ORIGINAL ARTICLE

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

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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

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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 (±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 FEV_1 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-naive 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 ± 12	36 ± 11	NS
Sex			
Female, n (%)	4 (36)	15 (54)	NS
Male, n (%)	7 (74)	13 (46)	
Baseline FEV_1, % predicted	104 ± 8	86 ± 12	<0.001
Baseline histamine PC_{20}, mg/ml	>16	1.26 ± 2.01	—
ICS use, n (%)	—	15 (53.6)	—
ICS daily dose, beclomethasone/equivalent, μg*	—	427 ± 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 ± SD unless otherwise stated.
*Mean ± SD data of steroid-treated subjects, n = 15.

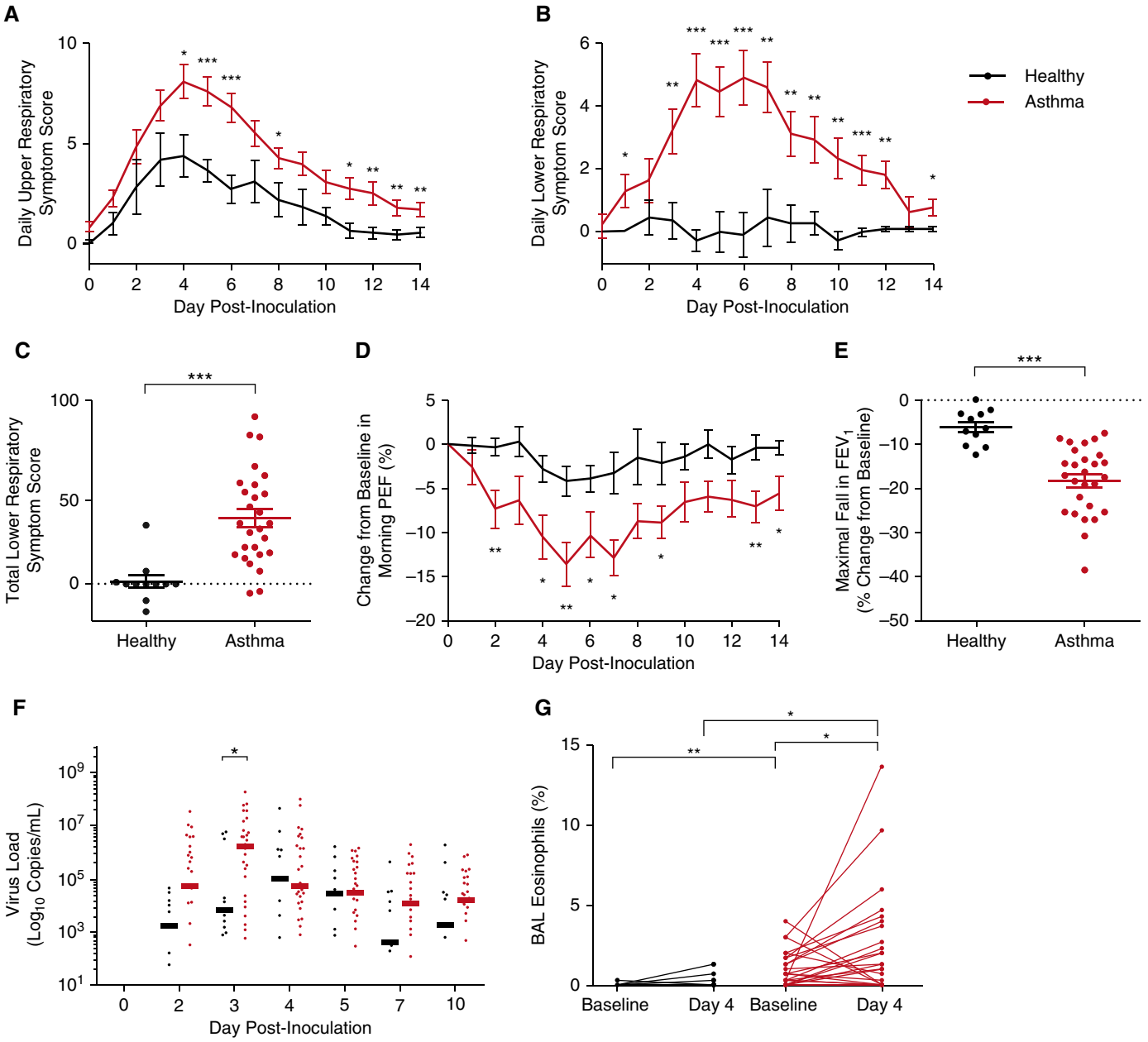

Figure 1. Rhinovirus infection results in more severe upper and lower respiratory tract involvement, greater viral loads, and bronchial eosinophilia in asthma. Shown are the daily change from baseline in upper (*A*) and lower (*B*) respiratory symptoms of subjects with asthma (*red*) and healthy volunteers (*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 FEV_1 (*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 ± 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-naive 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

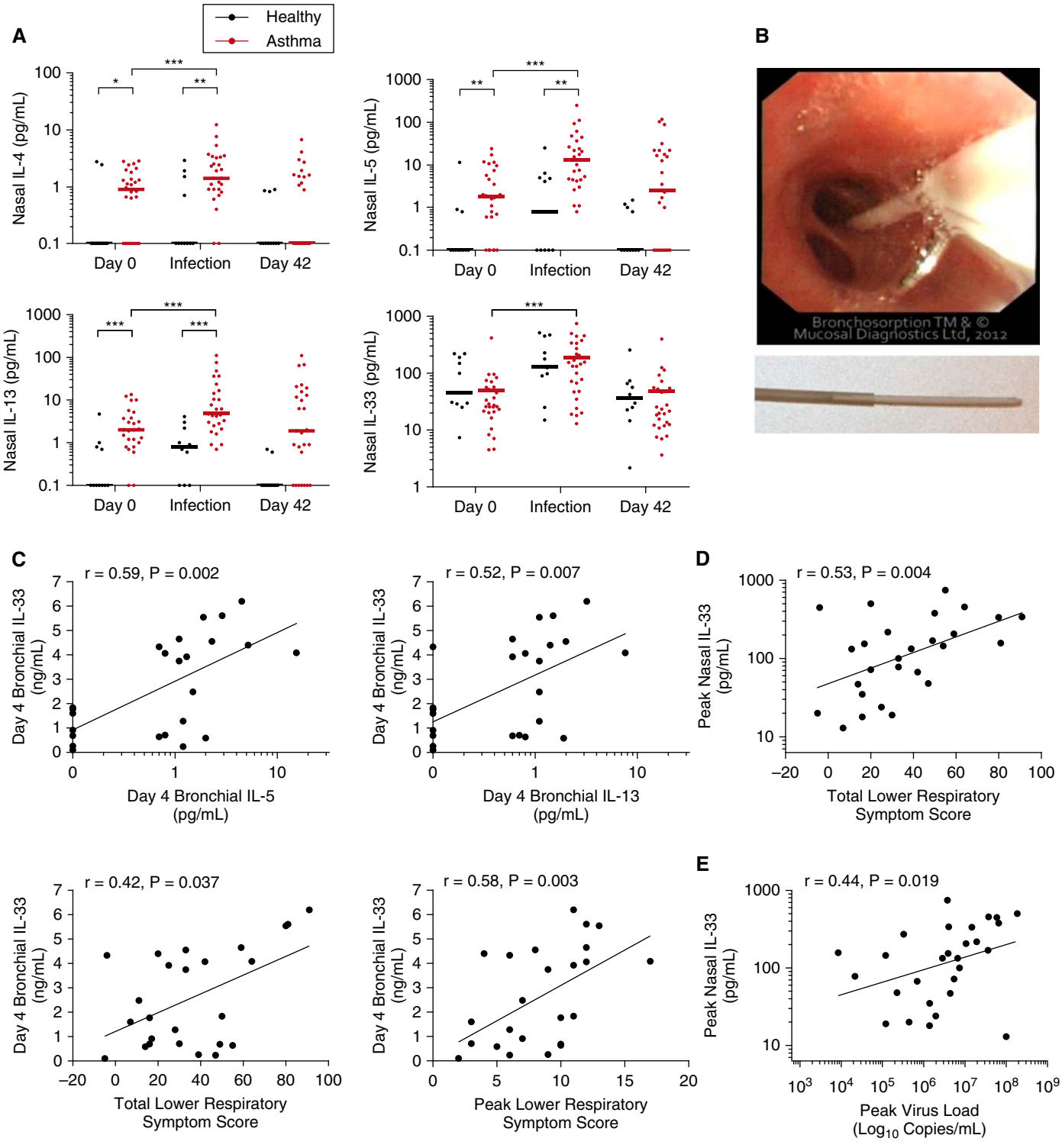

Figure 2. Rhinovirus infection in asthma leads to the induction of IL-33 and type 2 cytokines *in vivo*. Nasal levels of IL-33 and type 2 cytokines were measured by nasosorption (*A*) in subjects with asthma (*red*) and healthy subjects (*black*). The bronchosorption device (*B*) uses a strip of synthetic 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). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

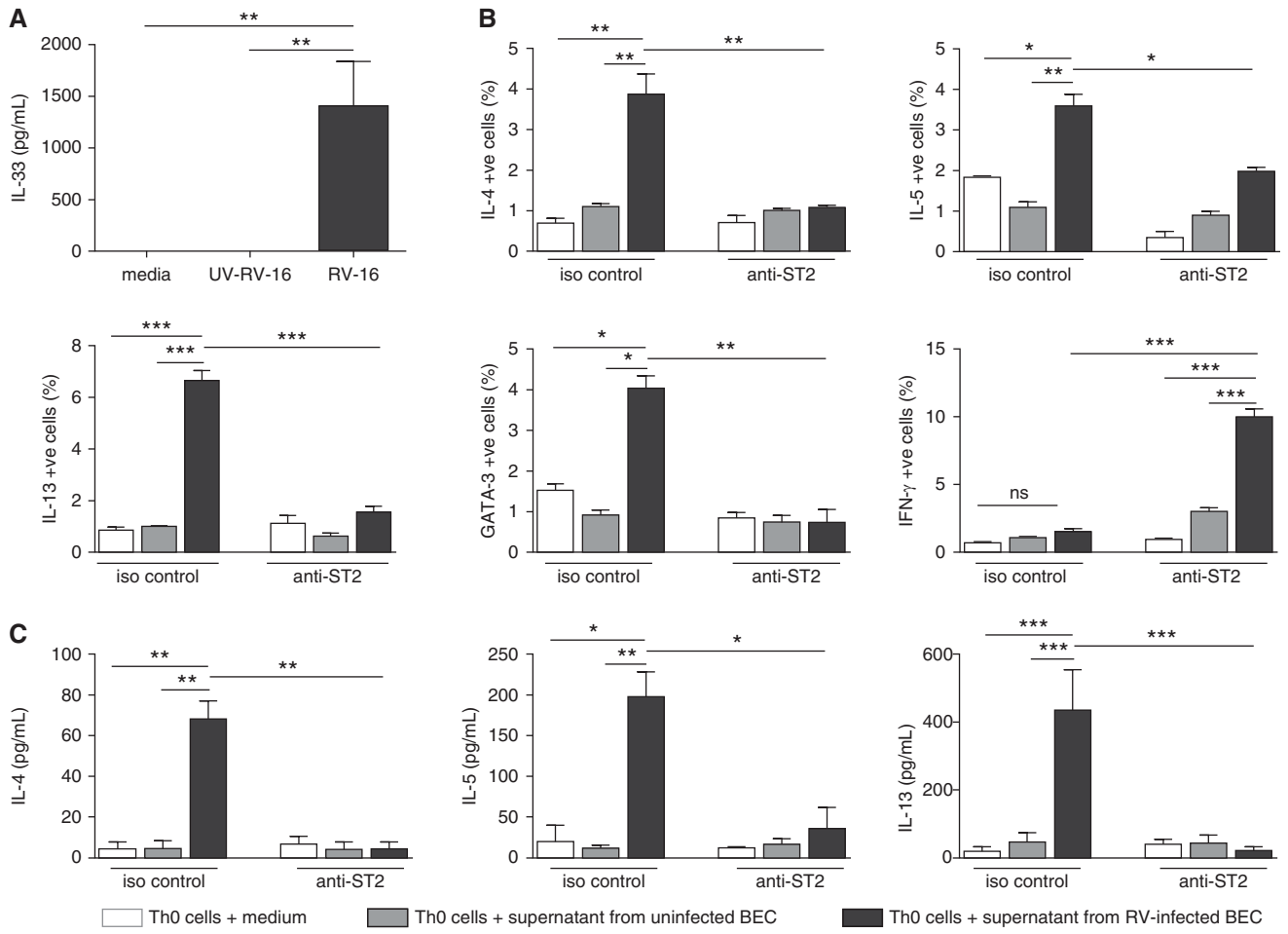

Figure 3. Rhinovirus infection of BECs induces IL-33, which subsequently induces type 2 cytokine production by human T cells and ILC2s. (*A*) Levels of IL-33 in BEC supernatants 24 hours after rhinovirus 16 infection or culture with ultraviolet light–inactivated rhinovirus 16 or medium control. To determine 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 (αST2) 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 ± 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,

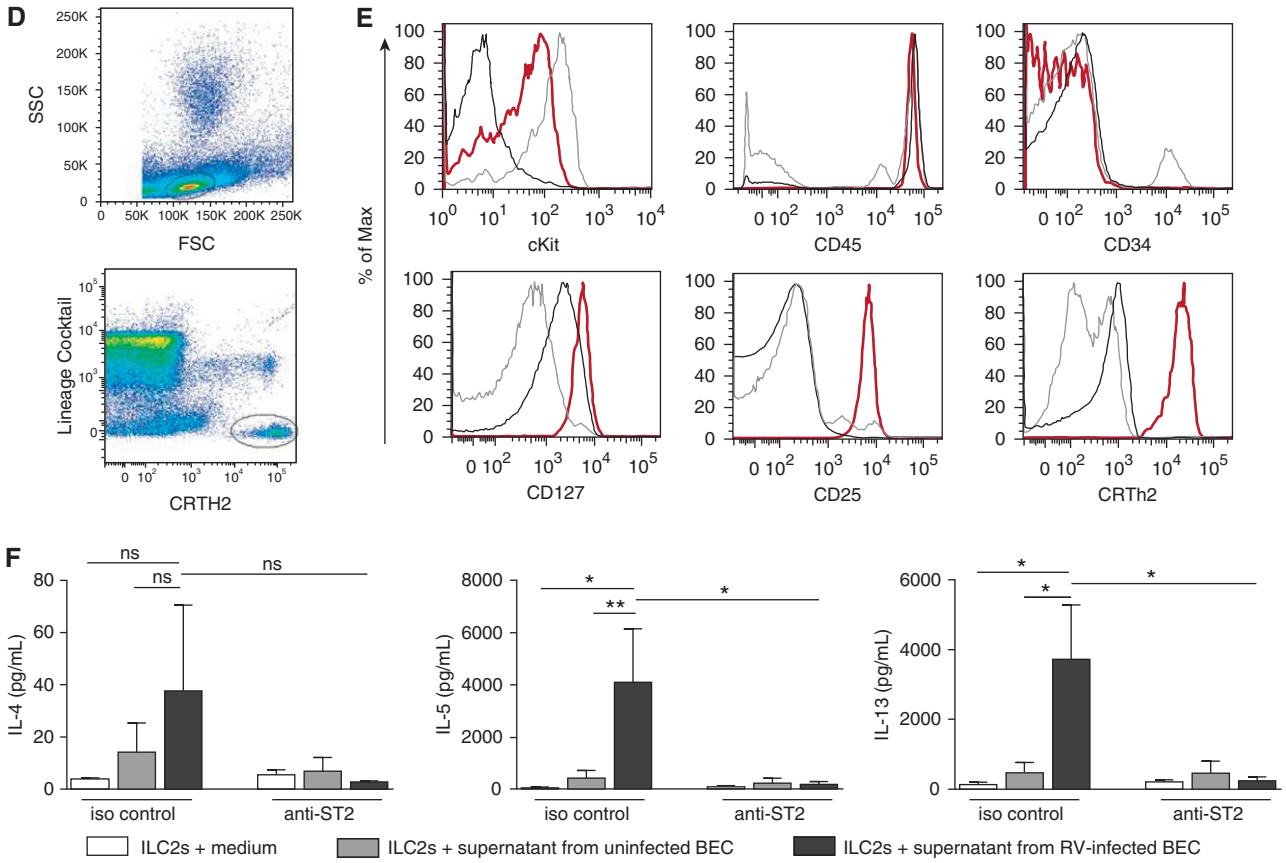

Figure 3. (Continued).

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 D_2 (PGD_2) 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, PGD_2, and IL-25 enhanced type 2 cytokine production by ILC2s in a synergistic manner (32), and Xue and colleagues demonstrated that ILC2 activation via PGD_2 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 PGD_2 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. ■

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