



IL-33–Dependent Type 2 Inflammation during Rhinovirus-induced Asthma Exacerbations *In Vivo*

David J. Jackson^{1,2,3*}, Heidi Makrinioti^{1,2*}, Batika M. J. Rana^{2,4*}, Betty W. H. Shamji⁵, Maria-Belen Trujillo-Torralbo^{1,2,3}, Joseph Footitt^{1,2,3†}, Jerico del-Rosario^{1,2,3}, Aurica G. Telcian^{1,2}, Alexandra Nikonova^{1,2,6}, Jie Zhu^{1,2}, Julia Aniscenko^{1,2}, Leila Gogsadze^{1,2}, Eteri Bakhsoliani^{1,2}, Stephanie Traub^{1,2}, Jaideep Dhariwal^{1,2,3}, James Porter^{1,2}, Duncan Hunt⁷, Toby Hunt⁷, Trevor Hunt⁷, Luminita A. Stanciu^{1,2}, Musa Khaitov⁶, Nathan W. Bartlett^{1,2}, Michael R. Edwards^{1,2}, Onn Min Kon³, Patrick Mallia^{1,2,3}, Nikolaos G. Papadopoulos⁸, Cezmi A. Akdis⁹, John Westwick⁵, Matthew J. Edwards⁵, David J. Cousins^{2,4*}, Ross P. Walton^{1,2*}, and Sebastian L. Johnston^{1,2,3*}

¹Airway Disease Infection Section, National Heart & Lung Institute, Imperial College London, London, United Kingdom; ²Medical Research Council (MRC) & Asthma UK Centre in Allergic Mechanisms of Asthma, London, United Kingdom; ³Imperial College Healthcare NHS Trust, London, United Kingdom; ⁴Division of Asthma, Allergy & Lung Biology, King's College London, London, United Kingdom; ⁵Novartis Institute for Biomedical Research, Horsham, United Kingdom; ⁶National Research Center, Institute of Immunology, Federal Medical and Biological Agency of the Russian Federation (FMBA), Moscow, Russian Federation; ⁷Hunt Developments (UK) Ltd, Midhurst, United Kingdom; ⁸Allergy Unit, Attikon General University Hospital, Athens, Greece; and ⁹Swiss Institute of Allergy and Asthma Research, University of Zurich, Davos, Switzerland

Abstract

Rationale: Rhinoviruses are the major cause of asthma exacerbations; however, its underlying mechanisms are poorly understood. We hypothesized that the epithelial cell–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 airways 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 viral 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 viral infection and exacerbation of asthma. IL-33 inhibition is a novel therapeutic approach for asthma exacerbations.

Keywords: ILC2; infection; Th2; virus

(Received in original form June 6, 2014; accepted in final form October 26, 2014)

*These authors contributed equally to this work.

†Deceased.

Supported by European Research Council Seventh Framework Programme (ERC FP7) grant 233015, a chair from Asthma UK (CH11SJ), Medical Research Council (MRC) Centre grant G1000758, Asthma UK grant 09/020, National Institute for Health Research Biomedical Research Centre (NIHR BRC) grant P26095, RSF grant 14-15-00894, Predicta FP7 Collaborative Project grant 260895 by NIHR BRCs at Imperial College London and King's College London, and a British Medical Association TV James Fellowship. Novartis Institutes for BioMedical Research funded the cytokine assays and development of bronchosorption by Mucosal Diagnostics, working in collaboration with Hunt Developments (UK) Ltd, Midhurst, United Kingdom.

Author Contributions: D.J.J. performed the clinical aspects of the study. M.-B.T.-T., J.F., J.d.-R., and J.D. assisted with screening volunteers and bronchoscopies. S.L.J., O.M.K., and P.M. supervised clinical aspects of the study and bronchoscopies. B.W.H.S., A.G.T., A.N., J.A., L.G., E.B., J.P., J.Z., and S.T. performed clinical sample processing. L.A.S., M.K., J.W., and M.J.E. supervised clinical sample processing. D.H., Toby Hunt, and Trevor Hunt developed the novel sampling techniques used in the study. H.M. performed T-cell work. R.P.W., N.G.P., C.A.A., and S.L.J. supervised T-cell work. B.M.J.R. performed ILC2 work. D.J.C. and S.L.J. supervised ILC2 work. S.L.J., N.W.B., and M.R.E. conceived of and designed the study.

Correspondence and requests for reprints should be addressed to Sebastian L. Johnston, M.B. B.S., Ph.D., Airway Disease Infection Section, National Heart & Lung Institute, Imperial College London, Norfolk Place, London W2 1PG, UK. E-mail: s.johnston@imperial.ac.uk

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

Am J Respir Crit Care Med Vol 190, Iss 12, pp 1373–1382, Dec 15, 2014

Copyright © 2014 by the American Thoracic Society

Originally Published in Press as DOI: 10.1164/rccm.201406-1039OC on October 28, 2014

Internet address: www.atsjournals.org

At a Glance Commentary

Scientific Knowledge on the

Subject: Rhinovirus infections are the most common trigger for asthma exacerbations. Data derived from mouse and *ex vivo* human models suggest that rhinovirus-induced augmentation of T helper type 2 (Th2) inflammation may play a role in the pathogenesis of exacerbation. However, the understanding of how a classic Th1 trigger—a virus—exacerbates a classic Th2 disease—allergic asthma—is unknown.

What This Study Adds to the

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

Immune responses to viral infections involve CD4⁺ IFN- γ -producing T helper type 1 (Th1) cells, regarded as the archetypal effector cell of antiviral immunity. In contrast, Th2 cells, which secrete IL-4, IL-5, and IL-13, are regarded as critical effector cells in allergic asthma. Furthermore, IL-4 and IFN- γ inhibit development of Th1 and Th2 subsets, respectively, thus creating polarized immune responses that counterregulate each other.

This fundamental understanding of T-cell biology is not aligned mechanistically

with the highly consistent finding that respiratory viral (mostly human rhinovirus) infections, an archetypal Th1 trigger, are the dominant cause of acute exacerbations of the Th2-mediated disease allergic asthma (1–3). Furthermore, studies reporting substantial reductions in asthma exacerbations using therapies targeting type 2 cytokines (4–8), as well as synergistic interactions between allergen exposure and viral infections that increase the risk of asthma exacerbations (9, 10), suggest strong interactions between viral infection and type 2 responses that are similarly unexplained mechanistically.

Type 2 innate lymphoid cells (ILC2s) provide a potent early innate source of the cytokines IL-5 and IL-13 in mice (11–13), and recent studies have demonstrated that similar cells are found in humans (14, 15). IL-33 is an epithelial cell-derived cytokine, and its receptor (ST2) is expressed on both Th2 cells and ILC2s, making it a potential target for inhibition of both innate and acquired type 2 inflammation in asthma (16). Polymorphisms in IL-33 and its receptor are associated with increased risk of asthma (17). Additionally, IL-33 is induced by the influenza virus in mice (18, 19), raising the possibility that IL-33 could be a bridging mediator between viral infection and type 2-driven disease. However, the role of IL-33 in virus-induced asthma exacerbations in humans is unknown, and no evidence exists that indicates that rhinovirus infection can induce IL-33. It is also unknown whether respiratory viral 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 nasosorption 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 “bronchosorption.”

Using 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 cell-derived IL-33 on human

T-cell and ILC2 cytokine production *ex vivo*. We thereby demonstrate a critical role for IL-33 in linking viral infection with induction of a type 2 immune response in asthma exacerbations. Some of the results of these studies have been reported previously in the form of an abstract (22). Additionally, baseline clinical characteristics, baseline bronchoalveolar lavage (BAL) fluid cell counts, and nasal IL-25 levels from the same study subjects described herein have been published elsewhere (23).

Methods

The study received ethical approval (St Mary's Hospital research ethics committee, 09/H0712/59), and informed consent was obtained from all subjects. Detailed methods are available in the online supplement.

Study Participants

We recruited nonsmoking patients with mild or moderately severe asthma and nonsmoking, nonatopic healthy volunteers aged 18–55 years and without a recent viral illness or serum neutralizing antibodies to rhinovirus 16 (RV16) at screening. Patients with asthma were excluded if they had severe disease (as defined by the Global Initiative for Asthma [24]), a recent asthma exacerbation, or current symptoms of allergic rhinitis. Full inclusion and exclusion criteria are available in the online supplement.

Study Design

Study volunteers who met the inclusion criteria underwent baseline sampling, including bronchoscopy 2–4 weeks before inoculation with RV16 (25). A second bronchoscopy was performed on Day 4 postinoculation. Daily diary cards of respiratory symptoms were commenced 2 weeks before baseline sampling and continued until 6 weeks after inoculation. Subjects were seen on Days 2, 3, 4, 5, 7, 10, and 42 postinoculation for clinical assessment and nasal sampling (see Figure E1 in the online supplement). As previously reported (25), lower respiratory symptom scores were corrected for baseline symptoms and the effects of the bronchoscopy (see Figure E2). Spirometry was performed using a PiKo-1 spirometer (nSpire Health, Hertford, UK). Rhinovirus was detected by polymerase chain reaction of nasal

lavage and BAL samples as described previously (25).

Bronchosorption 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 BAL. The bronchosorption device is passed down the operating port of the bronchoscope (*see* online supplement for further details). Nasosorption was performed as described (21, 26, 27). IL-4, IL-5, IL-13 and IL-33 were measured using the Meso Scale Discovery (MSD) (Rockville, MD) array platform.

In Vitro Studies

Human bronchial epithelial cells (BECs) (Lonza, Basel, Switzerland) were either infected with RV16 or treated with media for 24 hours (28). Supernatants were harvested, ultraviolet light irradiated, and filtered to inactivate and/or remove virus particles (28). Inactivation was confirmed by an absence of cytopathic effect of treated supernatants in HeLa cell titration assays (29). IL-33 was measured in supernatants by using the DuoSet ELISA Development Kit (R&D Systems, Minneapolis, MN).

Naive 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 (30). 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 polarized (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 (ab89741; Abcam, Cambridge, UK) or matched isotype control (ab81216; Abcam). Three hours later, Th0 cells were cultured at 1×10^6 cells/ml with media (RPMI 1640) (Sigma-Aldrich Corp., St. Louis, MO) alone or with media (four parts) plus supernatants (one part) from rhinovirus-infected or media-treated, uninfected BECs for 12 days before intracellular staining of T cells for IL-4, IL-5, IL-13, and GATA-3 (assessed using the LSRFortessa cell analyzer [BD Biosciences, San Jose, CA] with FlowJo v10 software [FlowJo, Ashland, OR]) 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-negative (CD2⁻, CD3⁻, CD14⁻, CD16⁻, CD19⁻, CD56⁻, CD235a⁻, and CD123⁻), CRTh2⁺, CD127⁺, and CD45⁺ cells (*see* Figure E4). ILC2s were then cultured under conditions identical to those used for the Th0 cells, but at 1×10^5 cells/ml, and cytokine production was measured on Day 7. A full description of the methods used is available in the online supplement.

Statistical Analysis

Data were analyzed using SPSS v20.0 software (IBM SPSS, Chicago, IL). Data are given as mean ($\pm$ SEM) if normally distributed or median (interquartile range [IQR]) if nonparametric. Differences between groups were analyzed by unpaired *t* or Mann-Whitney *U* tests. Within-group comparisons were analyzed with paired *t* tests or Wilcoxon's signed-rank test. Correlations were examined using Pearson's and Spearman's correlation tests for parametric and nonparametric data, respectively. Differences were considered significant at $P < 0.05$. All *P* values are two sided.

Results

Forty-six volunteers (32 with asthma and 14 healthy) were inoculated with RV16. Seven subjects failed to develop infections and were excluded. The baseline characteristics of subjects who were successfully infected are shown in Table 1 and have been reported previously (23). There were no subject withdrawals and no

requirement for systemic corticosteroids during this study.

Patients with Asthma Experience Greater Rhinovirus-induced Respiratory Morbidity and Viral Load than Healthy Subjects

Following inoculation with rhinovirus, subjects with asthma displayed significantly greater upper and lower respiratory symptoms and reductions in peak expiratory flow (PEF) and FEV₁ compared with healthy subjects (Figures 1A–1E). In addition, we observed increased viral loads in patients with asthma, with a notable earlier peak (Day 3) compared with healthy subjects (Day 4) (Figure 1F). At Day 3, viral load levels in patients with asthma were ~ 250 -fold greater than in healthy subjects (median [IQR] copies per milliliter: 1.68×10^6 [1.60×10^4 to 1.28×10^7] in subjects with asthma vs. 6.92×10^3 [1.50×10^3 to 3.21×10^6] in healthy volunteers; $P = 0.042$). In asthma, peak viral load correlated with exacerbation severity (peak reductions in PEF: $r = -0.463$, $P = 0.008$). Viral load in BAL fluid was measured at a single time point during infection (Day 4) and was not significantly different between groups on this day. There were no differences between steroid-treated and steroid-naïve subjects with asthma in terms of viral load (*see* Table E1).

Virus-induced Lower Airway Eosinophilia Is Increased in Asthma

BAL fluid cell counts showed a significant increase in eosinophil numbers from baseline during rhinovirus infection in

Table 1. Baseline Characteristics of Study Volunteers

Characteristics	Healthy (N = 11)	Asthma (N = 28)	P
Age, yr	31 $\pm$ 12	36 $\pm$ 11	NS
Sex			
Female, n (%)	4 (36)	15 (54)	NS
Male, n (%)	7 (74)	13 (46)	
Baseline FEV ₁ , % predicted	104 $\pm$ 8	86 $\pm$ 12	<0.001
Baseline histamine PC ₂₀ , mg/ml	>16	1.26 $\pm$ 2.01	—
ICS use, n (%)	—	15 (53.6)	—
ICS daily dose, beclomethasone/equivalent, μ g*	—	427 $\pm$ 71	—
IgE, IU/ml, median (IQR)	16 (14–19)	139 (70–448)	<0.001
BAL fluid eosinophilia, %, median (IQR)	0 (0)	0.5 (0–1.7)	0.002

Definition of abbreviations: BAL = bronchoalveolar lavage; ICS = inhaled corticosteroid; IQR = interquartile range; NS = not significant.

These baseline characteristics were reported previously in Reference 23. Data shown are mean $\pm$ SD unless otherwise stated.

*Mean $\pm$ SD data of steroid-treated subjects, n = 15.

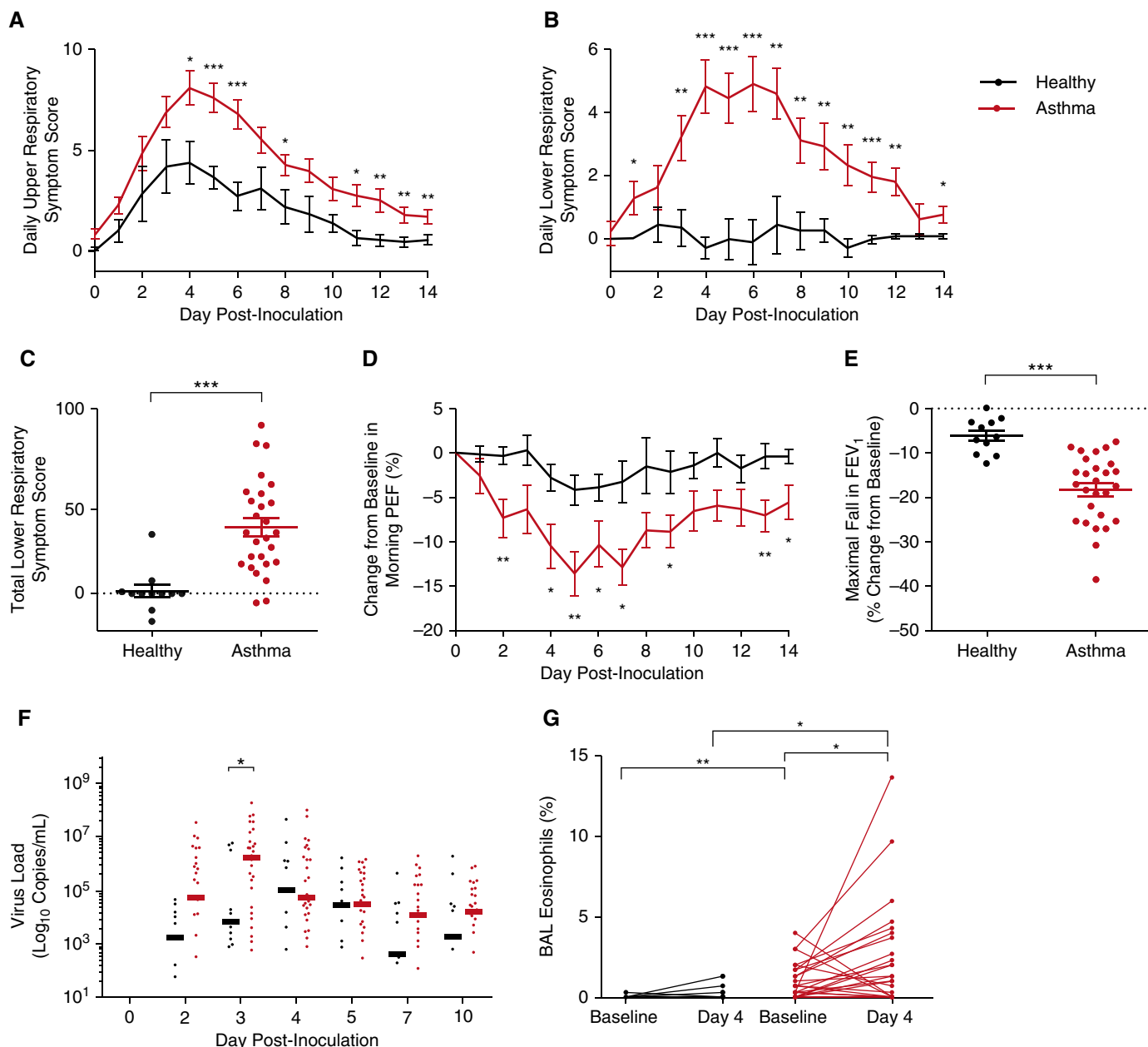


Figure 1. Rhinovirus infection results in more severe upper and lower respiratory tract involvement, greater viral loads, and bronchial eosinophilia in asthma. Shown are the daily change from baseline in upper (A) and lower (B) respiratory symptoms of subjects with asthma (red) and healthy volunteers (black). The total lower respiratory symptom score (C) equates to the summation of daily scores over the 14-day postinoculation period 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). Decreases in morning PEF are shown as percentage changes from baseline (D) following rhinovirus inoculation. The maximal decline in FEV₁ (E) represents the maximal change during the infection period for each subject. Viral load was measured at each study visit in nasal lavage (F). Bronchoalveolar lavage eosinophil counts were measured at baseline and on Day 4 postinoculation (G). Results shown are mean $\pm$ SEM (A–E); bars represent median values (F). Statistical comparisons between groups were performed at each time point, but have been left unmarked where nonsignificant to aid clarity. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

patients with asthma, but not in healthy subjects (median [IQR]: asthma at baseline = 0.5% [0.0–1.7] vs. asthma at Day 4 = 1.2% [0.0–3.8], $P = 0.025$) (Figure 1G). As previously reported (25), eosinophil

numbers during infection in the patients with asthma were significantly greater than in healthy subjects ($P = 0.046$). However, to our knowledge, 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 subjects with asthma. In addition, no

significant relationships between cell counts in BAL fluid on Day 4 and lower respiratory symptoms were identified. We believe that this may simply reflect the single time point (Day 4) on which counts during the exacerbation were possible rather than a true absence of a relationship between counts and symptoms. Data regarding further inflammatory cell counts are available online (see 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 subjects with asthma, both at baseline and upon infection (all $P < 0.05$). Significant induction of these cytokines during infection was observed only in the subjects with asthma (all $P < 0.001$) (Figure 2A; see also Table E3). Bronchial levels of IL-5 and IL-13 measured using bronchosorption (Figure 2B; see also Table E4) were also significantly greater in the subjects with asthma at baseline (all $P < 0.05$), with a significant increase in IL-5 levels from baseline to infection observed only for the subjects with asthma ($P < 0.05$; see also Table E4). Cell type 2 cytokine production in BAL fluid in response to nonspecific *ex vivo* stimulation has previously been reported to be increased in asthma and also to be related to the severity of the asthma exacerbation following subsequent rhinovirus challenge (25). However, we believe that our observation that rhinovirus infection directly drives type 2 responses in patients with asthma but not in healthy individuals *in vivo* is novel.

IL-33 Is Induced by Rhinovirus Infection *In Vivo* and Is Related to Type 2 Responses

Nasal IL-33 was significantly induced by rhinovirus infection in subjects with asthma ($P < 0.001$), with a trend toward induction in healthy subjects (Figure 2A). Given our current understanding of IL-33, these findings strongly suggest that rhinovirus-induced IL-33 may drive the type 2 responses observed during asthma exacerbations. Although bronchial IL-33 was not significantly induced in either group (see Table E4), measurements were possible only at a single time point (Day 4); IL-33 induction at alternative time points of infection are possible. In support of a role for IL-33 in promoting type 2 responses during asthma exacerbations, we identified

significant correlations between bronchial IL-33 and both IL-5 and IL-13. Again, these findings were exclusive to subjects with asthmatic (Figure 2C).

Type 2 Cytokines and IL-33 Correlate with Clinical Outcomes and Viral Load

We next investigated relationships between type 2 cytokines, exacerbation severity, and viral load. In asthma, IL-5 and IL-13 levels during infection both positively correlated with respiratory symptom severity ($P < 0.05$) (see Table E5). Similarly, in subjects with asthma, both nasal and bronchial IL-33 levels during infection correlated with asthma symptom severity (Figure 2D). The IL-33 level also significantly correlated with viral load (Figure 2E), which is in keeping with the respiratory epithelium being both the site of infection and the source of IL-33.

Rhinovirus Infection of Primary Human BECs *Ex Vivo* Induces IL-33 Secretion

Our clinical observations led us to hypothesize that virus-induced, BEC-derived IL-33 might promote type 2 responses by responsive immune cells, thereby driving virus-induced asthma exacerbations. We therefore performed functional analyses to test this hypothesis *ex vivo*. Rhinovirus infection of BECs significantly upregulated levels of IL-33 in culture supernatants ($P < 0.01$) (Figure 3A), demonstrating that rhinovirus infection of the 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, nonpolarized human CD4⁺ T cells (Th0 cells) with media alone and with supernatants from rhinovirus-infected or uninfected 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⁺ cells than the Th0 cells cultured with either medium alone or medium with supernatants from uninfected BECs (all $P < 0.05$) (Figure 3B). This induction was Th2-specific, as there was no similar induction of Th1 responses assessed on the basis of IFN- γ expression (Figure 3B). Moreover, this induction of

Th2 responses was dependent on IL-33, as it was completely inhibited by pretreatment 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 (Figure 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 uninfected BECs or with medium alone (all $P < 0.05$) (Figure 3C). This induction was also completely prevented by blocking the IL-33 receptor in these cultures ($P < 0.05$ vs. isotype control) (Figure 3C).

IL-33 in Rhinovirus-infected BEC Supernatants Directly Induces IL-5 and IL-13 Production by Human ILC2s

We next investigated whether IL-33 released from rhinovirus-infected BECs could induce type 2 cytokine production by human ILC2s. ILC2s, characterized as lineage negative, ckit^{int}, CD45⁺, CD127⁺, CD25⁺, and CRTH2⁺, were purified by flow cytometric sorting from peripheral blood (Figures 3D and 3E; see also 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 uninfected BECs) (Figure 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) (Figure 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 study is the first, to our knowledge, to demonstrate 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 have shown relationships between IL-33 and increased type 2 cytokines and between these cytokines and asthma exacerbation severity

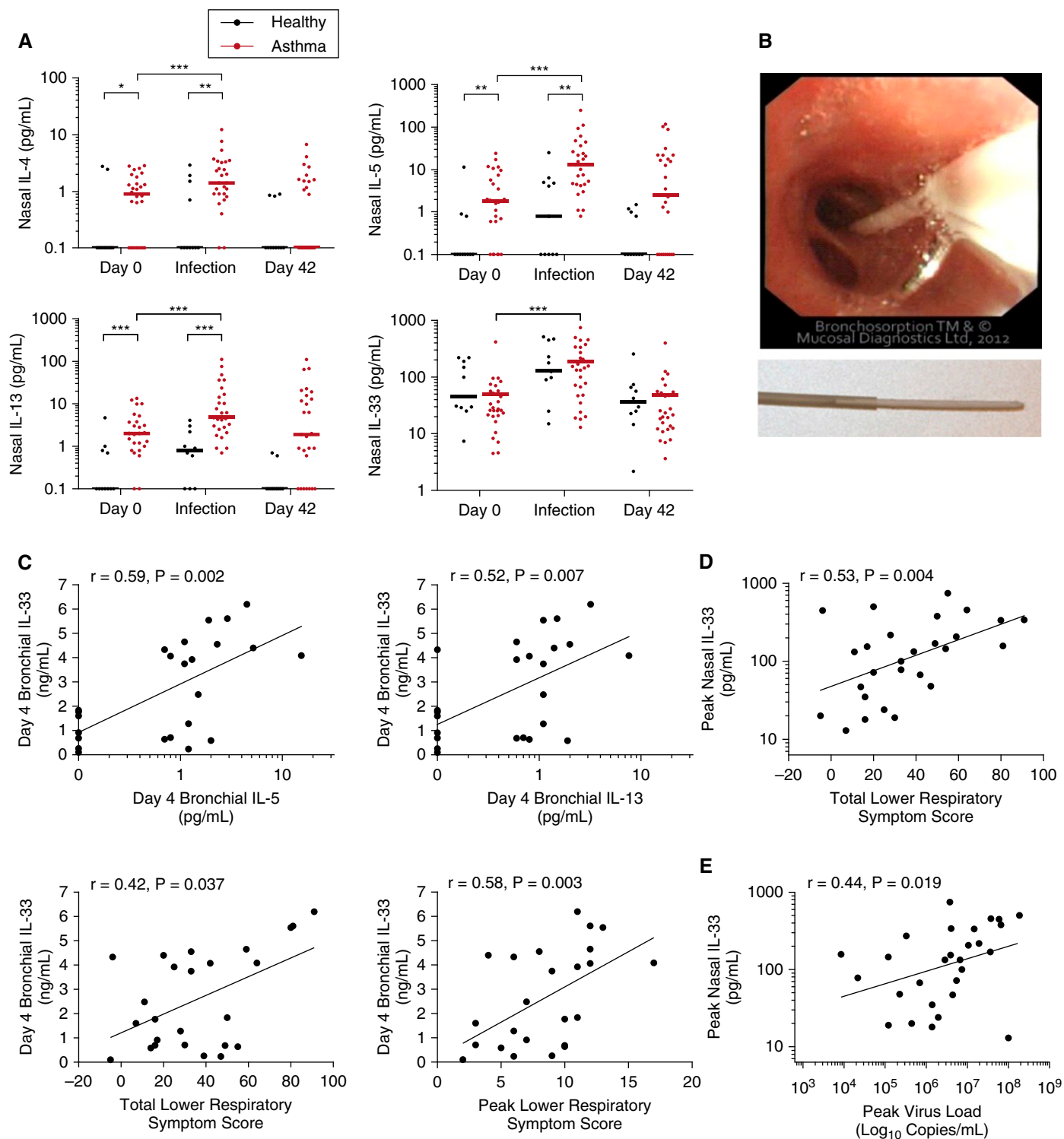


Figure 2. Rhinovirus infection in asthma leads to the induction of IL-33 and type 2 cytokines *in vivo*. Nasal levels of IL-33 and type 2 cytokines were measured by nasosorption (A) in subjects with asthma (red) and healthy subjects (black). The bronchosorption device (B) uses a strip of synthetic absorptive matrix similar to that used for nasosorption to sample bronchial mucosal lining fluid. In subjects with asthma, IL-33 levels were correlated with IL-5 and IL-13 levels (C), severity of the asthma exacerbation *in vivo* (D), and viral load (E). Bars represent median levels (A). The “infection” level (A) and “peak” levels (D and E) represent the greatest (maximal) level of induction during the infection for each subject. The correlations shown are for subjects with asthma only and are nonparametric (Spearman’s correlation). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

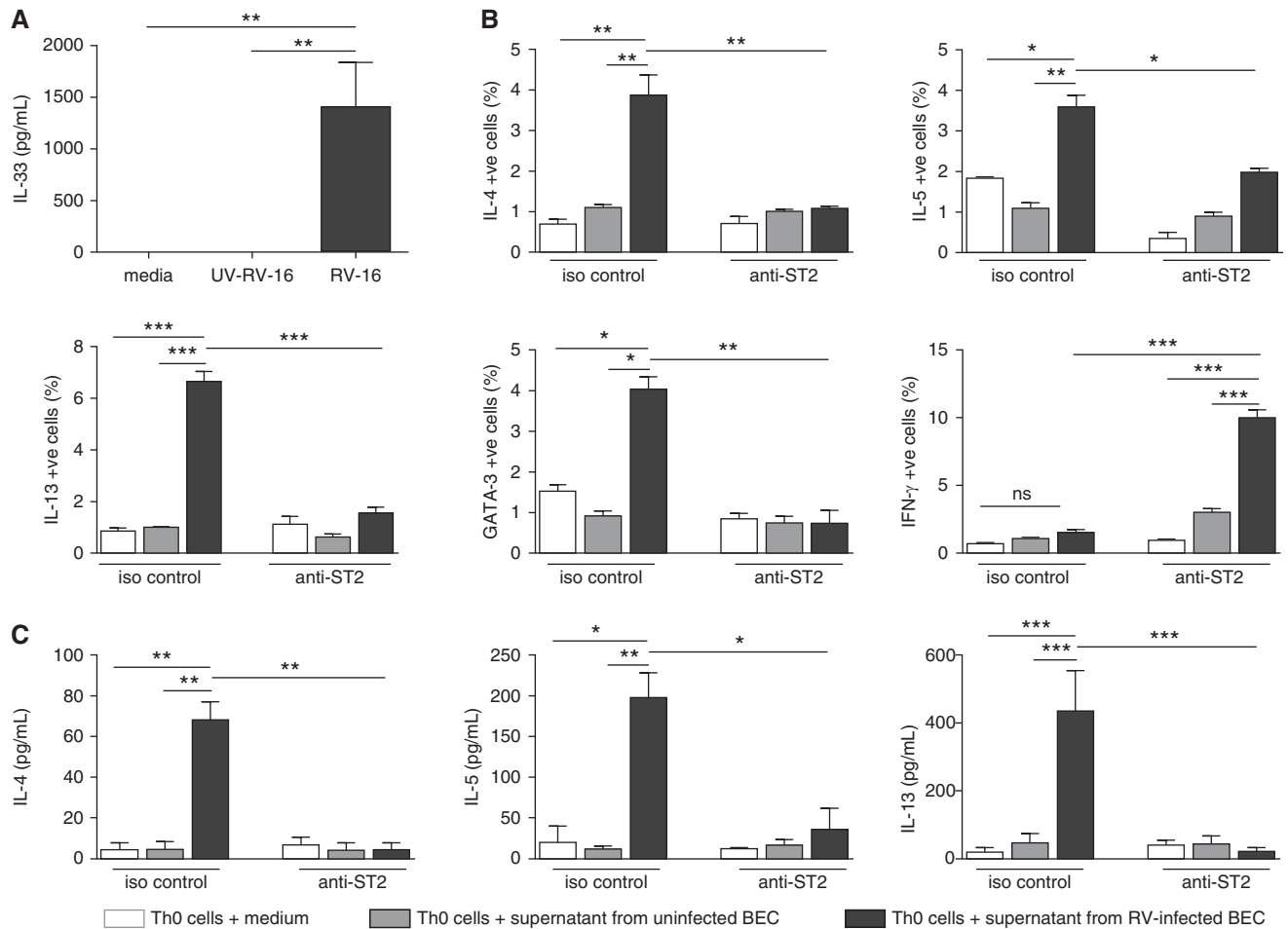


Figure 3. Rhinovirus infection of BECs induces IL-33, which subsequently induces type 2 cytokine production by human T cells and ILC2s. (A) Levels of IL-33 in BEC supernatants 24 hours after rhinovirus 16 infection or culture with ultraviolet light-inactivated rhinovirus 16 or medium control. To determine whether IL-33 present in rhinovirus-infected BEC supernatants could induce Th2 responses in human T cells, naive (CD45RO⁺), activated (anti-CD2/CD3/CD28-stimulated), nonpolarized human CD4⁺ T cells (Th0 cells) were cultured in the presence of medium alone or in 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, before 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 in medium plus supernatants from uninfected (gray) or in rhinovirus 16-infected (black) BECs, in the presence of isotype control or α -ST2 blocking antibody. (C) Levels of IL-4, IL-5, and IL-13 in supernatants from Th0 cells cultured in medium alone (white) or in medium plus supernatants from uninfected (gray) 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⁺ cells and were then flow-sorted as lymphocyte-sized, lineage-negative (CD2⁺, CD3⁺, CD14⁺, CD16⁺, CD19⁺, CD56⁺, CD235a⁺, and CD123⁺) CRTH2⁺ cells. The top panel shows the forward and side scatter characteristics of the sorted population, and the lower panel shows the distinct lineage-negative, CRTH2⁺ ILC2 population. (E) Surface marker expression of ILC2s (red), lineage-negative CRTH2⁺ (gray) cells, and lineage-positive CRTH2⁺ (black) cells were compared using flow cytometry. Histograms show sorted ILC2s to have a distinct phenotype (lineage-negative CD34⁺, CRTH2⁺, cKit⁺, CD45⁺, CD127⁺, and CD25⁺). (F) Human ILC2s were cultured in medium alone (white) or in medium plus supernatants from uninfected (gray) and rhinovirus 16-infected (black) BECs, in the presence of isotype control or α -ST2-blocking antibody and cytokine levels were measured in the ILC2 culture supernatants. Data are expressed as mean $\pm$ SEM. * P < 0.05, ** P < 0.01, *** P < 0.001, n = 6.

in humans. We have demonstrated 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 the 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.

Recent trials of anti-IgE (31), anti-IL-4 (4), anti-IL-5 (5, 6, 8), and anti-IL-13 (7) therapies have 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 patients with asthma *in vivo* underscores the validity of this approach. Our data suggest that blockade of IL-33 signaling 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,

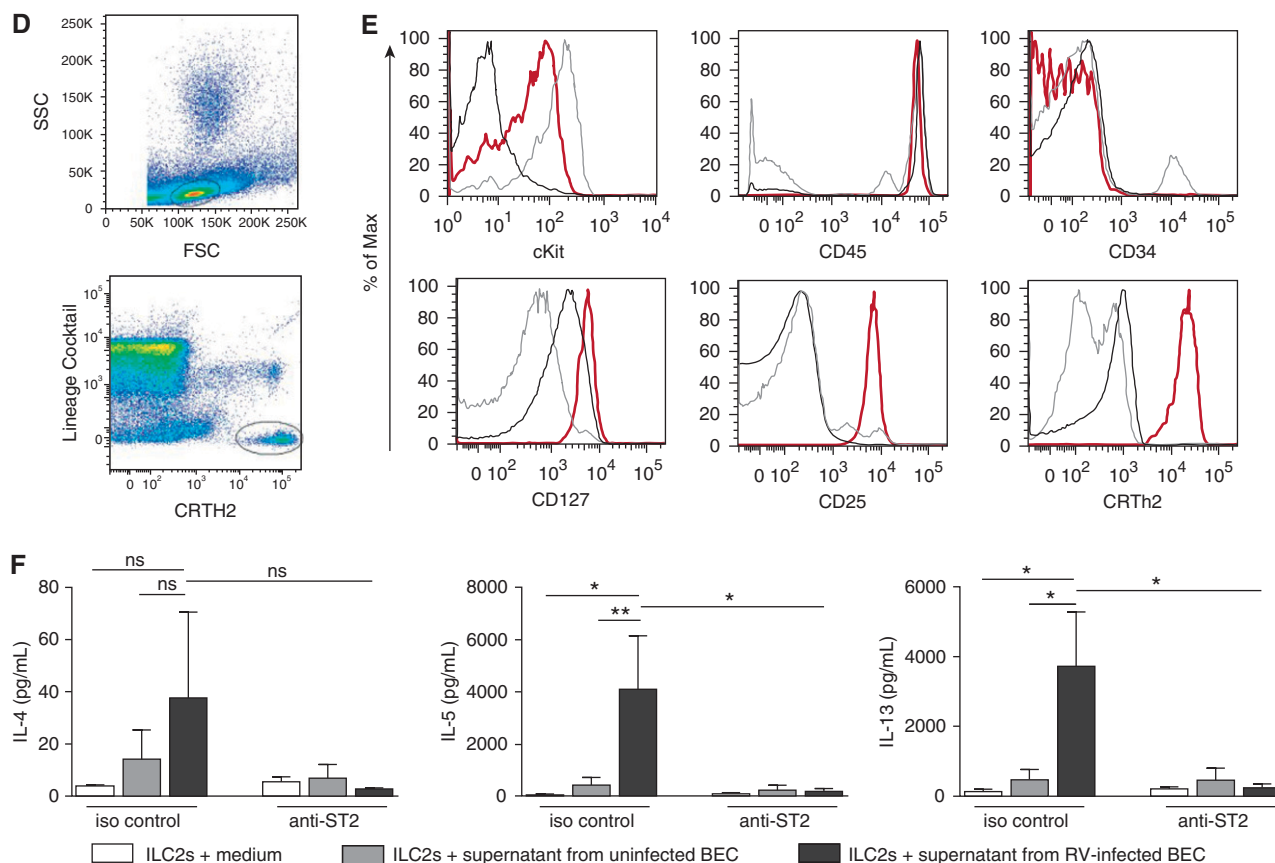


Figure 3. (Continued).

airway hyperresponsiveness, mucus hypersecretion, and airway remodeling associated with IL-13 production, as well as IgE class switching associated with both IL-4 and IL-13. Inhibitors of IL-33 should therefore be more effective than approaches that block only a single type 2 cytokine and/or receptor.

In this study, we have established a biological system that allowed us to measure the ability of rhinovirus-infected epithelial cells to produce IL-33 and mediate polarization of T-cell populations and ILC2 activation *ex vivo*. We cultured activated, but nonpolarized, CD4⁺ T cells in the presence of supernatants from rhinovirus-infected epithelial cells and observed increased production of IL-4, IL-5, and IL-13 compared with T cells cultured with supernatants from uninfected epithelial cells. We have clearly established an essential role for IL-33 in this epithelial cell–Th2–ILC2 axis, as blocking ST2 completely inhibited Th2 polarization, indicating that, in this system, rhinovirus-induced Th2 polarization was entirely

dependent on IL-33. Our data also suggest that ILC2s may be major innate sources 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, respectively, on a per-cell basis than T cells.

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 the induction of type 2 cytokines in our *ex vivo* system appeared to be entirely dependent upon IL-33, it is also possible that cell types other than epithelial cells might be important sources of cytokines such as thymic stromal

lymphopoietin (TSLP), IL-25, or prostaglandin D₂ (PGD₂) that are able to induce type 2 responses in responding cells, and/or that cosecretion of these other mediators may act synergistically with IL-33. For example, Barnig and colleagues recently showed that the combination of IL-33, PGD₂, and IL-25 enhanced type 2 cytokine production by ILC2s in a synergistic manner (32), and Xue and colleagues demonstrated that ILC2 activation via PGD₂ upregulated the expression of ST2 on ILC2s (15).

We recently reported that nasal IL-25 is induced by rhinovirus infection in both healthy individuals and patients with asthma, with nonsignificant trends for higher IL-25 protein levels in asthma (23). We also reported data from a separate study showing that IL-25 was induced by rhinovirus to a greater degree *ex vivo* in primary BECs from patients with asthma compared with healthy subjects. In a murine model, we showed that blocking rhinovirus-induced IL-25 is capable of suppressing induction of Th2/ILC2-type

inflammation during rhinovirus-induced exacerbation of allergic airway inflammation (23). Further studies in other *in vitro* and *in vivo* human and animal models, and eventually in clinical trials, are required to determine the relative importance of TSLP, IL-25, IL-33, and PGD₂ in the context of virus-induced asthma exacerbations.

Our finding of substantially higher levels of IL-33 in the lung than in the nose was unexpected. As this is, to our knowledge, the first study in which nasal and bronchial IL-33 were simultaneously measured 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 and bronchial mucosa) 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 correlates with both IL-5 and IL-13, as well as with exacerbation severity, suggest that our measurements are functionally relevant.

Taken together, our findings suggest that viral induction of BEC-derived IL-33 is centrally involved in the induction of the type 2 response we observed in virus-induced asthma exacerbations. This central role for IL-33 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 the reported synergistic interaction between allergen sensitization and/or exposure and viral infection in increasing the risk of asthma

exacerbations (9, 10), because an atopic individual with asthma who is exposed to allergens in parallel with viral infection may have increased frequencies of allergen-specific Th2 cells in the lung, with enhanced ST2 expression. Because 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, but elucidating the relative importance of these cell types requires further study.

Increased rhinovirus replication in asthmatic BECs has previously been observed *ex vivo* and related to delayed and impaired production of antiviral IFNs (34, 35), but to date increased viral load *in vivo* in asthma has not been observed (36, 37). The earlier and greater peak in viral load in asthma demonstrated in the present study is consistent with these *ex vivo* reports and suggests an impaired antiviral immune response in some patients with asthma. It is possible that these novel observations might be due to the inclusion of subjects with more severe asthma, as most previous rhinovirus infection studies limited inclusion to only subjects with mild asthma. 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.

In the present study, we also introduced bronchosorption as a new technique to sample bronchial mucosal lining fluid and permit detection of cytokines not normally detectable in BAL fluid. This technique offers great potential to advance the mechanistic understanding of many respiratory conditions.

In summary, this study provides evidence that rhinovirus infection leads to induction of IL-33 and type 2 cytokines in asthma *in vivo* and that levels of these mediators are related to the severity of asthma exacerbations. To our knowledge, this study is the first to show that rhinovirus infection of the 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 viral infection and asthma exacerbations. On the basis of the data presented here, we believe that the IL-33/ST2 axis is an exciting target for future therapeutic interventions for asthma exacerbations. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

Acknowledgment: The authors gratefully acknowledge both Dr. Trevor Hansel of Imperial College London and the Novartis Institute for Biomedical Research for aid in the development of bronchosorption by Mucosal Diagnostics, an Imperial College London spin-off company working in collaboration with Hunt Developments (UK) Ltd. The authors also acknowledge the National Heart and Lung Institute flow cytometry facility at Imperial College St Mary's campus for support and access to equipment.

References

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.
2. Grissell TV, Powell H, Shafren DR, Boyle MJ, Hensley MJ, Jones PD, Whitehead BF, Gibson PG. Interleukin-10 gene expression in acute virus-induced asthma. *Am J Respir Crit Care Med* 2005;172:433-439.
3. Jackson DJ, Johnston SL. The role of viruses in acute exacerbations of asthma. *J Allergy Clin Immunol* 2010;125:1178-1187.
4. Wenzel S, Wilbraham D, Fuller R, Getz EB, Longphre M. Effect of an interleukin-4 variant on late phase asthmatic response to allergen challenge in asthmatic patients: results of two phase 2a studies. *Lancet* 2007;370:1422-1431.
5. Nair P, Pizzichini MMM, Kjarsgaard M, Inman MD, Efthimiadis A, Pizzichini E, Hargreave FE, O'Byrne PM. Mepolizumab for prednisone-dependent asthma with sputum eosinophilia. *N Engl J Med* 2009;360:985-993.
6. Halder P, Brightling CE, Hargadon B, Gupta S, Monteiro W, Sousa A, Marshall RP, Bradding P, Green RH, Wardlaw AJ, *et al.* Mepolizumab and exacerbations of refractory eosinophilic asthma. *N Engl J Med* 2009;360:973-984.
7. Corren J, Lemanske RF, Hanania NA, Korenblat PE, Parsey MV, Arron JR, Harris JM, Scheerens H, Wu LC, Su Z, *et al.* Lebrikizumab treatment in adults with asthma. *N Engl J Med* 2011;365:1088-1098.
8. 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.
9. Murray CS, Poletti G, Kebabdz T, Morris J, Woodcock A, Johnston SL, Custovic A. Study of modifiable risk factors for asthma exacerbations: virus infection and allergen exposure increase the risk of asthma hospital admissions in children. *Thorax* 2006;61:376-382.
10. Green RM, Custovic A, Sanderson G, Hunter J, Johnston SL, Woodcock A. Synergism between allergens and viruses and risk of hospital admission with asthma: case-control study. *BMJ* 2002;324:763.

11. Walker JA, Barlow JL, McKenzie ANJ. Innate lymphoid cells—how did we miss them? *Nat Rev Immunol* 2013;13:75–87.
12. Neill DR, Wong SH, Bellosi A, Flynn RJ, Daly M, Langford TKA, Bucks C, Kane CM, Fallon PG, Pannell R, *et al.* Nuocytes represent a new innate effector leukocyte that mediates type-2 immunity. *Nature* 2010;464:1367–1370.
13. Halim TYF, Krauss RH, Sun AC, Takei F. Lung natural helper cells are a critical source of Th2 cell-type cytokines in protease allergen-induced airway inflammation. *Immunity* 2012;36:451–463.
14. Salimi M, Barlow JL, Saunders SP, Xue L, Gutowska-Owsiak D, Wang X, Huang LC, Johnson D, Scanlon ST, McKenzie AN, *et al.* A role for IL-25 and IL-33-driven type-2 innate lymphoid cells in atopic dermatitis. *J Exp Med* 2013;210:2939–2950.
15. Xue L, Salimi M, Panse I, Mjösberg JM, McKenzie ANJ, Spits H, Klennerman P, Ogg G. Prostaglandin D₂ activates group 2 innate lymphoid cells through chemoattractant receptor-homologous molecule expressed on T_H2 cells. *J Allergy Clin Immunol* 2014;133:1184–1194.
16. Kumar RK, Foster PS, Rosenberg HF. Respiratory viral infection, epithelial cytokines, and innate lymphoid cells in asthma exacerbations. *J Leukoc Biol* 2014;96:391–396.
17. Moffatt MF, Gut IG, Demenais F, Strachan DP, Bouzigon E, Heath S, von Mutius E, Farrall M, Lathrop M, Cookson WO; GABRIEL Consortium. A large-scale, consortium-based genomewide association study of asthma. *N Engl J Med* 2010;363:1211–1221.
18. Chang YJ, Kim HY, Albacker LA, Baumgarth N, McKenzie ANJ, Smith DE, Dekruff RH, Umetsu DT. Innate lymphoid cells mediate influenza-induced airway hyper-reactivity independently of adaptive immunity. *Nat Immunol* 2011;12:631–638.
19. Le Goffic R, Arshad MI, Rauch M, L'Helgoualc'h A, Delmas B, Piquet-Pellorce C, Samson M. Infection with influenza virus induces IL-33 in murine lungs. *Am J Respir Cell Mol Biol* 2011;45:1125–1132.
20. Gern JE, Vrtis R, Grindle KA, Swenson C, Busse WW. Relationship of upper and lower airway cytokines to outcome of experimental rhinovirus infection. *Am J Respir Crit Care Med* 2000;162:2226–2231.
21. Følsgaard NV, Chawes BL, Rasmussen MA, Bischoff AL, Carson CG, Stokholm J, Pedersen L, Hansel TT, Bønnelykke K, Brix S, *et al.* Neonatal cytokine profile in the airway mucosal lining fluid is skewed by maternal atopy. *Am J Respir Crit Care Med* 2012;185:275–280.
22. Jackson DJ, Johnston SL. Sampling airway mucosal lining fluid identifies roles for IL-33 and multiple inflammatory pathways in virus-induced asthma exacerbations [abstract]. *Am J Respir Crit Care Med* 2013;187:A1002.
23. Beale J, Jayaraman A, Jackson DJ, Macintyre JD, Edwards MR, Walton RP, Zhu J, Ching YM, Shamji B, Edwards M, *et al.* Rhinovirus-induced IL-25 in asthma exacerbation drives type 2 immunity and allergic pulmonary inflammation. *Sci Transl Med* 2014;6:ra134.
24. Global Initiative for Asthma (GINA). Global strategy for asthma management and prevention. Workshop report; 2004 [accessed 2014 Nov 12]. Available from: www.ginasthma.org/local/uploads/files/GINAw04clean2_1.pdf
25. Message SD, Laza-Stanca V, Mallia P, Parker HL, Zhu J, Keadze T, Contoli M, Sanderson G, Kon OM, Papi A, *et al.* 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 USA* 2008;105:13562–13567.
26. Chawes BLK, Edwards MJ, Shamji B, Walker C, Nicholson GC, Tan AJ, Følsgaard NV, Bønnelykke K, Bisgaard H, Hansel TT. A novel method for assessing unchallenged levels of mediators in nasal epithelial lining fluid. *J Allergy Clin Immunol* 2010;125:1387–1389.e3.
27. Nicholson GC, Kariyawasam HH, Tan AJ, Hohlfeld JM, Quinn D, Walker C, Rodman D, Westwick J, Jurcevic S, Kon OM, *et al.* The effects of an anti-IL-13 mAb on cytokine levels and nasal symptoms following nasal allergen challenge. *J Allergy Clin Immunol* 2011;128:800–807.e9.
28. Slater L, Bartlett NW, Haas JJ, Zhu J, Message SD, Walton RP, Sykes A, Dahdaleh S, Clarke DL, Belvisi MG, *et al.* Co-ordinated role of TLR3, RIG-I and MDA5 in the innate response to rhinovirus in bronchial epithelium. *PLoS Pathog* 2010;6:e1001178.
29. Papi A, Papadopoulos NG, Stanciu LA, Degitz K, Holgate ST, Johnston SL. Effect of desloratadine and loratadine on rhinovirus-induced intercellular adhesion molecule 1 upregulation and promoter activation in respiratory epithelial cells. *J Allergy Clin Immunol* 2001;108:221–228.
30. Burgler S, Ouaked N, Bassin C, Basinski TM, Mantel PY, Siegmund K, Meyer N, Akdis CA, Schmidt-Weber CB. Differentiation and functional analysis of human T_H17 cells. *J Allergy Clin Immunol* 2009;123:588–595.e7.
31. Busse WW, Morgan WJ, Gergen PJ, Mitchell HE, Gern JE, Liu AH, Gruchalla RS, Kattan M, Teach SJ, Pongracic JA, *et al.* Randomized trial of omalizumab (anti-IgE) for asthma in inner-city children. *N Engl J Med* 2011;364:1005–1015.
32. Barnig C, Cernadas M, Dutilleul S, Liu X, Perrella MA, Kazani S, Wechsler ME, Israel E, Levy BD. Lipoxin A₄ regulates natural killer cell and type 2 innate lymphoid cell activation in asthma. *Sci Transl Med* 2013;5:174ra26.
33. Halim TYF, Steer CA, Mathä L, Gold MJ, Martinez-Gonzalez I, McNagny KM, McKenzie AN, Takei F. Group 2 innate lymphoid cells are critical for the initiation of adaptive T helper 2 cell-mediated allergic lung inflammation. *Immunity* 2014;40:425–435.
34. Wark PAB, 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.
35. Contoli M, Message SD, Laza-Stanca V, Edwards MR, Wark PAB, Bartlett NW, Keadze T, Mallia P, Stanciu LA, Parker HL, *et al.* Role of deficient type III interferon-lambda production in asthma exacerbations. *Nat Med* 2006;12:1023–1026.
36. DeMore JP, Weisshaar EH, Vrtis RF, Swenson CA, Evans MD, Morin A, Hazel E, Bork JA, Kakamanu S, Sorkness R, *et al.* Similar colds in subjects with allergic asthma and nonatopic subjects after inoculation with rhinovirus-16. *J Allergy Clin Immunol* 2009;124:245–252.e3.
37. Kennedy JL, Shaker M, McMeen V, Gern J, Carper H, Murphy D, Lee WM, Bochkov YA, Vrtis RF, Platts-Mills T, *et al.* Comparison of viral load in individuals with and without asthma during infections with rhinovirus. *Am J Respir Crit Care Med* 2014;189:532–539.
38. Bonilla WV, Fröhlich A, Senn K, Kallert S, Fernandez M, Johnson S, Kreutzfeldt M, Hegazy AN, Schrick C, Fallon PG, *et al.* The alarmin interleukin-33 drives protective antiviral CD8⁺ T cell responses. *Science* 2012;335:984–989.

Online Data Supplement

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

David J. Jackson, Heidi Makrinioti, Batika M.J. Rana, Betty W.H. Shamji, Maria-Belen Trujillo-Torralbo, Joseph Footitt, Jerico del-Rosario, Aurica G. Telcian, Alexandra Nikonova, Julia Aniscenko, Leila Gogsadze, Eteri Bakhsoliani, Stephanie Traub, Jaideep Dhariwal, James Porter, Duncan Hunt, Toby Hunt, Trevor Hunt, Luminita A. Stanciu, Musa Khaitov, Nathan W. Bartlett, Michael R. Edwards, Onn Min Kon, Patrick Mallia, Nikolaos G. Papadopoulos, Cezmi A. Akdis, John Westwick, Matthew J. Edwards, David J. Cousins, Ross P. Walton, and Sebastian L. Johnston.

Supplementary Methods

Inclusion and Exclusion Criteria. Asthmatic subjects were eligible for inclusion if aged 18-55 years with a doctor diagnosis of asthma (of mild-to-moderate severity based on GINA criteria(2)), had objective airway hyperresponsiveness with a $(PC)_{20}$ histamine $< 8 \mu\text{g/mL}$, evidence of atopy on skin prick testing (≥ 1 positive skin prick test on a panel of 10 aeroallergens), and had a history of worsening asthma symptoms with infection since last change in asthma therapy. Subjects were excluded if they had a history of severe asthma or smoking history over the previous 6 months or > 5 pack year history, had current symptoms of allergic rhinitis, current or previous history of significant respiratory or other disease (other than asthma), or had experienced an asthma exacerbation or viral illness within the previous 6 weeks. Healthy, non-smoking subjects were eligible if they had no history of asthma, allergy or any other significant disease, had negative skin prick testing, and no objective airway hyperresponsiveness with a histamine $(PC)_{20} \geq 8 \mu\text{g/mL}$.

Subjects who had contact with infants or the elderly at home or at work as well as pregnant or breastfeeding women were excluded. Further exclusion criteria included evidence of serum antibodies to rhinovirus 16 at screening along with the use of any nasal or oral medication including anti-histamines, or nasal / oral corticosteroids.

Subjects were excluded from the study analysis if they failed to develop objective evidence of a rhinovirus infection defined as absent serum neutralising antibody to RV16 at 6 weeks and no detection of RV16 in nasal lavage samples throughout the infection period (day 2-10).

Study design. Volunteers underwent baseline sampling including bronchoscopy 2-4 weeks prior to inoculation with RV16(3). Subjects were seen on days 2,3,4,5,7,10 and 42 post-inoculation. Nasal lavage for virus load was performed at each visit. A second bronchoscopy was performed on day 4. The study design is summarised in Fig. 3.

Symptom Scores and Spirometry Recording. Symptoms were assessed by daily diary cards, from 2 weeks before baseline sampling until 6 weeks post-inoculation. The daily cold score was measured using the Jackson(4) scale and summated from individual scores (sneezing, headache, malaise, chilliness, nasal discharge, nasal obstruction, sore throat, cough, fever) graded 0 (absent) to 3 (severe). A clinical cold was defined as reported (3)(5). The daily lower respiratory score was calculated from symptom scores (cough on waking; wheeze on waking; daytime cough; daytime wheeze; daytime chest tightness; daytime shortness of breath; nocturnal cough, wheeze or shortness of breath), also graded 0–3 (absent, mild, moderate, severe)(3). The same diary cards recorded bronchodilator usage and home spirometry on waking each morning recording the best of three recordings of PEF and FEV₁ (Piko-1; nSpire Health, CO).

Analysis of Lower Respiratory Symptom Scores and Lung Function. As previously reported(3), lower respiratory symptom scores were analysed in 2-week blocks as the baseline and acute infection stages both contained a bronchoscopy on the fourth day of each 2-week block (Fig. 4). Daily symptom scores during infection, corrected for baseline symptoms and the effects of bronchoscopy, were calculated by subtracting scores obtained during the baseline 2-week block from the corresponding days of the acute infection 2-week block. Total lower respiratory scores were calculated by summing the corrected daily scores for the 2-week infection period. Peak symptom score was the maximum corrected daily score in the 2-week block after infection. The % change in morning FEV₁ and PEF from baseline during infection was calculated for each subject as the % fall from the mean of the 7 day period prior to inoculation. Maximal fall from baseline (%) for each subject represented the greatest fall from baseline over the 2 week period following inoculation.

Histamine Challenge. Histamine challenge (maximum concentration 16 mg/mL) was performed according to guidelines by using the 2-min tidal breathing method(6). Subjects used a MicroLab Spirometer V002ML3500. The provocative concentration of histamine causing a 20% reduction in

FEV₁ (PC)₂₀ was calculated. Asthmatic subjects were required to have a (PC)₂₀ < 8 mg/mL, and healthy subjects a (PC)₂₀ ≥ 8 mg/mL.

Skin Prick Testing. Atopy was determined by skin prick testing to common aeroallergens: six grass pollen mix; house dust mite; cat; dog; *Aspergillus fumigatus*; *Cladosporium herbarium*; *Alternaria alternata*; birch; three tree mix and nettle pollen (ALK Abello). At least one wheal ≥ 3 mm larger than the negative control was considered diagnostic of atopy.

Virus Inoculation. Rhinovirus-16(3) (RV-16) was diluted in 250 µL of 0.9% saline and inoculated into both nostrils (total dose 100 TCID₅₀) using an atomizer (no. 286; De Vilbiss Co., Heston, UK).

Nasal Lavage. Nasal lavage with sterile 0.9% sodium chloride was performed using a 10mL syringe attached to a hollow nasal adapter ('olive') used to obstruct the nostril and prevent leak of lavage fluid(7). A total of 5 mL of sterile normal saline was instilled into the left nostril and the fluid withdrawn into the 10mL syringe and flushed back into the nasal cavity 20 times over a 1 min period. Fluid was collected, aliquoted and stored at -80°C. Nasal lavage for virus load was measured on days 0, 2, 3, 4, 5, 7 and 10 post-inoculation.

Nasosorption™. This minimally invasive technique samples upper airway mucosal lining fluid and offers potential benefits over nasal lavage including improved sensitivity through avoidance of significant analyte dilution (inherent with lavage). Nasosorption™ was performed as described(7–9) prior to nasal lavage by placing strips of a hydrophilic polyester synthetic absorptive matrix (SAM) (Mucosal Diagnostics, Hunt Developments (UK) Ltd) measuring 7mm x 35mm into each nostril for a duration of 2 minutes(7). 100 µL of a PBS buffer containing 1% BSA and 1% Triton X (Sigma Aldrich, USA) was then applied to the SAM prior to elution by spin filter centrifugation (5 minutes at 16,000G at 4 °C) using a Spin-X centrifuge tube filter with 0.22µm Cellulose Acetate filter (Sigma-Aldrich, USA) to remove any mucus. The eluate was then immediately frozen at -80°C.

Bronchoscopy. All bronchoscopies were performed in the endoscopy unit at St Mary's Hospital, UK using an Olympus PF260 bronchoscope. Subjects were administered nebulised salbutamol (2.5mg) prior to the procedure and sedation was achieved with intravenous midazolam. The bronchoscope was passed via the mouth and not the nose. Bronchoalveolar lavage (BAL) was performed by instillation of 150 mL sterile normal saline in 5 x 30 mL aliquots with a 10 sec dwell time into the right upper lobe bronchus. An aliquot of unfiltered BAL was stored for qRT-PCR for virus load and the remaining BAL filtered, centrifuged and the supernatant stored. The BAL cell pellet was resuspended, a cell count was obtained and cytopins were prepared and stained using Shandon Diff-Quik kit (Thermo Shandon Ltd, Cheshire, UK), coded and counted blind to study status and time-point to obtain differential cell counts. Cell counts were expressed as a percentage of at least 400 inflammatory cells.

Bronchosorption™. Bronchosorption™ (Mucosal Diagnostics, Hunt Developments (UK) Ltd, Midhurst, UK) 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 bronchosorption™ device is passed down the operating port of the bronchoscope. The distal end of the inner probe incorporates a folded strip of polyester synthetic absorptive matrix (Mucosal Diagnostics) measuring 1.8mm x 30mm which is placed on the bronchial mucosa for a period of 30 seconds. Following sampling, the bronchosorption™ device is withdrawn back into its sheath and then the complete device is removed from the bronchoscope. The sampling end of the probe is then cut off and treated in an identical way to the nasosorption™ strips of absorptive matrix. Bronchosorption™ devices are supplied as individual packaged single-use sterile medical devices by Mucosal Diagnostics (mucosaldiagnosics.com), an Imperial College spin-off company, working in collaboration with Hunt Developments (UK) Ltd.

Cytokine and Chemokine Measurement from *in vivo* samples. Levels of IL-4, IL-5, IL-13 and IL-33 in the extracted upper and lower airway mucosal lining fluid were analysed using Ultrasensitive Meso Scale Discovery Multi-spot Human cytokine assays (Meso Scale Discovery, Gaithersburg, MD, USA). Samples were read using the Sector Imager 6000 (Meso Scale Discovery). The lower limit of detection was 0.6 pg/mL for IL-4, IL-5, and IL-13; and 3 pg/mL for IL-33. All samples with values under the detection limits were given a value of 0 pg/mL for the subsequent statistical analysis. The standard curve was derived using the recombinant protein provided with the multiplex soluble mediator assay (Mesoscale) as per their protocol.

The IL-33 assay measures total IL-33 (including every fragment of IL-33 trimmed by proteases published to date). We also specifically looked at whether the MSD assay detects IL-33 in the presence of sST2 and found that it did meaning we are reporting total IL-33 readout rather than just free IL-33.

Virologic Confirmation of RV16 Infection. All subjects were seronegative (absent neutralizing antibodies) for RV16 at screening and on repeat serology performed on day 0 prior to inoculation. Rhinovirus infection was confirmed by positive nasal lavage qPCR for rhinovirus or seroconversion defined as a titre of serum neutralizing antibodies to RV16 of at least 1:4 at 6 weeks post-inoculation. Serology was performed at screening, day 0, and day 42 post infection by microneutralization test for neutralizing antibody to RV16(3). RNA was extracted from samples (QIAamp viral RNA mini kit; Qiagen Ltd, Crawley, UK) and reverse-transcribed (omniscrypt RT kit, Qiagen) with random hexamers. qPCR was performed on 2 µl of cDNA to detect picornavirus in nasal lavage and unprocessed BAL, using AmplitaqGold DNA polymerase (PE Biosystems ABI Prism 7700)(10). A standard curve was produced by using serially diluted cloned product and results expressed as copies/mL. The sensitivity of this assay was 10^4 copies/mL.

Development of serum neutralising antibodies to RV16. All subjects were seronegative (absent neutralising antibodies) for RV16 on serology performed at baseline. At 6 weeks post-inoculation

serology showed a rise in RV16 neutralising antibody titre to $\geq 1:4$ for all but 3 subjects (2 healthy, 1 mild asthmatic). See Table E6 for full results.

PCR for Additional Respiratory Infection. Infection with bacteria/viruses other than RV was excluded by testing nasal lavage by PCR on random hexamer primed cDNA for *Mycoplasma* and *Chlamydomphila pneumoniae*, adenoviruses, respiratory syncytial virus, influenza AH1/AH3/B, parainfluenza 1–3, human metapneumoviruses (HMPV), and coronaviruses 229E and OC43 as described(3). All subjects were negative to the PCR panel for common respiratory viruses in nasal lavage at baseline and no additional respiratory viruses were detected by PCR panel in nasal lavage in any subject during the infection period of the study.

Infection of Bronchial Epithelial Cells with Rhinovirus. Normal human bronchial epithelial cells (NHBEs, Lonza; Lonza Ltd, Basel, Switzerland) from two different or separate batches were infected with RV16, or treated with UV-inactivated RV16 or media by using previously published methodology(11). Supernatants were harvested 24 hours later, were UV-irradiated for 10 minutes and filtered (Amicon Ultra 0.5 ml 30K filters, Millipore, 12000rpm, 5 minutes), in order to remove any replicating or not virus particles. The cytopathic effect of the supernatants on HeLa cells was assessed by means of visual assessment and examination of the continuity of the monolayer 96 hours later(12).

T Cell Isolation and Expansion. Peripheral blood mononuclear cells (PBMCs) were isolated from four healthy donors by Histopaque (Histopaque Sigma- 10771) density gradient centrifugation out of the Component Donation Leucocyte cone of healthy donors (CD Leukocyte Cone)(13). Cells were washed three times and resuspended in PBS media enriched with 0.5% bovine serum albumin (BSA) and 2mM EDTA. CD4⁺ CD45RA⁺ CCR7⁺ T cells were magnetically sorted twice for negative selection (Naive CD4⁺ T-cell isolation kit II with AutoMACS; Miltenyi Biotec #130-094-131) and assessed as >96% pure, by surface expression of CD4, CD45RA, CCR7 (Biolegend, #300521, #304108, #353206)(14).

CD4⁺ CD45RA⁺ CCR7⁺ T cells were expanded in supplemented RPMI media in the presence of recombinant human IL-2 (10ng/mL) (R&D, 202-IL-010) and anti-CD2, anti-CD3, anti-CD28 (50µg/mL, Sanquin Pelicuster, #M1651-2, Abcam #ab86883, eBioscience #16-0289) for 10 days. Dead cells were removed from the culture by using the Dead Cell Removal Kit (Miltenyi Biotech #130-090-101).

Culture of CD4⁺ T Cells with Bronchial Epithelial Cell Supernatants. Upon removal of dead cells, activated, but not polarised CD4⁺ T cells (Th0 cells at 10⁶ cells/mL in RPMI 1640) were stimulated overnight with recombinant human IL-2 and anti-CD2,-CD3,-CD28 and were treated with either the blocking anti-human ST2 antibody (#ab89741, Abcam, Cambridge, UK) at 10µg/mL or a matched isotype control (#ab81216). 3 hours later, Th0 cells were cultured with RPMI alone, or together with the supernatants from rhinovirus infected or media treated bronchial epithelial cells in a proportion 4 to 1 (4 parts RPMI : 1 part epithelial cell supernatant). T cells remained in culture for 12 days, by renewing 1/10 of the media every 4 days.

Intracellular levels of IL-4, IL-5, IL-13, IFN-γ, FOXP3 and GATA3. After 12 days T cells were stimulated with 50 ng/mL PMA, 0.5 µg/mL ionomycin and 5 ng/mL Brefeldin A for 6 hours. Cells were first stained with anti-CD4, then with a live-dead dye (Invitrogen, #L10119). Cells were fixed and permeabilised (Biolegend, #320118) for intracellular staining with anti-IL-4, IL-5, IL-13, IFN-γ, GATA-3 and FOXP3 antibodies (Biolegend #500822, #500904, #501907, #341117, eBioscience #12-9966, Biolegend #320118).

The samples were processed using a BD LSR Fortessa and data was analysed with FlowJo Version 10.

ILC2 cell isolation and culture. Anti-coagulated peripheral blood was obtained from 4 healthy donors with ethical approval from the research ethics committee of Guy's Hospital, London, UK (REC reference number 09/H804/077). PBMCs were isolated using Lymphoprep (Axis-Shield, Oslo, Norway) according to the manufacturer's instructions. Prior to flow cytometric sorting, depletion of the majority of lineage positive cells was performed using antibodies recognizing CD3 (clone OKT3, in

house), CD14, CD16 and CD19 (mouse anti-human; all eBioscience, Hatfield, UK) and Pan Mouse IgG Dynabeads (Life Technologies, Paisley, UK) according to the manufacturer's instructions. Remaining cells were stained using a human Hematopoietic Lineage FITC cocktail (CD2, CD3, CD14, CD16, CD19, CD56, CD235a; eBioscience) with the addition of anti-CD123-FITC (eBioscience) to identify basophils. CRTh2-AlexaFluor 647 (Biolegend, London, UK), CD127-Brilliant Violet 570 (Biolegend) CD45-AlexaFluor 700 (Biolegend), CD34-Brilliant Violet 421 (Biolegend), CD25 Brilliant Violet 605 (Biolegend) and c-kit-PE (eBioscience) were used to phenotype and identify ILC2s. ILC2 cells were sorted as Lineage- CRTh2⁺ CD127⁺ on a FACS Aria II (BD Biosciences, Oxford, UK), cells were collected into RPMI 1640 containing 10% FBS (Life Technologies).

ILC2 cells were cultured at 10,000 cells/mL in RPMI 1640 supplemented with 10% FBS and penicillin/streptomycin/L-glutamine (Life Technologies) with rhIL-2 (50U/mL; Eurocetus, Amsterdam, The Netherlands) and rhIL-7 (50ng/mL; R&D Systems, Abingdon, UK). Cells were incubated for 1hr with anti-ST2 (Abcam) prior to *in vitro* culture. Where indicated bronchial epithelial cell supernatants were added at a ratio of 1:4 and cells were cultured for seven days.

Measurement of IL-4, IL-5, IL-13, and IL-33 levels in culture supernatants. Levels of IL-4, IL-5, and IL-13 were assessed in the supernatants of the T cell and ILC2 cultures by MSD. The sensitivity levels were 0.35 pg/mL for IL-4 and IL-5 and 1.57pg/mL for IL-13. These sensitivity levels were slightly different from those observed when the in-vivo airway samples were measured (see earlier) and is likely to reflect minor differences in the groups of cytokines multiplexed together on each plate in these two settings. IL-33 was measured in supernatants by R&D DuoSet ELISA with a lower limit of detection of 23.4 pg/mL.

References

1. Beale J, Jayaraman A, Jackson DJ, Macintyre J, Edwards MR, Walton RP, et al. Rhinovirus-induced IL-25 in asthma exacerbation drives type 2 immunity and allergic pulmonary inflammation. *Sci Transl Med*. 2014;6(256) *In Press*.
2. Global Initiative for Asthma (GINA). Global strategy for asthma management and prevention. Workshop report. 2004. Available from www.ginasthma.com:Accessed December 2011.
3. Message SD, Laza-Stanca V, Mallia P, Parker HL, Zhu J, Keadze T, et al. 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 Sep 9;105(36):13562–7.
4. Jackson GG, Dowling HF, Spiesman IG, Boand AV. Transmission of the common cold to volunteers under controlled conditions. I. The common cold as a clinical entity. *AMA Arch Intern Med*. 1958 Feb;101(2):267–78.
5. Mallia P, Message SD, Gielen V, Contoli M, Gray K, Keadze T, et al. Experimental rhinovirus infection as a human model of chronic obstructive pulmonary disease exacerbation. *Am J Respir Crit Care Med*. 2011 Mar 15;183(6):734–42.
6. Sterk PJ, Fabbri LM, Quanjer PH, Cockcroft DW, O’Byrne PM, Anderson SD, et al. Airway responsiveness. Standardized challenge testing with pharmacological, physical and sensitizing stimuli in adults. Report Working Party Standardization of Lung Function Tests, European Community for Steel and Coal. Official Statement of the European Respiratory Society. *Eur Respir J Suppl*. 1993 Mar;16:53–83.
7. Nicholson GC, Kariyawasam HH, Tan AJ, Hohnfeld JM, Quinn D, Walker C, et al. The effects of an anti-IL-13 mAb on cytokine levels and nasal symptoms following nasal allergen challenge. *J Allergy Clin Immunol*. 2011 Oct;128(4):800–7.e9.
8. Chawes BLK, Edwards MJ, Shamji B, Walker C, Nicholson GC, Tan AJ, et al. A novel method for assessing unchallenged levels of mediators in nasal epithelial lining fluid. *J Allergy Clin Immunol*. 2010 Jun;125(6):1387–9.e3.
9. Følsgaard NV, Chawes BL, Rasmussen MA, Bischoff AL, Carson CG, Stokholm J, et al. Neonatal cytokine profile in the airway mucosal lining fluid is skewed by maternal atopy. *Am J Respir Crit Care Med*. 2012 Feb 1;185(3):275–80.
10. Pevear DC, Tull TM, Seipel ME, Groarke JM. Activity of pleconaril against enteroviruses. *Antimicrob Agents Chemother*. 1999 Sep;43(9):2109–15.
11. Slater L, Bartlett NW, Haas JJ, Zhu J, Message SD, Walton RP, et al. Co-ordinated role of TLR3, RIG-I and MDA5 in the innate response to rhinovirus in bronchial epithelium. *PLoS Pathog*. 2010;6(11):e1001178.
12. Papi A, Papadopoulos NG, Stanciu LA, Degitz K, Holgate ST, Johnston SL. Effect of desloratadine and loratadine on rhinovirus-induced intercellular adhesion molecule 1 upregulation and promoter activation in respiratory epithelial cells. *J Allergy Clin Immunol*. 2001 Aug;108(2):221–8.
13. Néron S, Thibault L, Dussault N, Côté G, Ducas E, Pineault N, et al. Characterization of mononuclear cells remaining in the leukoreduction system chambers of apheresis instruments

after routine platelet collection: a new source of viable human blood cells. *Transfusion (Paris)*. 2007 Jun;47(6):1042–9.

14. Burgler S, Ouaked N, Bassin C, Basinski TM, Mantel P-Y, Siegmund K, et al. Differentiation and functional analysis of human T(H)17 cells. *J Allergy Clin Immunol*. 2009 Mar;123(3):588–95, 595.e1–7.

Figure E1. Study visits and assessments.

Day	-15	-14	0	1	2	3	4	5	6	7	8	9	10	42
Symptom diaries														
Home spirometry														
Virus inoculation			X											
Bronchoscopy		X					X							
Serum			X				X			X				X
Nasal lavage		X	X		X	X	X	X		X			X	X
Nasosorption™		X	X		X	X	X	X		X			X	X
Clinic Spirometry	X	X	X		X	X	X	X		X			X	X
Histamine challenge	X									X				

Figure E2. Baseline asthma symptom scores and any increase in symptoms following the first bronchoscopy were subtracted from the equivalent day symptom scores during the 2 week infection block. This was performed to allow an analysis of virus-induced symptoms rather than those either already present in some asthmatics or those induced by bronchoscopy itself. It is therefore possible to have a negative score assigned to a study subject if baseline symptoms exceeded those during the acute infection block.

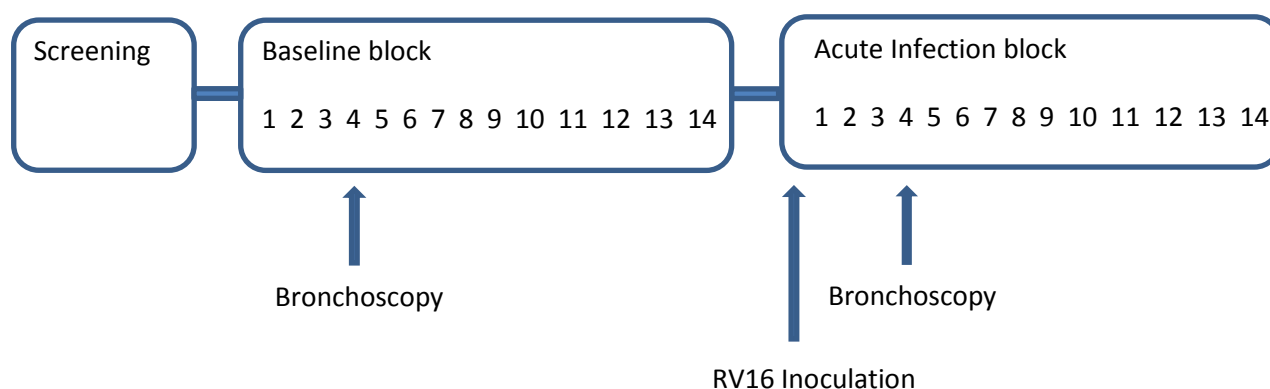


Table E1. Virus Load. Values represent medians and interquartile range (copies/mL). *, $P < 0.05$ compared to healthy subjects.

	Peak NL VL	Day 2 NL	Day 3 NL	Day 4 NL	Day 5 NL	Day 7 NL	Day 10 NL
Healthy N=11	1.23×10^6 (3.98×10^4 - 5.85×10^6)	5722 (0 - 1.52×10^4)	6917 (1501 - 3.21×10^6)	9.93×10^4 (622 - 1.28×10^6)	2.71×10^4 (751 - 2.03×10^5)	395 (0 - 3.25×10^5)	1763 (0 - 3.11×10^5)
All Asthma N=28	3.99×10^6 (5.07×10^5 - 1.81×10^7)	5.39×10^4 (0 - 1.52×10^6)	* 1.68×10^6 (1.61×10^4 - 1.28×10^7)	5.43×10^4 (8811 - 3.10×10^6)	2.96×10^4 (548 - 3.97×10^5)	1.22×10^4 (287 - 1.60×10^5)	1.52×10^4 (0 - 1.01×10^5)
Severity							
Mild N=11	3.92×10^6 (4.44×10^5 - 1.93×10^7)	1.90×10^5 (1.24×10^4 - 1.69×10^6)	8.32×10^5 (2.30×10^4 - 1.93×10^7)	3.90×10^4 (1.22 - 4.85×10^6)	1.77×10^5 (0 - 4.38×10^5)	1.11×10^4 (119 - 6.95×10^5)	4962 (0 - 2.81×10^4)
Moderate N=17	4.06×10^6 (8.05×10^5 - 2.56×10^7)	1.33×10^4 (0 - 2.42×10^6)	2.85×10^6 (8907 - 1.07×10^7)	2.27×10^5 (5266 - 2.47×10^6)	4.16×10^4 (805 - 4.35×10^5)	1.34×10^4 (395 - 1.17×10^5)	2.52×10^4 (241 - 1.16×10^5)
Treatment							
Steroid naïve N=13	3.92×10^6 (3.86×10^5 - 2.84×10^7)	1.90×10^5 (7258 - 2.80×10^6)	8.32×10^5 (1.58×10^4 - 2.84×10^7)	3.90×10^4 (7665 - 3.31×10^6)	1.77×10^4 (0 - 3.02×10^5)	1.19×10^4 (702 - 5.22×10^6)	1.34×10^4 (0 - 1.58×10^5)
ICS N=15	4.06×10^6 (1.38×10^6 - 1.48×10^7)	1.33×10^4 (0 - 1.01×10^6)	2.85×10^6 (1.38×10^4 - 6.66×10^6)	2.98×10^5 (7668 - 3.54×10^6)	5.53×10^4 (1317 - 5.96×10^5)	1.25×10^4 (0 - 7.39×10^4)	1.70×10^4 (0 - 6.93×10^4)

Table E2 Changes in BAL inflammatory cell proportions during RV infection. Data shown as median percentages (interquartile range). (*, $P<0.05$; **, $P<0.01$; ***, $P<0.001$ compared to healthy subjects; +, $P<0.05$ day 4 compared to baseline).

	Eosinophils		Neutrophils		Lymphocytes	
	Baseline	Day 4	Baseline	Day 4	Baseline	Day 4
Healthy N=11	0 (0-0)	0 (0-0.7)	2.0 (0-3.0)	1.3 (0.3-3.3)	2.0 (1.0-3.0)	3.9 (1.5-8.9)
All Asthma N=26	0.5** (0-1.7)	1.2+* (0-3.8)	1.3 (0.7-2.7)	1.9 (1.2-4.0)	3.0 (1.3-6.5)	4.3 (2.0-8.2)
Severity						
Mild N=11	0.7** (0-1.7)	1.0 (0-3.3)	2.3 (1-3.7)	1.7 (0.3-4.0)	3.0 (1.0-4.3)	4.4 (1.7-6.5)
Moderate N=15	0.3** (0-1.7)	1.2+ (0-3.9)	1.2 (0-2.3)	2.0 (1.3-4.2)	6.0 (1.3-7.3)	4.3 (2.2-8.7)
Treatment						
Steroid naïve N=12	0.5** (0-1.5)	1.3 (0-2.3)	2.0 (1.0-3.6)	1.7 (0.3-4.0)	3.0 (1.2-4.8)	5.0+ (2.0-8.0)
ICS N=14	0.5** (0-1.8)	1.0+ (0-4.0)	1.3 (0.2-2.3)	2.0 (1.3-2.7)	6.2 (1.3-7.8)	4.0 (2.0-8.7)

The baseline inflammatory cell proportions have been previously reported(1).

Table E3. Nasal levels of cytokines measured by nasosorption™. Values represent median and interquartile range. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$ compared to healthy subjects; +, $P<0.05$; ++, $P<0.01$; +++, $P<0.001$ day 4 compared to baseline.

	IL-33 (pg/mL)		IL-4 (pg/mL)		IL-5 (pg/mL)		IL-13 (pg/mL)	
	Baseline	Peak	Baseline	Peak	Baseline	Peak	Baseline	Peak
Healthy N=11	45.5 (29.0-190.4)	129.3 (90.0-451.0)	0 (0-0)	0 (0-1.5)	0 (0-0.8)	0.8 (0-5.0)	0 (0-0.8)	0.8 (0-2.2)
All Asthma N=28	26.5 (20.4-56.8)	+++139.0 (47.3-319.3)	*0.9 (0-1.7)	+++*,** 1.8 (0.9-3.3)	** 1.8 (0.6-7.0)	+++*,** 13.0 (4.5-33.9)	*** 2.0 (0.8-3.8)	+++*,*** 4.9 (2.6-16.0)
Severity								
Mild N=11	25.5 (16.4-95.2)	+100.0 (47.0-272.0)	* 1.1 (0-1.8)	+*,**1.1 (0.9-3.3)	* 3.5 (0.6-7.4)	++*,** 10.1 (5.3-26.4)	** 2.8 (1.0-3.8)	++*,*** 7.7 (3.0-14.3)
Moderate N=17	28.7 (20.9-54.4)	++145.0 (41.5-337.0)	0.8 (0-1.7)	++*,**2.0 (0.9-3.3)	* 1.7 (0.3-5.8)	+++*,** 13.1 (3.6-54.6)	** 1.3 (0.8-4.7)	+++*,*** 4.3 (2.2-36.4)
Treatment								
Steroid naïve N=13	27.1 (18.7-82.8)	++145.0 (57.1-360.0)	* 1.1 (0-1.9)	+*,**1.1 (0.8-3.4)	** 3.5 (1.1-9.5)	++*,** 15.4 (6.2-31.4)	** 2.8 (1.2-4.5)	++*,*** 7.7 (3.6-15.5)
ICS N=15	26.0 (20.2-51.9)	++133.0 (35.0-335.0)	0.8 (0-1.3)	++*,**2.0 (1.1-3.2)	* 1.1 (0-1.9)	++*,**12.8 (3.0-47.8)	** 1.2 (0.8-3.7)	++*,**4.3 (1.8-36.3)

Note: One day 2 nasosorption™ sample was missing from a moderate asthmatic. One day 7 nasal sample was missing from a different moderate asthmatic subject.

Table E4. Bronchial levels of cytokines measured by bronchosorption™. Values represent median and interquartile range. (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ compared to healthy subjects; (+, $P < 0.05$; ++, $P < 0.01$; +++, $P < 0.001$ day 4 compared to baseline).

	IL-33 (ng/mL)		IL-4 (pg/mL)		IL-5 (pg/mL)		IL-13 (pg/mL)	
	Baseline	Day 4	Baseline	Day 4	Baseline	Day 4	Baseline	Day 4
Healthy N=11 (baseline) N=10 (day 4)	1.50 (1.13-3.02)	2.35 (1.87-4.22)	0 (0-0.8)	0 (0-0)	0 (0-0.6)	0 (0-0.8)	0 (0-0)	0 (0-1.5)
All Asthma N=25	1.83 (1.16-4.62)	1.84 (0.69-4.37)	0 (0-0.9)	0 (0-1.0)	0.7 (0-1.3)	+* 1.1 (0-1.9)	0.6 (0-1.2)	** 0.7 (0-1.2)
Severity								
Mild N=10 (baseline) N=9 (day 4)	2.18 (1.26-4.45)	3.83 (0.83-4.35)	0 (0-1.2)	* 0.7 (0-1.9)	0.8 (0-2.5)	* 1.1 (0.5-2.0)	* 0.9 (0-1.4)	* 1.0 (0-1.5)
Moderate N=15 (baseline) N=16 (day 4)	1.76 (1.03-5.68)	1.77 (0.68-4.65)	0 (0-0.6)	0 (0-0.9)	0.3 (0-1.0)	+ 0.8 (0-1.9)	0 (0-0.8)	* 0.6 (0-1.1)
Treatment								
Steroid naïve N=12 (baseline) N=11 (day 4)	2.00 (1.11-4.33)	3.92 (0.92-4.33)	0 (0-1.2)	* 0.7 (0-2.1)	0.7 (0-2.2)	* 1.2 (0.7-2.3)	* 0.8 (0-1.4)	** 1.1 (0-1.9)
ICS N=13 (baseline) N=14 (day 4)	1.79 (1.12-5.89)	1.69 (0.67-4.87)	0 (0-0.6)	0 (0-0.8)	0.6 (0-1.0)	0.8 (0-1.6)	0 (0-0.9)	* 0.6 (0-1.1)

Note: Baseline bronchosorption™ samples were not obtained from 3 asthmatics; day 4 bronchosorption™ samples were not obtained from 3 asthmatic and 1 healthy subject (numbers for each subgroup shown in table).

Table E5. Correlations between type 2 cytokines and respiratory symptom severity. The correlations shown are for asthmatics only. The 'peak' nasal level refers to the maximal level for each subject during the infection period. The 'total' respiratory score refers to the summation of scores over the 14 days post-inoculation (as seen in manuscript figure 1A and B) and represents the severity of the cold or exacerbation.

	Total Upper Respiratory Symptoms	Total Lower Respiratory Symptoms
Peak Nasal IL-5	$r = 0.55, P = 0.003$	$r = 0.37, P = 0.057$
Peak Nasal IL-13	$r = 0.47, P = 0.014$	$r = 0.20, P = 0.321$
Day 4 Bronchial IL-5	$r = 0.43, P = 0.034$	$r = 0.38, P = 0.059$
Day 4 Bronchial IL-13	$r = 0.51, P = 0.009$	$r = 0.42, P = 0.036$

Note: The symptom diary was missing from one asthmatic subject.

Table E6. Individual serum rhinovirus-16 antibody titres.

Subject ID	Asthma Status	Day 0 RV16 Antibody titre	Day 42 RV16 Antibody titre
40	Healthy	0	1:16
45	Healthy	0	1:4
48	Healthy	0	1:32
47	Healthy	0	0
56	Healthy	0	>1:64
101	Healthy	0	1:32
100	Healthy	0	>1:64
81	Healthy	0	0
114	Healthy	0	1:32
119	Healthy	0	>1:64
116	Healthy	0	1:32
39	Asthma	0	1:64
67	Asthma	0	1:64
92	Asthma	0	1:16
121	Asthma	0	1:8
111	Asthma	0	>1:64
129	Asthma	0	1:16
124	Asthma	0	0
97	Asthma	0	1:64
142	Asthma	0	1:64
139	Asthma	0	1:32
144	Asthma	0	1:4
148	Asthma	0	1:16
146	Asthma	0	1:16
21	Asthma	0	1:16
75	Asthma	0	1:16
61	Asthma	0	1:16
73	Asthma	0	1:4
85	Asthma	0	>1:64
86	Asthma	0	1:64
60	Asthma	0	1:32
105	Asthma	0	1:64
104	Asthma	0	1:32
126	Asthma	0	>1:64
136	Asthma	0	1:32
113	Asthma	0	>1:64
125	Asthma	0	>1:64
134	Asthma	0	>1:64
132	Asthma	0	1:4