Published in final edited form as:
*Sci Transl Med*. 2014 October 1; 6(256): 256ra134. doi:10.1126/scitranslmed.3009124.

# Rhinovirus induced IL-25 in asthma exacerbation drives type-2 immunity and allergic pulmonary inflammation

**Janine Beale**[#1,2,3], **Annabelle Jayaraman**[#1,2,3], **David J. Jackson**[#1,2,3,4], **Jonathan D. R. Macintyre**[1,2,3,4], **Michael R. Edwards**[1,2,3], **Ross P. Walton**[1,2,3], **Jie Zhu**[1,2,3], **Yee Man Ching**[1,2,3], **Betty Shamji**[5], **Matt Edwards**[5], **John Westwick**[5], **David J. Cousins**[2,6], **You Yi Hwang**[7], **Andrew McKenzie**[7], **Sebastian L. Johnston**[#1,2,2,4], and **Nathan W. Bartlett**[#1,2,3,~]

[1]Airway Disease Infection Section, National Heart and Lung Institute, Imperial College London, London, UK

[2]MRC and Asthma UK Centre in Allergic Mechanisms of Asthma, London, UK

[3]Centre for Respiratory Infections, Imperial College London, London, UK

[4]Imperial College Healthcare National Health Service Trust, London, UK

[5]Novartis Institute for Biomedical Research, Horsham, UK

[6]Division of Asthma, Allergy & Lung Biology, King College London, UK

[7]MRC Laboratory for Molecular Biology, Cambridge, UK

[#] These authors contributed equally to this work.

## Abstract

Rhinoviruses are the most common cause of virally-induced asthma exacerbations which continue to account for the greatest burden in terms of morbidity, mortality and cost associated with this disease. IL-25 activates type-2-driven inflammation and is potentially important in virally-induced asthma exacerbations. Rhinovirus-infected cultured asthmatic bronchial epithelial cells exhibited a heightened intrinsic capacity for IL-25 expression which correlated with donor atopic status. *In vivo* human IL-25 expression was greater in asthmatics at baseline and during experimental rhinovirus infection. In mice rhinovirus infection induced IL-25 expression and augmented allergen-induced IL-25. Blockade of the IL-25 receptor reduced many RV-induced exacerbation-specific responses including type-2 cytokine expression, mucus production and recruitment of eosinophils, neutrophils, basophils, T and non-T type-2 cells. We have identified that asthmatic epithelial cells possess increased intrinsic capacity for expression of a pro-type-2 cytokine in

[~]Correspondence should be addressed to: Dr Nathan Bartlett BSc(Hons)PhD, Airway Disease Infection Section, National Heart and Lung Institute, Imperial College London, St. Mary's Campus, Norfolk Place, London W2 1PG. UK. tel: +44-207-594-3775. fax: +44-207-262-8913 n.bartlett@ic.ac.uk.

**Author Contributions:** NWB, SLJ, JB, AJ, AM, DJC, MRE and RPW devised and/or conducted mouse studies, analysed the data and prepared the manuscript. SLJ devised and JDRM conducted the human bronchial epithelial cell study and provided associated data. SLJ devised and DJJ conducted the human experimental infection study and analysed and provided associated data. JZ conducted the immunohistochemical analyses. YMC and YYH provided flow cytometric analyses. BS, ME and JW provided human IL-25 protein data. All authors revised and approved the final version of the manuscript.

**Competing Interests:** The authors declare that they have no competing interests.


Europe PMC Funders Author Manuscripts

response to a viral infection and identify IL-25 as a key mediator in RV-induced exacerbations of pulmonary inflammation.

## Introduction

Asthma attacks (exacerbations) are the most clinically and economically important form of this disease. Respiratory viruses are the most common triggers of exacerbations, accounting for approximately 85% of cases, and rhinoviruses (RV) represent the great majority of viruses detected(1). At present our understanding of asthma is such that targeted therapies for exacerbations tailored to immunopathogenic mechanisms are lacking(2). What is clear is that allergen driven type-2 immunity is central to the immunopathogenesis of allergic asthma. Many studies have detected increased type-2 cells(3, 4) and type-2 cytokines(5, 6) in asthma. Genetic studies have linked numerous type-2-associated genes with asthma (IL-4, IL-13, IL-4Rα, STAT6)(7, 8) and asthma exacerbations (IL-4 and IL-4Rα)(9, 10). We studied exacerbations in a human experimental RV infection model in allergic asthmatic and normal volunteers and demonstrated that asthmatics had more severe lower respiratory tract symptoms, reductions in lung function and increases in bronchial hyper-reactivity. Exacerbation severity was strongly correlated with BAL $CD4^+$ Th2 cell cytokine production and viral load(11). Mouse models of allergic asthma exacerbation have also demonstrated the capacity of RV infection to augment Th2 responses and associated features of allergic airways disease such as airway hyperreactivity (AHR) and mucus production(12, 13). While Th2-mediated responses are clearly implicated in exacerbations, it is unclear how responses to RV infection interact with Th2 immunity to enhance disease in allergic asthma to cause exacerbations. Bronchial epithelium is likely to be critical for this interaction since it responds to both allergen(14) and RV infection(15) and has the capacity to produce cytokines that are potent activators of type-2 immunity such as IL-25(16).

The IL-17 family member, IL-25 (IL-17E), has been identified as an initiator and regulator of type-2 immunity and plays a role in asthma pathogenesis(17-19). Clinical studies have demonstrated increased IL-25 gene expression together with its receptor, IL-17RB, in tissue from patients with asthma(20) and atopic dermatitis(21) while eosinophils, mast cells and the airway epithelium(17, 22) have been reported as sources within the lung. Blocking IL-25 in a mouse model of allergic airway disease prior to allergen sensitisation and/or challenge resulted in a striking reduction in allergic inflammation, AHR and type-2 cytokine (IL-5 and IL-13) production(18). IL-25 can augment type-2 cytokine production via activation of IL-17RB-expressing immune cells including Th2 cells(20) and type 2 innate lymphoid cells (ILC-2)(23).

Respiratory syncytial virus (RSV) infection has been shown to induce IL-25 in mouse lungs(24), but the great majority of asthma exacerbations are precipitated by RV infections, with only a small minority caused by RSV infections. The role of IL-25 in RV induced asthma exacerbations is unknown. We therefore hypothesised that RV-induced IL-25 was required for the immune cascade leading to increased type-2 immune responses and allergic airways inflammation during RV-induced asthma exacerbations.




Europe PMC Funders Author Manuscripts

We used human *in vitro* and *in vivo* RV infection studies using asthmatic and healthy volunteers to show that asthmatics possess a greater capacity to express IL-25 which correlated with atopic status. Studies using mouse models confirmed that RV infection can induce lung IL-25 expression and augment allergen-driven IL-25 in an asthma exacerbation model characterised by increased type-2 cytokine production and recruitment of cells associated with type-2 immunity such as basophils ($CD3^-$ $CD4^-$, $Fc\varepsilon RI^+$, $CD49b^+$, $ST2^+$ and $IL\text{-}4^+$) and IL-25-receptor expressing non-T type 2 cells ($CD3^-$, $CD4^-$, $CD49b^-$, $ICOS^+$ and $ST2^+$ and $IL\text{-}17RB^+$) and Th2 cells ($CD3^+$ $CD4^+$, and $IL\text{-}4^+$). Using an IL-25 receptor blocking antibody we showed that RV augmented IL-25 caused increased innate and adaptive type-2 immune responses and associated pulmonary allergic inflammation. We have identified that heightened IL-25 expression occurs during rhinovirus infection in asthma. Supporting mouse studies have defined a role for IL-25 in the immunopathogenesis of virally induced exacerbation of pulmonary allergic inflammation.

## Results

### Asthmatic bronchial epithelial cells express increased IL-25 in response to RV infection *in vitro*

Human bronchial epithelial cells (BECs) were obtained from 10 atopic asthmatic and 10 non-atopic non-asthmatic healthy volunteers and infected with RV-1B *in vitro* (inclusion criteria described in Supplementary Table 1.). There were no significant differences between uninfected (Media control, M) asthmatic and healthy *Il25* mRNA expression levels. Significant up-regulation of *Il25* gene expression was detected in RV infected asthmatic BECs at 8 h post-infection (p.i.) compared to mock-infected cells. Approximately 10-fold greater *Il25* mRNA levels in RV infected BECs from asthmatic donors compared to those from healthy controls were observed. By 24 h p.i. *Il25* mRNA levels had returned to baseline (Fig. 1A). The peak of *Il25* mRNA at 8 h was followed by significantly increased IL-25 protein levels in the supernatant of RV-infected asthmatic BECs 24 h p.i., whilst no significant increase over uninfected cells was observed with RV infected healthy BECs at 8 or 24 h p.i. (Fig. 1B).

To assess if RV-induced IL-25 production by BECs *in vitro* was related to markers of allergy or of disease severity, correlation analyses with serum IgE levels, skin prick test positivity (SPT), $FEV_1$/FVC ratio, $PC_{20}$ and the number of recorded asthma exacerbations per year were performed. Of these, increased RV-induced IL-25 expression was associated with sensitization to a greater number of common environmental allergens as indicated by positive wheal and flare responses in positive SPT scores (Fig. 1C) and there was a trend towards greater IL-25 levels in those with more frequent asthma exacerbations ($r=0.61$, $P=0.067$, Supplementary Table 2).

### Experimental rhinovirus infection in vivo induces high levels of IL-25 expression in asthmatics

Next we investigated IL-25 expression in the airways of 28 mild to moderate atopic asthmatic subjects and 11 non-asthmatic (healthy) subjects (inclusion criteria and baseline percentages of BAL eosinophils, neutrophils and lymphocytes for all subjects in

Supplementary Table 3) during *in vivo* experimental RV-16 infection. Secreted IL-25 in the nasal mucosal fluid was sampled by the nasosorption™ technique before infection (baseline) and on several days up to 10 days after infection. Baseline IL-25 levels were higher in asthmatics, but this difference was not statistically significant ($P$=0.220, n=28 asthmatic and 11 healthy subjects, Mann Whitney test). During RV infection 61.0% (17/28) of asthmatics had increased levels of IL-25 and the increase was highly significant ($P$<0.001, n=28, Wilcoxon Rank Sum test). The non-asthmatic subjects also had a small increase in IL-25 during infection ($P$=0.018, n= 11, Wilcoxon Rank Sum test), and peak levels of IL-25 during infection were much higher in asthmatics compared to healthy volunteers but this difference was not statistically significant ($P$=0.351, n=28 and 11, Mann Whitney test) (Fig. 1D).

## Rhinovirus infection augments IL-25 expression and allergic pulmonary inflammation

Having identified a link between RV infection, IL-25 expression and disease severity in human asthmatic subjects we moved to mouse models to identify potential mechanisms. We used a previously reported model of RV exacerbated pulmonary allergic inflammation involving systemic ovalbumin (OVA) protein sensitisation followed by intranasal OVA challenges and RV-1B infection(12). Sensitised mice were either challenged with OVA protein or sham challenged with PBS, once daily over 3 consecutive days. Following the third OVA/PBS treatment each group was either infected with rhinovirus 1B (RV) or mock infected with UV-inactivated RV (UV). We measured inflammatory cells (macrophages, eosinophils and lymphocytes) in the BAL to confirm viral induced exacerbation of airways inflammation in the model (Supplementary Figure 1). OVA challenged groups (RV-OVA and UV-OVA) had elevated *Il25* mRNA levels compared to non-allergic controls (RV-PBS and UV-PBS) (Fig. 2A). The combination of RV infection during allergic airway inflammation in RV-OVA mice further significantly enhanced *Il25* gene expression by approximately 28-fold at 10 h p.i. compared to UV-OVA mice. Increased gene expression coincided with elevated lung protein which was significant at day 1 p.i. with a trend for prolonged increased expression observed at 2 and 4 days p.i. (Fig. 2A). Although at significantly lower levels to that in allergen challenged mice, we observed that RV infection alone (RV-PBS) induced IL-25 protein expression above that in unchallenged/uninfected (UV-PBS) negative controls which was significant on day 4.

To determine the cellular source of IL-25 in the lung during allergen challenge and RV infection, lung sections were taken at 2 days p.i. for immunohistochemistry (IHC) analysis. Lung sections probed with an isotype control antibody instead of anti-IL-25 showed no IL-25 staining (Supplementary Figure 2A). Epithelial expression of IL-25 was significantly higher in the lungs of RV infected mice (RV-OVA and RV-PBS). Whilst epithelium in large airways exhibited the strongest IL-25 expression, bronchiolar- and alveolar epithelium also expressed IL-25 during RV-1B infection (Supplementary Figure 2B). Sub-epithelial IL-25+ inflammatory cells were frequently detectable in the lungs of OVA-challenged mice (RV-OVA and UV-OVA) and when quantitated were significantly increased in RV-OVA mice compared to non-allergen challenged groups (Fig. 2B) These data are consistent with maximal IL-25 gene and protein observed in RV-OVA mice linked to combined expression by bronchial epithelial cells and infiltrating immune cells.

Exacerbated IL-25 was associated with increased pulmonary type-2 cytokine expression. IL-4, IL-5 and IL-13 were only detectable following allergen challenge and RV-1B infection, with significantly enhanced levels of IL-4 and IL-13 at 10 h and IL-5 in the bronchoalveolar lavage (BAL) fluid at 24 h p.i. as compared to UV-OVA mice (Fig. 2C). Type-2 cytokines in lung tissue exhibited a prolonged expression profile similar to IL-25 with increased IL-4 and IL-5 levels evident at day 4 p.i. (Fig. 2D). Basophils are a key component of allergic responses, present in asthmatic lungs and a source of type-2 cytokines. Basophils were identified as $CD3^{-}$ $CD4^{-}$ $Fc\varepsilon RI^{+}$ $CD49b^{+}$ and $ST2^{+}$ cells. $IL\text{-}4^{+}$ basophils peaked at 1 day p.i. in OVA challenged groups with very few detected in unchallenged groups, and RV-1B infection resulted in significantly greater accumulation of $IL4^{+}$ basophils in the airways of OVA-challenged mice (Fig. 2E). Asthmatics are susceptible to more severe viral infections(25) and our model replicated this with increased viral loads detected in mice with allergic airways inflammation (RV-OVA) compared to non-allergic, infected mice (RV-PBS) (Fig. 2F).

## Rhinovirus-induces accumulation of IL-17RB$^{+}$ T and non-T type-2- cells

Using the same mouse model, we detected IL-25 receptor ($IL\text{-}17RB^{+}$) expressing cells in total leukocyte preparations from lung tissue and BAL (representative flow plots for each shown in Fig. 3A). We observed that virus infection exacerbated $IL\text{-}17RB^{+}$ lung cells at early (day 1) and late (day 7) times after infection of OVA challenged mice. Significantly increased $IL\text{-}17RB^{+}$ BAL cells in this group were observed at day 1 only (Fig. 3A). To determine if IL-25 receptor expressing Th2 cells contributed to this response we measured $CD3^{+}$, $CD4^{+}$, $IL\text{-}4^{+}$ T cells. At day 1 IL-4-expressing Th2 cells constituted 5% of the total $IL\text{-}17RB^{+}$ signal in the lung. By day 7 Th2 cells accounted for approximately 30% of $IL\text{-}17RB^{+}$ lung cells and were significantly higher in virally exacerbated mice. At day 1 BAL $IL\text{-}17RB^{+}$ Th2 cell were exacerbated in of RV-infected, OVA challenged mice. Similar to the lung, $IL\text{-}4^{+}$ Th2 cells accounted for around 5% of BAL $IL\text{-}17RB^{+}$ cells at day 1 rising to around 30% by day 7 (Fig. 3B). Excluding T cells, which express ICOS, and NK cells (CD3-, CD4-, DX5-) and selecting $ICOS^{+}$ and IL-33 receptor expressing ($ST2^{+}$) cells, allowed us to identify non-T type-2 cells. These cells were significantly more abundant in the lungs of RV-OVA treated mice at day 1 compared to other groups (Fig. 4A). Approximately 10% of these cells were $IL\text{-}17RB^{+}$, with significantly greater numbers observed in the lung tissue and airways of virally infected, allergic mice compared to non-infected allergic controls at day 1 (Fig. 4B). To determine if these cells were likely to include ILC-2s we analysed ICOS expression by lung- and mediastinal-lymph node leukocytes following allergen challenge and observed that almost all (>90%) ICOS positive cells were either T cells or ILC-2s (Supplementary Figure 6).

## IL-25 is necessary for rhinovirus-exacerbated allergic pulmonary inflammation

We used an IL-25 receptor blocking mouse monoclonal antibody (α-IL-17RB, clone D9.2(23)) to define the role of IL-25 in the exacerbated inflammatory responses observed in OVA-challenged, RV-infected mice. Mice were treated with antibody after OVA challenges, before and during RV-1B infection in order to specifically target virally augmented IL-25 and assess IL-25 regulation of inflammatory mediators. Blocking the IL-25 receptor reversed the RV-exacerbated expression of IL-4, IL-5 and IL-13 to levels similar to allergen

challenged, uninfected mice (Fig. 5A). IL-6 has also been associated with type-2 immune responses(26). Consistent with this we observed that OVA-challenge alone induced IL-6 protein expression which was markedly increased (10 fold) by RV infection. However in contrast to prototypic type-2 cytokines (IL-4, IL-5 and IL-13) IL-25R blockade further increased IL-6 expression in RV exacerbated mice (Fig. 5B). RV-enhanced expression of the eosinophil-recruiting chemokines CCL11 and CCL24 in allergen challenged mice was partially reduced with IL-17RB blocking, and a smaller reduction in CCL24 levels was also observed in UV-OVA treated mice (Fig. 5C).

Like IL-25, IL-33 and thymic stromal lymphopoietin (TSLP) are type-2 immunity-promoting cytokines. For RV-OVA mice IL-25 protein levels in lung tissue were reduced to UV-OVA levels with IL-17RB blocking by 24 h p.i. (Fig. 5D). Lung protein levels of IL-33 and TSLP were also reduced, however this effect was observed in all OVA-challenged groups regardless of RV infection (Fig. 5D). Increased IgE contributes to heightened allergen driven inflammation. Allergen challenged RV infected mice had the highest levels of serum IgE at 7 days p.i. and a trend for reduction was observed with IL-17RB blocking however, the differences observed were not statistically significant (Fig. 5E). In addition to effector functions that drive allergic inflammation, type-2 cytokines can interfere with anti-viral responses(27). It was possible that the increased viral loads observed in RV-infected allergic mice were due to IL-25 augmented type-2 responses suppressing anti-viral immunity. In support of this we observed decreased levels of viral RNA with inhibition of IL-25 signalling (Fig. 5F). MUC5ac protein is a principal constituent of respiratory mucus and is induced by type-2 cytokines and associated with asthma exacerbations(28). Virally enhanced MUC5ac was blocked by anti-IL-17RB such that expression was restricted to levels similar to allergen challenged mice (Fig. 5G). These data identify the role of virus-induced IL-25 in the exacerbation of allergen driven cellular inflammation and type-2 mediator expression and effector function.

## IL-17RB blockade ablates viral recruitment of innate and adaptive type-2 cell populations

We next assessed the impact of IL-17RB blockade on RV-exacerbated cellular pulmonary inflammation. Exacerbated airway neutrophil, eosinophil and lymphocyte responses are features of RV-induced asthma exacerbations. Blocking IL-17RB significantly reduced airway neutrophilia in RV-OVA treated mice and inhibited the RV-exacerbated airway eosinophil and lymphocyte responses to levels that were not statistically different to that observed in UV-OVA treated mice (Fig. 6A). IL-$4^+$ basophil numbers were also significantly reduced by α-IL-17RB in the exacerbated airways of RV-OVA mice (Fig. 6B)

The α-IL-17RB antibody used to block the IL-25 receptor was the same clone used in previous experiments to detect IL-17RB expression by flow cytometry and as a result we were unable to measure IL-17RB expression in these experiments. However, we detected non-T type-2 cells by gating on CD4 negative cells that co-expressed ICOS and ST2. As early as 8 h we observed a significant decrease in virally exacerbated numbers of these cells following IL-25 receptor blocking (Fig. 6C). Similar results were observed later at day 7 for IL-4-expressing $CD4^+$ T (Th2) cells (Fig. 6D). These data demonstrate the role of RV-

enhanced IL-25 signalling in the exacerbation of both early and late innate and adaptive type-2 cellular responses in mice with allergic pulmonary inflammation.

## Discussion

Most studies investigating IL-25 have demonstrated expression in response to type-2 inducing stimuli such as parasitic helminth infections and allergen. No study to date has undertaken a detailed analysis of IL-25 protein expression *in vivo* during respiratory virus infection despite the role viruses play in allergic diseases such as asthma. Over 160 genetically distinct rhinovirus types belonging to 3 species (A, B and C) are now recognised(29). We used species A viruses (RV16 and RV1B) for these studies as these have been shown to be more frequently associated with lower respiratory tract disease compared to viruses belonging to species B. RV C viruses have been detected at a similar rate to species A viruses during severe respiratory disease. RV C were not used in this study because these viruses cannot be grown and purified using conventional virological techniques(30).

Using a human infection model we were able to show that RV was capable of inducing IL-25 in the upper respiratory tract. Nasal mucosa protein analyses revealed that 20 out of 22 asthmatic subjects had detectably increased IL-25 during experimental RV infection. Viral induction of IL-25 was clearly less robust in healthy subjects. Baseline and infection peak levels were also significantly higher in asthma indicating that whilst RV infection could induce IL-25 expression in healthy and asthmatic subjects, asthmatic airways possessed a significantly increased propensity to produce this cytokine. To investigate if greater IL-25 expression was a consequence of the *in vivo* environment characteristic of asthma such as increased type-2 immunity or atopy we conducted *in vitro* studies using serially passaged BECs from healthy and asthmatic donors. RV infected asthmatic BECs possessed greater intrinsic capacity for IL-25 gene and protein expression compared to non-asthmatic cells. To further assess this possibility, IL-25 induction by BECs was related to markers of allergy or of asthma severity. IL-25 levels were significantly related to SPT positivity and there was a trend towards greater IL-25 levels in those with more frequent asthma exacerbations. As we only had 10 subjects with asthma in this part of the study, we feel that the lack of statistically significant correlations of IL-25 with markers of true asthma severity, rather than SPTs which are a marker of allergy severity, likely relates to the small number of subjects studied. Whilst several studies using this *in vitro* RV infection model have identified deficiencies in expression anti-viral molecules(31-33), this is the first report of increased expression of a pro-type-2 cytokine by virally infected cultured asthmatic BECs. The fact that increased expression was observed directly *in vivo* and *in vitro* raises the possibility that both extrinsic (such as immune) and intrinsic (such as epigenetic) factors present in asthma and are involved in the heightened IL-25 response.

Treatment of mice with recombinant IL-25 induced type-2 cytokine expression by T- and non-T cell populations(34). Forced expression of IL-25 by transgenic mice has been reported to enhance allergen-induced type-2 responses and pulmonary allergic inflammation(35). The OVA mouse model is designed to investigate type-2 driven allergic asthma which represents at least 50% of adult asthmatics based on gene expression studies

of bronchial epithelial cells(8). The mouse model is different to human asthma in that the mice are systemically sensitised to a model allergen that is not a common aeroallergen in humans. However it does provide a model system in which to investigate the interaction of type-2 immunity and RV infection *in vivo* which we believe is mechanistically important in the pathogenesis of exacerbations for many asthmatics. To begin to better understand the role of IL-25 during viral exacerbations we examined IL-25 receptor-expressing cell populations. We examined an early (day 1 p.i.) time point for acute responses at the peak of infection and later time point (day 7 p.i.) to determine the duration of the response. At both times we observed significantly increased numbers of IL-17RB-expressing lung leukocytes in virally exacerbated mice. At day 1 IL-4-expressing T cells accounted for approximately 5% of the IL-17RB expressing leukocytes. By day 7 these cells represented around 30% of the IL-25 responsive cells in the lung suggesting that Th2 cells were more important for later responses mediated by IL-25, potentially as a consequence of ongoing amplification of type-2 immune cascade. Whilst our focus was IL-4 expression as a marker type-2 T cell responses it is possible that IL-17RB$^+$ CD4$^+$ T cells expressing other type-2 cytokines such as IL-5 and IL-13 were also contributing to virally exacerbated type-2 cytokine production.

We also observed that non-T type 2 cells expressing IL-25 receptor were associated with virally exacerbated pulmonary inflammation. Previous studies have demonstrated that ILC-2 cells do not express any conventional lineage markers, but do express ICOS and the IL-33 receptor (ST2)(36, 37). To investigate the role of non-T type-2 cells we identified a population of cells that were not T cells (CD3-, CD4-) and expressed ICOS and ST2. Although we did not use an exhaustive lineage panel to identify ILC-2 cells the fact that ICOS is expressed almost exclusively on T cells (particularly Th2)(38-40) and ILC-2 cells meant we could approximate the number of ILC-2 cells by identifying the CD3$^-$, CD4$^-$, ICOS$^+$ ST2$^+$ population. In our model approximately 10% of these cells co-expressed the IL-25 receptor and at day 1, and were significantly increased during viral exacerbation. We have previously reported that repeated dosing with recombinant IL-25 protein expands ILC-2 numbers in naïve mice(23). In this study RV induced IL-25 did not induce similar levels of non-T type 2 cell expansion (most likely due to the amount of recombinant IL-25 administered in previous studies far exceeded that produced *in vivo* in our studies). We did observe evidence of expansion as the number of lung leukocytes expressing IL-25 receptor on day 7 was double that observed on day 1. We have observed that RV-1B infection during allergic airways inflammation augments expression of leukocyte-recruiting chemokines such as CCL5, CCL17 and CCL22(15). This provides a possible mechanism for augmented recruitment of ILC-2s and later Th2 cells, which we report here to express the IL-25 receptor and be sensitive to IL-25 receptor blockade. Thus we propose that virally increased pulmonary IL-25 can promote survival, activation and up-regulated type 2 cytokine expression by these cells which are central to asthma exacerbation pathogenesis.

Whilst further studies are needed to identify all IL-25-receptor expressing cells, we provide good evidence that rhinovirus augmented IL-25 influences multiple T and non-T type-2 cellular responses.

Other studies have begun to investigate mechanisms linking anti-viral immunity and type-2 responses via deletion of key anti-viral mechanisms. Depletion of NK cells and associated

loss of IFN-γ expression lead to emergence of type-2 responses including IL-25 production during respiratory syncytial virus (RSV) infection(22). Neonatal mice lacking TLR7 infected with pneumonia virus of mice also exhibited exaggerated type-2 responses which included prolonged IL-25 protein in the BAL(41). In both paramyxovirus infection models viral induction of IL-25 was dependent on suppression of anti-viral immunity. If you consider that asthma is characterised by chronic activation of type-2 immunity and therefore represents a state of anti-viral immune suppression then our observation of increased rhinovirus load associated with increased IL-25 and type-2 immune responses (which was reversed with blocking IL-25 receptor) is consistent with the two studies mentioned above. That is there is critical balance between type-2- and anti-viral immunity which underpins the immunopathogenesis of virally exacerbated allergic diseases.

Blocking IL-25 had the dual effect of suppressing type-2 responses and viral infection, new evidence that IL-25 and type-2 immunity have two important immune-pathogenic roles: promote allergic inflammation and interfere with anti-viral responses. This paradigm has important implications for allergic diseases that are exacerbated by viral infection such as asthma. Targeting type-2 promoting cytokines such as IL-25 has the potential to simultaneously suppress allergic inflammation and promote anti-viral immunity. This paradigm dictates that therapies that either promote interferon production or inhibit type-2 responses could have therapeutic benefit. Both have recently been reported by separate human asthma clinical studies observing reduced exacerbation frequency or severity with either antibody-mediated blocking of Th2 cytokines(42, 43) or treatment with IFN-β (Synairgen, press release 2013).

This study had a number of limitations and caveats. The *in vitro* studies used submerged BEC monolayers which are not differentiated. Air-liquid interface (ALI) differentiated cell cultures contain fully differentiated ciliated epithelial cells which may provide better insight into the interaction of RV with the asthmatic airway. It will be interesting to investigate whether cytokines differentially expressed by submerged culture asthmatic epithelial cells are similarly dysregulated in ALI cultures during RV infection. The use of ALI cultures might also enable RV-C viruses to be studied to determine if common pathogenic mechanisms such as modulation of type-2 immunity by IL-25 are important across RV species. We showed infection augmented IL-25 was associated with increased numbers of basophils, T- and non-T type 2 cells all of which potentially contributed to increased type-2 cytokine expression. Additional studies involving specific blockade or depletion of these cell populations will determine how IL-25 regulates pulmonary type-2 cytokine expression. The antibody that we used for these studies blocks IL-17RB. This receptor specifically binds both IL17B and IL17E/IL-25, thus there is a possibility that certain effects observed could be mediated through blocking IL-17B binding to IL-17BR, as well as through blocking IL-17E/IL-25 binding to IL-17RB. Members of our lab have been unable to identify a robust IL-17B bioassay in which we could assess the effect of the anti-IL-17BR-antibody on IL-17B function *in vitro* or *in vivo*. However, we have as yet unpublished data that suggests that ablation of the IL-17BR in mice may have a slightly more potent anti-inflammatory effect than the ablation of the IL-25 ligand, raising the possibility that blocking IL-17RB, and therefore both the now quite well characterized IL-25 pathway and the largely unknown

IL-17B pathway may have added value therapeutically, above blocking the IL-25 ligand alone. Further studies will be needed to shed light on this.

Our studies identify blockade of IL-25 or its receptor as an attractive target for development of novel therapies for asthma exacerbations. This approach should potentially be more effective than the above approaches targeting single Th2 cytokines, as anti-IL-25 should block induction of all Th2 cytokines. We believe such therapies should urgently be investigated in the human experimental RV-induced asthma exacerbation model to determine if RV-induced asthma exacerbations are indeed impacted upon as our data suggests they should be. Further, as RSV infection has also been shown to induce IL-25(24) it is likely that anti-IL-25 therapy would impact upon asthma exacerbations induced by other virus types, as well as those precipitated by RV infections, thus making this a broadly applicable potential novel therapy.

In summary we provide substantial new insight into the biological activity of IL-25. Our data supports a mechanism whereby rhinovirus up-regulates IL-25 expression by susceptible asthmatic epithelial cells. Augmented IL-25 can potently amplify the type-2 immune cascade involving activation of IL-25-receptor expressing non-T type 2 cells and Th2 cells. This study also provides evidence to support therapies that target virally induced mediators such as IL-25 that are upstream of type-2 cytokine expression.

## Materials and Methods

### Study Design

The study objective was to use human and mouse *in vivo* models of RV infection in asthma to investigate virus-induced and augmented IL-25 expression in allergic airways and its role in the exacerbation of type 2 immune responses, and identify IL-25 as a potential therapeutic target. Human subjects from whom bronchial epithelial cells were obtained and those who took part in the experimental *in vivo* RV infection study were either healthy or asthmatic volunteers. Clinical characteristics of volunteers recruited by strict inclusion/exclusion criteria are summarised in Supplementary Table 1 and 3.

For all work, human and mouse samples were blinded to the scientists assessing/quantifying the results (e.g. counting stained cells from cytoslides). All controlled mouse experiments were replicated as indicated. Five mice per experimental group permitted reliable statistical analysis of data by two-way ANOVA, in addition to complying with the UK Home Office regulations to reduce total numbers of animals used where possible. The time points were selected based on preliminary studies. No data was excluded (including outliers).

### Viruses

Rhinovirus-16 (RV-16) for the human challenge study was obtained from clinical isolates as previously described(11, 44) and rhinovirus-1B (RV-1B) and -16 (American Type Culture Collection) were grown and titrated in HeLa cells (European Collection of Cell Cultures) by standard methods. For *in vivo* use in mice, virus was purified as previously described(12). Virus was inactivated by exposure to UV light at 1,200 mJ/cm$^2$ for 30 min.

### Rhinovirus infection of human bronchial epithelial cells

Bronchial epithelial cells (BECs) were obtained from 10 moderate atopic asthmatics (according to the BTS and GINA guidelines(45, 46)) and 10 non-atopic non-asthmatic healthy volunteers. Recorded clinical characteristics of the volunteers are summarised in the Supplementary Table 1. In brief, a subsection of the bronchial wall was scraped 5-10 times to obtain BECs that were seeded into flasks in BEGM with supplements according to the suppliers recommended protocol (Clonetics). At passage 2-3 cells were seeded into 12-well plates. At 80% confluency cells were infected with RV-1B (MOI 2) or treated with PBS as a control for 1 h with shaking, after which medium was replaced and supernatants and lysates harvested at the relevant time points post-infection (p.i.). All subjects gave written informed consent and the St. Mary's Hospital Ethics Committee approved the study. Bronchoscopies were carried out at St. Mary's Hospital, London, in accordance with standard guidelines(11).

### Human experimental rhinovirus infection

28 mild and moderate atopic asthmatics and 11 non-asthmatic healthy volunteers, baseline clinical characteristics summarised in Supplementary Table 3, were infected with RV-16, (100 $TCID_{50}$) diluted in 250μL of 0.9% saline, via an atomizer into both nostrils. Soluble mediators in the fluid lining the nasal mucosa was sampled with the Nasosorption™ technique prior to infection (baseline) and at days 2, 3, 4, 5, 7 and 10 post-inoculation. All subjects gave written informed consent and the St. Mary's Hospital Ethics Committee approved the study. Bronchoscopies were carried out at St. Mary's Hospital, London, in accordance with standard guidelines(11).

### Mouse model of RV-induced exacerbation of allergic airway inflammation

All experiments involving mice were in accordance with legislation outlined by the Home Office. We purchased 6-8-week-old female BALB/c mice (Charles River). The model used was previously described(12). In brief, mice were sensitized intraperitoneally (i.p.) with 50μg of ovalbumin (OVA) (Calbiochem) and 2mg aluminium hydroxide in 200μL PBS on d -13. Lightly anesthetized (isofluorane) mice were challenged intranasally (i.n.) with 50μg OVA in 30μL PBS or PBS alone on d -2, -1 and 0, immediately followed by infection with 50μL ($2\times10^6$ $TCID_{50}$) RV-1B or UV-RV-1B on d0. Mice were sacrificed at various time p.i. for end-point analyses. To block RV-induced IL-25 signalling in the mouse model of RV-induced exacerbation of allergic airway inflammation, 0.5mg of anti-mouse IL-17RB antibody (clone D9.2) (provided by Prof. Andrew McKenzie, MRC Laboratory of Molecular Biology [LMB]) was administered i.p. in 200μl PBS 4 h prior to infection and on 3 d and 5 d p.i. As a control 0.5mg of anti-c-myc mouse IgG1 isotype control antibody (Clone 9e10.2) (also provided by Prof. Andrew McKenzie, MRC LMB) was used. Airway cells were isolated from the bronchoalveolar lavage (BAL) fluid after brief centrifugation followed by red blood cell lysis using ACK buffer, washing and re-suspension in 1mL RPMI. Cells were stained with Quick Diff (Reagena) for differential cell counts.

### Flow cytometry

Mouse lungs were crudely dissociated using the GentleMACS™ tissue dissociator (Miltenyi Biotech) and digested upon incubation at 37°C in RPMI containing 1mg/mL collagenase

Type XI and 80 units/mL Bovine Pancreatic DNase Type IV (both Sigma-Aldrich). A single cell suspension was attained upon further GentleMACS™ dissociation followed by treatment with ACK lysis buffer and filtration through a 100μm cell strainer. For IL-4 expression analysis by intracellular cytokine staining (ICS), BAL and lung cells were stimulated for 4 h with PMA (50ng/mL) and ionomycin (500ng/mL) (both Sigma-Aldrich) in the presence of monensin (BD GolgiStop, BD Biosciences). Staining with the LIVE/DEAD Fixable Aqua Dead Cell Stain Kit identified dead cells (Invitrogen). Cells were incubated with FcBlock (BD Biosciences) prior to staining for surface markers using anti-mouse antibodies for CD3 (500A2, eBiosciences), CD4 (RM4-5, Biologend), CD49b (DX5, Biolegend), FcεR1 (MAR-1, eBiosciences), ICOS (C398.4 A, Biologend), T1/ST2 (DJB, MD Biosciences), and IL-17RB (D9.2, obtained from Andrew McKenzie, MRC LMB). Intracellular cytokine staining was performed by standard techniques and according to manufacturers' instructions (BD Biosciences Fix/Perm kit) and cells were stained with anti-mouse IL-4 (11B11, Biolegend) antibody. Data was collected on a BD Biosciences Fortessa flow cytometer and analysed with FlowJo software (Tree Star).

### Ragweed administration and identification of ICOS+ cells

Wildtype C57Bl/6 mice were lightly anaesthetised with isoflurane and immunised with 50 μl of short ragweed pollen (100 μg/dose of protein, Greer Laboratories) intranasally for four consecutive days. Mice were euthanized 24 hours after final administration and tissues were collected for analysis. Lung tissues were chopped into small pieces then incubated with 720 μg/ml collagenase D (Amersham, Bucks) for 1 hour. Lung and mediastinal lymph node tissues were mechanically passed through 70 μm cell strainers to obtain single cell suspensions. Tissue cell suspensions were incubated with anti-Fc receptor blocking antibody (anti-CD16/32, eBioscience, 14-0161-85), then stained with the following antibody panel: CD19-PerCP-Cy5.5 (eBioscience, 45-0193-82), ICOS-Alexa Fluor 647 (Biolegend, 313516), CD4-Alexa Fluor 700 (eBioscience, 56-0041-80), Fixable Viability Dye eFluor780 (eBioscience, 65-0865-14), CD8-Brilliant Violet 421 (Biolegend, 100738) and Lineage-PeCy7 (CD3 (eBioscience, 25-0031-82), CD11b (eBioscience, 25-0112-82), CD11c (eBioscience, 25-0114-82), FcεR1 (eBioscience, 25-5898-82), Gr-1 (eBioscience, 25-5931-82), NK1.1 (eBioscience, 25-5941-82), Ter-119 (eBioscience, 25-5921-82)).

### Protein quantification

IL-25 in the supernatant of bronchial epithelial cell cultures was measured with a human IL-25 ELISA developed in-house by Novartis. IL-25 sampled from the nasal mucosal fluid of experimentally infected human volunteers was analysed by Meso-Scale Discovery (MSD) platform, with the limit of detection at 10 pg/mL. Mouse IFN-α, IFN-β, IFN-λ2/3, IL-4, IL-5, IL-6, IL-13, CCL11 and CCL24 in the BAL fluid and IL-25, IL-33 and TSLP in lung homogenate supernatant were quantified by ELISA (R&D Systems) according to manufacturers' specifications. IgE in mouse serum was quantified using the BD OptEIA™ Mouse IgE Set (BD Biosciences) according to manufacturers' specifications.

### Immunohistochemistry

Single IHC staining on formalin-fixed and paraffin-embedded mouse lung sections was performed as previously described(15). Formalin-fixed, paraffin-embedded lungs were

deparaffinised and antigen unmasking was carried out by immersing sections in citrate buffer. Sections were then covered with 3% hydrogen peroxidase and left for 5 min before washing with PBS. Cell membranes were permeabilised with 0.1% saponin in PBS and non-specific staining blocked with 5% rabbit serum. Sections were incubated with 60μg/mL rat anti-mouse IL-25 antibody (clone 35b) (Biolegend) or a rat IgG isotype control (Sigma-Aldrich). Sections were then washed and incubated with HRP labelled rabbit anti-rat secondary antibody (Dako) for 1 h. After a further wash with PBS, sections were incubated with ABC reagent (Vector ABC Kit) and the reaction visualised with chromogen-fast diaminobenzidine5 (DAB) as a chromogenic substrate. Slides were counterstained with haematoxylin to provide nuclear and morphological detail, and mounted in DPX Mountant (Sigma-Aldrich). The sections of whole lungs (right + left) were stained for IL-25. All large airways ($2^{nd}$ & $3^{rd}$ generations) were scored for epithelial positivity. The average score from all airways was used to express epithelial IL-25 staining intensity for each lung, which was scored semi-quantitatively from 0 to 1.5 (0 = negative; 0.5 = weak staining, 1 = moderate staining, and 1.5 = strong staining). The length of five airways (1 airways per lobe of 2nd & 3rd generation airways) were measured and counted for numbers of subepithelial inflammatory cells expressing IL-25. Infiltrating IL-25+ inflammatory cells in the airway lamina propria were quantified and expressed as number of positive inflammatory cells per mm length of the reticular basement membrane. All counts on histology sections were performed by one investigator blinded to the treatment protocol.

### mRNA quantification

Total RNA was extracted from bronchial epithelial cells (RNeasy kit, Qiagen) and 2μg was reverse transcribed for cDNA synthesis (Omniscript RT kit, Qiagen). Total RNA was also extracted from mouse apical lung lobes stored in RNA later (Qiagen), followed by cDNA synthesis as above. Quantitative PCR was performed using specific primers and probes for each gene and Quantitect Probe PCR master mix (Qiagen). Mouse IL-25 primers and probe sequences: sense 5′-CAC ACC CAC CAC GCA GAA T-3′ 300nm, antisense 5′-CAA CTC ATA GCT CCA AGG AGA GAT G-3′ 300nm and probe 5′-FAM-CCA GCA AGG ATG GCC CCC TCA-TAMRA-3′ 100nm. Human IL-25 primers and probe sequences: sense 5′-GAG ATA TGA GTT GGA CAG AGA CTT GAA-3′ 300nm, antisense 5′-CCA TGT GGG AGC CTG TCT GTA-3′ 300nm and probe 5′-FAM-CTC CCC CAG GAC CTG TAC CAC GC-TAMRA-3′ 100nm. RV-1B genomic RNA primes and probe sequences: sense 5′-GTG AAG AGC CSC RTG TGC T-3′ 50nm, antisense 5′-GCT SCA GGG TTA AGG TTA GCC-3′ 300nm and probe-5′-FAM-TGA GTC CTC CGG CCC CTG AAT G-TAMRA-3′ 100nM. An ABI 7500 Taqman (ABI) was used to analyse PCR reactions. Each gene was normalised to 18s rRNA, quantified using a standard curve generated by amplification of plasmid DNA and is expressed as copies per μL of cDNA reaction.

### Statistical analysis

Human data was analysed by one-way ANOVA using the Kruskal-Wallis test with a 95% confidence interval with Dunn's multiple comparison test. Correlations were assessed with linear regression and Spearman's coefficient (r) value. All data from mouse studies are presented as means ± s.e.m. Studies were conducted with 5-6 mice per group, and data is representative of at least 2 independent experiments unless indicated as combined

Europe PMC Funders Author Manuscripts

experiments. Where groups were greater than 2 and/or multiple comparisons were analysed, results were analyzed by ANOVA and differences between groups identified using Bonferroni's post-test with 95% confidence using GraphPad Prism 6 software. When only two groups were analysed and one condition was variable a 2 tailed unpaired t test was used to compare groups.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

Rebecca Beavil at the MRC Protein Purification (Kings College London, UK) unit for providing purified anti-IL-17RB and isotype control antibodies.

**Funding:** This work was supported by Medical Research Council (MRC) Grant numbers G0601236 and G1100168, by ERC FP7 grant 233015, a Chair from Asthma UK (CH11SJ), MRC Centre grant G1000758, NIHR BRC Centre grant P26095, Predicta FP7 Collaborative Project grant 260895 and by the NIHR BRC at Imperial College London. Novartis Institute for Biomedical Research funded the IL-25 nasosorption assays.

## References and notes

1. Johnston SL, Pattemore PK, Sanderson G, Smith S, Lampe F, Josephs L, Symington P, O'Toole S, Myint SH, Tyrrell DA, et al. Community study of role of viral infections in exacerbations of asthma in 9-11 year old children. Bmj. 1995; 310:1225–1229. [PubMed: 7767192]
2. Botturi K, Langelot M, Lair D, Pipet A, Pain M, Chesne J, Hassoun D, Lacoeuille Y, Cavailles A, Magnan A. Preventing asthma exacerbations: what are the targets? Pharmacol Ther. 2011; 131:114–129. [PubMed: 21440000]
3. Larche M, Robinson DS, Kay AB. The role of T lymphocytes in the pathogenesis of asthma. J Allergy Clin Immunol. 2003; 111:450–463. quiz 464. [PubMed: 12642820]
4. Christodoulopoulos P, Cameron L, Nakamura Y, Lemiere C, Muro S, Dugas M, Boulet LP, Laviolette M, Olivenstein R, Hamid Q. TH2 cytokine-associated transcription factors in atopic and nonatopic asthma: evidence for differential signal transducer and activator of transcription 6 expression. J Allergy Clin Immunol. 2001; 107:586–591. [PubMed: 11295643]
5. Berry MA, Parker D, Neale N, Woodman L, Morgan A, Monk P, Bradding P, Wardlaw AJ, Pavord ID, Brightling CE. Sputum and bronchial submucosal IL-13 expression in asthma and eosinophilic bronchitis. J Allergy Clin Immunol. 2004; 114:1106–1109. [PubMed: 15536417]
6. Saha SK, Berry MA, Parker D, Siddiqui S, Morgan A, May R, Monk P, Bradding P, Wardlaw AJ, Pavord ID, Brightling CE. Increased sputum and bronchial biopsy IL-13 expression in severe asthma. J Allergy Clin Immunol. 2008; 121:685–691. [PubMed: 18328894]
7. Kabesch M, Schedel M, Carr D, Woitsch B, Fritzsch C, Weiland SK, von Mutius E. IL-4/IL-13 pathway genetics strongly influence serum IgE levels and childhood asthma. J Allergy Clin Immunol. 2006; 117:269–274. [PubMed: 16461126]
8. Woodruff PG, Modrek B, Choy DF, Jia G, Abbas AR, Ellwanger A, Koth LL, Arron JR, Fahy JV. Th2-driven Inflammation Defines Major Sub-phenotypes of Asthma. Am J Respir Crit Care Med. 2009
9. Sandford AJ, Chagani T, Zhu S, Weir TD, Bai TR, Spinelli JJ, Fitzgerald JM, Behbehani NA, Tan WC, Pare PD. Polymorphisms in the IL4, IL4RA, and FCERIB genes and asthma severity. J Allergy Clin Immunol. 2000; 106:135–140. [PubMed: 10887316]
10. Wenzel SE, Balzar S, Ampleford E, Hawkins GA, Busse WW, Calhoun WJ, Castro M, Chung KF, Erzurum S, Gaston B, Israel E, Teague WG, Curran-Everett D, Meyers DA, Bleecker ER. IL4R alpha mutations are associated with asthma exacerbations and mast cell/IgE expression. Am J Respir Crit Care Med. 2007; 175:570–576. [PubMed: 17170387]

11. Message SD, Laza-Stanca V, Mallia P, Parker HL, Zhu J, Kebadze T, Contoli M, Sanderson G, Kon OM, Papi A, Jeffery PK, Stanciu LA, Johnston SL. Rhinovirus-induced lower respiratory illness is increased in asthma and related to virus load and Th1/2 cytokine and IL-10 production. Proc Natl Acad Sci U S A. 2008; 105:13562–13567. [PubMed: 18768794]
12. Bartlett NW, Walton RP, Edwards MR, Aniscenko J, Caramori G, Zhu J, Glanville N, Choy KJ, Jourdan P, Burnet J, Tuthill TJ, Pedrick MS, Hurle MJ, Plumpton C, Sharp NA, Bussell JN, Swallow DM, Schwarze J, Guy B, Almond JW, Jeffery PK, Lloyd CM, Papi A, Killington RA, Rowlands DJ, Blair ED, Clarke NJ, Johnston SL. Mouse models of rhinovirus-induced disease and exacerbation of allergic airway inflammation. Nat Med. 2008; 14:199–204. [PubMed: 18246079]
13. Collison A, Hatchwell L, Verrills N, Wark PA, de Siqueira AP, Tooze M, Carpenter H, Don AS, Morris JC, Zimmermann N, Bartlett NW, Rothenberg ME, Johnston SL, Foster PS, Mattes J. The E3 ubiquitin ligase midline 1 promotes allergen and rhinovirus-induced asthma by inhibiting protein phosphatase 2A activity. Nat Med. 2013
14. Hammad H, Chieppa M, Perros F, Willart MA, Germain RN, Lambrecht BN. House dust mite allergen induces asthma via Toll-like receptor 4 triggering of airway structural cells. Nat Med. 2009; 15:410–416. [PubMed: 19330007]
15. Bartlett NW, Slater L, Glanville N, Haas JJ, Caramori G, Casolari P, Clarke DL, Message SD, Aniscenko J, Kebadze T, Zhu J, Mallia P, Mizgerd JP, Belvisi M, Papi A, Kotenko SV, Johnston SL, Edwards MR. Defining critical roles for NF-kappaB p65 and type I interferon in innate immunity to rhinovirus. EMBO Mol Med. 2012; 4:1244–1260. [PubMed: 23165884]
16. Licona-Limon P, Kim LK, Palm NW, Flavell RA. TH2, allergy and group 2 innate lymphoid cells. Nat Immunol. 2013; 14:536–542. [PubMed: 23685824]
17. Corrigan CJ, Wang W, Meng Q, Fang C, Eid G, Caballero MR, Lv Z, An Y, Wang YH, Liu YJ, Kay AB, Lee TH, Ying S. Allergen-induced expression of IL-25 and IL-25 receptor in atopic asthmatic airways and late-phase cutaneous responses. J Allergy Clin Immunol. 2011; 128:116–124. [PubMed: 21570719]
18. Ballantyne SJ, Barlow JL, Jolin HE, Nath P, Williams AS, Chung KF, Sturton G, Wong SH, McKenzie AN. Blocking IL-25 prevents airway hyperresponsiveness in allergic asthma. J Allergy Clin Immunol. 2007; 120:1324–1331. [PubMed: 17889290]
19. Barlow JL, Bellosi A, Hardman CS, Drynan LF, Wong SH, Cruickshank JP, McKenzie AN. Innate IL-13-producing nuocytes arise during allergic lung inflammation and contribute to airways hyperreactivity. J Allergy Clin Immunol. 2012; 129:191–198. e191–194. [PubMed: 22079492]
20. Wang YH, Angkasekwinai P, Lu N, Voo KS, Arima K, Hanabuchi S, Hippe A, Corrigan CJ, Dong C, Homey B, Yao Z, Ying S, Huston DP, Liu YJ. IL-25 augments type 2 immune responses by enhancing the expansion and functions of TSLP-DC-activated Th2 memory cells. J Exp Med. 2007; 204:1837–1847. [PubMed: 17635955]
21. Hvid M, Vestergaard C, Kemp K, Christensen GB, Deleuran B, Deleuran M. IL-25 in atopic dermatitis: a possible link between inflammation and skin barrier dysfunction? J Invest Dermatol. 2011; 131:150–157. [PubMed: 20861853]
22. Kaiko GE, Phipps S, Angkasekwinai P, Dong C, Foster PS. NK cell deficiency predisposes to viral-induced Th2-type allergic inflammation via epithelial-derived IL-25. J Immunol. 2010; 185:4681–4690. [PubMed: 20855881]
23. Neill DR, Wong SH, Bellosi A, Flynn RJ, Daly M, Langford TK, Bucks C, Kane CM, Fallon PG, Pannell R, Jolin HE, McKenzie AN. Nuocytes represent a new innate effector leukocyte that mediates type-2 immunity. Nature. 2010
24. Petersen BC, Dolgachev V, Rasky A, Lukacs NW. IL-17E (IL-25) and IL-17RB promote respiratory syncytial virus-induced pulmonary disease. J Leukoc Biol. 2014
25. Tan WC. Viruses in asthma exacerbations. Curr Opin Pulm Med. 2005; 11:21–26. [PubMed: 15591884]
26. Rincon M, Anguita J, Nakamura T, Fikrig E, Flavell RA. Interleukin (IL)-6 directs the differentiation of IL-4-producing CD4+ T cells. J Exp Med. 1997; 185:461–469. [PubMed: 9053446]

27. Sriram U, Biswas C, Behrens EM, Dinnall JA, Shivers DK, Monestier M, Argon Y, Gallucci S. IL-4 suppresses dendritic cell response to type I interferons. J Immunol. 2007; 179:6446–6455. [PubMed: 17982033]

28. Hewson CA, Haas JJ, Bartlett NW, Message SD, Laza-Stanca V, Kebadze T, Caramori G, Zhu J, Edbrooke MR, Stanciu LA, Kon OM, Papi A, Jeffery PK, Edwards MR, Johnston SL. Rhinovirus induces MUC5AC in a human infection model and in vitro via NF-kappaB and EGFR pathways. The European respiratory journal. 2010; 36:1425–1435. [PubMed: 20525715]

29. McIntyre CL, Knowles NJ, Simmonds P. Proposals for the classification of human rhinovirus species A, B and C into genotypically assigned types. J Gen Virol. 2013; 94:1791–1806. [PubMed: 23677786]

30. Lee WM, Lemanske RF Jr. Evans MD, Vang F, Pappas T, Gangnon R, Jackson DJ, Gern JE. Human rhinovirus species and season of infection determine illness severity. Am J Respir Crit Care Med. 2012; 186:886–891. [PubMed: 22923659]

31. Wark PA, Johnston SL, Bucchieri F, Powell R, Puddicombe S, Laza-Stanca V, Holgate ST, Davies DE. Asthmatic bronchial epithelial cells have a deficient innate immune response to infection with rhinovirus. J Exp Med. 2005; 201:937–947. [PubMed: 15781584]

32. Contoli M, Message SD, Laza-Stanca V, Edwards MR, Wark PA, Bartlett NW, Kebadze T, Mallia P, Stanciu LA, Parker HL, Slater L, Lewis-Antes A, Kon OM, Holgate ST, Davies DE, Kotenko SV, Papi A, Johnston SL. Role of deficient type III interferon-lambda production in asthma exacerbations. Nat Med. 2006; 12:1023–1026. [PubMed: 16906156]

33. Laza-Stanca V, Message SD, Edwards MR, Parker HL, Zdrenghea MT, Kebadze T, Kon OM, Mallia P, Stanciu LA, Johnston SL. The role of IL-15 deficiency in the pathogenesis of virus-induced asthma exacerbations. PLoS Pathog. 2011; 7:e1002114. [PubMed: 21779162]

34. Fort MM, Cheung J, Yen D, Li J, Zurawski SM, Lo S, Menon S, Clifford T, Hunte B, Lesley R, Muchamuel T, Hurst SD, Zurawski G, Leach MW, Gorman DM, Rennick DM. IL-25 induces IL-4, IL-5, and IL-13 and Th2-associated pathologies in vivo. Immunity. 2001; 15:985–995. [PubMed: 11754819]

35. Tamachi T, Maezawa Y, Ikeda K, Kagami S, Hatano M, Seto Y, Suto A, Suzuki K, Watanabe N, Saito Y, Tokuhisa T, Iwamoto I, Nakajima H. IL-25 enhances allergic airway inflammation by amplifying a TH2 cell-dependent pathway in mice. J Allergy Clin Immunol. 2006; 118:606–614. [PubMed: 16950278]

36. Barlow JL, Peel S, Fox J, Panova V, Hardman CS, Camelo A, Bucks C, Wu X, Kane CM, Neill DR, Flynn RJ, Sayers I, Hall IP, McKenzie AN. IL-33 is more potent than IL-25 in provoking IL-13-producing nuocytes (type 2 innate lymphoid cells) and airway contraction. J Allergy Clin Immunol. 2013

37. Spits H, Artis D, Colonna M, Diefenbach A, Di Santo JP, Eberl G, Koyasu S, Locksley RM, McKenzie AN, Mebius RE, Powrie F, Vivier E. Innate lymphoid cells - a proposal for uniform nomenclature. Nat Rev Immunol. 2013; 13:145–149. [PubMed: 23348417]

38. Hutloff A, Dittrich AM, Beier KC, Eljaschewitsch B, Kraft R, Anagnostopoulos I, Kroczek RA. ICOS is an inducible T-cell co-stimulator structurally and functionally related to CD28. Nature. 1999; 397:263–266. [PubMed: 9930702]

39. Yoshinaga SK, Whoriskey JS, Khare SD, Sarmiento U, Guo J, Horan T, Shih G, Zhang M, Coccia MA, Kohno T, Tafuri-Bladt A, Brankow D, Campbell P, Chang D, Chiu L, Dai T, Duncan G, Elliott GS, Hui A, McCabe SM, Scully S, Shahinian A, Shaklee CL, Van G, Mak TW, Senaldi G. T-cell co-stimulation through B7RP-1 and ICOS. Nature. 1999; 402:827–832. [PubMed: 10617205]

40. Tesciuba AG, Subudhi S, Rother RP, Faas SJ, Frantz AM, Elliot D, Weinstock J, Matis LA, Bluestone JA, Sperling AI. Inducible costimulator regulates Th2-mediated inflammation, but not Th2 differentiation, in a model of allergic airway disease. J Immunol. 2001; 167:1996–2003. [PubMed: 11489981]

41. Kaiko GE, Loh Z, Spann K, Lynch JP, Lalwani A, Zheng Z, Davidson S, Uematsu S, Akira S, Hayball J, Diener KR, Baines KJ, Simpson JL, Foster PS, Phipps S. Toll-like receptor 7 gene deficiency and early-life Pneumovirus infection interact to predispose toward the development of asthma-like pathology in mice. J Allergy Clin Immunol. 2013; 131:1331–1339. e1310. [PubMed: 23561801]

42. Pavord ID, Korn S, Howarth P, Bleecker ER, Buhl R, Keene ON, Ortega H, Chanez P. Mepolizumab for severe eosinophilic asthma (DREAM): a multicentre, double-blind, placebo-controlled trial. Lancet. 2012; 380:651–659. [PubMed: 22901886]

43. Wenzel S, Ford L, Pearlman D, Spector S, Sher L, Skobieranda F, Wang L, Kirkesseli S, Rocklin R, Bock B, Hamilton J, Ming JE, Radin A, Stahl N, Yancopoulos GD, Graham N, Pirozzi G. Dupilumab in persistent asthma with elevated eosinophil levels. N Engl J Med. 2013; 368:2455–2466. [PubMed: 23688323]

44. Mallia P, Message SD, Gielen V, Contoli M, Gray K, Kebadze T, Aniscenko J, Laza-Stanca V, Edwards MR, Slater L, Papi A, Stanciu LA, Kon OM, Johnson M, Johnston SL. Experimental rhinovirus infection as a human model of chronic obstructive pulmonary disease exacerbation. Am J Respir Crit Care Med. 2011; 183:734–742. [PubMed: 20889904]

45. Bateman ED, Hurd SS, Barnes PJ, Bousquet J, Drazen JM, FitzGerald M, Gibson P, Ohta K, O'Byrne P, Pedersen SE, Pizzichini E, Sullivan SD, Wenzel SE, Zar HJ. Global strategy for asthma management and prevention: GINA executive summary. Eur Respir J. 2008; 31:143–178. [PubMed: 18166595]

46. Statement by the British Thoracic Society; Brit. Paediatric Association; Research Unit of the Royal College of Physicians of London; King's Fund Centre; National Asthma Campaign; Royal College of General Practitioners; General Practitioners in Asthma Group; Brit. Assoc. of Accident and Emergency Medicine; Brit. Paediatric Respiratory Group. Guidelines on the management of asthma. Thorax. 1993; 48:S1–24. [PubMed: 8322228]

Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts

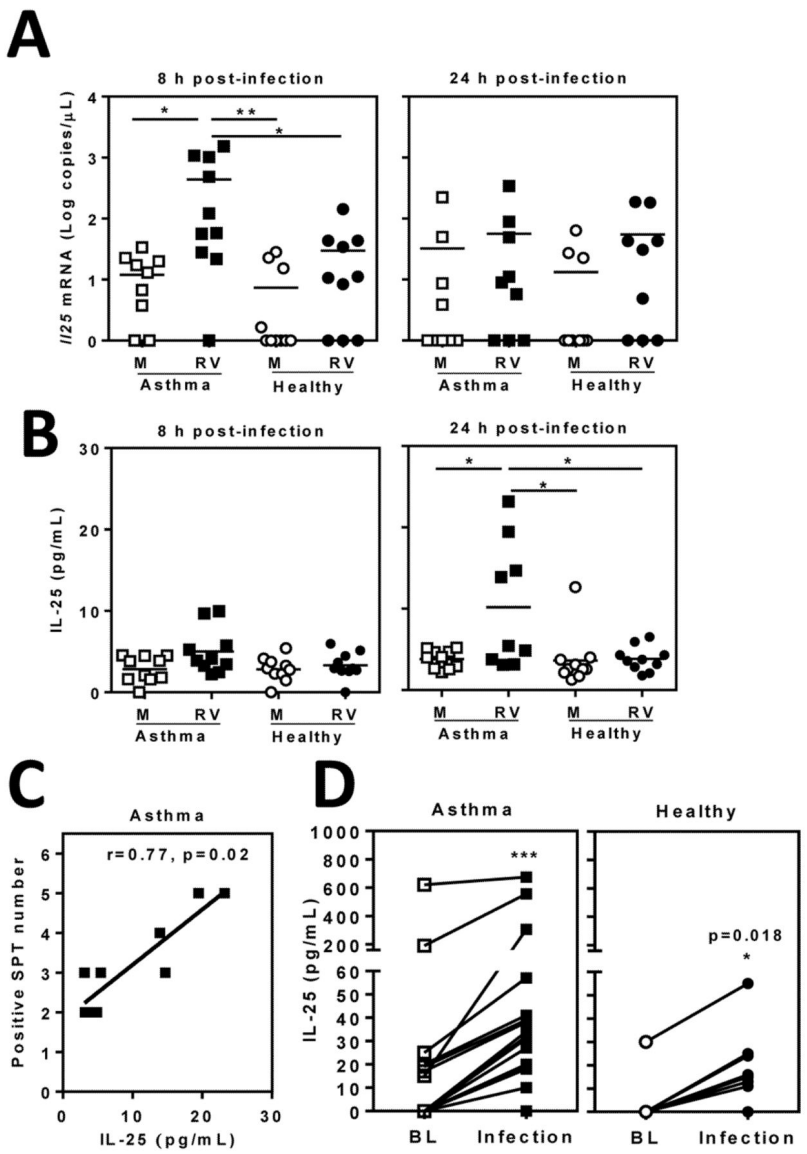


**Figure 1. Asthmatics expressed greater levels of IL-25 in response to rhinovirus infection *in vitro* and *in vivo***

Cultured bronchial epithelial cells obtained from 10 asthmatic and 10 healthy volunteers were infected with RV-1B (RV) or mock infected (M). Quantification of IL-25 (**A**) mRNA and (**B**) secreted protein levels at 8 h and 24 h post-infection, as assessed by qRT-PCR and ELISA respectively. (**C**) Correlation of IL-25 protein levels at 24 h post-infection with the number of positive skin prick tests (SPT) in asthmatics. In another study, 28 asthmatic and 11 healthy human volunteers were experimentally infected with RV-16. (**D**) Baseline (BL) and peak IL-25 protein levels in the nasal mucosal fluid after RV-16 infection, quantified by MSD platform. Any value under 10 pg/mL was given a value of zero. **P*<0.05 and ***P*<0.01 as indicated (A & B) and **P*<0.05 and ****P*<0.001 baseline vs infection peak (D). All data represent mean±s.e.m. qRT-PCR and ELISA data analysed by 1 away ANOVA with Bonferroni post-test. Baseline compared to peak infection IL-25 protein data

were analysed by Wilcoxon Signed Rank test. Correlations were assessed with linear regression and Spearman's coefficient (r) value.



Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts

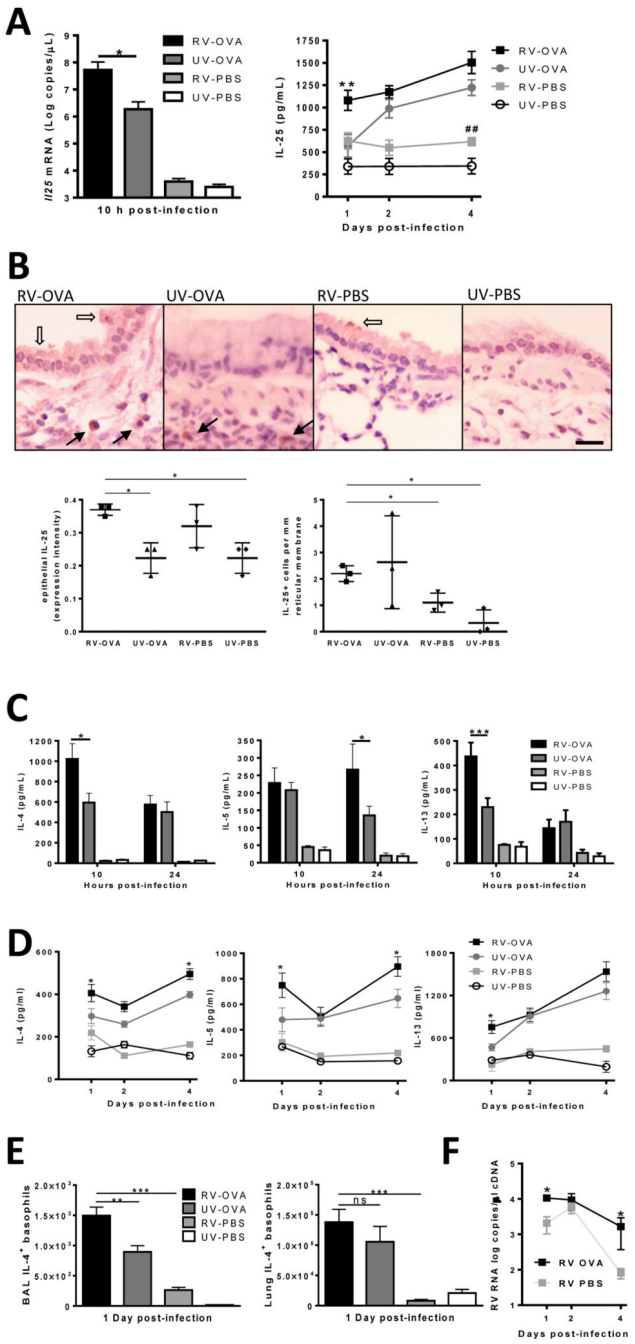


**Figure 2. Rhinovirus infection increased pulmonary IL-25 expression which is associated with increased type-2 cytokine production, basophil recruitment and increased viral load in mice with allergic pulmonary inflammation**

OVA-sensitized mice were challenged intranasally with OVA or PBS prior to infection with RV-1B (RV-OVA or RV-PBS) or UV-inactivated RV-1B (UV-OVA or UV-PBS) (*n* = 5 per group). (**A**) Quantification of IL-25 mRNA and protein levels in lung tissue at the indicated time points post-infection, as assessed by qRT-PCR and ELISA. (**B**) IL-25 protein expression in lung sections at day 2 post-infection, as assessed by immunohistochemistry. Black arrows indicate sub-epithelial IL-25+ inflammatory cells in OVA challenged mice.

Europe PMC Funders Author Manuscripts

Open arrows indicate areas of IL-25+ epithelium in RV infected mice. Scale bar: 20 μm. Epithelial IL-25 staining intensity and number of IL-25+ inflammatory cells were measured and plotted. Immunohistochemistry data represent mean±s.e.m for three mice per treatment group. **P*<0.05 (unpaired t-test) for RV-OVA vs indicated group. (**C,D**) Level of the type-2 cytokines IL-4, IL-5 and IL-13 protein levels in (**C**) BAL fluid and (**D**) lung homogenate by ELISA. (**e**) Total number of BAL and lung IL-4 expressing basophils at 1 day post-infection, as assessed by flow cytometry. (**f**) Viral RNA in lung tissue for RV infected mice, quantified by qRT-PCR. **P*<0.05, ***P*<0.01 and ****P*<0.001 for RV-OVA vs UV-OVA, $^{\#\#}P$<0.01 for RV-PBS vs UV-PBS and ns = not significantELISA and qPCR results were analyzed by ANOVA and differences between groups identified using Bonferroni's post-test

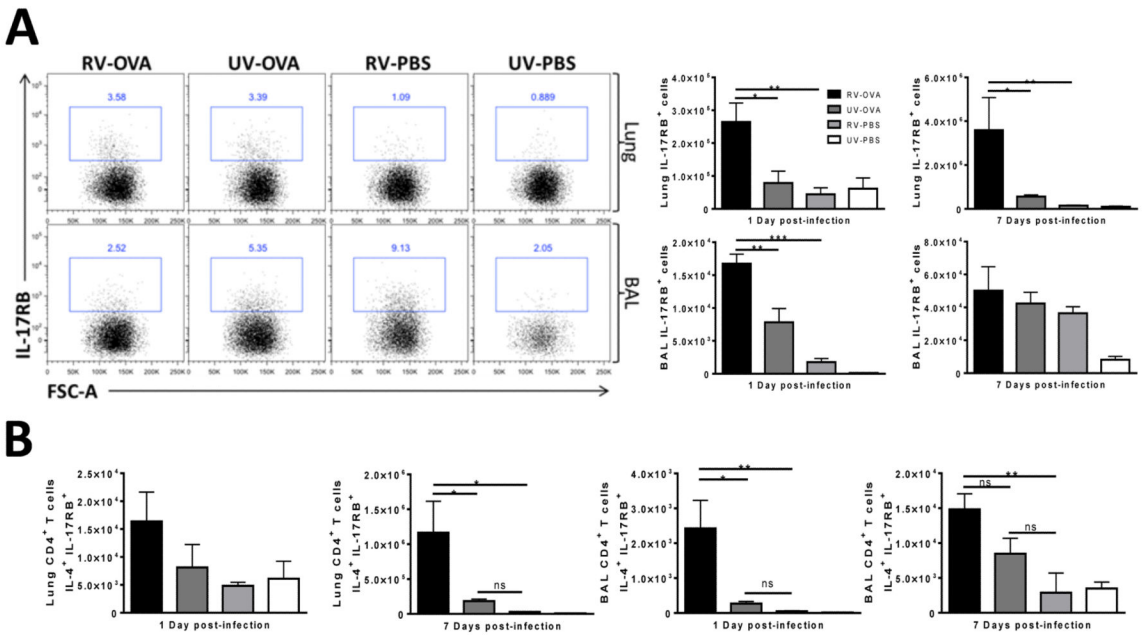


**Figure 3. Rhinovirus-induced accumulation of IL-25-responsive cells in mice with allergic pulmonary inflammation**

OVA-sensitized mice were challenged intranasally with OVA or PBS prior to infection with RV-1B (RV-OVA or RV-PBS) or UV-inactivated RV-1B (UV-OVA or UV-PBS) (*n* = 5 per group). (**A**) Representative flow plots of total IL-17RB$^+$ leukocytes in lung and BAL and enumerated at day 1 and day 7. (**B**) IL-4-expressing IL-17RB$^+$ CD4$^+$ T cells at 1 and 7 days post-infection in lung and BAL measured by flow cytometry. **P*<0.05, ***P*<0.01, ****P*<0.001 and ns = not significant. All data anlayzed by ANOVA and differences between groups identified using Bonferroni's post-test and represent mean±s.e.m and are representative of 2-3 studies.

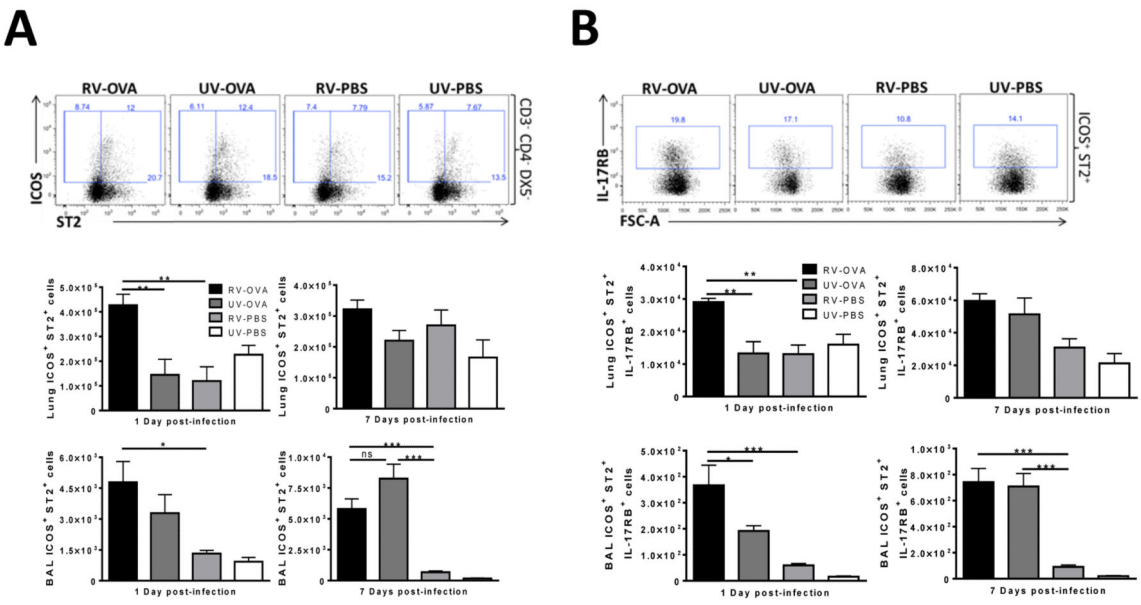


**Figure 4. Rhinovirus-induced accumulation of IL-25-responsive non-T type-2- cells in mice with allergic pulmonary inflammation**

OVA-sensitized mice were challenged intranasally with OVA or PBS prior to infection with RV-1B (RV-OVA or RV-PBS) or UV-inactivated RV-1B (UV-OVA or UV-PBS) (*n* = 5 per group). (**A, B**) Representative flow cytometry plots and total BAL and lung numbers of (**A**) non-T/non-NK cells (from $CD3^-$, $CD4^-$ and $DX5^-$ gated cells) $ICOS^+/ST2^+$ cells and (**B**) $IL\text{-}17RB^+$ non-T type 2 cells at 1 and 7 days post-infection. **P*<0.05, ***P*<0.01, ****P*<0.001 and ns = not significant. All data anlayzed by ANOVA and differences between groups identified using Bonferroni's post-test and represent mean±s.e.m and are representative of 2-3 studies.

Europe PMC Funders Author Manuscripts

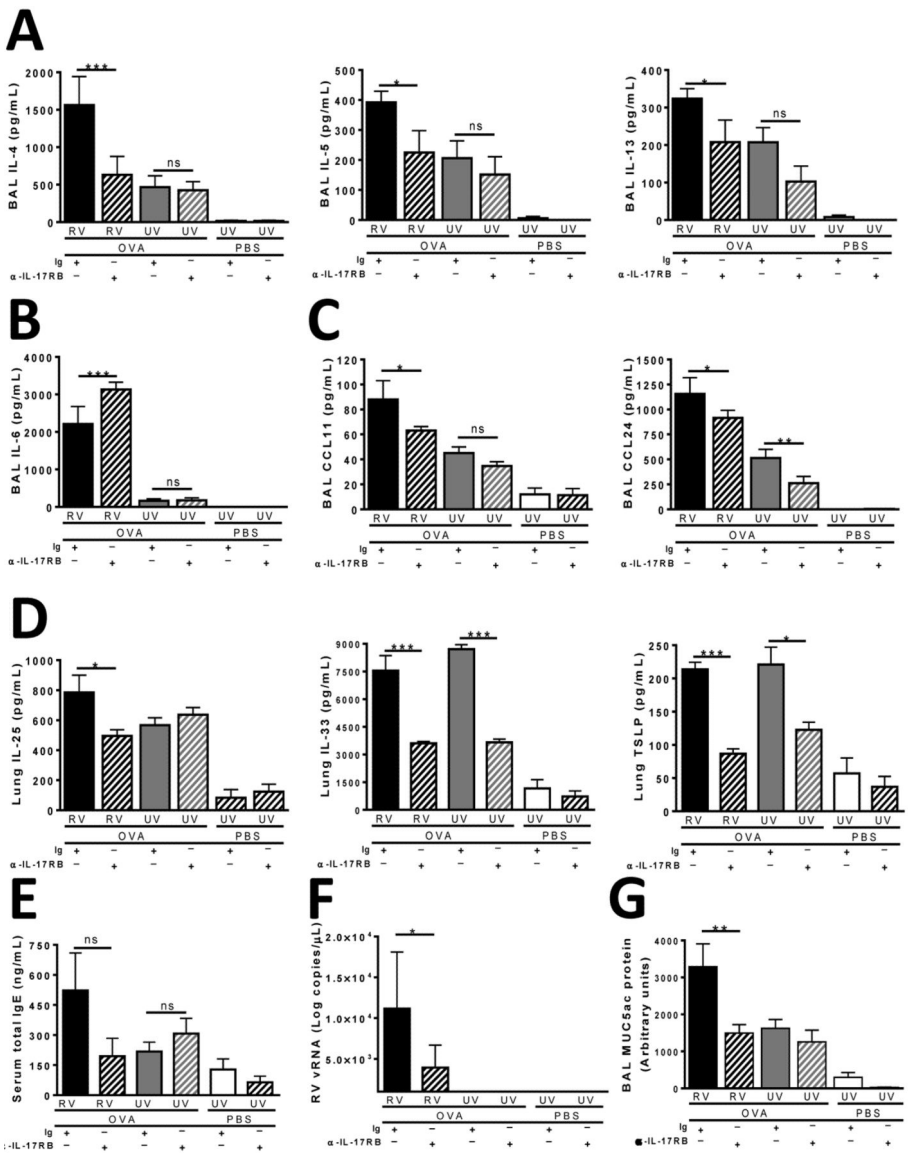


**Figure 5. Blocking IL-25 signalling attenuated OVA-induced and rhinovirus-exacerbated expression of type-2 mediators in mice with allergic pulmonary inflammation**

OVA-sensitized mice were challenged intranasally with OVA or PBS followed by intraperitoneal injection of anti-IL-17RB blocking antibody or isotype control (Ig) 4 h before and 3 and 5 days post-infection with RV-1B or UV inactivated RV-1B ($n$ = 5 per group). (**A**) Type-2 cytokines IL-4, IL-5 and IL-13 at 8 post-infection (**B**) proinflammatory cytokine IL-6 at 8 h post-infection and (**C**) eosinophil recruiting chemokines CCL11 and CCL24 at 8 and 24 h post-infection respectively. (**D**) Type-2-associated epithelial-derived cytokines IL-25, IL-33 and TSLP in lung homogenate supernatant at 24 h post-infection. (**E**) Total serum IgE levels at 7 days post-infection by ELISA. (**F**) Viral RNA in lung homogenate at 10 h post-infection, as assessed by qRT-PCR. (**G**) MUC5ac protein in the BAL fluid at 7 days post-infection. All protein mediators in BAL and lung homogenate were assessed by ELISA. *$P$<0.05, **$P$<0.01, ***$P$<0.001 and ns = not significant. All data

anlayzed by ANOVA and differences between groups identified using Bonferroni's post-test and represent mean±s.e.m and are representative of 2-3 studies.



Europe PMC Funders Author Manuscripts

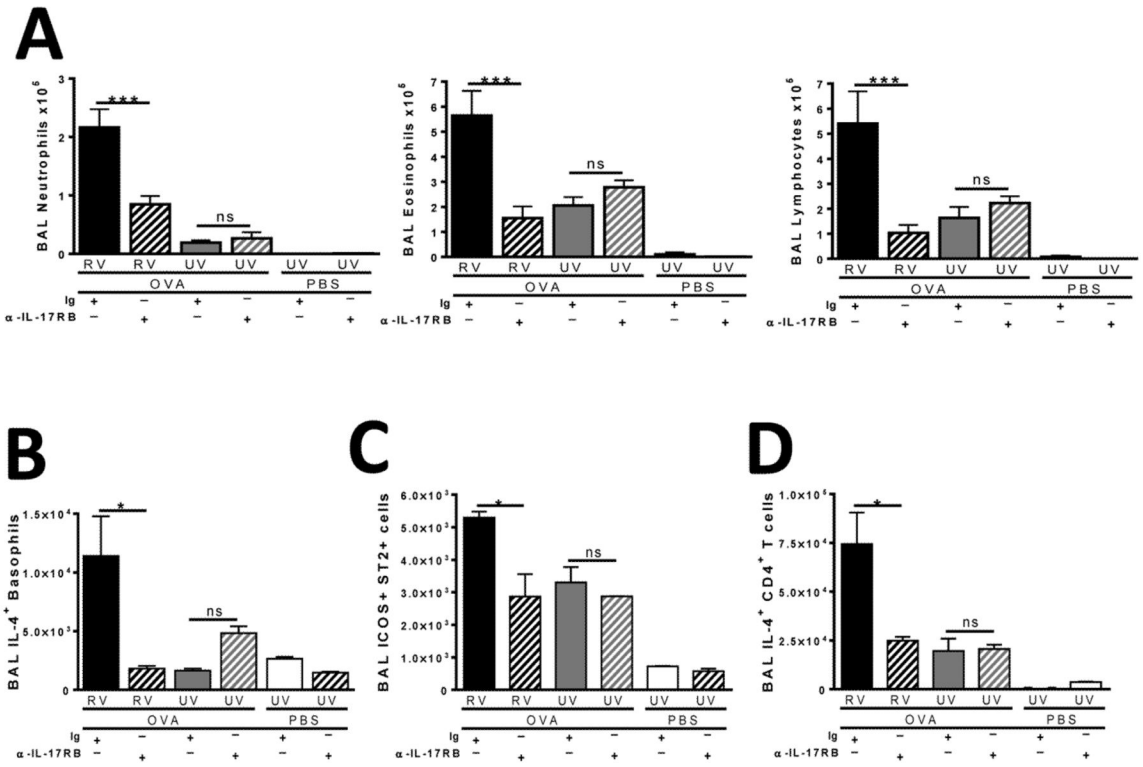


**Figure 6. Blocking IL-25 signalling ablated rhinovirus-exacerbated type-2 leukocytic airways inflammation**

OVA-sensitized mice were challenged intranasally with OVA or PBS followed by intraperitoneal injection of anti-IL-17RB blocking antibody or isotype control (Ig) 4 h before and 3 and 5 days post-infection with RV-1B or UV-RV-1B (*n* = 5 per group). (**A**) Total number of BAL neutrophils and eosinophils at day 1 post-infection and lymphocytes at day 7 post-infection, as assessed by differential cell counts. (**B-D**) Total numbers of BAL (**B**) IL-4$^+$ basophils 1 day post-infection, (**C**) Non-T (from CD3$^-$, CD4$^-$ and DX5$^-$ gated cells) ICOS$^+$/ST2$^+$ cells at 8 h post-infection and (**D**) IL-4$^+$ CD4$^+$ T cells at 7 days post-infection, as assessed by flow cytometry. **P*<0.05, ***P*<0.01 ****P*<0.001 and ns = not significant. All data anlayzed by ANOVA and differences between groups identified using Bonferroni's post-test represent mean±s.e.m. and are representative of 2-3 studies.