosciences). IL-6 and TNF were measured by cytokine bead array.

Statistical analysis

Results are expressed as the mean ± s.e.m. Statistical significance of differences was assessed by Student's *t*-test and one-way ANOVA. *P* <0.05 was set as the level of significance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

RvE1 dampens the development of allergic airway inflammation. **a**, Schematic diagram showing the protocol used to induce allergic airway inflammation. Mice were sensitized with OVA (i.p.) on day 0 and 7 and challenged with aerosolized OVA on days 14–17. **b,c** On protocol days 18 (white), 21 (gray) and 25 (black), mice were sacrificed and total BALF cells **b** and leukocyte subsets **c** were counted. **d,e** To some animals, RvE1 (200ng) (black) or vehicle (white) was given 30 min before aerosol challenge (days 14–17). On day 18, total BALF cells **d** and leukocyte subsets **e** were counted. Values represent the mean $\pm$ s.e.m. for $n \geq 3$ mice, in ≥ 3 independent experiments *, $P < 0.05$ by Student's t-test.

Figure 2.

RvE1 promotes resolution of airway inflammation. **a**, Schematic diagram showing the protocol used to determine the impact of RvE1 (100ng, i.v., days 18-20) or vehicle on the resolution of airway inflammation. **b,c** On day 21, total BALF cells **b** and leukocyte subsets **c** were counted. **d**, Using the mean number of BALF leukocytes on day 18, 21 and 25, the resolution interval (R_i) was calculated for RvE1 and vehicle cohorts. Values represent the mean $\pm$ s.e.m. for $n \geq 8$ in ≥ 4 independent experiments, *, $P < 0.05$ by Student's t-test.

Figure 3.

RvE1 improves lung inflammation, airway mucus and airway hyper-reactivity. Representative ($n \geq 3$) lung tissue sections from day 21 mice given RvE1 (100 ng) or vehicle were obtained from fixed, paraffin embedded lung tissue, prepared and stained with hematoxylin and eosin (**a-b**) or Periodic Acid Schiff (PAS) (**c-d**). Original magnification $\times 100$ and $\times 200$ as indicated. Br; bronchus, V; vein. Arrowheads denote mucus (magenta) containing Goblet cells. **e**, RvE1 (200ng) (filled circles) or vehicle (open circles) was administered on days 18-20 and AHR to methacholine was measured on day 21. Lung resistance was measured by flexiVent, and the change in mean lung resistance was plotted as a percentage of baseline (PBS alone). **f**, Log ED_{200} (the effective dose of MCh required to increase mean lung resistance 200% from

baseline) for RvE1 (black) and vehicle (white) exposed cohorts. Values represent the mean $\pm$ s.e.m. for $n \geq 6$ in ≥ 3 independent experiments *, $P < 0.05$ by one-way ANOVA.

Figure 4.

RvE1 selectively regulates cytokine and lipid mediators. **a-i**, Cytokines in day 21 BALF from vehicle (V) or RvE1 (100ng) treated mice were coded and measured by cytokine bead array. **j,k** BALF LTB₄ and CysLTs **j** and LXA₄ **k** concentrations were determined by ELISA. Results are expressed as mean $\pm$ s.e.m. for $n \geq 3$ in ≥ 3 independent experiments, * $P < 0.05$ by Student's t-test.

Figure 5.

RvE1 regulates pathogenic T cell IL-17A and IFN- γ production. **a,b** Representative FACS plots showing IFN- γ and IL-17A in CD4⁺ T cells from spleen, MLN and lung of day 21 mice treated with RvE1 or vehicle. Percentages of IFN- γ ⁺ and IL-17A⁺ **a** and the ratio of IFN- γ ⁺ to IL-17A⁺ **b** CD4⁺ T cells in spleen, MLN and lung are indicated after treatment with vehicle (white) or RvE1 (black). **c**, BALF cells at day 21 were counted after anti-IL-17A, rmIL-17A or RvE1 plus rmIL-17A (left) and in separate experiments after RvE1, rmIL-23 or RvE1 plus rmIL-23 (right) (See Methods) **d**, Mice were given IL-6, anti-IFN- γ alone or in combination with RvE1 on days 18-20 and BALF cells were enumerated. **e**, Representative lung tissue sections stained with H&E from day 21 mice treated with an isotype control antibody, anti-

IL-17A, RvE1 or RvE1 plus anti-IFN- γ (see Methods). Original magnification $\times 100$. Br; bronchus, V; vein. *, $P < 0.05$ compared to vehicle or isotype control; † , $P < 0.05$ compared to anti-IL-17, #, $P < 0.05$ compared to RvE1 by one-way ANOVA for $n \geq 4$ in ≥ 2 independent experiments.

Figure 6.

RvE1 and LXA₄ utilize distinct mechanisms to promote resolution of airway inflammation. **a**, Total BALF leukocytes from mice given RvE1, ATLa or RvE1 plus ATLa. **b-d**, Concentrations of IL-17, IL-23 and IFN- γ in BALF were measured by cytokine bead array. **e**, The inhibitory effect of increasing concentrations of RvE1 on IL-23 release from LPS-stimulated BMDCs was measured as a percentage of maximum (89.5 pg/ml/million cells). The LXA₄ receptor antagonist BOC-2 (10 μ M) was added where indicated. Results are expressed as mean $\pm$ s.e.m. *, $P < 0.05$. **f**, Concentrations of IL-6 and TNF released from LPS-activated BMDCs treated with vehicle or 1ng/ml RvE1 were measured by cytokine bead array. Results

are expressed as mean $\pm$ s.e.m for $n \geq 3$ in ≥ 2 independent experiments. *, $P < 0.05$ by one-way ANOVA.