Clinical Science (2014) **126**, 805–814 (Printed in Great Britain) doi: 10.1042/CS20130623

Original Paper

Resolvin E1 promotes resolution of inflammation in a mouse model of an acute exacerbation of allergic asthma

Rylie P. FLESHER*, Cristan HERBERT* and Rakesh K. KUMAR*

*Inflammation and Infection Research Centre, School of Medical Sciences, University of New South Wales, Sydney, NSW 2052, Australia

Abstract

Endogenous mediators, such as RvE1 (resolvin E1), promote resolution of an inflammatory response and have potential as novel therapeutic agents. In the present study, we investigated the activity of RvE1 in a model of an acute exacerbation of chronic allergic asthma in mice. Animals sensitized to OVA (ovalbumin) received controlled low-level challenge with aerosolized antigen for 4 weeks, followed by a single moderate-level challenge to simulate an allergen-induced exacerbation of asthmatic inflammation. Induction of an exacerbation was associated with rapid recruitment of neutrophils, lymphocytes and eosinophils, together with increased levels of Th2 and pro-inflammatory cytokines. When administered before the final moderate-level challenge, RvE1 had only a modest effect on airway inflammation. To assess its effects when administered after induction of an exacerbation, we first characterized the cellular and molecular events associated with spontaneous resolution of airway inflammation over the following 96 h. Subsequently, we showed that administration of RvE1 at 2 and 8 h after the final challenge accelerated this process significantly. Specifically, RvE1 promoted a decline in the number of inflammatory cells, concentration of cytokines in lavage fluid and expression of mRNA for cytokines by macrophages, confirming its pro-resolution activity. *In vitro*, RvE1 had no apparent effect on lymphocytes, but suppressed significantly cytokine production by pulmonary macrophages, with evidence of down-regulation of the nuclear translocation of NF-κB (nuclear factor κB) p65 in these cells. The present study provides novel evidence that RvE1 can facilitate resolution of airway inflammation in a clinically relevant model of an acute exacerbation of asthma, possibly via its effects on activated pulmonary macrophages.

Key words: anti-inflammatory agent, asthma, cytokine, inflammation mediator, macrophage

INTRODUCTION

Asthma is characterized by chronic allergic inflammation of the airways with superimposed episodes of acute inflammation [1]. These acute exacerbations of asthma, which can be life threatening, account for a large proportion of the health care costs associated with this illness [2]. Exacerbations are usually triggered by respiratory viral infections, but may also be related to acute exposure to allergen or to various non-allergic irritants [3,4]. An acute exacerbation of asthma is characterized by intense inflammation which extends into the distal airways, with recruitment of eosinophils and a significant number of neutrophils, as well as worsening airflow obstruction [5,6].

Exacerbations often do not respond satisfactorily to standard therapy [7,8], so there is a need for better or additional treatments. In this context, the resolution of inflammation is now recognized as being an actively regulated process [9]. Notably, endogenous lipid-derived mediators produced during spontaneous resolution of inflammation have been demonstrated to exhibit anti-inflammatory and pro-resolving actions, which potentially could be exploited for therapeutic intervention [10,11]. One such mediator is RvE1 (resolvin E1), which is enzymatically derived from eicosapentaenoic acid via aspirin-acetylated COX2 (cyclooxygenase 2) and 5-lipoxygenase [12,13]. RvE1 signals via the receptor CMKLR1 (chemokine-like receptor 1), also known as ChemR23, as well as binding to the leukotriene receptor BLT1

Abbreviations: AM, alveolar macrophage; BAL, bronchoalveolar lavage; CCL, chemokine (C-C motif) ligand; CMKLR1, chemokine-like receptor 1; CXCL, chemokine (C-X-C motif) ligand; HPRT, hypoxanthine–guanine phosphoribosyltransferase; IL, interleukin; NF-κB, nuclear factor κB; OVA, ovalbumin; PBLN, peribronchial lymph node; RvE1, resolvin E1; TNFα, tumour necrosis factor α.

Correspondence: Professor Rakesh K. Kumar (email R.Kumar@unsw.edu.au).

[also known as LTB_4R (leukotriene B_4 receptor)] to inhibit responses to leukotriene B_4 [14].

RvE1 has been shown to inhibit the migration of neutrophils and the production of pro-inflammatory cytokines. It suppresses inflammation in several models of disease, including experimental colitis and short-term models of allergic airway inflammation [15–17]. However, most such studies have assessed the effects of RvE1 administered as a pre-treatment, before induction of an inflammatory response. We sought to undertake a more clinically relevant investigation of the therapeutic potential of RvE1 using an animal model of an acute exacerbation of mild chronic allergic asthma and comparing treatment before or after induction of acute airway inflammation. The experimental model is based on our well-characterized model of chronic asthmatic inflammation of the airways of mice [18], in which animals systemically sensitized to OVA (ovalbumin) receive long-term inhalational challenge with a controlled low mass concentration of aerosolized OVA. Acute inflammatory changes simulating an asthmatic exacerbation are then induced by exposing the chronically challenged mice to a single moderate-level challenge with aerosolized OVA, which triggers rapid and enhanced accumulation of eosinophils and neutrophils around intrapulmonary airways [19].

In the present study, we first assessed the effect of the administration of RvE1 in the 24 h before induction of an experimental exacerbation. Then, to be able to assess the capacity of RvE1 to promote resolution, we defined the cellular and molecular events associated with spontaneous resolution of inflammation in our model. Thereafter we assessed the potential of RvE1 to accelerate this process when administered following induction of an experimental acute exacerbation.

MATERIALS AND METHODS

Animals and treatments

The protocols used for sensitization and inhalational challenge have been described previously [19]. Briefly, specific pathogen-free female 7–8-week-old BALB/c mice (Animal Resources Centre, Perth, Australia) were systemically sensitized by intraperitoneal injection of 50 μg of alum-precipitated chicken egg OVA (grade V; $\geqslant$98% pure; Sigma) 21 and 7 days before inhalational challenge, then exposed to aerosolized OVA in a whole-body inhalation exposure chamber (Unifab). Chronic low-level challenge involved exposure to $\approx$3 mg/m^3 aerosolized OVA for 30 min/day 3 days per week for 4 weeks to induce changes of mild chronic asthma. At the end of this period, a single moderate-level challenge ($\approx$30 mg/m^3) was used to induce an experimental acute exacerbation. Particle concentration within the chamber was continuously monitored using a DustTrak^{TM} 8520 instrument (TSI). All experimental procedures complied with the requirements of the Animal Care and Ethics Committee of the University of New South Wales (study reference number AEC 11/48A). Animals were randomly allocated to cages and cages were randomly allocated to experimental groups, which comprised seven animals per group for the assessment of spontaneous resolution and six animals per group for treatment with RvE1.

In the initial experiments to assess the effects of treatment before induction of an acute exacerbation, mice were administered 1 μg of RvE1 (Resolvyx Pharmaceuticals) in saline per animal, or vehicle alone, by intraperitoneal injection at 24 and 2 h before the final moderate-level challenge. As a control, the anti-inflammatory drug dexamethasone (cyclodextrin compound; equivalent to 1 mg/kg of body mass; Sigma) was administered by gavage at the same time points (we have shown previously that this dose maximally suppresses inflammation in our model [20]). Animals were killed 4 h after the final challenge (Figure 1A). To define the time course of resolution of acute asthmatic inflammation, groups of mice were killed at 4, 12, 24, 36, 48, 72 and 96 h after the moderate-level challenge (Figure 1B). In subsequent experiments to assess the pro-resolution effects of the drug treatment, mice were administered 1 μg of RvE1, 1 mg of dexamethasone/kg of body mass or vehicle alone at 2 and 8 h after the final moderate-level challenge. Groups of mice were killed at 4, 12 and 24 h (Figure 1C).

Assessment of inflammatory response

Mice were killed by exsanguination following an overdose of sodium pentobarbital. Lungs were perfused with saline to remove blood from the pulmonary capillary bed. BAL (bronchoalveolar lavage) was performed by inflating the lungs with 1 ml of PBS three times. A differential count of the leucocytes in the BAL fluid was performed on at least 200 cells in Leishman-stained smears. Pulmonary AMs (alveolar macrophages) were purified from BAL cells from individual animals by adherence for 30 min. Non-adherent cells were removed by washing and cells were either lysed using Tri Reagent (Sigma) for extraction of RNA or used for *in vitro* experiments. Accumulation of eosinophils in the lung was quantified using a colorimetric assay for eosinophil peroxidase [21], which we have shown provides a reliable estimate of the numbers of these cells in tissues [22].

In vitro stimulation and treatment of cells

AMs were isolated from naïve mice and incubated for 48 h in RPMI 1640 medium with 10% FBS, supplemented with M-CSF (macrophage colony-stimulating factor; 20 ng/ml) as well as IL (interleukin)-4 and IL-13 (both 10 ng/ml) to simulate a Th2-biased cytokine environment. Cells were washed and then stimulated for 4 h with IL-33 (20 ng/ml) with or without either RvE1 (1000 nM) or dexamethasone (100 nM) as a positive control. Similarly, the murine macrophage cell lines MH-S and RAW264.7 (A.T.C.C.; Manassas, VA, U.S.A.) were cultured in medium for 24 h, washed and stimulated for 4 h with IL-33, with or without either RvE1 or dexamethasone. PBLN (peribronchial lymph node) cells were collected from mice that had been sensitized to OVA and received chronic low-level challenge for 4 weeks. Tissue was passed through a 70 μm cell strainer to release the PBLN cells, predominantly lymphocytes, which were resuspended in medium and incubated for 16 h with CD3/CD28 beads (Dynabeads; Life Technologies), with or without either RvE1 or dexamethasone. At the end of each experiment, cells were lysed using Tri Reagent for the extraction of RNA.

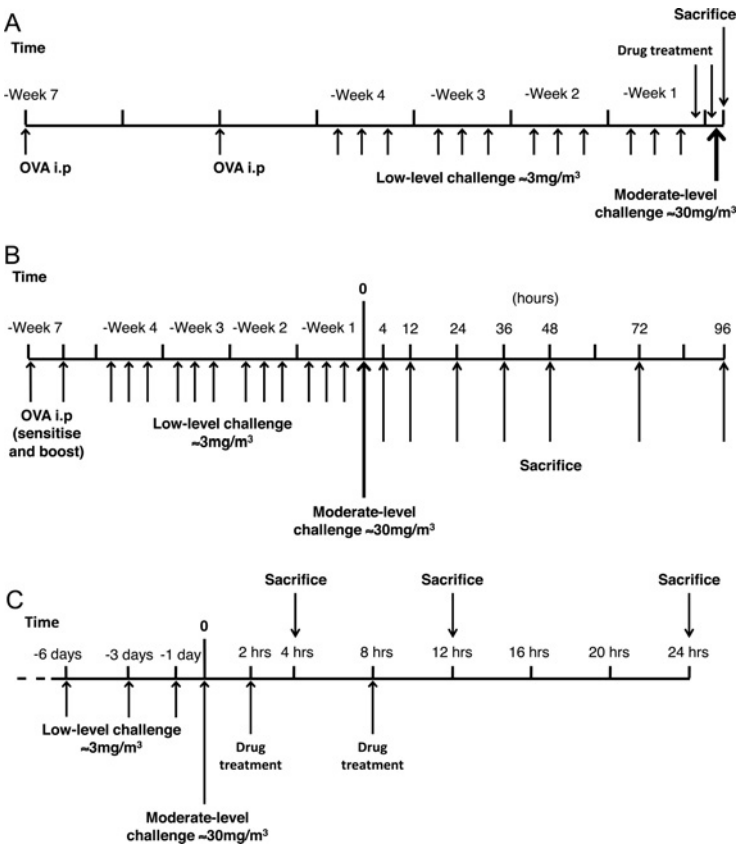

Figure 1 Timeline for assessment of the effect of RvE1 on resolution of inflammation in the model of an acute exacerbation of chronic asthma
i.p, intraperitoneal.

Cytokine analysis

Extracted RNA samples were treated with DNase (Turbo DNase; Ambion) and reverse-transcribed into cDNA using Superscript III (Invitrogen). Quantitative real-time PCR was used to assess the expression of cytokines, with detection of amplified products using SYBR Green (Bioline). Expression was normalized to *Hprt* (hypoxanthine–guanine phosphoribosyltransferase).

The concentrations of cytokines in the BAL fluid were assessed by multiplex immunoassay (mouse 23-plex panel; Bio-Rad Laboratories) according to the manufacturer's instructions.

Expression of CMKLR1

Expression of the receptor for RvE1 on MH-S and RAW264.7 cells was assessed by staining with a fluorochrome-conjugated monoclonal antibody (R&D Systems). A FACSCalibur flow cytometer (Becton Dickinson) was used to acquire the fluorescence data. FlowJo (version 8.8.6; Treestar) was used to quantify the median fluorescence intensity.

Nuclear translocation of NF-κB (nuclear factor κB) p65

RAW264.7 cells cultured in eight-well chamber slides (Nunc) were treated with IL-33 and either RvE1 or dexamethasone for 15 min. Fixed cells were permeabilized, blocked and incubated overnight with an anti-(NF-κB p65) antibody (Cell Signaling Technology) at 4 °C. Control cells were incubated without the primary antibody. Detection was with an Alexa Fluor™ 568-labelled anti-(rabbit IgG) antibody (Invitrogen) for 90 min. Slides were washed, coverslipped with Prolong® Gold Antifade Reagent and DAPI (Life Technologies), and visualized using an Olympus BX51 microscope with a DP70 camera. ImageJ software (http://rsbweb.nih.gov/ij/) was used to identify the nuclei as regions of interest, on the basis of DAPI staining, and then to quantify the mean red fluorescence intensity per unit area for each sample.

Statistical analysis

Results are means ± S.E.M. For studies of spontaneous resolution, multiple comparisons were performed using one-way ANOVA followed by a Holm–Sidak test for all groups, or a Kruskal–Wallis test followed by a Dunn's test for non-parametric data, as appropriate. For studies of the effect of RvE1, post-test comparisons were between the control and RvE1-treated groups. For the study of receptor expression, an unpaired Student's *t* test was used to compare cell lines. The software package GraphPad Prism 6.02 (GraphPad Software) was used for all data analysis and preparation of the graphs.

Table 1 Effects on leucocyte recruitment of drug treatment before the induction of an experimental acute exacerbation
Results are the percentage of cells in BAL fluid (neutrophils and lymphocytes) or peroxidase activity in lung tissue measured as A_{450} (eosinophils) and are shown as means ± S.E.M. ($n = 7$). $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with naïve animals and $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ compared with the vehicle-treated group.

Cellular response	Naïve	Vehicle	Dexamethasone	RvE1
Neutrophils	2.4 ± 0.6	34.0 ± 3.1***	2.5 ± 0.8###	20.1 ± 4.5##
Lymphocytes	4.0 ± 0.4	33.8 ± 5.3***	9.1 ± 1.8###	25.1 ± 4.3
Eosinophils	1.06 ± 0.11	1.74 ± 0.17**	1.04 ± 0.13##	2.02 ± 0.07

Table 2 Effects on cytokine concentrations in BAL fluid of drug treatment before the induction of an experimental acute exacerbation
Results are mean ± S.E.M. pg/ml ($n = 7$). $^{***}P < 0.001$ compared with naïve animals and $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ compared with the vehicle-treated group.

Cytokine	Naïve	Vehicle	Dexamethasone	RvE1
IL-4 (pg/ml)	6.7 ± 2.2	110.7 ± 23.5***	14.7 ± 2.92###	61.7 ± 11.1#
IL-5 (pg/ml)	18.9 ± 5.7	64.1 ± 6.9***	32.7 ± 3.0#	48.4 ± 5.1
IL-1β (pg/ml)	40.9 ± 7.2	186.4 ± 10.8***	126.2 ± 7.9	148.7 ± 7.8
IL-6 (pg/ml)	3.6 ± 2.0	25.9 ± 2.5***	7.8 ± 1.7##	18.2 ± 2.1

RESULTS

No adverse events occurred during these studies.

RvE1 as anti-inflammatory therapy

In the initial experiments, we investigated the effects of RvE1 on the development of inflammation when treatment was administered before induction of the experimental acute exacerbation of asthma. We found that RvE1 modestly diminished the increase in the percentage of neutrophils in BAL fluid, but had little effect on the accumulation of other inflammatory cells. In contrast, dexamethasone suppressed markedly the number of neutrophils and lymphocytes in BAL fluid, as well as the accumulation of eosinophils in the lung tissue (Table 1).

Similarly, RvE1 modestly inhibited the increase in the concentration of IL-4 in BAL fluid, but had little effect on other cytokines of interest (IL-5, IL-1β and IL-6), whereas the increase in concentrations of IL-4, IL-5 and IL-6 was suppressed markedly by treatment with dexamethasone (Table 2).

Time course of the resolution of inflammation

To be able to investigate the effects of RvE1 as a pro-resolution treatment, when administered after induction of an experimental acute exacerbation, we defined the pattern of resolution of the inflammatory response so that optimal time points could be selected. Following the induction of an acute exacerbation, the percentage of neutrophils in BAL fluid increased sharply to reach a maximum at 12 h, and then declined relatively rapidly returning to near baseline levels by 36 h (Figure 2A). In contrast, the percentage of lymphocytes increased within 4 h, but the high proportion of these cells was maintained for longer and did not subside to baseline until 96 h (Figure 2B). The number of eosinophils in lung tissue, as assessed by the eosinophil peroxidase assay, peaked at 12 h after the induction of an acute exacerbation, but declined to baseline by 48 h (Figure 2C).

The concentration of various pro-inflammatory cytokines in BAL fluid was also increased. Typically, the maximum concentration was observed at 4 h, although IL-5 peaked at 12 h after induction of an acute exacerbation (Figure 3). The concentration of most cytokines then declined relatively rapidly, reaching near baseline by 36 h. However, levels of IL-5 (Figure 3) and CXCL1 [chemokine (C-X-C motif) ligand 1; results not shown] remained elevated for longer. Relative expression by AMs of mRNA for various pro-inflammatory cytokines was similarly increased sharply at 4 h and then declined rapidly to reach baseline by 36 h (Figure 4).

RvE1 as 'pro-resolution' therapy

On the basis of the above results, we investigated the effects of RvE1 on the resolution of inflammation during the first 24 h, with treatment administered at 2 and 8 h after induction of the acute exacerbation of asthmatic inflammation and assessment at 4, 12 and 24 h. We found that treatment with RvE1 decreased significantly the percentage of neutrophils at 12 h (Figure 5A) and lymphocytes at 24 h (Figure 5B) in BAL fluid. In contrast, RvE1 did not affect the accumulation of eosinophils in the lung tissue (Figure 5C). In this setting, the effects of RvE1 were similar to those of dexamethasone, which, as expected, caused a marked suppression of the inflammatory response. Therefore we investigated further the possible mechanisms of the action of RvE1. We found that RvE1 decreased significantly the concentrations of various cytokines in BAL fluid over the time period of interest. In addition to the decrease in the concentration of Th2 cytokines (IL-4 and IL-5) and pro-inflammatory cytokines (IL-1β and IL-6) shown in Figure 6, there were also significantly reduced concentrations of IL-9, IL-13, IL-17, GM-CSF (granulocyte/macrophage colony-stimulating factor), IFNγ (interferon γ), CCL4 [chemokine (C-C motif) ligand 4], CCL5 and CCL11 (Supplementary Figures S1 and S2 at http://www.clinsci.org/cs/126/cs1260805add.htm). However, there was little effect of RvE1 on BAL fluid levels of TNFα (tumour necrosis factor α) or CXCL1 (Supplementary Figure S2).

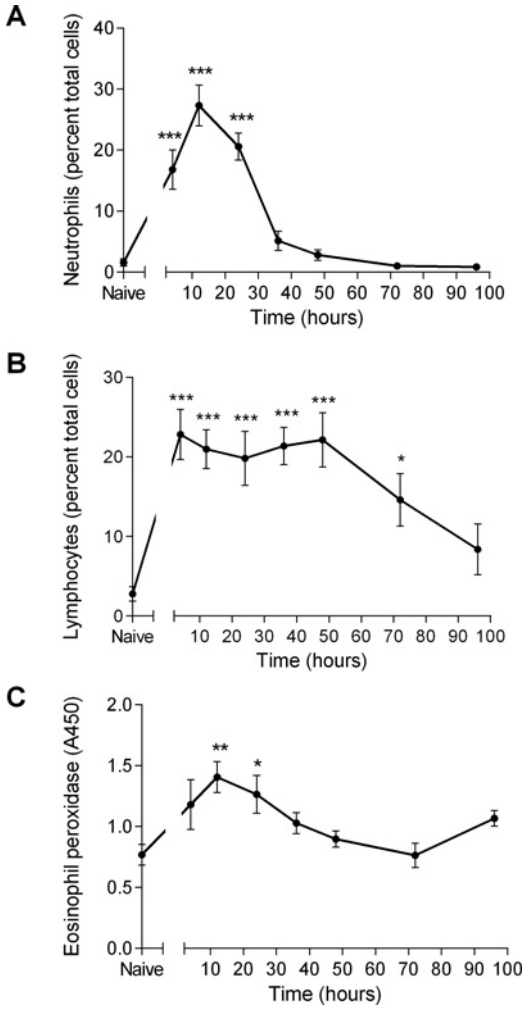

Figure 2 Leucocyte recruitment after induction of an experimental acute exacerbation

Percentage of neutrophils (**A**) and lymphocytes (**B**) in BAL fluid. (**C**) Eosinophil peroxidase activity as an indicator of numbers of eosinophils in lung tissue. Results are means ± S.E.M. ($n = 7$ samples per group). $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P <$ 0.001 compared with naïve animals.

Importantly, treatment with RvE1 decreased significantly the relative expression of mRNA for pro-inflammatory cytokines by pulmonary macrophages (Figure 7).

Effects of RvE1 *in vitro*

RvE1 decreased significantly the expression of pro-inflammatory cytokines by Th2 cytokine-primed IL-33-stimulated AMs *in vitro*, with a lesser effect on TNFα and a greater effect on CXCL1 than dexamethasone (Figure 8). In contrast, RvE1 had no effect on the levels of mRNA expression for Th2 cytokines by antigen-primed CD3/CD28-restimulated lymphocytes *in vitro*, whereas dexamethasone suppressed these completely (Supplementary Figure S3 at http://www.clinsci.org/cs/126/cs1260805add.htm).

To investigate further the effect of RvE1 on macrophages, we used two macrophage-derived cell lines. Unexpectedly,

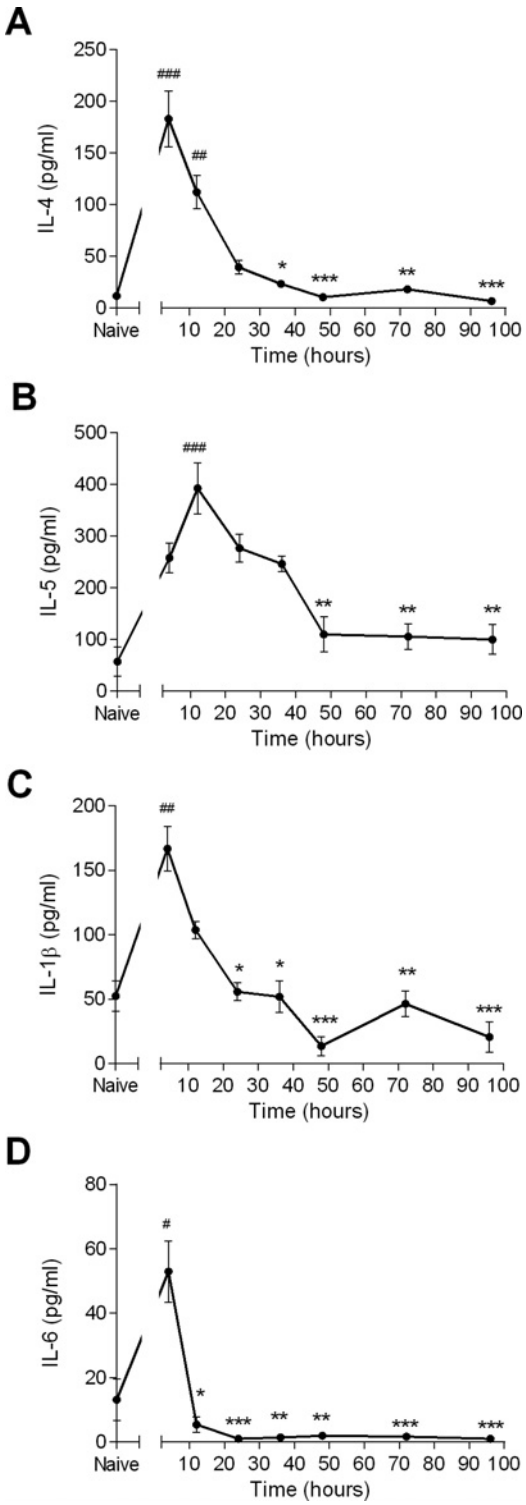

Figure 3 Concentrations of Th2 and pro-inflammatory cytokines in BAL fluid after induction of an experimental acute exacerbation

(**A**) IL-4. (**B**) IL-5. (**C**) IL-1β. (**D**) IL-6. Results are means ± S.E.M. ($n = 7$ samples per group). $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ and $^{\#\#\#}P <$ 0.001 compared with naïve animals and $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with the peak.

we found that RvE1 had no effect on the expression of pro-inflammatory cytokines by IL-33-stimulated MH-S cells, but

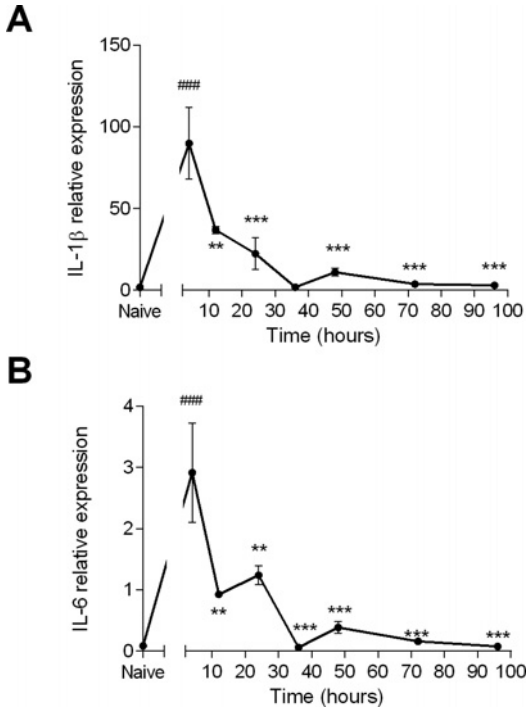

Figure 4 Relative expression of mRNA for pro-inflammatory cytokines by AMs after induction of an experimental acute exacerbation

(**A**) *Il-1b*. (**B**) *Il-6*. Results are means ± S.E.M. ($n = 7$ samples per group) relative to *Hprt*. $^{###}P < 0.001$ compared with naïve animals and $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with the peak.

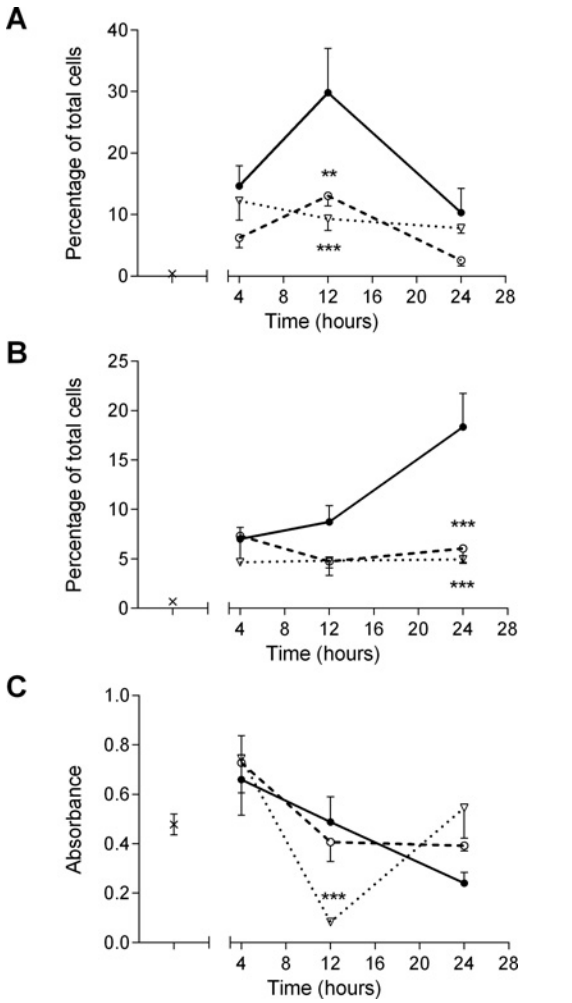

Figure 5 Effects of treatment with RvE1 on leucocyte recruitment

Percentage of neutrophils (**A**) and lymphocytes (**B**) in BAL fluid. (**C**) Eosinophil peroxidase activity as an indicator of numbers of eosinophils in lung tissue. ●, vehicle-treated mice; ○, RvE1-treated mice; ▽, dexamethasone-treated mice; ×, naïve mice. Results are means ± S.E.M. ($n = 6$ samples per group). $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with the corresponding vehicle-treated group.

did decrease significantly their expression by IL-33-stimulated RAW 264.7 cells (Supplementary Figure S4, at http://www.clinsci.org/cs/126/cs1260805add.htm, shows data for *Tnfa* and *Il-1b*; similar results were obtained for *Cxcl1*, results not shown). In seeking a possible explanation for this, we examined the expression of the CMKLR1, a receptor for RvE1, in these two cell lines. Flow cytometry demonstrated that RvE1-responsive RAW264.7 cells had a significantly higher median fluorescence intensity of staining for the CMKLR1 than RvE1-unresponsive MH-S cells (2.92 ± 0.05 for MH-S cells compared with 12.53 ± 0.50 for RAW264.7 cells; $P < 0.001$).

Inhibition by RvE1 of the nuclear translocation of NF-κB

We assessed the role of the NF-κB pathway, as a potential mechanism of the action of RvE1 on macrophages, by immunocytochemistry of RAW 264.7 cells. Nuclear staining for NF-κB p65 was increased markedly within 15 min in cells stimulated with IL-33. Cells that were treated simultaneously with RvE1 exhibited significantly reduced nuclear staining (Figure 9) and quantification of fluorescence intensity revealed that this decrease was comparable with that observed as a result of treatment with dexamethasone (unstimulated, 16.04 ± 1.49; IL-33-stimulated, 41.80 ± 1.36; dexamethasone-treated, 31.29 ± 1.62; RvE1-treated, 31.35 ± 0.32; $P < 0.001$ for both treatment groups compared with the IL-33-stimulated group).

DISCUSSION

In the present study, we assessed the ability of RvE1 to suppress airway inflammation in an experimental model of an acute exacerbation of chronic allergic asthma. We found that RvE1 had limited anti-inflammatory activity when administered before moderate-level challenge with OVA, which simulates induction of an allergen-induced exacerbation. In contrast, when administered in the clinically relevant setting of treatment following induction of an experimental exacerbation, RvE1 accelerated significantly the decline in the numbers of leucocytes and the levels of pro-inflammatory cytokines. These findings were in contrast with treatment with the glucocorticoid dexamethasone, which exhibited strong activity in both settings. The results of the present study are thus consistent with previous reports [11,23] suggesting that RvE1 functions as a pro-resolution mediator. Moreover, in this model the effects of

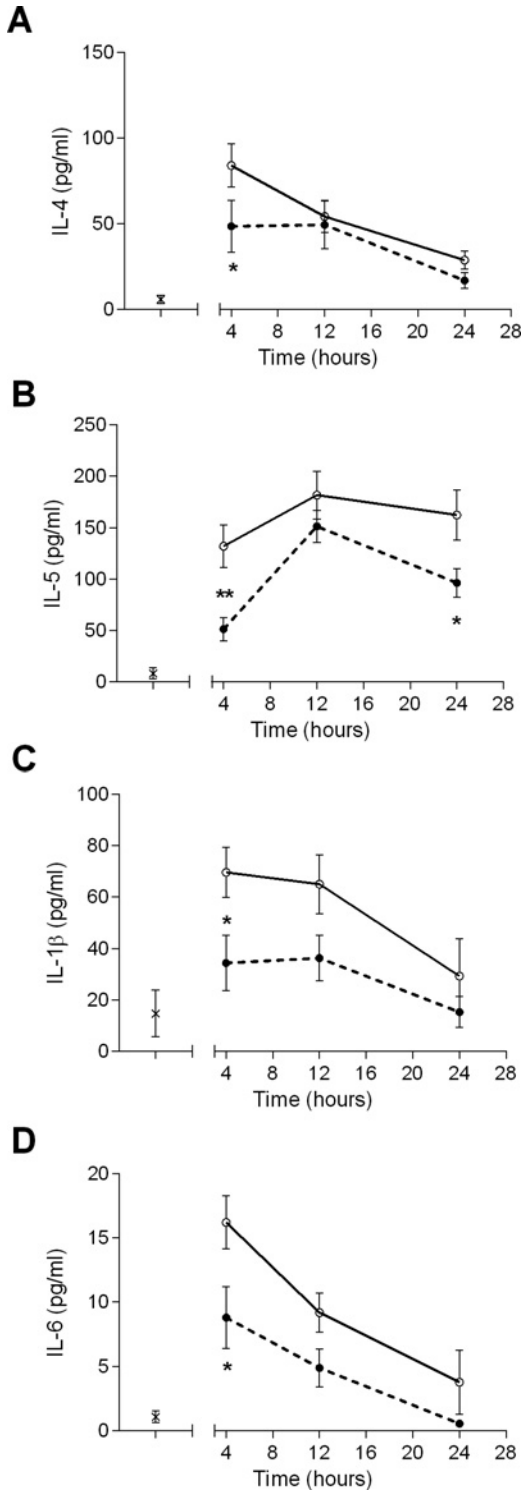

Figure 6 Effects of treatment with RvE1 on concentrations of Th2 and pro-inflammatory cytokines in BAL fluid

(**A**) IL-4. (**B**) IL-5. (**C**) IL-1β. (**D**) IL-6. ●, vehicle-treated mice; ○, RvE1-treated mice; ×, naïve mice. Results are means ± S.E.M. ($n = 6$ samples per group). $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with the corresponding vehicle-treated group.

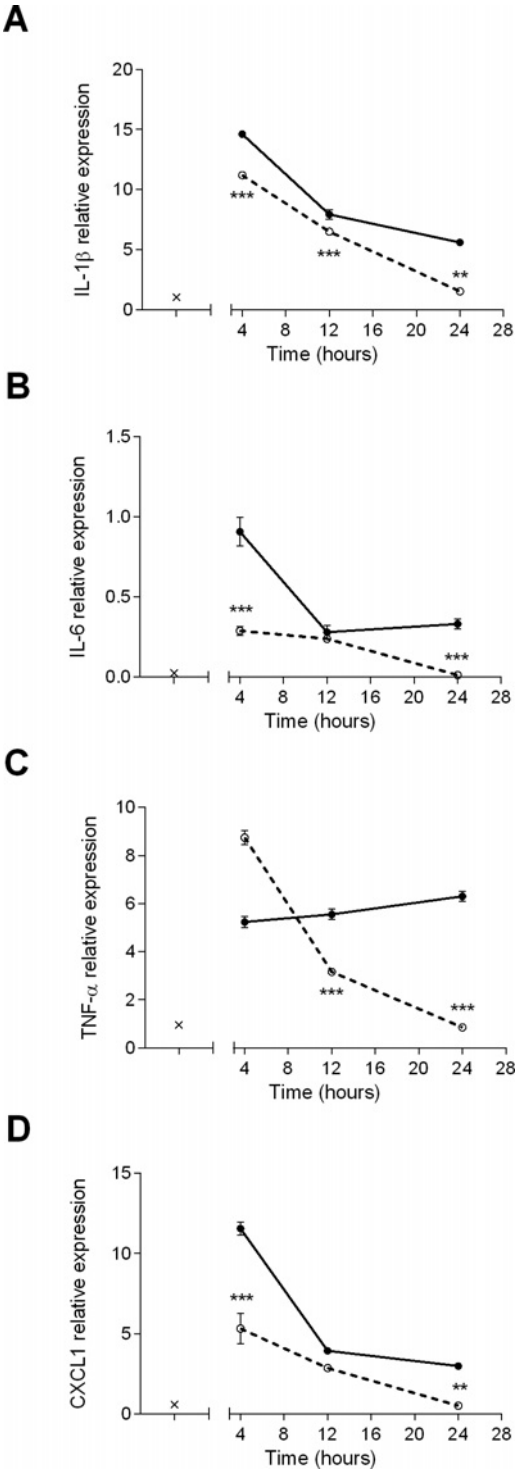

Figure 7 Effects of treatment with RvE1 on relative expression of mRNA for pro-inflammatory cytokines by AMs

(**A**) *Il-1b*. (**B**) *Il-6*. (**C**) *Tnfa*. (**D**) *Cxcl1*. ●, vehicle-treated mice; ○, RvE1-treated mice; ×, naïve mice. Results are means ± S.E.M. ($n = 6$ samples per group). $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with the corresponding vehicle-treated group.

RvE1 appear to be mediated, at least in part, via suppression of the activation of pulmonary macrophages, with evidence of down-regulation of the nuclear translocation of NF-κB p65 in these cells.

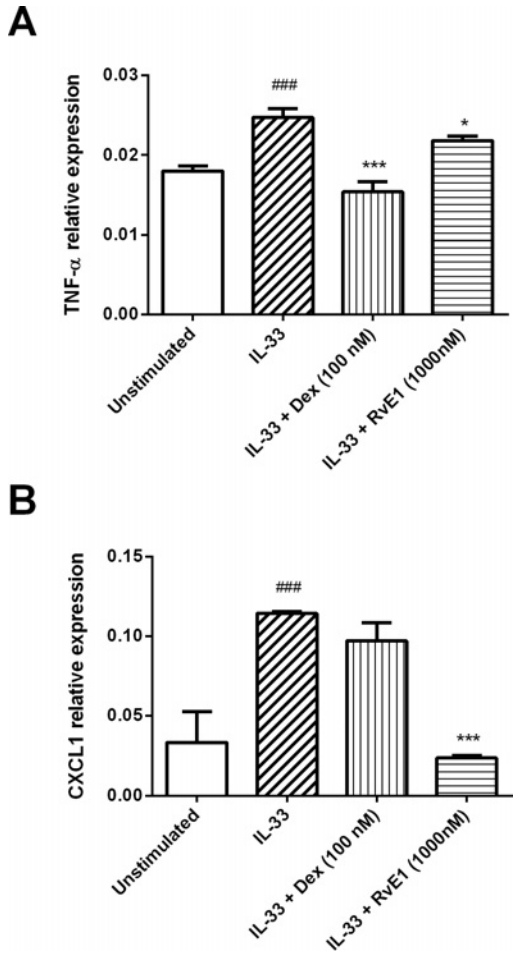

Figure 8 Effects of treatment with RvE1 on the relative expression of mRNA for pro-inflammatory cytokines by AMs pre-treated with IL-4/IL-13 and stimulated with IL-33, compared with treatment with dexamethasone as a positive control

(**A**) *Tnfa*. (**B**) *Cxcl1*. Results are means ± S.E.M. ($n = 4$ samples per group). $^{###}P < 0.001$ compared with the unstimulated group and $^{*}P < 0.05$ and $^{***}P < 0.001$ compared with the IL-33-stimulated group.

Our animal model exhibits many of the characteristic features of human asthma, including a background of chronic inflammation, goblet cell hyperplasia/metaplasia and subepithelial fibrosis following low-level chronic challenge, with development of increased and more distal airway inflammation following the single moderate-level challenge used to simulate an allergen-induced exacerbation. The model has therefore been acknowledged to represent a significant improvement in terms of the fidelity with which it reproduces features of human asthma [24,25]. In the present study, we showed that spontaneous resolution of inflammation commences within 24 h and is complete by 96 h, with numbers of recruited neutrophils and eosinophils declining more rapidly than lymphocytes. The associated increase in the levels of Th2 and pro-inflammatory cytokines in BAL fluid returns to baseline in a shorter period, typically 24–36 h. These results enabled us to select the optimal time points for assessment of pro-resolution activity. Our finding that RvE1 is effective in promoting resolution of airway inflammation has the potential to be translated to the management of acute severe asthma. However, we acknowledge that for use as a therapeutic agent, RvE1 will need to be assessed further, including for its efficacy following delivery via the respiratory tract.

We recognize that most clinical exacerbations of asthma develop in the setting of respiratory viral infections, notably by rhinovirus [3,4]. Nevertheless, a proportion of exacerbations is initiated by exposure to allergen, and there is synergism between allergic and non-allergic triggers as well as cross-talk between the inflammatory mechanisms, which converge [26,27]. It therefore seems probable that the results we have obtained using this animal model would also be relevant to acute exacerbations of asthmatic inflammation involving other trigger factors.

Neutrophilic inflammation is a feature of asthmatic exacerbations [3], so it is noteworthy that RvE1 diminished the number of neutrophils in BAL fluid. The present study does not indicate whether this effect was primarily via inhibition of neutrophil recruitment or via accelerated clearance, by way of apoptosis and phagocytosis. However, given the rapidity with which neutrophils are recruited in any acute inflammatory response and the fact that treatment with RvE1 commenced at 2 h after induction of the experimental exacerbation, it appears probable that clearance mechanisms would have been important. Consistent with this, we observed that the effects of RvE1 on levels of potentially relevant chemoattractants in BAL fluid correlated relatively poorly with changes in the numbers of neutrophils or eosinophils in BAL fluid and tissue. Previous studies have shown that RvE1 reduces transendothelial migration and promotes apoptosis of neutrophils [12,28], as well as facilitating the non-phlogistic phagocytosis of apoptotic neutrophils by macrophages [29].

In this model, we have demonstrated previously that activation of pulmonary macrophages plays an important role in driving the airway inflammation associated with an acute exacerbation [30]. Our finding that treatment with RvE1 suppressed significantly and/or accelerated the decline in the levels of expression of pro-inflammatory cytokines by pulmonary macrophages is thus of particular interest. In our previous work, we had shown that these macrophages have a key role in activating the production of Th2 cytokines in sensitized chronically challenged animals [30]. This may help to explain why, using this model of an acute exacerbation of pre-existing mild asthmatic inflammation, we found decreased levels of Th2 cytokines in BAL fluid, whereas this was not observed in a previous study [16]. However, consistent with earlier studies, we did demonstrate decreased levels of several pro-inflammatory cytokines in BAL fluid.

In further *in vitro* experiments, we showed that although RvE1 had no direct effect on lymphocytes, it suppressed production of pro-inflammatory cytokines by macrophages and macrophage-derived cell lines, apparently by signalling via the CMKLR1 receptor. These findings are consistent with published results about the expression of this receptor on leucocytes [14,31]. We also demonstrated that the effects of RvE1 on macrophages are likely to be related to inhibition of the NF-κB pathway, because we were able to show that treatment with RvE1 markedly reduced translocation of p65 protein into the nucleus. This novel finding in macrophages is consistent with earlier studies of CMKLR1 signalling using HeLa and embryonic kidney cells transfected with the receptor [14,32].

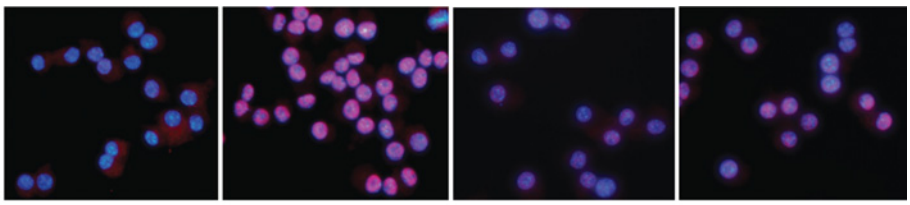

Figure 9 **Inhibition by RvE1 and dexamethasone of IL-33-induced translocation of NF-κB p65 in RAW264.7 cells**
Representative immunofluorescence micrographs of nuclear staining for DAPI (blue) and cytoplasmic or nuclear staining for p65 (red in cytoplasm and purple when translocated to nuclei).

In conclusion, the results of the present study support the notion that pro-resolving endogenous mediators, such as RvE1, may have potential for the treatment of pulmonary inflammatory disorders, such as acute exacerbations of asthma [23]. Although resolvins are inactivated relatively rapidly *in vivo* [10,33], repeated administration may be feasible during an acute exacerbation, whereas analogues with greater stability and/or longer half-lives may become available in the future.

CLINICAL PERSPECTIVES

- Acute exacerbations of asthma often do not respond satisfactorily to standard therapy. We investigated whether RvE1, an endogenous lipid-derived mediator produced during spontaneous resolution of inflammation, could promote resolution of airway inflammation in a mouse model of an acute exacerbation of chronic asthma.
- We found that RvE1 accelerated significantly the decline in numbers of inflammatory cells and levels of pro-inflammatory cytokines, possibly via its effects on activated pulmonary macrophages.
- RvE1 thus appears to have potential as a novel therapeutic agent for pulmonary inflammatory disorders, such as acute exacerbations of asthma.

AUTHOR CONTRIBUTION

Rylie Flesher conducted the majority of the experiments, analysed the data and wrote the first draft of the paper; Cristan Herbert performed some of the experimental work and reviewed the paper before submission; and Rakesh Kumar designed the study, supervised the project and edited the paper before submission.

ACKNOWLEDGEMENTS

Resolvin E1 was a gift from Resolvyx Pharmaceuticals, Bedford, MA. U.S.A.

FUNDING

This work was supported by the National Health and Medical Research Council of Australia [grant number 568616].

REFERENCES

1 Kumar, R. K. and Jeffery, P. K. (2013) Pathology of asthma. In Middleton's Allergy (N. F. Adkinson, B. S. Bochner, W. Burks, W. W. Busse, S. T. Holgate, R. F. Lemanske and R. E. O'Hehir, eds), pp. 986–999, Elsevier, Cambridge
2 Holgate, S. T. (2005) Exacerbations: the asthma paradox. Am. J. Respir. Crit. Care Med. **172**, 941–943
3 Dougherty, R. H. and Fahy, J. V. (2009) Acute exacerbations of asthma: epidemiology, biology and the exacerbation-prone phenotype. Clin. Exp. Allergy **39**, 193–202
4 Jackson, D. J., Sykes, A., Mallia, P. and Johnston, S. L. (2011) Asthma exacerbations: origin, effect, and prevention. J. Allergy Clin. Immunol. **128**, 1165–1174
5 Martin, R. J. (2002) Therapeutic significance of distal airway inflammation in asthma. J. Allergy Clin. Immunol. **109**, S447–S460
6 Qiu, Y., Zhu, J., Bandi, V., Guntupalli, K. K. and Jeffery, P. K. (2007) Bronchial mucosal inflammation and upregulation of CXC chemoattractants and receptors in severe exacerbations of asthma. Thorax **62**, 475–482
7 McFadden, Jr, E. R. (2003) Acute severe asthma. Am. J. Respir. Crit. Care Med. **168**, 740–759
8 Carroll, C. L., Bhandari, A., Schramm, C. M. and Zucker, A. R. (2006) Chronic inhaled corticosteroids do not affect the course of acute severe asthma exacerbations in children. Pediatr. Pulmonol. **41**, 1213–1217
9 Serhan, C. N., Brain, S. D., Buckley, C. D., Gilroy, D. W., Haslett, C., O'Neill, L. A., Perretti, M., Rossi, A. G. and Wallace, J. L. (2007) Resolution of inflammation: state of the art, definitions and terms. FASEB J. **21**, 325–332
10 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
11 Levy, B. D. (2010) Resolvins and protectins: natural pharmacophores for resolution biology. Prostaglandins Leukot. Essent. Fatty Acids **82**, 327–332
12 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
13 Arita, M., Bianchini, F., Aliberti, J., Sher, A., Chiang, N., Hong, S., Yang, R., Petasis, N. A. and Serhan, C. N. (2005) Stereochemical assignment, antiinflammatory properties, and receptor for the omega-3 lipid mediator resolvin E1. J. Exp. Med. **201**, 713–722
14 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

15 Arita, M., Yoshida, M., Hong, S., Tjonahen, E., Glickman, J. N., Petasis, N. A., Blumberg, R. S. and Serhan, C. N. (2005) Resolvin E1, an endogenous lipid mediator derived from omega-3 eicosapentaenoic acid, protects against 2,4,6-trinitrobenzene sulfonic acid-induced colitis. Proc. Natl. Acad. Sci. U.S.A. **102**, 7671–7676
16 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
17 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
18 Temelkovski, J., Hogan, S. P., Shepherd, D. P., Foster, P. S. and Kumar, R. K. (1998) An improved murine model of asthma: selective airway inflammation, epithelial lesions and increased methacholine responsiveness following chronic exposure to aerosolised allergen. Thorax **53**, 849–856
19 Siegle, J. S., Hansbro, N., Herbert, C., Yang, M., Foster, P. S. and Kumar, R. K. (2006) Airway hyperreactivity in exacerbation of chronic asthma is independent of eosinophilic inflammation. Am. J. Respir. Cell Mol. Biol. **35**, 565–570
20 Ito, K., Herbert, C., Siegle, J. S., Vuppusetty, C., Hansbro, N., Thomas, P. S., Foster, P. S., Barnes, P. J. and Kumar, R. K. (2008) Steroid-resistant neutrophilic inflammation in a mouse model of an acute exacerbation of asthma. Am. J. Respir. Cell Mol. Biol. **39**, 543–550
21 Schneider, T. and Issekutz, A. C. (1996) Quantitation of eosinophil and neutrophil infiltration into rat lung by specific assays for eosinophil peroxidase and myeloperoxidase. Application in a Brown Norway rat model of allergic pulmonary inflammation. J. Immunol. Methods **198**, 1–14
22 Bunting, M. M., Shadie, A. M., Flesher, R. P., Nikiforova, V., Garthwaite, L., Tedla, N., Herbert, C. and Kumar, R. K. (2013) Interleukin-33 drives activation of alveolar macrophages and airway inflammation in a mouse model of acute exacerbation of chronic asthma. Biomed. Res. Int. **2013**, 250938
23 Uddin, M. and Levy, B. D. (2011) Resolvins: natural agonists for resolution of pulmonary inflammation. Prog. Lipid Res. **50**, 75–88
24 Fulkerson, P. C., Rothenberg, M. E. and Hogan, S. P. (2005) Building a better mouse model: experimental models of chronic asthma. Clin. Exp. Allergy **35**, 1251–1253
25 Nials, A. T. and Uddin, S. (2008) Mouse models of allergic asthma: acute and chronic allergen challenge. Dis. Model. Mech. **1**, 213–220
26 Green, R. M., Custovic, A., Sanderson, G., Hunter, J., Johnston, S. L. and Woodcock, A. (2002) Synergism between allergens and viruses and risk of hospital admission with asthma: case-control study. BMJ **324**, 763
27 Holt, P. G. and Strickland, D. H. (2010) Interactions between innate and adaptive immunity in asthma pathogenesis: new perspectives from studies on acute exacerbations. J. Allergy Clin. Immunol. **125**, 963–972
28 El Kebir, D., Gjorstrup, P. and Filep, J. G. (2012) Resolvin E1 promotes phagocytosis-induced neutrophil apoptosis and accelerates resolution of pulmonary inflammation. Proc. Natl. Acad. Sci. U.S.A. **109**, 14983–14988
29 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
30 Herbert, C., Scott, M. M., Scruton, K. H., Keogh, R. P., Yuan, K. C., Hsu, K., Siegle, J. S., Tedla, N., Foster, P. S. and Kumar, R. K. (2010) Alveolar macrophages stimulate enhanced cytokine production by pulmonary $CD4^+$ T-lymphocytes in an exacerbation of murine chronic asthma. Am. J. Pathol. **177**, 1657–1664
31 Luangsay, S., Wittamer, V., Bondue, B., De Henau, O., Rouger, L., Brait, M., Franssen, J. D., de Nadai, P., Huaux, F. and Parmentier, M. (2009) Mouse ChemR23 is expressed in dendritic cell subsets and macrophages, and mediates an anti-inflammatory activity of chemerin in a lung disease model. J. Immunol. **183**, 6489–6499
32 Ishida, T., Yoshida, M., Arita, M., Nishitani, Y., Nishiumi, S., Masuda, A., Mizuno, S., Takagawa, T., Morita, Y., Kutsumi, H. et al. (2010) Resolvin E1, an endogenous lipid mediator derived from eicosapentaenoic acid, prevents dextran sulfate sodium-induced colitis. Inflamm. Bowel Dis. **16**, 87–95
33 Arita, M., Oh, S. F., Chonan, T., Hong, S., Elangovan, S., Sun, Y. P., Uddin, J., Petasis, N. A. and Serhan, C. N. (2006) Metabolic inactivation of resolvin E1 and stabilization of its anti-inflammatory actions. J. Biol. Chem. **281**, 22847–22854

Received 30 September 2013/29 November 2013; accepted 18 December 2013
Published as Immediate Publication 18 December 2013, doi: 10.1042/CS20130623

Clinical Science (2014) **126**, 805–814 (Printed in Great Britain) doi: 10.1042/CS20130623

Original Paper

SUPPLEMENTARY ONLINE DATA

Resolvin E1 promotes resolution of inflammation in a mouse model of an acute exacerbation of allergic asthma

Rylie P. FLESHER*, Cristan HERBERT* and Rakesh K. KUMAR*

*Inflammation and Infection Research Centre, School of Medical Sciences, University of New South Wales, Sydney, NSW 2052, Australia

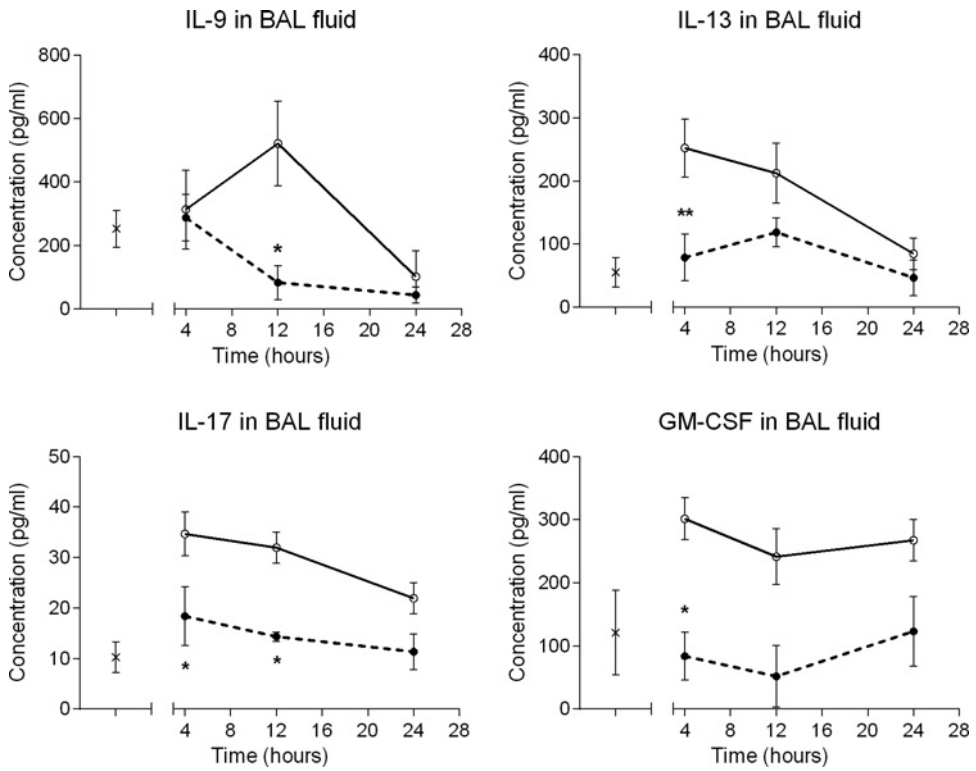

Figure S1 **Effects of treatment with RvE1 on the concentrations of cytokines in BAL fluid**
GM-CSF, granulocyte/macrophage colony-stimulating factor. ●, vehicle-treated mice; ○, RvE1-treated mice; ×, naïve mice. Results are means ± S.E.M. ($n = 6$ samples per group). $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with the corresponding vehicle-treated group.

Correspondence: Professor Rakesh K. Kumar (email R.Kumar@unsw.edu.au).

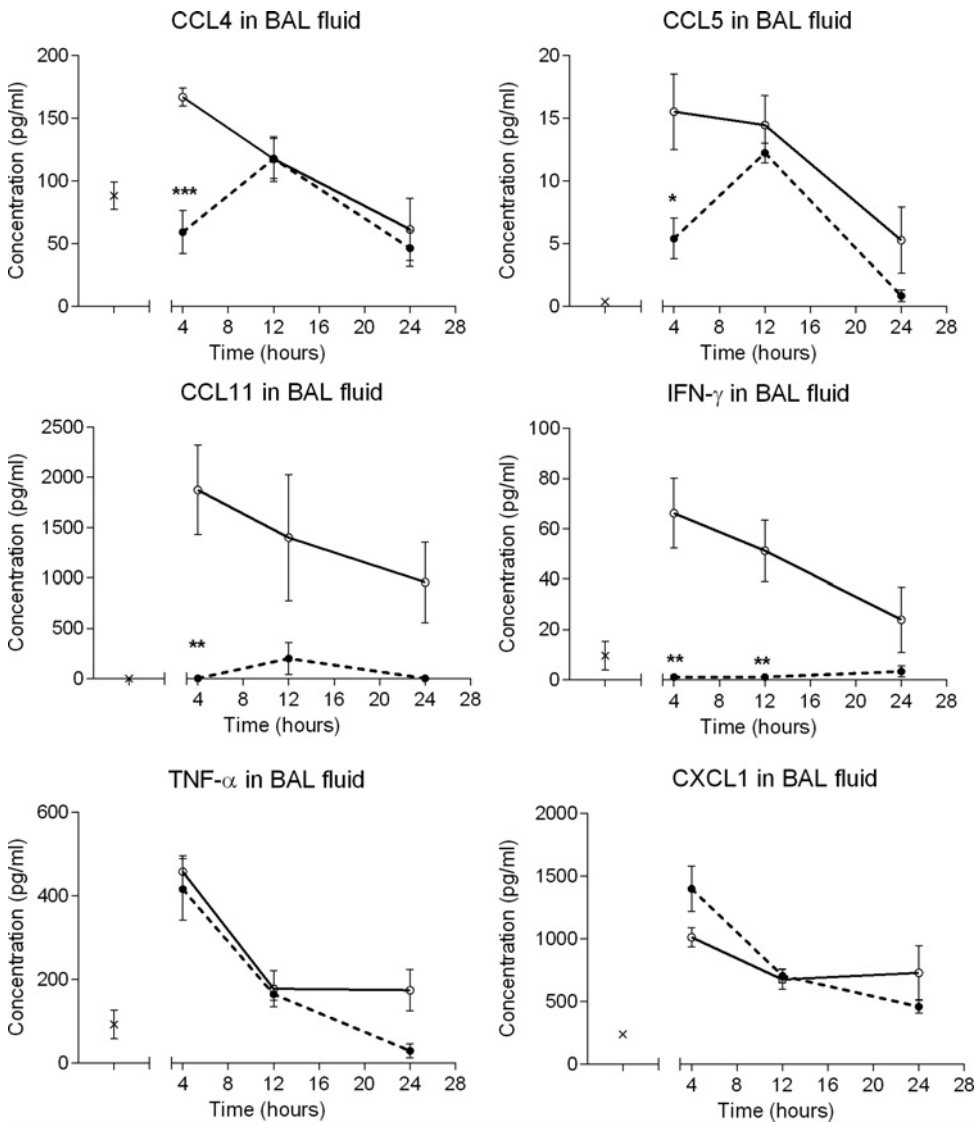

Figure S2 Effects of treatment with RvE1 on the concentrations of cytokines in BAL fluid

●, vehicle-treated mice; ○, RvE1-treated mice; ×, naïve mice. Results are means ± S.E.M. ($n = 6$ samples per group). $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with the corresponding vehicle-treated group.

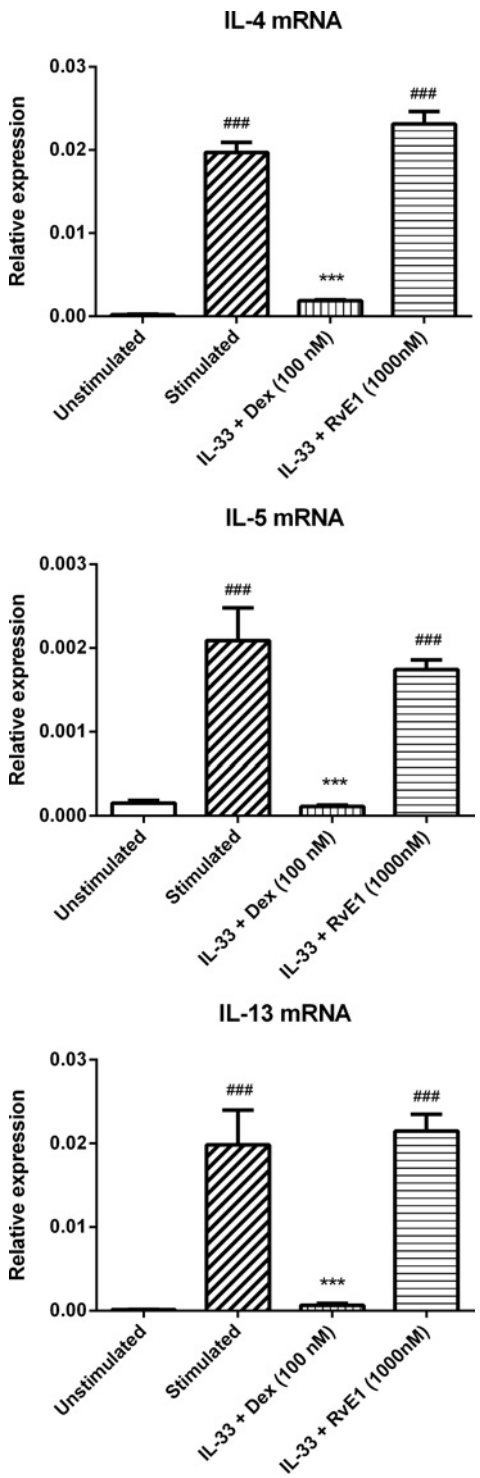

Figure S3 Effects of treatment with RvE1 on relative expression of mRNA for Th2 cytokines by antigen-primed lymphocytes restimulated with CD3/CD28 compared with treatment with dexamethasone

Results are means ± S.E.M. ($n = 4$ samples per group). $^{###}P < 0.001$ compared with the unstimulated group and $^{***}P < 0.001$ compared with the IL-33-stimulated group.

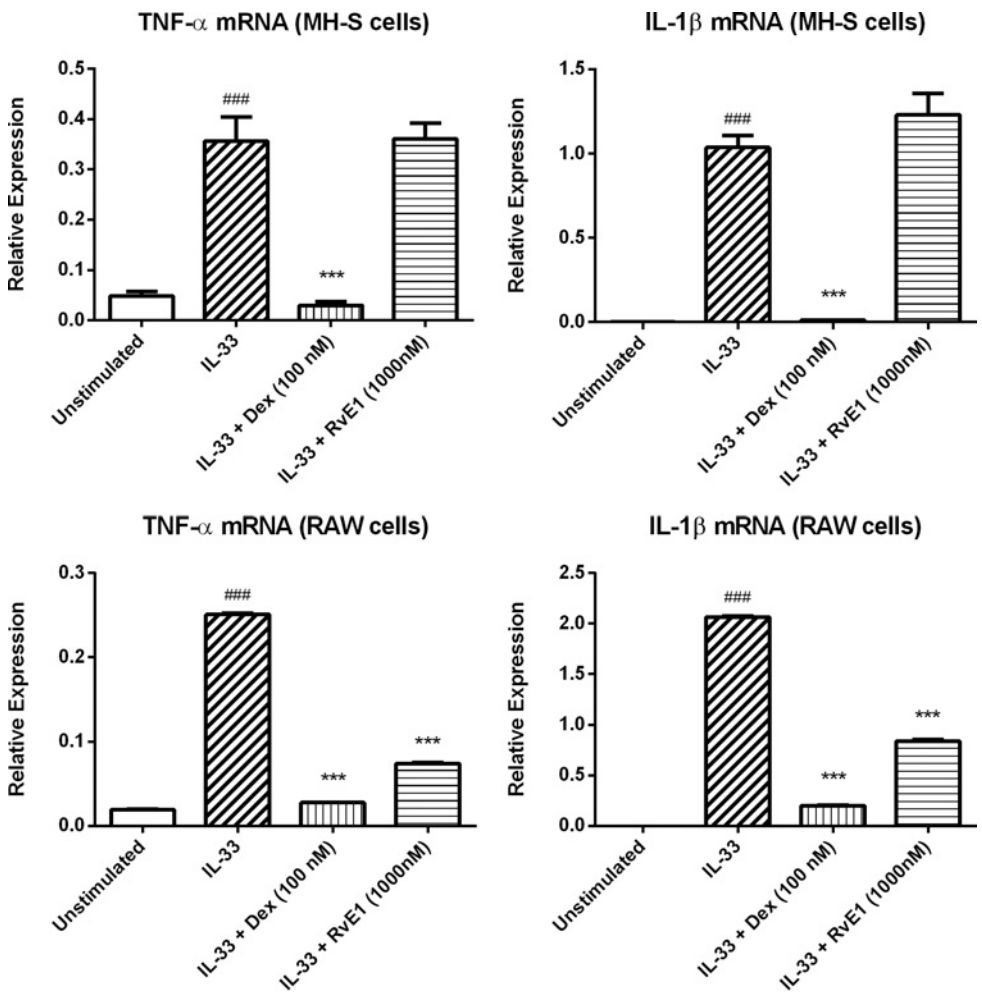

Figure S4 Differential effects of treatment with RvE1 on the relative expression of *Tnfa* and *Il-1b* mRNA by MH-S and RAW264 cells

Differential effects of treatment with RvE1 on the relative expression of *Tnfa* and *Il-1b* mRNA by MH-S and RAW264.7 macrophage cell lines stimulated with IL-33 compared with treatment with dexamethasone. Results are mean ± S.E.M. ($n = 4$ samples per group). $^{###}P < 0.001$ compared with the unstimulated group and $^{***}P < 0.001$ compared with the IL-33-stimulated group.

Received 30 September 2013/29 November 2013; accepted 18 December 2013
Published as Immediate Publication 18 December 2013, doi: 10.1042/CS20130623