

IL-33-dependent type 2 inflammation during rhinovirus-induced asthma exacerbations in vivo

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23 **Authors contributions:**

24 DJ carried out the clinical aspects of this study with assistance in screening volunteers and
25 bronchoscopies from MBT, JF, JDR and JD with supervision from SJ, OK, and PM. Clinical
26 sample processing was carried out by BS, AT, AN, JA, LG, EB and ST with supervision from LS,
27 MK, JW and MJE. Development of the novel sampling techniques used in this study was
28 performed by DH, TH and TH. The T cell work was carried out by HM and supervised by RW,
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45 **At a Glance Commentary**

46 **Scientific Knowledge on the Subject:** Rhinovirus infections are the most common trigger for
47 asthma exacerbations. Data from mouse and ex-vivo human models suggest rhinovirus-
48 induced augmentation of Th2 inflammation may play a role in exacerbation pathogenesis.
49 However our understanding of how a classic Th1 trigger – a virus – exacerbates a classic Th2
50 disease – allergic asthma - is unknown.

51 **What This Study Adds to the Field:** IL-33 is an inducer of type 2 inflammation in mouse
52 models. We show for the first time that IL-33 and the type 2 cytokines IL-4, IL-5 and IL-13
53 are induced by rhinovirus in the asthmatic airway in vivo and that their levels relate to
54 exacerbation severity. We also show that IL-33 is strongly induced by rhinovirus infection of
55 primary human bronchial epithelial cells in vitro. We further show that type 2 cytokine
56 production by human T cells and type 2 innate lymphoid cells is induced by supernatant
57 from rhinovirus-infected human bronchial epithelial cells and this induction is completely
58 inhibited by blocking the IL-33 receptor. These findings highlight IL-33 as a key mechanistic
59 link between rhinovirus infection and amplification of type 2 inflammation in asthma
60 exacerbations and identify IL-33 inhibition as a novel therapeutic approach for treating
61 asthma exacerbations.

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63 This article has an online data supplement, which is accessible from this issue's table of
64 content online at www.atsjournals.org

Abstract

Rationale: Rhinoviruses are the major cause of asthma exacerbations, however mechanisms are poorly understood. We hypothesised that the epithelial-derived cytokine IL-33 plays a central role in exacerbation pathogenesis through augmentation of type 2 inflammation.

Objectives: To assess whether rhinovirus induces a type 2 inflammatory response in asthma *in vivo* and to define a role for IL-33 in this pathway.

Methods: We used a human experimental model of rhinovirus infection and novel airway sampling techniques to measure IL-4, IL-5, IL-13, and IL-33 levels in the asthmatic and healthy airway during a rhinovirus infection. Additionally we cultured human T cells and type 2 innate lymphoid cells (ILC2s) with the supernatants of rhinovirus-infected bronchial epithelial cells (BECs) to assess type 2 cytokine production in the presence or absence of IL-33 receptor blockade.

Measurements and Main Results: IL-4, IL-5, IL-13 and IL-33 are all induced by rhinovirus in the asthmatic airway *in vivo* and relate to exacerbation severity. Further, induction of IL-33 correlates with virus load and IL-5 and IL-13 levels. Rhinovirus infection of human primary BECs induced IL-33 and culture of human T cells and ILC2s with supernatants of rhinovirus-infected BECs strongly induced type 2 cytokines. This induction was entirely dependent on IL-33.

Conclusions: IL-33 and type 2 cytokines are induced during a rhinovirus-induced asthma exacerbation *in vivo*. Virus induced IL-33 and IL-33 responsive T cells and ILC2s are key mechanistic links between virus infection and exacerbation of asthma. IL-33 inhibition is a novel therapeutic approach for asthma exacerbations.

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88 Introduction

89 Immune responses to viral infections involve CD4⁺ IFN- γ -producing T helper 1 (Th1) cells,
90 regarded as the archetypal effector cell of anti-viral immunity. In contrast, Th2 cells
91 secreting IL-4, IL-5, and IL-13 are regarded as critical effector cells in allergic asthma.
92 Furthermore IL-4 and IFN- γ inhibit development of Th1 and Th2 subsets respectively so
93 creating polarised immune responses that counter-regulate each other.

94 This fundamental understanding of T cell biology is not aligned mechanistically with the
95 highly consistent finding that respiratory virus (mostly human rhinovirus) infections, an
96 archetypal Th1 trigger, are the dominant cause of acute exacerbations of the Th2-mediated
97 disease allergic asthma(1–3). Further, studies reporting substantial reductions in asthma
98 exacerbations using therapies targeting type 2 cytokines(4–8), and synergistic interactions
99 between allergen exposure and virus infections increasing the risk of asthma
100 exacerbations(9,10), suggest strong interactions between virus infection and type 2
101 responses that are similarly unexplained mechanistically.

102 Type 2 innate lymphoid cells (ILC2s) provide a potent early innate source of the cytokines IL-
103 5 and IL-13 in mice(11–13), and recent studies have demonstrated that similar cells are
104 found in humans(14,15). IL-33 is an epithelial-derived cytokine and its receptor (ST2) is
105 expressed on both Th2 cells and ILC2s making it a potential target for inhibition of both
106 innate and acquired type 2 inflammation in asthma(16). Polymorphisms in IL-33 and its
107 receptor are associated with increased risk of asthma(17). Additionally, IL-33 is induced by
108 influenza virus in mice(13,14), raising the possibility that IL-33 could be a bridging mediator
109 between virus infection and type 2-driven disease. However, the role of IL-33 in virus-
110 induced asthma exacerbations in man is unknown and no evidence exists that rhinovirus

infection can induce IL-33. It is also unknown whether respiratory virus infection in asthma leads to amplification of type 2 inflammation *in vivo* as measuring type 2 cytokines in human airway samples is difficult leading to reliance upon indirect measures such as RNA levels(20), eosinophils (for IL-5)(5,6) or periostin (for IL-13)(7). The technique of NasosorptionTM uses an absorptive matrix to sample nasal mucosal lining fluid undiluted(21). We adapted this method to sample bronchial mucosal lining fluid and have termed this technique BronchosorptionTM.

Utilising these novel sampling techniques along with experimental rhinovirus infection in asthma we investigated IL-33 and type 2 cytokine production during virus-induced asthma exacerbations *in vivo*. Furthermore, we examined the functional role of rhinovirus-induced, bronchial epithelial-derived IL-33 on human T cell and ILC2 cytokine production *ex vivo*. We thereby demonstrate a critical role for IL-33 in linking virus infection with induction of a type 2 immune response in asthma exacerbations. Some of the results of these studies have been previously reported in the form of an abstract(22).

Methods

The study received ethical approval (09/H0712/59) and informed consent was obtained from all subjects. Detailed methods are available in the online supplement.

Study participants

Non-smoking mild and moderately severe asthmatic and non-atopic, healthy volunteers aged 18-55 years without a recent viral illness or serum neutralising antibodies to rhinovirus 16 (RV16) at screening were recruited. Asthmatic volunteers were excluded if they had

severe disease (defined by GINA(23)), a recent asthma exacerbation or current symptoms of allergic rhinitis. Full inclusion/exclusion criteria are available in the online supplement.

Study design

Study volunteers meeting inclusion criteria underwent baseline sampling including bronchoscopy 2-4 weeks prior to inoculation with RV16(24). A second bronchoscopy was performed on day 4 post-inoculation. Daily diary cards of respiratory symptoms were commenced 2 weeks prior to baseline sampling and continued until 6 weeks after inoculation. Subjects were seen on days 2,3,4,5,7,10 and 42 post-inoculation for clinical assessment and nasal sampling (online supplement Fig. E1). As previously reported(24) lower respiratory symptom scores were corrected for baseline symptoms and the effects of bronchoscopy (Fig. E2). Spirometry was performed on waking using a Piko-1 spirometer (nSpire Health, CO). Rhinovirus was detected by polymerase chain reaction (PCR) of nasal lavage and bronchoalveolar lavage (BAL) as described(24).

BronchosorptionTM is a technique to sample bronchial mucosal lining fluid. The main benefit of this novel technique is the measurement of previously undetectable mediators through avoidance of the significant analyte dilution associated with bronchoalveolar lavage. The bronchosorptionTM device is passed down the operating port of the bronchoscope. (see online supplement for further details). NasosorptionTM was performed as described (21,25,26). IL-4, IL-5, IL-13 and IL-33 were measured using the Meso-Scale Discovery (MSD) platform.

***In vitro* studies**

Human bronchial epithelial cells (BECs) (Lonza, Basel, Switzerland) were infected with RV16, or treated with media for 24 hours(27). Supernatants were harvested, UV-irradiated and filtered to inactivate/remove virus particles(27). Inactivation was confirmed by absence of

cytopathic effect of treated supernatants in HeLa cell titration assays(28). IL-33 was measured in supernatants by R&D Duoset ELISA.

Naïve human CD4⁺ T cells from peripheral blood were isolated by negative selection, expanded and assessed as >96% pure by surface expression of CD4, CD45RA and CCR7(29). To determine the importance of IL-33 in the induction of Th2 cytokines, activated (to induce ST2 and the ability to respond to IL-33) but not polarised (equal low expression of IL-4, IL-5, IL-13, IFN- γ and FOXP3, data not shown) CD4⁺ T cells (Th0 cells) were treated with blocking anti-ST2 antibody (Abcam, #ab89741) or matched isotype control (#ab81216). Three hours later, Th0 cells were cultured at 1×10^6 cells/mL with media (RPMI 1640) alone, or with media (4 parts) plus supernatants (1 part) from rhinovirus-infected, or media-treated uninfected BECs for 12 days prior to intracellular staining of T cells for IL-4, IL-5, IL-13 and GATA-3 (on a BD LSR Fortessa with data analysed using FlowJo V10) or measurement of Th2 cytokines in the culture supernatants using the MSD platform.

Human ILC2s were isolated from peripheral blood using flow cytometric sorting of Lineage (CD2, CD3, CD14, CD16, CD19, CD56, CD235a and CD123) negative, CRTh2⁺, CD127⁺ and CD45⁺ cells (Fig. S4). ILC2s were then cultured under identical conditions to the Th0 cells, but at 1×10^5 cells/mL and cytokine production was measured at day 7. A full description of methods used is available online.

Statistical analysis

Data were analysed using SPSS v20.0 (IBM Corp, NY). Data are mean (\pm SEM) if normally distributed or median (interquartile range) if nonparametric. Differences between groups were analysed by unpaired *t* or Mann-Whitney tests. Within-group comparisons were

analysed with paired *t* tests or Wilcoxon's signed rank test. Correlations were examined using Pearson's/Spearman's correlation tests for nonparametric/parametric data. Differences were considered significant at *P* values <0.05. All *P* values are two-sided.

Results

Forty-six (32 asthmatic and 14 healthy) volunteers were inoculated with RV16. 7 subjects failed to develop infection and were excluded. Baseline characteristics of successfully infected subjects are shown in Table 1. There were no subject withdrawals or requirement for systemic corticosteroid therapy during this study.

Asthmatics experience greater rhinovirus-induced respiratory morbidity and virus loads than healthy subjects. Following inoculation with rhinovirus, asthmatics reported significantly greater upper and lower respiratory symptoms and reductions in peak expiratory flow (PEF) and FEV₁ compared to healthy subjects (Fig 1A-E). In addition we observed increased virus loads in asthmatics with a notable earlier peak (day 3) compared to healthy subjects (day 4) (Fig 1F). At day 3 virus levels in asthma patients were ~250-fold greater than in healthy subjects (median copies/mL: 1.68×10^6 [1.60×10^4 - 1.28×10^7] vs 6.92×10^3 [1.50×10^3 - 3.21×10^6], *P*=0.042). In asthma, peak virus loads correlated with exacerbation severity (peak reductions in PEF, *r*=-0.463, *P*=0.008). Virus load in BAL was measured on a single time-point during infection (day 4) and was not significantly different on this day between groups. There were no differences between steroid-treated and steroid-naive asthmatics in terms of virus load (Table E1).

Virus induced lower airway eosinophilia is increased in asthma. Bronchoalveolar lavage (BAL) cell counts showed a significant increase in eosinophil numbers from baseline during rhinovirus infection in asthmatic but not healthy subjects (asthma baseline 0.5% [0.0-1.7] vs asthma day 4 1.2% [0.0-3.8], $P=0.025$) (Fig. 1G). As previously reported(24), eosinophil numbers during infection in asthma were significantly greater than in healthy subjects ($P=0.046$) however this is the first time a significant rhinovirus-induced eosinophilia has been demonstrated in asthma. No statistically significant differences were observed in cell counts between steroid-treated and steroid-naïve asthmatics. In addition, no significant relationships between BAL cell counts on day 4 and lower respiratory symptoms were identified. We believe this may simply reflect the single time-point (day 4) that counts during the exacerbation were possible rather than a true absence of a relationship between counts and symptoms. Further inflammatory cell counts available online (Table E2).

Type 2 cytokines are induced by rhinovirus infection in asthma *in vivo*. Nasal levels of IL-4, IL-5 and IL-13 were significantly elevated in asthmatic subjects, both at baseline and upon infection (all $P<0.05$). Significant induction of these cytokines during infection was only observed in asthma (all $P<0.001$, Fig. 2A and supplementary Table E3). Bronchial levels of IL-5 and IL-13 measured using Bronchosorption (Fig 2B and supplementary Table E4) were also significantly greater in asthma at baseline (all $P<0.05$) with a significant increase in IL-5 levels from baseline to infection observed for asthma only ($P<0.05$, supplementary Table E4). BAL cell type 2 cytokine production in response to non-specific *ex vivo* stimulation has previously been reported to be increased in asthma and related to the severity of the asthma exacerbation following subsequent RV challenge(24). However, our observation that

221 rhinovirus infection directly drives type 2 responses in asthmatic but not healthy individuals
222 *in vivo* is novel.

223 **IL-33 is induced by rhinovirus infection *in vivo* and is related to type 2 responses.** Nasal IL-
224 33 was significantly induced by rhinovirus infection in asthma ($P<0.001$) with a trend
225 towards induction in healthy subjects (Fig. 2A). Given our current understanding of IL-33,
226 these findings strongly suggest rhinovirus induced IL-33 may drive the type 2 responses
227 observed during asthma exacerbations. Although bronchial IL-33 was not significantly
228 induced in either group (Table E4), measurements were only possible at a single time-point
229 (day 4) and IL-33 induction at alternative time points of infection are possible. In support of
230 a role for IL-33 promoting type 2 responses during asthma exacerbations, we identified
231 significant correlations between bronchial IL-33 and both IL-5 and IL-13. Again these findings
232 were exclusive to asthmatic subjects (Fig. 2C).

233 **Type 2 cytokines and IL-33 correlate with clinical outcomes and virus load.** We next
234 investigated relationships between type 2 cytokines, exacerbation severity and virus load. In
235 asthma, IL-5 and IL-13 during infection both positively correlated with respiratory symptom
236 severity ($P<0.05$) (Table E5). Similarly, in asthma both nasal and bronchial IL-33 levels during
237 infection correlated with asthma symptom severity (Fig. 2D). IL-33 also significantly
238 correlated with virus load (Fig. 2E) in keeping with the respiratory epithelium being both the
239 site of infection and the source of IL-33.

240 **Rhinovirus-infection of primary human BECs *ex vivo* induces IL-33 secretion.** Our clinical
241 observations led us to hypothesize that virus-induced, BEC-derived IL-33 promoted type 2
242 responses by responsive immune cells, thereby driving virus-induced asthma exacerbations.
243 We therefore performed functional analyses to test this hypothesis *ex vivo*. Rhinovirus

infection of BECs significantly up-regulated levels of IL-33 in culture supernatants (Fig. 3A, $P<0.01$), demonstrating that rhinovirus infection of bronchial epithelium leads to the release of large amounts of IL-33.

IL-33 present in rhinovirus-infected BEC supernatants directly induces Th2 responses in human T cells. To test the functional role of IL-33 released from rhinovirus infected bronchial epithelium in inducing Th2 responses, we cultured activated, non-polarised human CD4-positive T cells (Th0 cells) with media alone, and with supernatants from rhinovirus-infected or un-infected BECs. The Th0 cells cultured with supernatants from rhinovirus-infected BECs had significantly higher frequencies of IL-4, IL-5, IL-13 and GATA-3 positive cells than the Th0 cells cultured with either medium alone or medium with supernatants from un-infected BECs (all $P<0.05$, Fig. 3B). This induction was Th2-specific, as there was no similar induction of Th1 responses assessed by IFN- γ expression (Fig. 3B). Moreover, this induction of Th2 responses was dependent on IL-33 as it was completely inhibited by pre-treatment of the Th0 cells with anti-ST2 monoclonal antibody ($P<0.05$ vs isotype control for IL-4, IL-5, IL-13 and GATA-3). In contrast, blocking the actions of IL-33 in these cultures potentiated Th1 responses (Fig. 3B).

In addition, levels of secreted type 2 cytokines were significantly higher following culture of Th0 cells with supernatants from rhinovirus-infected BECs compared with those cultured with supernatants from un-infected BECs or with medium alone (all $P<0.05$, Fig. 3C). This induction was also completely prevented by blocking the IL-33 receptor in these cultures ($P<0.05$ vs isotype control, Fig. 3C).

IL-33 in rhinovirus-infected BEC supernatants directly induces IL-5 and IL-13 production by human ILC2s. Finally we investigated whether IL-33 released from rhinovirus-infected BECs

could induce type 2 cytokine production by human ILC2s. ILC2s, characterized as lineage⁻, ckit^{int}, CD45⁺, CD127⁺, CD25⁺, CRTH2⁺, were purified by flow cytometric sorting from peripheral blood (Fig 3D &E and supplementary methods). We observed striking induction of both IL-5 and IL-13 by human ILC2s cultured with supernatants from rhinovirus-infected BECs ($P<0.05$ vs ILC2s cultured with media alone or with supernatants from un-infected BECs, Fig. 3F). Cytokine levels were ~200 and ~100 times greater on a per cell basis respectively than those from Th0 cells. Critically, this IL-5 and IL-13 induction was again completely blocked by anti-ST2 treatment ($P<0.05$ vs isotype control, Fig. 3F), demonstrating IL-33 as the key factor in this pathway. This rhinovirus trigger of IL-33 is therefore likely to drive an early and robust type 2 response via these innate cells.

Discussion

Our findings demonstrate for the first time that rhinovirus induces IL-33 and the type 2 cytokines IL-4, IL -5 and IL-13 during a virus-induced asthma exacerbation *in vivo*. We also show relationships between IL-33 and increased type 2 cytokines (which were exclusive to asthmatic subjects), and between these cytokines and asthma exacerbation severity in humans. We demonstrate that rhinovirus infection of BECs strongly induces IL-33 release *in vitro*, which activates both human T cells and human ILC2s to produce type 2 cytokines in a manner dependent upon IL-33. These observations have important implications for our understanding of virus-induced asthma exacerbations and offer a mechanism through which the classic Th1 trigger - a virus – promotes type 2 inflammation in susceptible individuals.

Trials of anti-IgE,(30) anti-IL-4(4), anti-IL-5(5,6,8) and anti-IL-13(7) therapies have recently shown the potential of blocking individual type 2 molecules to reduce asthma exacerbations. Our observations that type 2 cytokines and airway eosinophilia are both induced by rhinovirus infection in human asthmatics *in vivo* underscores the validity of this approach. Our data suggest blockade of IL-33 signalling may be considerably more effective than blocking individual type 2 cytokines in view of the potential to inhibit eosinophilic inflammation consequent upon IL-5 production, airway hyperresponsiveness, mucus hypersecretion and airway remodelling associated with IL-13 production, as well as IgE class switching associated with both IL-4 and IL-13 in asthma. Inhibitors of IL-33 should therefore be more effective than approaches that only block a single type 2 cytokine/receptor.

In this study we established a biological system allowing us to measure the ability of rhinovirus-infected epithelial cells to produce IL-33 and mediate polarisation of T cell populations and ILC2 activation *ex vivo*. We cultured activated, but non-polarised CD4⁺ T cells in the presence of supernatants from rhinovirus-infected epithelial cells and demonstrated increased production of IL-4, IL-5 and IL-13 compared to T cells cultured with supernatants from uninfected epithelial cells. We clearly established an essential role for IL-33 in this process, as blocking ST2 completely inhibited Th2 polarisation, indicating that it was entirely dependent upon IL-33. Our data also suggests that ILC2s may be a major innate source of type 2 cytokines in response to rhinovirus infection, as they are capable of producing ~200 and ~100 times the amount of IL-5 and IL-13 on a per cell basis than T cells respectively. Although we provide evidence that virus-induced IL-33 can directly and potently induce type 2 responses by two critical immune cells, we observed similar levels of IL-33 in both healthy and asthmatic airways *in vivo* suggesting that it may not be virus-

induced IL-33 levels that are discriminatory but rather the number of cells able to respond to IL-33 when it is released. This may relate to quantitative and/or qualitative differences in the numbers of ST2-expressing cells present in exacerbating asthma such as Th2 cells, ILC2s, basophils and mast cells. Although in our *ex-vivo* system, the induction of type 2 cytokines appeared entirely dependent upon IL-33 in the RV-infected BEC supernatant, it is also possible that *in vivo* co-secretion of other mediators such as TSLP, IL-25, or PGD₂ may act synergistically with IL-33. For example, Barnig recently showed that the combination of IL-33, PGD₂ and IL-25 enhanced type 2 cytokine production by ILC2s in a synergistic manner(31) whilst Xue demonstrated that ILC2 activation via PGD₂ upregulated the expression of ST2 on ILC2s(32). Further studies will be required to investigate these possibilities in the context of virus-induced asthma.

The finding of substantially higher levels of IL-33 in the lung than in the nose was unexpected. As this is the first study to simultaneously measure nasal and bronchial IL-33 in asthma using our novel sampling techniques we cannot relate our values to other studies. However we used similar sampling methods in both compartments (absorptive matrices placed on the nasal / bronchial mucosa respectively), and the same assays for IL-33 analysis (MSD). Thus we must assume that bronchial mucosal IL-33 is present in greater quantities than nasal IL-33. Our findings that bronchial IL-33 in asthma correlates with both IL-5 and IL-13, as well as exacerbation severity suggests that our measurements are functionally relevant.

Taken together, our findings suggest that virus-induction of BEC-derived IL-33 is centrally involved in the induction of the type 2 response we have observed in virus-induced asthma exacerbations. This central role for IL-33 in the type 2 response is supported by recent work

by Halim and colleagues who demonstrated that ILC2 activation and Th2 cell differentiation in papain-treated mice was also IL-33 dependent(33). Our findings also suggest a mechanism for reports of synergistic interaction between allergen sensitisation/exposure and virus infection in increasing risk of asthma exacerbations(9,10): as an atopic asthmatic individual exposed to allergen in parallel with viral infection may have increased frequencies of allergen-specific Th2 cells in the lung, with enhanced ST2 expression. Since Th2 cells, along with other type 2 cells, are recruited and activated by allergen exposure these patients may exhibit an increased capacity to respond to virus-induced IL-33 by the release of type 2 cytokines. A similar pathway may be invoked in relation to ILC2s, though elucidating the relative importance of these cell types requires further study.

Increased rhinovirus replication in asthmatic BECs has been previously observed *ex vivo* and related to delayed and impaired production of anti-viral interferons(34,35), but to date increased virus load *in vivo* in asthma has not been observed (36,37). The earlier and greater peak in virus load in asthma reported here is consistent with these *ex-vivo* reports and suggest an impaired anti-viral immune response in some asthmatics. It is possible that these novel observations may be due to the inclusion of more severe asthmatic subjects, as most previous rhinovirus infection studies limited inclusion to mild subjects only. However, the precise nature of how this relates to the augmented type 2 inflammation we observed requires further study. Additionally, Bonilla and colleagues reported that IL-33 is necessary for potent CD8+ T cell responses to both RNA and DNA viruses in mice(38) highlighting a need to investigate the effect of IL-33 blockade on viral replication in future work.

Finally, this study also introduced Bronchosorption as a new technique to sample bronchial mucosal lining fluid and permit detection of cytokines not normally detectable in bronchial

lavage. This technique offers great potential to advance our mechanistic understanding of many respiratory conditions.

In summary, this study provides the first evidence that rhinovirus infection leads to induction of IL-33 and type 2 cytokines in asthma *in vivo* and that levels of these mediators relate to severity of asthma exacerbations. This study is also the first to show that rhinovirus infection of bronchial epithelium can directly activate human T cells and human ILC2s to produce large quantities of type 2 cytokines, a process found to be completely dependent on IL-33. We therefore identify IL-33 ligation to its receptor as a mechanistic link between virus infection and asthma exacerbations. Based on data presented here, the IL-33/ST2 axis is an exciting target for future therapeutic intervention in asthma exacerbations.

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Figure 1 Rhinovirus infection results in more severe upper and lower respiratory tract involvement, greater virus loads, and bronchial eosinophilia in asthma. Shown are the daily change from baseline in upper (A) and lower (B) respiratory symptoms of asthmatic (red) and healthy (black) volunteers. The total lower respiratory symptom score (C) equates to the summation of daily scores over the 14 days post inoculation and represents the severity of the exacerbation. As symptom scores were corrected for baseline and bronchoscopy-induced symptoms a small number of subjects had a negative score (see online supplement for further details). Falls in morning peak expiratory flow (PEF) are shown as a percentage change from baseline (D) following rhinovirus inoculation. The maximal fall in FEV₁ (E) represents the maximal change during the infection period for each subject. Virus load was measured at each study visit in nasal lavage (F). Bronchoalveolar lavage eosinophil counts were measured at baseline and day 4 post-inoculation (G). Results shown are mean±SEM (A-E); bars represent median values (F). Statistical comparisons between groups were performed at each time-point but where non-significant have been left unmarked to aid clarity. *,*P*<0.05; **,*P*<0.01; ***,*P*<0.001

Figure 2 Rhinovirus infection in asthma leads to the induction of IL-33 and type 2 cytokines *in vivo*. Nasal levels of IL-33 and type 2 cytokines were measured by Nasosorption (A) in asthmatic (red) and healthy (black) subjects. The Bronchosorption device (B) utilises a similar strip of synthetic absorptive matrix as for Nasosorption to sample bronchial mucosal lining fluid. In asthmatic subjects only, IL-33 levels correlated with IL-5 and IL-13 levels (C), severity of the asthma exacerbation *in vivo* (D), and virus load (E). Bars represent median levels (A). The ‘infection’ level (A) and ‘peak’ levels (D & E) represents the greatest

(maximal) level of induction during the infection for each subject. The correlations shown are for asthmatics only and are non-parametric (Spearman).
*, $P<0.05$; **, $P<0.01$; ***, $P<0.001$

Figure 3 Rhinovirus infection of BECs induces IL-33, which subsequently induces type 2 cytokine production by human T cells and ILC2s. (A) Levels of IL-33 in BEC supernatants 24 hours after rhinovirus-16 infection or culture with UV-inactivated rhinovirus-16 or medium control. To determine whether IL-33 present in rhinovirus-infected BEC supernatants could induce Th2 responses in human T cells, naïve (CD45RO⁻), activated (anti-CD2/CD3/CD28 stimulated) non-polarised human CD4⁺ T cells (Th0 cells) were cultured in the presence of medium alone, or medium plus supernatant from either uninfected or rhinovirus-16-infected BECs, in the presence of blocking antibody to the IL-33 receptor (α ST2), or isotype control antibody, prior to flow cytometric analysis. (B) Intracellular levels of IL-4, IL-5, IL-13, GATA-3 and IFN- γ in Th0 cells cultured in medium alone (white), or medium plus supernatants from uninfected (grey) or rhinovirus-16-infected (black) BECs, in the presence of isotype control or α -ST2 blocking antibody. (C) Levels of IL-4, IL-5, IL-13 in supernatants from Th0 cells cultured in medium alone (white), or medium plus supernatants from uninfected (grey) or rhinovirus-16-infected (black) BECs in the presence of isotype control or α -ST2 blocking antibody. (D) Human peripheral blood mononuclear cells were enriched for ILC2s by magnetic depletion of CD3, CD14, CD16 and CD19 positive cells and were then flow sorted as lymphocyte sized, Lineage⁻ (CD2⁻, CD3⁻, CD14⁻, CD16⁻, CD19⁻, CD56⁻, CD235a⁻, CD123⁻), CRTH2⁺ cells. The top panel shows the forward and side scatter characteristics of the sorted population, the lower panel shows the distinct Lineage⁻CRTH2⁺ ILC2 population. (E) Surface marker expression of ILC2s (red), Lineage⁻CRTH2⁻ (grey) cells and Lineage⁺ CRTH2⁻

535 (black) cells were compared using flow cytometry. Histograms show sorted ILC2s to have a
536 distinct phenotype (Lineage⁻CD34⁻CRTH2⁺ cKit⁺CD45⁺CD127⁺CD25⁺). (F) Human ILC2s were
537 cultured in medium alone (white), or medium plus supernatants from uninfected (grey) and
538 rhinovirus-16-infected (black) BECs, in the presence of isotype control or α -ST2 blocking
539 antibody and cytokine levels were measured in the ILC2 culture supernatants. Data are
540 expressed as mean \pm SEM, * P <0.05, ** P <0.01, *** P <0.001, n=6.

541

542 Table 1. Baseline characteristics of study volunteers.

Characteristic	Healthy (N=11)	Asthma (N=28)	<i>P</i> 543
Age (yr)	31±12	36±11	<i>NS</i>
Sex (%)			
Female	4 (36)	15 (54)	<i>NS</i>
Male	7 (74)	13 (46)	
Baseline FEV ₁ (Percent predicted)	104±8	86±12	<0.001
Baseline histamine PC ₂₀ (mg/mL)	>16	1.26±2.01	-
ICS use (%)	-	15 (53.6)	-
ICS daily dose			
Beclometasone/equivalent (mcg) (mean of steroid-treated subjects, n = 15)	-	427±71	-
IgE IU/mL			
Median (IQR)	16 (14-19)	139 (70-448)	<0.001
BAL eosinophilia (%)			
Median (IQR)	0 (0)	0.5 (0-1.7)	0.002

544

545 ICS=inhaled corticosteroids; BAL = bronchoalveolar lavage; IQR=interquartile range