

Enhanced Pro-Inflammatory Response of Macrophages to Interleukin-33 in an Allergic Environment

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Keywords

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Abstract

Background: Allergic asthma is common in childhood and is associated with a T-helper type 2 (Th2)-biased immunological response. Exacerbations of asthma are characterised by increased inflammation of the airways, which appears to be driven by interleukin (IL)-33 that can activate pulmonary macrophages. The Th2 cytokine environment of allergic asthma may contribute to exaggerated airway inflammation. **Objectives:** To test this, we assessed whether production of pro-inflammatory cytokines by IL-33-stimulated macrophages was enhanced in cells pre-treated with the key Th2 cytokines IL-4 and IL-13. We also investigated whether this was associated with altered expression of regulatory microRNAs (miRNAs). **Methods:** RAW264.7 cells cultured with IL-4 and IL-13 for 48 h were stimulated with IL-33 for 4 h. Pro-inflammatory mediators were assessed using quantitative real-time PCR (RT-PCR). Expression of miRNAs was assessed using microarrays and RT-PCR. In further experiments, we examined whether resolvin E1 (RvE1), which

promotes the resolution of experimental asthmatic inflammation in vivo, could suppress the enhanced response by treating cells with RvE1 concurrently with IL-33 stimulation. **Results:** In cells pre-treated with IL-4 and IL-13, expression of mRNA for *Ccl3*, *Ccl5*, *Ccl17*, *Ccl24*, and *Il1b* in response to IL-33 stimulation was significantly increased. This was paralleled by up-regulated expression of miR-155-5p, a miRNA that is predicted to regulate several aspects of allergic inflammation. RvE1 suppressed the enhanced production of *Ccl3*, *Ccl5*, *Ccl24*, and *Il1b*. **Conclusions:** We conclude that IL-33-activated macrophages may contribute to the exaggerated airway inflammation in exacerbations of allergic asthma, and that RvE1 has potential as a therapeutic agent that targets macrophages.

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Introduction

Asthma is one of the most common chronic diseases affecting children, particularly in industrialised societies. Most childhood asthma is allergic, with inflammation in

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the airways being driven by T-helper type 2 (Th2) cells producing cytokines such as interleukin (IL)-4, IL-5, and IL-13 [1]. A substantial proportion of the health care costs associated with asthma are related to hospitalisation, resulting from severe episodes referred to as acute exacerbations [2, 3]. These exacerbations are most often triggered by respiratory viral infections, but may also result from acute exposure to high levels of allergens or ambient particulate matter [4–6]. Importantly, asthmatics develop an exaggerated inflammatory response to respiratory viral infections [7].

Macrophages are increasingly being recognised as important in the inflammation associated with allergic asthma [8]. In experimental allergen-induced acute exacerbations of asthmatic inflammation in mice, we have previously shown that macrophages secrete increased quantities of various pro-inflammatory cytokines, while at the same time exhibiting elevated expression of markers associated with alternative activation [9, 10]. More recently, we have demonstrated that in a mouse model of a virally induced acute exacerbation of allergic asthma, exaggerated airway inflammation is dependent on macrophages and their cytokine products [11]. Notably, this inflammatory response is relatively resistant to glucocorticoid therapy, which is a recognised issue in the management of exacerbations of asthma.

Human and animal studies have established that a potentially important driver of airway inflammation in asthma is IL-33, which is produced by a variety of cell types, notably airway epithelial cells [12]. Studies in mouse models, including those from our laboratory, have demonstrated that this cytokine has a key role in exacerbations triggered by exposure to allergen [10, 13] or particulate matter [14], while human studies have similarly demonstrated its importance in asthmatic exacerbations caused by rhinovirus infection [15]. Furthermore, we have shown that IL-33 directly activates macrophages to secrete pro-inflammatory mediators [10]. Thus it seems plausible that IL-33 functions as a “final common pathway” in promoting severe airway inflammation in patients with allergic asthma [16], possibly via macrophage activation.

The mechanisms regulating this activation of macrophages remain poorly defined. However, there is much current interest in the potential role of microRNAs (miRNAs), which are 20- to 24-nucleotide non-coding RNAs, that, when hybridised to their target mRNAs, cause either translational repression or degradation of mRNA by an exonuclease [17, 18]. Notably, individual miRNAs have the capacity to regulate entire families of mRNA and thus to determine inflammatory outcomes [18, 19].

With respect to control of airway inflammation, considerable attention has recently been given to the possibility of enhancing the process of resolution of inflammation. Prominent among pro-resolution mediators are lipid-derived molecules [20], of which one family referred to as the resolvins appears to have considerable therapeutic potential. Resolvin E1 (RvE1) is an endogenous mediator which is enzymatically derived from eicosapentaenoic acid via aspirin-acetylated COX2 (cyclo-oxygenase 2) and 5-lipoxygenase [21]. RvE1 signals via the receptor ChemR23 expressed on macrophages and can bind to the leukotriene B4 receptor (BLT1) on neutrophils, resulting in suppression of neutrophil migration and reduced production of inflammatory cytokines [22]. Previously, we have shown that RvE1 can accelerate resolution in an allergen-driven model of an acute exacerbation of asthma and that this effect appears to be primarily via the action of RvE1 on macrophages [23].

In this investigation, we examined how the production of pro-inflammatory cytokines by macrophages in response to stimulation with IL-33 was influenced by a Th2 cytokine environment, which prevails in allergic asthma. We also examined how the altered response might be regulated by altered expression of miRNAs. Furthermore, we assessed whether the cytokine response could be suppressed by treatment with RvE1.

Material and Methods

Cell Culture

RAW264.7 mouse macrophage-like cells (American Type Culture Collection, Manassas, VA, USA) were cultured at 5×10^5 cells per well in 6-well plates in RPMI 1640 medium (Gibco, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum, L-glutamine, and antibiotics. Cells were maintained for 48 h either in the absence or the presence of 10 ng/mL of recombinant mouse IL-4 and IL-13 (R&D Systems, Minneapolis, MN, USA) and were then stimulated with 20 ng/mL of recombinant mouse IL-33 (eBioscience, San Diego, CA, USA) for 4 h. In some experiments, stimulated cells were concurrently treated with 1 μ M RvE1 (Cayman Chemical, Ann Arbor, MI, USA). Six independent experiments were performed for the initial assessment of cytokine expression following stimulation with IL-33, and 4 independent experiments were performed for all other comparisons. At the end of the incubation period, total RNA was isolated using TriReagent (Sigma-Aldrich, St Louis, MO, USA) and was assessed using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). A sample of total RNA was taken for microarray analysis (Ramaciotti Centre for Genomics, University of New South Wales, Sydney, NSW, Australia).

Microarray and Data Analysis

A mouse miRNA microarray (Release 19.0, 8 × 60K; Agilent, Santa Clara, CA, USA) was used to identify changes in the expression of miRNAs under various culture conditions. Array data were imported into the Partek Genomics Suite 6.6 (Partek Inc, St. Louis, MO, USA) for analysis. Background noise correction was applied to raw probe-level data, and quantile normalisation was performed according to the RMA (robust mean average) procedure. An analysis of variance (ANOVA) was performed with a false discovery rate adjustment, and the fold change was determined for the following comparisons (1) media versus IL-33, (2) IL-33 versus Th2 + IL-33, (3) media versus Th2, and (4) Th2 versus Th2 + IL-33. Differentially expressed miRNAs were defined as having a fold change of ± 1.5 with an unadjusted $p < 0.05$. Microarray data were uploaded to the Gene Expression Omnibus (GEO) repository (accession No.: GSE109372).

Assessment of mRNA Expression

Expression of mRNA for 10 selected pro-inflammatory markers, *Ccl2*, *Ccl3*, *Ccl4*, *Ccl5*, *Ccl17*, *Ccl24*, *Cxcl2*, *Tnf*, *Il6*, and *Il1b*, was assessed by quantitative real-time PCR (RT-PCR). These mediators were selected because of their important role in chemotaxis and activation of neutrophils, eosinophils, and monocytes during exacerbations of allergic asthma [24]. Extracted RNA samples were treated with TURBO DNase (Ambion, Scoresby, VIC, Australia) and reverse transcribed into cDNA using Superscript III (Life Technologies, Carlsbad, CA, USA). RT-PCR was performed using a Roche LightCycler 480 (Roche Diagnostics, Indianapolis, IN, USA) with detection of amplified products using SYBR green (BioLine, Tauton, MA, USA). Gene expression was normalised to the housekeeping gene HPRT (hypoxanthine-guanine phosphoribosyltransferase). Relative levels of expression were logarithmically transformed prior to analysis.

Assessment of miRNA Expression

Total RNA was reverse transcribed into cDNA using a TaqMan miRNA reverse transcription kit (Life Technologies), and a TaqMan miRNA assay kit (Thermo Fisher Scientific, Waltham, MA, USA) was used to assess expression. PCR was performed using a Roche LightCycler 480, with expression normalised to the small nucleolar (sno) RNA control gene *sno234*.

Statistical Analysis

Data are presented as arithmetic means $\pm$ SEM. RT-PCR data were analysed using GraphPad Prism 6.07. To assess significant differences between unstimulated and stimulated cells cultured in the absence or presence of Th2 cytokines, repeated-measure 2-way ANOVA, followed by a Holm-Sidak post hoc test, was employed. To assess the effect of RvE1, repeated-measure 1-way ANOVA, followed by a Holm-Sidak post hoc test, was employed.

Results

Pro-Inflammatory Responses

Pre-treatment of RAW264.7 cells with IL-4 and IL-13 led to marked up-regulation of the relative expression of the gene *Arg1*, from 0.00005 ± 0.00001 to 0.14 ± 0.05

($n = 4$, $p < 0.001$). This increased expression of arginase-1 confirmed that the cells exhibited phenotypic changes consistent with alternative activation.

In cells that were pre-treated with IL-4 and IL-13, there was significantly increased baseline expression of pro-inflammatory mediators *Ccl5*, *Ccl17*, *Ccl24*, and *Il1b* compared to cells grown in media alone (Fig. 1). IL-33 stimulation increased the expression of all the pro-inflammatory cytokines that were assessed. In cells that were pre-treated with IL-4 and IL-13, then stimulated with IL-33, there was significantly enhanced expression of the pro-inflammatory mediators *Ccl3*, *Ccl5*, *Ccl17*, *Ccl24*, and *Il1b* compared to cells stimulated with IL-33 without Th2 cytokine pre-treatment (Fig. 1). Although expression of *Tnf* was up-regulated by IL-33 stimulation, this was not altered by pre-treatment with IL-4 and IL-13 (Fig. 1). Similarly, expression of *Il6*, *Ccl2*, *Ccl4*, and *Cxcl2* was up-regulated in IL-33-stimulated cells, but was unchanged by pre-treatment with Th2 cytokines (not shown).

Differential Expression of miR-155-5P

Microarray analysis provided raw probe data for 1,247 miRNAs. Data were initially filtered to identify miRNA for which there was a ± 1.5 -fold change in expression following stimulation with IL-33 compared to media alone. We then focused on miRNAs for which the IL-33-induced change in expression was significantly enhanced (± 1.5 -fold change; $p < 0.05$) in cells pre-treated with Th2 cytokines compared to cells that had not been pre-treated (Fig. 2a). After filtering for miRNAs with an unadjusted $p < 0.05$, only 5 miRNAs exhibited significantly increased levels of expression between these groups including mmu-miR-155 ($p = 0.006$), mmu-miR-22-5p ($p = 0.027$), mmu-miR-146b-5p ($p = 0.016$), mmu-miR-199b-5p ($p < 0.001$), and mmu-miR-511-3p ($p < 0.001$). Remarkably, of these, only mmu-miR-155-5p was also significantly up-regulated in IL-33-stimulated cells that had not been pre-treated when compared to unstimulated cells ($p = 0.0045$). There was also significant down-regulation of mmu-miR-711 in IL-33-stimulated cells pre-treated with Th2 cytokines compared to cells that had not been pre-treated ($p = 0.049$). However, this miRNA was not significantly down-regulated by IL-33 stimulation in the absence of Th2 cytokine pre-treatment ($p = 0.27$).

Therefore, we focused our attention on miR-155 and performed RT-PCR to confirm the microarray evidence of up-regulation. This demonstrated >3 -fold up-regulation of expression of this miRNA by IL-33-stimulated cells pre-treated with IL-4 and IL-13 compared to cells that had not been pre-treated (Fig. 2b).

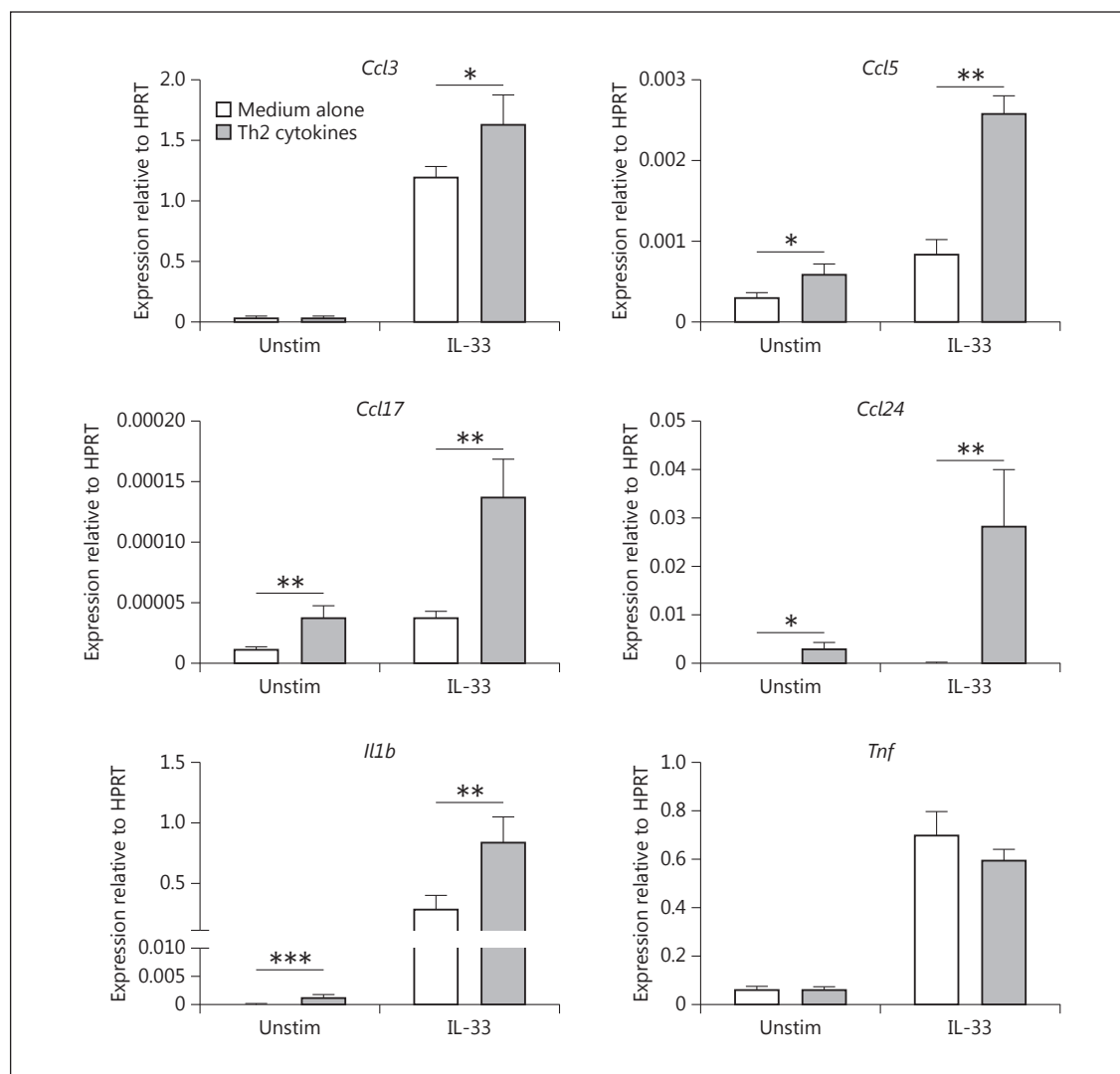


Fig. 1. Relative expression of mRNA for various chemokines and cytokines by RAW264.7 macrophages. Data show means $\pm$ SEM from 6 independent experiments. Open bars represent cells cultured in medium alone, grey bars represent cells cultured with Th2 cytokines for 48 h. Unstim, unstimulated cells; IL-33, cells stimulated with IL-33 for 4 h; HPRT, hypoxanthine-guanine phosphoribosyltransferase. Analysis was by repeated-measure 2-way ANOVA and Holm-Sidak post hoc test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Effect of RvE1

Addition of RvE1 to the cells that were pre-treated with IL-4 and IL-13, concurrent with stimulation with IL-33, suppressed the enhanced expression of *Ccl3*, *Ccl5*, *Ccl24*, and *Il1b* by these cells. However, expression of *Ccl17* or *Tnf* was not significantly affected by treatment with RvE1 (Fig. 3).

Discussion/Conclusion

In this study, we have demonstrated that when RAW264.7 macrophage-like cells are cultured in the presence of IL-4 and IL-13, they exhibit significantly augmented expression of several cytokines following stimulation by IL-33 in comparison to cells cultured in the absence of IL-4 and IL-13. Notably, these up-regulated cytokines included various CC chemokines and IL-1 β , which have the potential to recruit and activate Th2 lym-

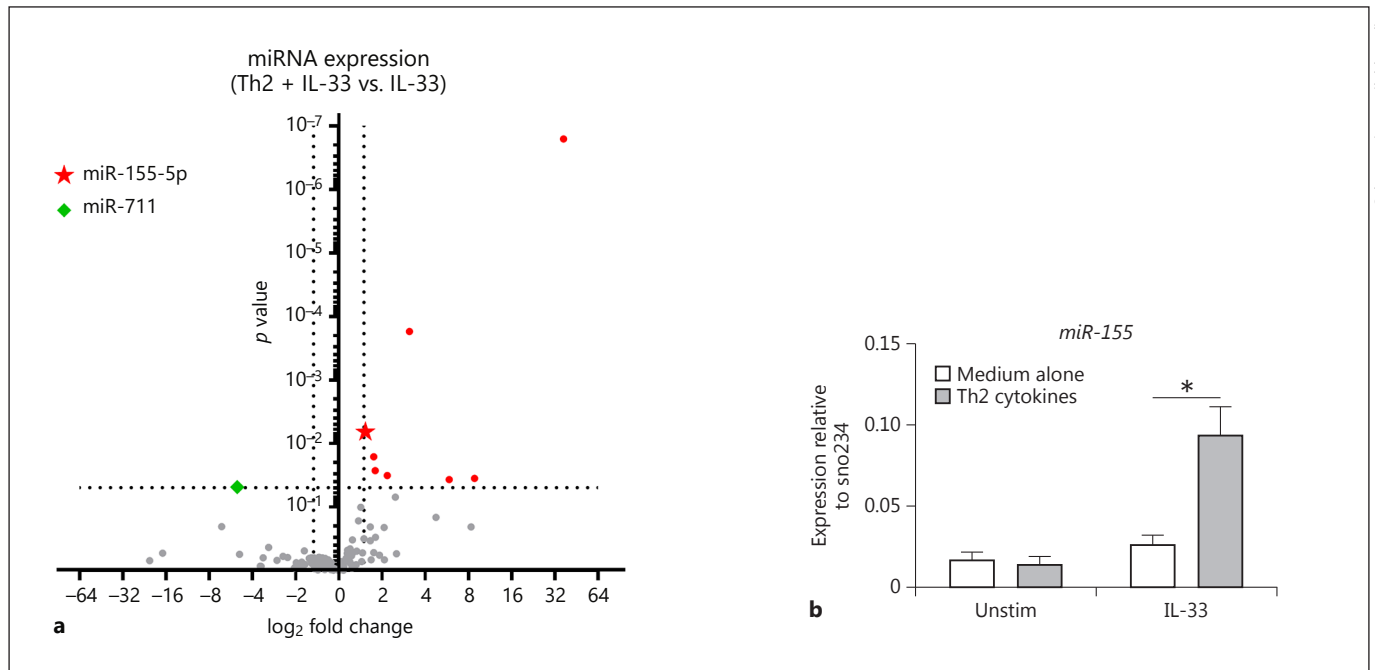


Fig. 2. **a** Volcano plot showing differential expression of microRNAs between IL-33-stimulated RAW264.7 macrophages with and without pre-treatment with Th2 cytokines. **b** Relative expression of microRNA-155 by RAW264.7 macrophages assessed by real-time PCR. Data show means $\pm$ SEM from 4 independent ex-

periments. Open bars represent cells cultured in medium alone, grey bars represent cells cultured with Th2 cytokines for 48 h. Unstim, unstimulated cells; IL-33, cells stimulated with IL-33 for 4 h. Analysis was by repeated-measure 2-way ANOVA and Holm-Sidak post hoc test. * $p < 0.05$.

phocytes, eosinophils, and neutrophils. The ability of IL-33 to stimulate macrophages is well recognised [10, 25], but our study is the first demonstration of an enhanced pro-inflammatory response of IL-33-stimulated macrophages in a Th2 cytokine environment. These results are likely to be biologically significant, because IL-33 appears to play a key role in driving airway inflammation in asthmatic exacerbations, regardless of whether the trigger is respiratory viral infection or exposure to high levels of allergen or ambient particulate matter [10, 13–15].

In the airways of asthmatics, macrophages typically express markers of so-called alternative activation, which is associated with prior exposure to Th2 cytokines [26]. Such macrophages are also referred to as having an M2 phenotype, in contrast to so-called classically activated or M1 macrophages, which are associated with prior exposure to interferon- γ and other relevant stimuli. Although there has been much debate about whether alternatively activated/M2 macrophages are pro-inflammatory, evidence is accumulating that under the appropriate circumstances, M2 macrophages can make an important contribution to driving inflammation, for

example in host defence against viral or fungal infections [27, 28]. Moreover, it is becoming increasingly clear that categorising macrophages into M1/M2 and further subdivisions is an oversimplification, in part because these cells exhibit marked phenotypic plasticity [29] and in part because newly recruited monocytes/macrophages may have quite different functional characteristics to resident tissue macrophages [8]. Indeed, a comprehensive analysis of human macrophage activation in response to a diverse range of stimuli identified a spectrum of activation with at least 9 distinct activation patterns [30]. In our experiments, RAW264.7 cells pre-treated with IL-4 and IL-13 expressed markers of alternative activation that are typical of mouse macrophages, including *Arg1*, *Ccl17*, and *Ccl24*. However, in response to stimulation with IL-33, these cells up-regulated expression of *Il1b* to a significantly greater extent than macrophages which had not been pre-treated with Th2 cytokines. Our findings strongly support the concept that alternatively activated macrophages are likely to play a major role in promoting airway inflammation in acute exacerbations of asthma.

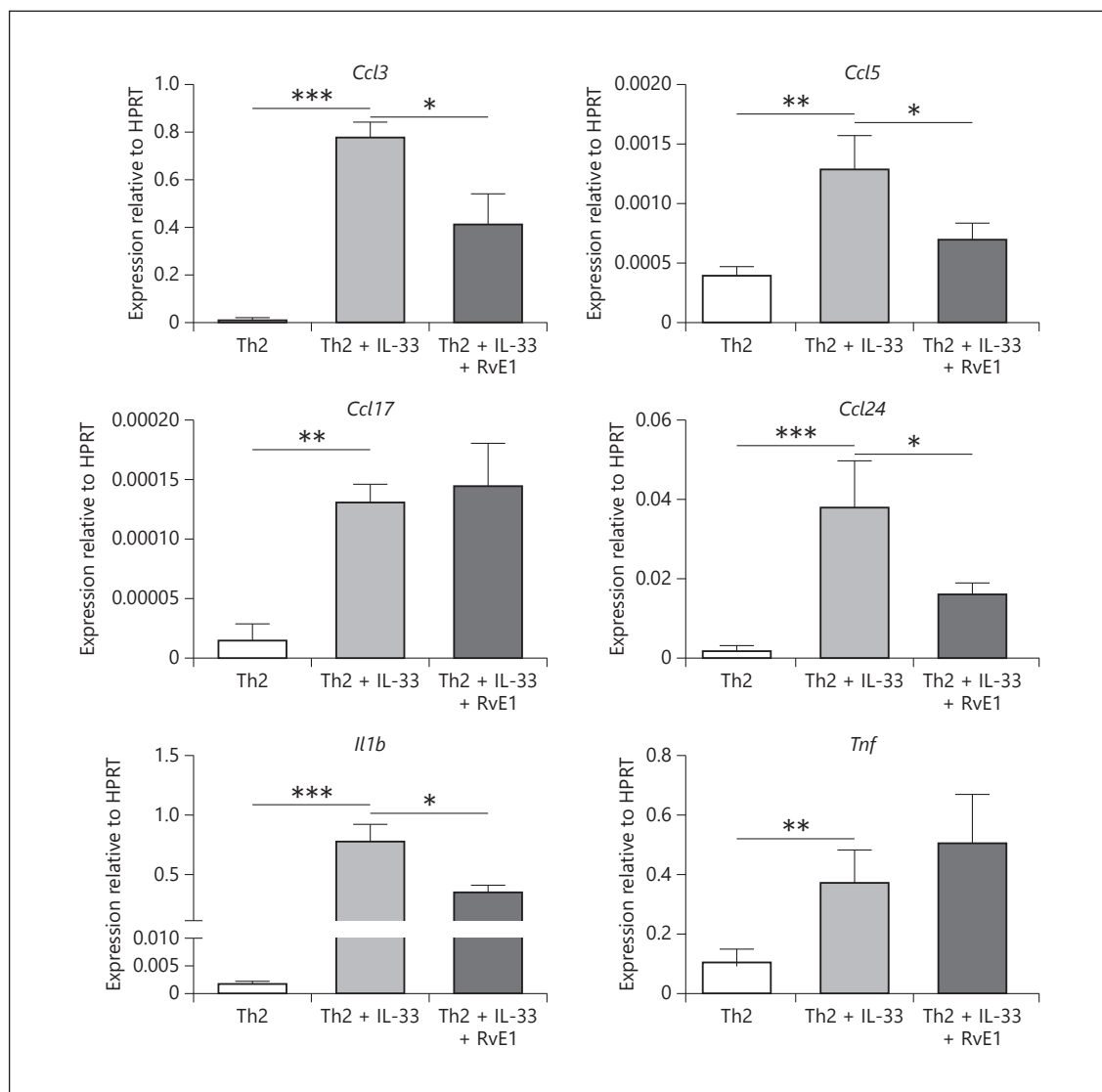


Fig. 3. Relative expression of mRNA for various chemokines and cytokines by RAW264.7 macrophages, cultured in the presence of Th2 cytokines for 48 h, then stimulated with IL-33 for 4 h, in the absence or presence of RvE1. Data show means $\pm$ SEM from 4 independent experiments. HPRT, hypoxanthine-guanine phosphoribosyltransferase. Analysis was by repeated-measure 1-way ANOVA and Holm-Sidak post hoc test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, vs. cells stimulated with IL-33.

In this context, the demonstration of selective up-regulation of miR-155 is of considerable interest. miRNA-155 is well documented to be important in regulating the inflammatory response [19]. In macrophages, it represses expression of the negative regulators of cytokine signalling *Socs1* and *Shp1* and thus has pro-inflammatory activity [31]. Furthermore, it suppresses *Bcl6*, a transcription factor that counter-regulates activation of NF- κ B, and induces expression of *Ccl2* by macrophages

[32]. Thus, it is considered to be a potential therapeutic target in a variety of inflammatory diseases including rheumatoid arthritis [33], although studies in animal experimental models have reported variable success to date [34–36].

That miR-155 is up-regulated following stimulation of macrophages in a Th2 environment is a novel finding of potential significance. At baseline, classically activated/M1 macrophages exhibit markedly increased expression

of miR-155. In contrast, alternatively activated/M2 macrophages do not [37]. However, our results clearly show that macrophages cultured in the presence of IL-4 and IL-13, which exhibit little or no change in the expression of miR-155 at baseline, are primed to undergo striking up-regulation of this miRNA following stimulation by IL-33.

Although we have demonstrated that the enhanced IL-33-induced expression of mRNA for pro-inflammatory cytokines is associated with increased expression of miR-155, we have not established a direct link. Future in vitro experiments using miRNA inhibitors will be required to confirm a role for miR-155 in the enhanced inflammatory response of macrophages to IL-33 in an allergic cytokine environment. miR-155 may also contribute to the regulation of allergic inflammation in other ways. For example, it may have a feedback effect upon the M1/M2 balance in macrophages by targeting the $\alpha 1$ chain of the IL-13 receptor [38]. In addition, miR-155 appears to be essential for the development of an eosinophil response, apparently via its capacity to inhibit expression of the transcription factor PU.1, which is a negative regulator of Th2 cytokine production [39]. Furthermore, miR-155 is strikingly up-regulated in type 2 innate lymphoid cells in response to IL-33 and appears to contribute to their expansion [40].

In acute exacerbations of asthma, resistance to treatment with glucocorticoids is a significant clinical problem [41], which is at least in part related to inflammatory activation of macrophages that are unresponsive to glucocorticoids [11, 42]. Current research on alternative approaches to controlling inflammation includes a focus on mechanisms underlying resolution of inflammation and the lipid-derived mediators associated with this [20]. Among these, there is particular interest in the family of molecules known as resolvins, which appear to have considerable therapeutic potential [43, 44]. In studies in an animal model of an allergen-driven acute exacerbation of asthma, we previously demonstrated that RvE1 accelerated resolution. This effect appeared to be primarily via the ability of RvE1 to suppress cytokine expression by macrophages, by inhibiting NF- κ B [23].

Therefore, we examined whether RvE1 could also suppress the enhanced cytokine responses of RAW264.7 cells following IL-33 stimulation in an allergic environment. Consistent with our in vivo findings, we showed that RvE1 suppressed the expression of *Ccl3*, *Ccl5*, *Ccl24*, and *Il1b*, although it did not suppress expression of *Ccl17*. The latter may be because expression of *Ccl17* involves a separate pathway that is not targeted by RvE1. In this

context, it is of interest that the up-regulated expression of *CCL17* in macrophages from patients with allergic asthma is also not readily suppressed, being resistant to treatment with glucocorticoids [45]. Despite these limitations, our results support the possibility that RvE1 may be useful in treating exacerbations of allergic asthma. However, similar experiments using primary human macrophages, and cells from relevant models of allergic airway inflammation, would be needed to confirm our findings.

In summary, the present study has shown that macrophage-like cells, primed in an allergic cytokine environment, exhibit an enhanced pro-inflammatory response to IL-33. This exaggerated response is potentially regulated by the increased expression of miR-155, which may inhibit various negative regulators of inflammation. The novel pro-resolution compound RvE1 can effectively suppress the enhanced activation of these macrophages and may be useful as a therapeutic agent for acute exacerbations of asthma.

Statement of Ethics

The authors have no ethical conflicts to disclose.

Disclosure Statement

The authors have no conflicts of interest to declare.

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Author Contributions

N.C. performed experimental work and contributed to the analysis and interpretation of data. R.K.K. conceived the study, contributed to the analysis and interpretation of the data, and drafted the manuscript. P.S.F. contributed to the analysis and interpretation of data. C.H. contributed to the experimental work and to the analysis and interpretation of data. All authors provided intellectual content and contributed to manuscript revision.

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