

Airway epithelial cells activate T_H2 cytokine production in mast cells through IL-1 and thymic stromal lymphopoietin

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Background: Airway epithelial cells are important regulators of innate and adaptive immunity. Although mast cells are known to play a central role in manifestations of allergic inflammation and are found in the epithelium in patients with T_H2-related diseases, their role is incompletely understood.

Objectives: The objective of this study was to investigate the role of airway epithelial cells in the production of T_H2 cytokines in mast cells.

Methods: Normal human bronchial epithelial (NHBE) cells were stimulated with TNF, IL-4, IFN- γ , IL-17A, and double-stranded RNA (dsRNA) alone or in combination. Human mast cells were stimulated with epithelial cell-derived supernatants or cocultured with NHBE cells. T_H2 cytokine responses were blocked with neutralizing antibodies.

Results: Supernatants from IL-4- and dsRNA-stimulated NHBE cells significantly enhanced T_H2 cytokine production from mast cells. The combination of IL-4 and dsRNA itself or supernatants from NHBE cells stimulated with other cytokines did not activate mast cells, suggesting that mast cell responses were induced by epithelial cell factors that were only induced by IL-4 and dsRNA. Epithelial supernatant-dependent T_H2 cytokine production in mast cells was suppressed by anti-IL-1 and anti-thymic stromal lymphopoietin (TSLP) and was enhanced by anti-IL-1 receptor antagonist. Similar results were observed in coculture experiments. Finally, we found dsRNA-dependent production of IL-1, TSLP, and IL-1 receptor antagonist in NHBE cells was regulated by T_H cytokines, and their ratio in NHBE cells correlated with T_H2 cytokine production in mast cells.

Conclusions: Pathogens producing dsRNA, such as respiratory viral infections, might amplify local T_H2 inflammation in asthmatic patients through the production of TSLP and IL-1 by epithelial cells and subsequent activation of T_H2 cytokine

production by mast cells in the airways. (J Allergy Clin Immunol 2012;■■■:■■■-■■■.)

Key words: Epithelial cells, mast cells, virus, asthma, T_H2 cytokine, IL-1, thymic stromal lymphopoietin

Asthma is a chronic airway disease affecting 8.2% of the US population¹ and is characterized by increased airway inflammation, mucus oversecretion, airway remodeling, and increased bronchoconstrictor responses to provoking agonists.² Respiratory viruses that bind to the airway epithelium and initiate airway inflammation are key triggers of asthma exacerbations in children and adults, although the precise triggering mechanisms are not known.³⁻⁵ Among the infiltrating inflammatory cells, eosinophils, T_H2 cells, and mast cells are classical features of asthma.⁶⁻⁸ Of these, mast cells, are well known to express the high-affinity IgE receptor Fc ϵ RI.⁹ Upon cross-linking of Fc ϵ RI, mast cells degranulate and release a wide variety of mediators, such as histamine and cysteinyl leukotrienes, promoting immediate allergic responses, and cytokines that induce late-phase inflammation, including eosinophilia and mucus production.^{9,10} Mast cells can also be activated by non-IgE-mediated mechanisms, including Toll-like receptor (TLR) ligands, neuropeptides, and proinflammatory cytokines.¹¹⁻¹³ Upon activation, these agents enhance the production of cytokines and mediators that can subsequently augment airway inflammation. Recently, mast cells have been documented to accumulate in the airway epithelium of T_H2-high asthmatic patients and correlate with severity of the disease.^{14,15} However, the interaction of mast cells with airway epithelial cells and their role in virus-induced airway inflammation are not well understood.

Airway epithelial cells are among the first responders to invading allergens and viruses and mount an immediate innate response to counter an infection.¹⁶ Respiratory virus-infected airway epithelial cells have been reported to produce IL-8, IL-6, and eotaxins,¹⁶⁻¹⁸ indicating that apart from being a natural barrier to pathogens, epithelial cells play a seminal role in the initiation and amplification of innate immune responses to viral infections. We have previously shown that the cytokine thymic stromal lymphopoietin (TSLP) is synergistically induced by the combination of the T_H2 cytokine IL-4 and either double-stranded RNA (dsRNA), a TLR3 ligand, and a viral mimic or rhinovirus infection in primary human bronchial epithelial cells in a TLR3- and signal transducer and activator of transcription 6-dependent manner.¹⁹ TSLP is an IL-7-like cytokine derived mainly from epithelial cells that activates immature dendritic cells to express the OX40 ligand and causes preferential differentiation of naive T cells into T_H2 cells.^{16,20-22} TSLP expression has been associated

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Abbreviations used

dsRNA: Double-stranded RNA
 IL-1Ra: IL-1 receptor antagonist
 IMDM: Iscove modified Dulbecco medium
 IV: Influenza virus
 2-ME: 2-Mercaptoethanol
 MOI: Multiplicity of infection
 NHBE: Normal human bronchial epithelial
 SCF: Stem cell factor
 TLR: Toll-like receptor
 TSLP: Thymic stromal lymphopoietin

with T_H2-mediated diseases, including asthma, colocalizing to the bronchial epithelium of patients.^{20,23} Although TSLP alone cannot stimulate human mast cells *in vitro*, TSLP synergistically enhances IL-5 and IL-13 release in mast cells that are exogenously treated with IL-1 and TNF.¹¹ IL-1 α and IL-1 β are proinflammatory cytokines derived primarily from monocytes and macrophages, can be produced from airway epithelial cells, and bind to a common heterodimeric receptor consisting of the IL-1R1 and IL-1 receptor accessory protein.²⁴⁻²⁶ IL-1 receptor antagonist (IL-1Ra) negatively regulates the activity of IL-1 α and IL-1 β by binding IL-1R1 and preventing heterodimerization of the receptor, thus inhibiting signal transduction.²⁵

In light of the above studies, it is conceivable that epithelial cells can create a microenvironment that profoundly activates mast cells to produce cytokines. The specific role of the epithelium in the direct activation of mast cells in the context of a T_H2 environment and a viral stimulus has not been investigated. In the current study we demonstrate that epithelial cells can produce a combination of cytokines that directly activates human mast cells in an allergic (ie, T_H2) environment. Our findings aim to provide a possible mechanism for increased airway inflammation in asthmatic patients after exposure to a respiratory virus infection.

METHODS**Cell culture**

Primary normal human bronchial epithelial (NHBE) cells (purchased from Lonza, Walkersville, Md) were seeded in collagen-coated plates and maintained in serum-free bronchial epithelial cell growth medium (Lonza).¹⁹ Human peripheral blood-derived mast cells were obtained as described previously,²⁷ and details can be found in the **Methods** section in this article's Online Repository at www.jacionline.org. Submerged NHBE cells were stimulated with IL-4, TNF, IL-17A (100 ng/mL each), or IFN- γ (10 ng/mL) alone or in combination with dsRNA (5 μ g/mL) for 72 hours. The cell-free supernatants (20% volume) were overlaid on mast cells (5 $\times$ 10⁵ cells/mL) for 48 hours. In some experiments mast cells were stimulated with IL-1 α or IL-1 β (10 ng/mL each) alone or in combination with TSLP (10 ng/mL). In select experiments supernatants from IL-4 plus dsRNA-stimulated NHBE cells (10% volume) were overlaid on mast cells along with neutralizing antibodies to IL-1 α (10 μ g/mL), IL-1 β (10 μ g/mL), TSLP (32 μ g/mL), IL-1Ra (10 μ g/mL), or control IgG for 48 hours. In coculture experiments mast cells were cultured with NHBE cells in medium containing 50% bronchial epithelial cell growth medium and 50% mast cell culture medium and then stimulated with IL-4 alone or in combination with dsRNA or influenza A virus strain A/Udorn/1972 (H3N2; multiplicity of infection [MOI], 0.1).

ELISA and cytometric bead array

The concentrations of IL-1 α , IL-1 β , IL-5, and IL-13 in cell-free supernatants were measured by using cytometric bead array flex sets from BD

Biosciences (San Jose, Calif). The limit of detection for each is 2.5 pg/mL. The concentrations of IL-1Ra and TSLP were assayed by using ELISA (R&D Systems, Minneapolis, Minn), and the limits of detection are 39.1 and 15.6 pg/mL, respectively.

Statistical analysis

All data are reported as means $\pm$ SEMs unless otherwise noted. Differences between groups were analyzed by using 1-way ANOVA or the paired Student *t* test, where indicated, and considered significant at a *P* value of less than .05 unless otherwise indicated. Correlations were assessed by using Spearman rank correlation.

RESULTS**Soluble factors from airway epithelial cells induce T_H2 cytokine production in mast cells**

NHBE cells were stimulated with cytokine alone or in combination with dsRNA for 72 hours. We found that only the supernatants from IL-4 plus dsRNA-stimulated NHBE cells significantly enhanced production of IL-5 (87.1 $\pm$ 58.2 pg/mL) and IL-13 (16.3 $\pm$ 6.8 pg/mL) by human mast cells compared with the medium control (undetectable; *P* < .05, *n* = 9; Fig 1, A and B). It has previously been reported that TLR3 and IL-4 receptors are expressed on human mast cells.^{12,28} We therefore next examined whether IL-4 and dsRNA would directly promote T_H2 cytokine production in mast cells. Interestingly, enhancement of IL-5 production in mast cells was only observed in IL-4 plus dsRNA-stimulated NHBE cell supernatants but not after direct stimulation with IL-4, dsRNA, or the combination (*n* = 3-5; Fig 1, C). We next examined whether IL-4 plus dsRNA-stimulated NHBE cell supernatants also induced degranulation of mast cells and found that these supernatants did not induce β -hexosaminidase release (*n* = 3, see Fig E1 in this article's Online Repository at www.jacionline.org). These results suggest that only soluble factors from NHBE cells directly enhance T_H2 cytokine release by mast cells, and hence we focused on cytokines produced by NHBE cells.

IL-1 is a critical factor for NHBE cell-dependent T_H2 cytokine production in mast cells

It has been reported that IL-1 α and IL-1 β can amplify T_H2 cytokine production in mast cells¹¹; we therefore used neutralizing antibodies to IL-1 α and IL-1 β . We found that anti-IL-1 α significantly suppressed IL-5 production (2.3 $\pm$ 0.7 pg/mL) in mast cells compared with the IL-4 plus dsRNA-stimulated NHBE cell supernatant alone (25.4 $\pm$ 10.5 pg/mL) and the goat control IgG (79.7 $\pm$ 17.6 pg/mL), suggesting that IL-1 α from stimulated NHBE cells was required for the IL-5 response in mast cells (*P* < .05, *n* = 7; Fig 2, A, and see Fig E2 in this article's Online Repository at www.jacionline.org). Similarly, anti-IL-1 β antibody significantly suppressed NHBE cell supernatant-dependent IL-5 induction in mast cells (9.9 $\pm$ 2.0 pg/mL) compared with IL-4 plus dsRNA-stimulated NHBE cell supernatant alone (14.2 $\pm$ 1.3 pg/mL) and the goat control IgG (55.9 $\pm$ 26.1 pg/mL), indicating that IL-1 β was also required for the epithelial cell-induced IL-5 response (*P* < .05, *n* = 6; Fig 2, B, and see Fig E2). We also found that IL-4 plus dsRNA-stimulated NHBE cell supernatant-dependent IL-13 production was significantly inhibited by anti-IL-1 α and anti-IL-1 β in mast cells (*n* = 4, data not shown).

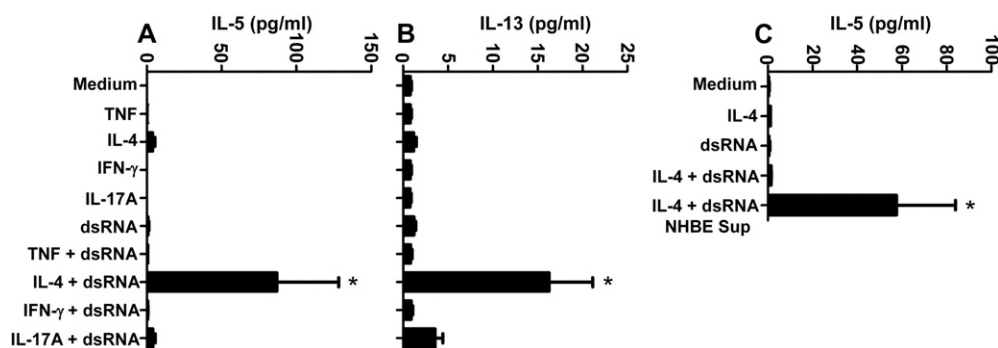


FIG 1. IL-4 plus dsRNA-stimulated NHBE cells promote T_H2 cytokine production in mast cells. NHBE cells were stimulated with 100 ng/mL TNF, 100 ng/mL IL-4, 10 ng/mL IFN- γ , and 100 ng/mL IL-17A alone or in combination with 5 μ g/mL dsRNA for 72 hours. Mast cells were then stimulated with derived supernatants (20% volume) for 48 hours (A and B). Mast cells were stimulated directly with IL-4 and dsRNA or with NHBE supernatants (NHBE Sup; C). IL-5 (Fig 1, A and C) and IL-13 (Fig 1, B) production was assessed by using the cytometric bead array. Results shown are means $\pm$ SEMs of 9 (Fig 1, A and B) or 3 to 5 (Fig 1, C) independent experiments with 3 NHBE cell donors and 3 mast cell donors. * $P < .05$ compared with all other groups, 1-way ANOVA.

Although airway epithelial cells are known to release IL-1 α and IL-1 β , regulation of these cytokines is not well elucidated. We therefore determined whether IL-1 α and IL-1 β were induced by dsRNA and cytokines in NHBE cells. We found that production of IL-1 α and IL-1 β was not upregulated by dsRNA (IL-1 α , 176.6 $\pm$ 22.6 pg/mL; IL-1 β , 35.5 $\pm$ 10.1 pg/mL) or induced by the individual cytokines tested compared with the medium control (IL-1 α , 61.2 $\pm$ 11.8 pg/mL; IL-1 β , 4.1 $\pm$ 0.6 pg/mL; $n = 8$, Fig 3). Interestingly, TNF (370.6 $\pm$ 79.9 pg/mL), IL-4 (318.1 $\pm$ 44.0 pg/mL), IFN- γ (908.3 $\pm$ 177.8 pg/mL), and IL-17A (617.7 $\pm$ 63.8 pg/mL) significantly enhanced dsRNA-dependent IL-1 α production in NHBE cells ($P < .05$, $n = 8$; Fig 3, A). In contrast to the induction of IL-1 α , only IL-17A plus dsRNA significantly induced IL-1 β production (128.2 $\pm$ 38.3 pg/mL) in NHBE cells ($P < .05$, $n = 8$; Fig 3, B).

TSLP enhances NHBE cell-dependent T_H2 cytokine response in mast cells

It has been previously reported that TSLP enhances IL-1-dependent T_H2 cytokine production from mast cells (Fig 2, A).¹¹ To determine whether TSLP contributes to the IL-5 response in mast cells induced by supernatants from IL-4 plus dsRNA-stimulated NHBE cells, we examined the effect of a neutralizing antibody against TSLP. We found that IL-4 plus dsRNA-stimulated NHBE cell supernatant-dependent IL-5 production was significantly attenuated by anti-TSLP but not by murine control IgG₁ in mast cells ($P < .05$, $n = 4$; Fig 2, C). This result suggests that TSLP is one of the factors amplifying the IL-5 response in mast cells. We confirmed our previous findings¹⁹ that IL-4 synergistically enhanced dsRNA-dependent TSLP production in NHBE cells (354.2 $\pm$ 39.6 pg/mL, $P < .05$, $n = 6$; Fig 3, D). Interestingly, IL-17A and IFN- γ significantly suppressed dsRNA-induced TSLP production.

IL-1/IL-1Ra ratio in airway epithelial cells is a critical factor for T_H2 cytokine production in mast cells

On the basis of the above results, we determined whether the concentration of IL-1 and TSLP in supernatants of IL-4 plus

dsRNA-stimulated NHBE cells correlated with IL-5 responses in mast cells. We found no individual correlation between the levels of TSLP, IL-1 α , or IL-1 β in NHBE cells and the levels of IL-5 production in mast cells (Fig 4, A-C). In addition, the observed increases in IL-1 α and IL-1 β levels in NHBE cells stimulated with dsRNA plus IFN- γ , IL-17A, or TNF did not correlate with the corresponding IL-5 levels in mast cells (Figs 1 and 3). We hypothesized that the activity of IL-1 might be regulated by an inhibitor, IL-1Ra, simultaneously produced by epithelial cells. We found that IL-1Ra production in NHBE cells was significantly induced by TNF plus dsRNA (59.4 $\pm$ 7.3 ng/mL) and IFN- γ plus dsRNA (45.2 $\pm$ 7.3 ng/mL) but not by dsRNA alone or the cytokines tested compared with the medium control (3.9 $\pm$ 0.3 ng/mL; $P < .05$, $n = 9$; Fig 3, C). The concentration of IL-1Ra in IL-4 plus dsRNA-stimulated NHBE cell supernatants significantly and inversely correlated with the IL-5 response in mast cells ($P = .015$, $r = -0.758$; Fig 4, D). These results suggest that the induction of IL-1Ra could be involved in negatively regulating the activity of IL-1 in NHBE cell supernatants, thus inhibiting IL-5 production in mast cells treated with dsRNA plus IFN- γ or dsRNA plus TNF-stimulated NHBE cell supernatants. Furthermore, the ratio of IL-1 α /IL-1Ra ($P < .001$, $r = 0.951$) and IL-1 β /IL-1Ra ($P = .016$, $r = 0.733$) in NHBE cells correlated strongly and positively with IL-5 production in mast cells (Fig 4, E and F). This suggests that the ratio of IL-1 to IL-1Ra in NHBE cell supernatants is a key determinant and that TSLP is a potent enhancer of the induction of IL-5 in mast cells. Only supernatants from IL-4 plus dsRNA-stimulated NHBE cells contain the positive IL-1/IL-1Ra ratio and sufficient TSLP to induce T_H2 cytokine production in mast cells.

IL-1 and TSLP enhance IL-5 response in mast cells when cocultured with NHBE cells

To verify whether mast cells could be activated by NHBE cells, we grew NHBE cells to confluence, added mast cells, and cultured them together for 72 hours. The combination of IL-4 and dsRNA significantly and synergistically enhanced IL-5 production (632.3 $\pm$ 152.8 pg/mL, $P < .05$, $n = 6$) compared with medium (0.79 $\pm$ 0.26 pg/mL), IL-4 (140.1 $\pm$ 35.9 pg/mL), or dsRNA

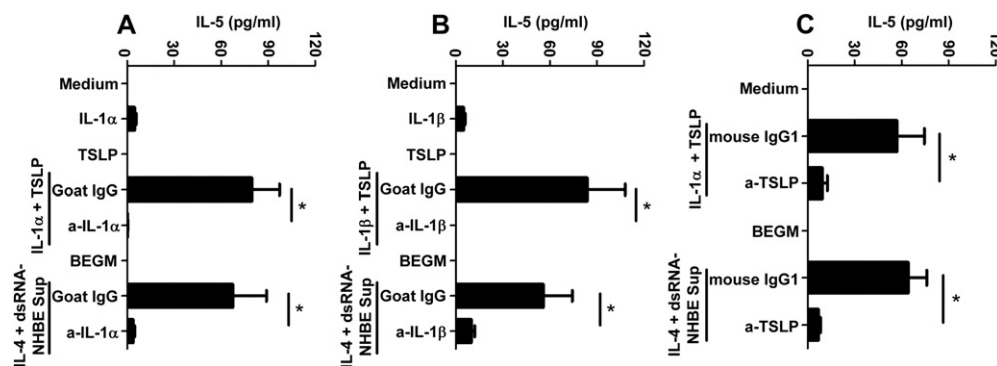


FIG 2. Neutralization of IL-1 and TSLP suppresses NHBE cell supernatant-induced IL-5 production in mast cells. Mast cells were stimulated with 10 ng/mL IL-1 α , 10 ng/mL IL-1 β , and 10 ng/mL TSLP; epithelial culture medium (bronchial epithelial cell growth medium [BEGM], 10% volume); or IL-4 plus dsRNA-stimulated NHBE cell supernatants (NHBE Sup; 10% volume) for 48 hours along with neutralizing antibodies against IL-1 α (10 μ g/mL, **A**), IL-1 β (10 μ g/mL, **B**), TSLP (32 μ g/mL, **C**), or respective isotype controls. IL-5 responses were measured by using the cytometric bead array. Results shown are means $\pm$ SEMs of 7 (Fig 2, **A**), 6 (Fig 2, **B**), or 4 (Fig 2, **C**) independent experiments with 2 NHBE cell donors and 6 mast cell donors. * P < .05, paired Student t test.

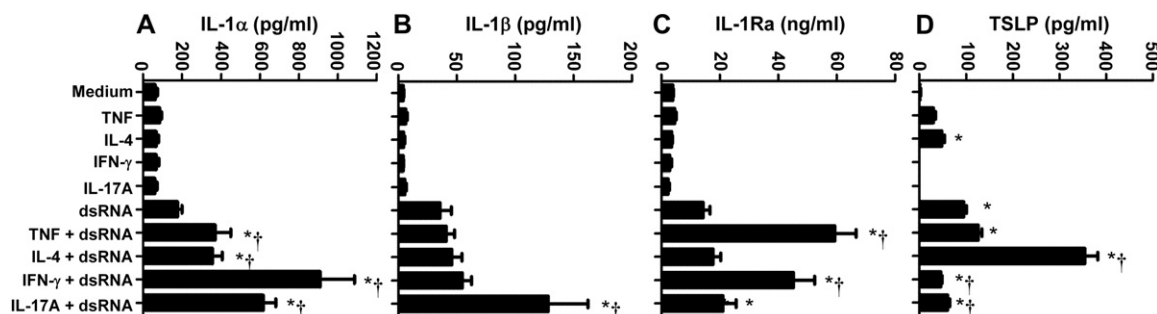


FIG 3. Induction of cytokines after NHBE cell stimulation. NHBE cells were stimulated with 100 ng/mL IL-4, 10 ng/mL IFN- γ , 100 ng/mL IL-17A, and 100 ng/mL TNF alone or in combination with 5 μ g/mL dsRNA for 72 hours. Concentrations of cytokines were measured by using the cytometric bead array (IL-1 α [**A**] and IL-1 β [**B**]) or ELISA (IL-1Ra [**C**] and TSLP [**D**]). Results shown are means $\pm$ SEMs of 8 (Fig 3, **A** and **B**), 9 (Fig 3, **C**), and 6 (Fig 3, **D**) independent experiments with 5 NHBE cell donors. * P < .05, different from medium; † P < .05, different from dsRNA, 1-way ANOVA

(34.8 $\pm$ 14.2 pg/mL) treatment (Fig 5, **A**). We also found that IL-4 plus dsRNA dose-dependently enhanced IL-5 production (Fig 5, **B**). Importantly, a concentration of IL-4 as low as 1 ng/mL was sufficient to enhance IL-5 production in the presence of dsRNA. To determine whether IL-1 and TSLP play an important role in the IL-5 response in the coculture system, we used neutralizing antibodies. As expected, we observed a significant reduction in the IL-5 response by neutralization of IL-1 α , IL-1 β , and TSLP and significant enhancement by anti-IL-1Ra compared with the control IgG (P < .05, n = 4–5; Fig 6). To test the effect of influenza virus (IV) infection, NHBE cells were infected with IV in the presence or absence of IL-4 and then cocultured with mast cells. We found that the combination of IL-4 and IV significantly enhanced IL-5 production in mast cells (P < .05, n = 5; Fig 7, **A**). Because we found that IV significantly induced IL-1Ra (see Fig E3 in this article's Online Repository at www.jacionline.org), we applied anti-IL-1Ra antibody and found that the combination of IL-4 and IV further enhanced IL-5 production in the presence of anti-IL-1Ra (P < .05, n = 5; Fig 7, **B**). These data suggest that in the context of a viral infection in a T_H2 -rich environment, which is typically prevalent in asthmatic patients, epithelial cells

could directly activate mast cells through the production of factors including TSLP, IL-1 α , and IL-1 β , which could further exacerbate airway inflammation. The data also suggest that IL-1Ra is an important mediator in negatively regulating this response.

DISCUSSION

The current study demonstrates that exposure of epithelial cells to IL-4 and dsRNA, a mimic of viral infection in a T_H2 environment, leads to production of factors that drive IL-5 and IL-13 secretion in mast cells (Fig 1). Our studies show that the mechanism of this response is through epithelial production of soluble factors, including IL-1 α , IL-1 β , and TSLP, which drive T_H2 cytokine production in mast cells. We also found that the combination of IL-4 and IV enhanced IL-5 production in mast cells cocultured with NHBE cells, suggesting a clinical situation in which this can occur (Fig 7). Finally, we show that the response is modulated by the production of IL-1Ra by the epithelial cells. The role of IL-5 in the accumulation, survival, and activation of eosinophils in the tissues and the roles of eosinophils in the pathophysiology of asthma are well known.^{29–31} IL-13 also activates eosinophils,

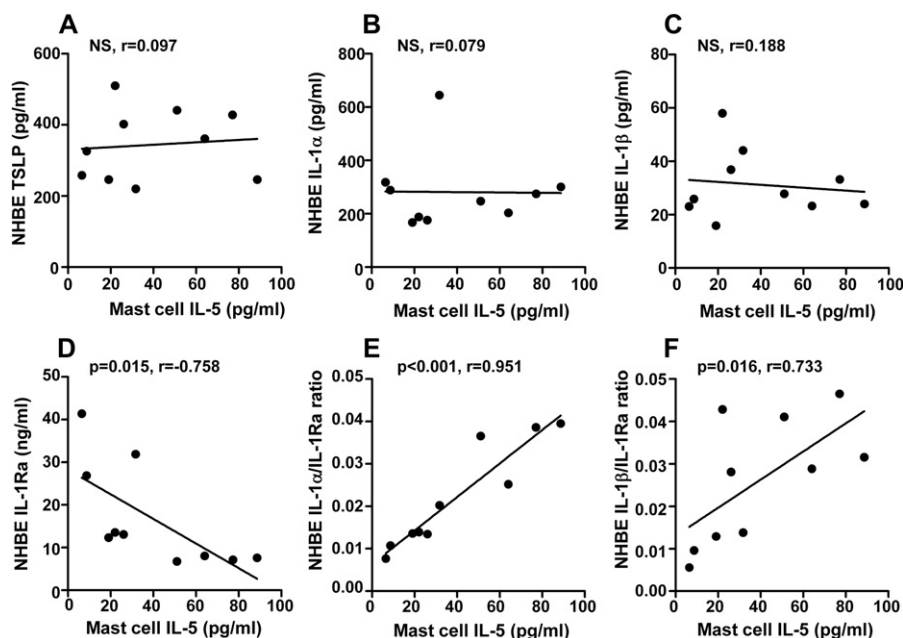


FIG 4. Correlation of cytokines TSLP (A), IL-1 α (B), IL-1 β (C), and the ratio of IL-1Ra (D) and IL-1/IL-1Ra (E and F) in supernatants from IL-4 plus dsRNA-stimulated NHBE cells with IL-5 production in mast cells. Data are representative of 2 mast cell donors and 3 NHBE cell donors ($n = 5$). The correlations were assessed by using the Spearman rank correlation. NS, Not significant.

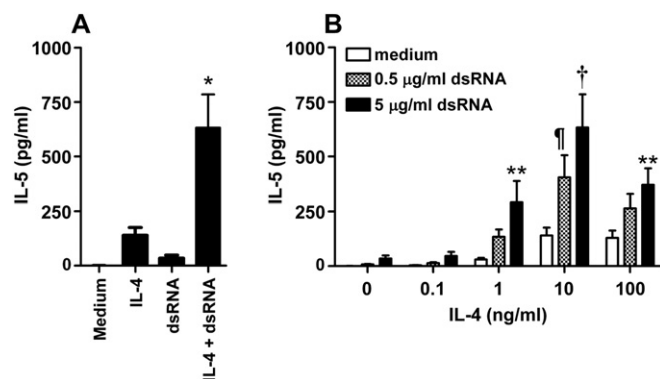


FIG 5. IL-4 and dsRNA dose-dependently and synergistically enhance IL-5 production in mast cells when cocultured with epithelial cells. Mast cells were overlayed on NHBE cells and then stimulated with 10 ng/mL IL-4 and 5 μ g/mL dsRNA (A) or at indicated concentrations (B) for 72 hours. Production of IL-5 was measured by using the cytometric bead array. Results shown are means $\pm$ SEMs of 6 independent experiments with 4 NHBE cell donors and 3 mast cell donors. * $P < .05$, 1-way ANOVA (Fig 5, A) and different from all groups; †different from medium, 0.1, 1, and 100 ng/mL IL-4; ** $P < .05$, compared with medium and 0.1 ng/mL IL-4; ‡ $P < .05$, compared with medium and 1 ng/mL IL-4, repeated-measures 1-way ANOVA Newman-Keuls test (Fig 5, B).

upregulates eotaxin expression, and plays a pivotal role in upregulating mucus secretion in the airways, one of the key features of exacerbations.³²⁻³⁴ Thus in the context of a T_H2 cytokine environment in which a viral infection occurs, asthmatic patients might be highly susceptible to the exacerbation of allergic inflammation through the production of factors including IL-1 and TSLP released from the airway epithelium, which further enhance IL-5 and IL-13 production in mast cells. We found that IL-5 production was 2.7- to 10-fold higher in studies using coculture of mast cells

and epithelial cells compared with exposure of mast cells to epithelial supernatants (Figs 1 and 5). These results can be explained by the fact that only 10% to 20% of the volume of NHBE cell supernatants was used to stimulate the mast cells. However, we cannot discount the effects of cell-to-cell contact, which could also result in the upregulation of receptors or mediators that might enhance IL-5 production.

In our studies we investigated the role of IL-1 and IL-1Ra in the activation of IL-5 production in mast cells by epithelial supernatants. Blocking IL-1 significantly attenuated the mast cell IL-5-inducing ability of supernatants from IL-4 plus dsRNA-stimulated NHBE cells (Figs 2 and 6). IL-1 α and IL-1 β are primarily derived from monocytes and, to a smaller extent, from epithelial and endothelial cells.²⁵ IL-1 β is expressed at higher levels in bronchoalveolar lavage fluid of patients with symptomatic asthma and is also detected in the bronchial epithelia of asthmatic patients.^{35,36} Additionally, IL-1 β levels are also increased in nasal secretions during rhinovirus-induced colds and in airway epithelial cells.^{37,38} Under normal conditions, IL-1 β synthesis and release are tightly controlled and cannot be detected in the circulation. In contrast to IL-1 β , the precursor to IL-1 α is constitutively expressed and serves as an autocrine growth factor.²⁵ During cellular injury, such as occurs during viral infections, necrotic cells release mature IL-1 α , which serves as a danger signal and promotes subsequent inflammation. Bronchial epithelial cells infected with respiratory viruses have been reported to release IL-1 α *in vitro*.^{24,26} In our studies IL-4 plus dsRNA-stimulated NHBE cells showed significantly higher levels of IL-1 α compared with cells stimulated with either IL-4 or dsRNA alone (Fig 3). However, production of IL-1 α and IL-1 β in NHBE cells did not correlate with IL-5 production in mast cells (Fig 4). Thus we investigated the role of IL-1Ra, which negatively regulates the activity of IL-1 α and IL-1 β . IL-1Ra has been reported to be significantly downregulated at baseline in nasal lavage samples of asthmatic

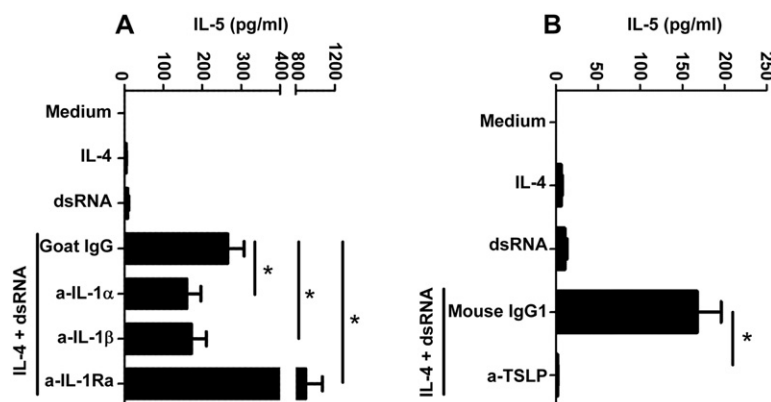


FIG 6. IL-4 and dsRNA enhance IL-5 production in mast cells when cocultured with NHBE cells in an IL-1- and TSLP-dependent manner. Mast cells were cocultured with NHBE cells and then stimulated with 10 ng/mL IL-4 and 5 μ g/mL dsRNA, along with neutralizing antibodies against IL-1 α , IL-1 β , IL-1Ra (all from **A**), TSLP (**B**), or isotype controls for 72 hours. IL-5 response was measured by using the cytometric bead array. Results shown are means $\pm$ SEMs of 5 (Fig 6, **A**) or 4 (Fig 6, **B**) independent experiments with 2 NHBE cell donors and 3 mast cell donors. * P < .05, paired Student t test.

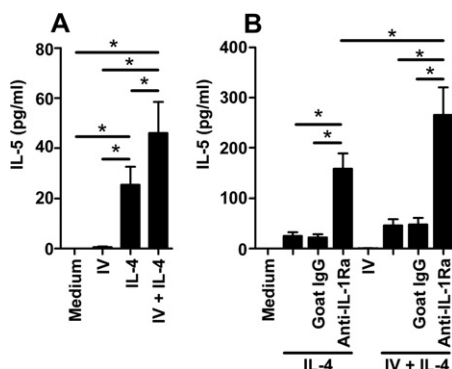


FIG 7. The combination of IL-4 and IV enhances IL-5 production from mast cells cocultured with NHBE cells. NHBE cells were stimulated with IV at an MOI of 0.1 and 10 ng/mL IL-4 along with neutralizing antibody to IL-1Ra at 20 μ g/mL or with 20 μ g/mL control goat IgG for 72 hours. Results shown are means $\pm$ SEMs of 5 independent experiments with 4 NHBE cell donors and 3 mast cell donors. * P < .05, 1-way ANOVA.

patients, suggesting that alterations of the ratio of IL-1 to IL-1Ra can occur in asthmatic patients.^{25,38} Interestingly, TNF and IFN- γ enhanced dsRNA-induced IL-1Ra production in NHBE cells (Fig 3), and these supernatants did not induce IL-5 production in mast cells (Fig 1). In addition, a strong positive correlation of the IL-5 response in mast cells with both the IL-1 α /IL-1Ra and IL-1 β /IL-1Ra ratios in IL-4 plus dsRNA-stimulated NHBE cells was observed. Finally, application of the anti-IL-1Ra antibody significantly enhanced the IL-5 response in mast cells after exposure to IL-4 plus dsRNA-stimulated NHBE cell supernatants, suggesting a role for endogenous IL-1Ra in NHBE cells in the regulation of IL-5 production by mast cells (Fig 6). This interplay between IL-1 and the IL-1Ra from epithelial cells and its role in regulation of T_H2 cytokines from mast cells is a novel mechanism by which viruses might modulate airway inflammation in asthmatic patients.

The finding that TSLP plays a role in the epithelial activation of an IL-5 response in mast cells is also of clinical interest. TSLP has been associated with airway obstruction in asthmatic patients and correlates with disease severity.²³ We have shown previously that

IL-4 synergistically enhances dsRNA- and rhinovirus-dependent TSLP expression in NHBE cells.¹⁹ Although the mechanisms are still not clear, published data suggest that synergistic effects could be the result of upregulation of the IL-4 receptor by dsRNA, the influence of a composite binding element for signal transducer and activator of transcription 6 and nuclear factor κ B in the TSLP promoter, or both.^{19,39} Bronchial epithelial cells from asthmatic patients produce higher amounts of TSLP in the response to dsRNA compared with cells from healthy subjects.⁴⁰ In our studies blocking TSLP caused a significant attenuation of the IL-4 plus dsRNA-stimulated NHBE cell-dependent IL-5 responses in mast cells (Figs 2 and 6). We note that low quantities (as little as 10% volume) of the stimulated NHBE cell supernatants, containing only 20 to 50 pg/mL TSLP, were sufficient to enhance T_H2 cytokine production in mast cells. This suggests that a modest induction of TSLP by the combination of an allergic stimulus and a viral infection could have important clinical implications for symptom worsening. On the other hand, IL-17A and IFN- γ strongly suppressed dsRNA-induced TSLP production in NHBE cells, which is consistent with studies performed in keratinocytes⁴¹ and nasal epithelial cells.⁴² Thus even with high levels of IL-1, the absence of TSLP production resulted in an insufficient activation of T_H2 cytokine responses in mast cells.

As discussed above, TSLP expression is increased in asthmatic bronchial epithelial cells, upon dsRNA stimulation,⁴⁰ and IL-1Ra has been reported to be downregulated at baseline in asthmatic patients with experimental rhinovirus infection.³⁸ It is therefore conceivable that bronchial epithelial cells in a T_H2 microenvironment in patients with asthma might be able to induce stronger IL-5 responses in mast cells than cells in healthy subjects during viral infection. Future studies will be required to determine the comparative effect of airway epithelial cells from patients with asthma in the interaction with mast cells. Our observations provide insight into possible therapeutic strategies to reduce allergic airways disease, including blockade of IL-1 α , IL-1 β , or TSLP, which are released from the airway epithelium in the presence of a viral stimulus and a T_H2 environment or maneuvers to increase IL-1Ra. Recombinant IL-1Ra (anakinra) has been approved for treatment of autoinflammatory diseases.⁴³ However,

as far as we are aware, there is no information about the utility of this drug in the prevention of exacerbations of asthma. Our findings suggest that targeting IL-1, TSLP or its receptors clinically in the treatment of asthma will be worthy of detailed investigation.

The present study also underscores the importance of recent reports of mast cells located in the bronchial epithelium of asthmatic patients. Dougherty et al¹⁵ observed accumulation of mast cells in the bronchial epithelium of T_H2-high asthmatic patients, whereas Balzar et al¹⁴ observed that the density of mast cells in epithelial cells correlated with the severity of the asthma. Importantly, Dougherty et al¹⁵ identified T_H2-high asthma by using the levels of 3 IL-13-inducible genes, including periostin in the epithelium, but did not identify the T_H2 cytokine-producing cells.¹⁵ Earlier studies showed that IL5 mRNA⁺ mast cell numbers were increased in bronchial biopsy specimens of atopic asthmatic patients.⁴⁴ The role of mast cells in T_H2 cytokine production in asthmatic patients, particularly in those with asthma exacerbations, is therefore worthy of future investigation.

In summary, we report here that human bronchial epithelial cells directly enhanced T_H2 cytokine production in mast cells by secretion of factors including IL-1 and TSLP after stimulation with IL-4 and dsRNA (or respiratory virus). Because IL-1Ra competitively inhibited the activity of IL-1 α and IL-1 β , the ratio of IL-1 and IL-1Ra from epithelial cells also controlled T_H2 cytokine production in mast cells. These results provide a novel mechanism for the susceptibility of asthmatic patients to viral infections and could point the way toward novel therapeutic interventions in the treatment of exacerbations.

Clinical implications: Specific therapies that target TSLP or IL-1, such as antibodies, soluble receptors, or IL-1Ra, might have value in the treatment or prevention of virus-induced airway inflammation in T_H2 airways diseases, such as asthma.

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METHODS

Reagents

Recombinant human TNF, IL-1 α , IL-1 β , TSLP, IL-4, IFN- γ , and IL-17A and neutralizing goat antibodies to IL-1 α , IL-1 β , and IL-1Ra were purchased from R&D systems. Recombinant human IL-3, IL-6, and stem cell factor (SCF) were purchased from Peprotech (Rocky Hill, NJ), and dsRNA (poly(I:C)) was purchased from InvivoGen (San Diego, Calif). Murine anti-human TSLP mAb (clone: M385, IgG₁) was a kind gift from Amgen (Seattle, Wash).

Generation of mast cells from CD34 progenitor cells

Buffy coats from human peripheral blood were obtained from the American Red Cross (St Paul, Minn). CD34+ hematopoietic progenitor cells were separated from human PBMCs by using the magnetic cell sorting (MACS) system (Miltenyi Biotec, Auburn, Calif) with anti-CD34 MicroBeads (Miltenyi Biotec), according to the manufacturer's instructions. The cells were suspended in serum-free Iscove methylcellulose medium (MethoCult SFBIT H4236; Stemcell Technologies, Vancouver, Canada) containing 200 ng/mL SCF, 50 ng/mL IL-6, 5 ng/mL IL-3, 11 μ mol/L 2-mercaptoethanol (2-ME; Invitrogen, Carlsbad, Calif), 100 U/mL penicillin, and 100 μ g/mL streptomycin (Invitrogen) and then incubated at 37°C in 5% CO₂. At 2 weeks of culture, fresh methylcellulose medium containing 200 ng/mL SCF, 50 ng/mL IL-6, 11 μ mol/L 2-ME, 100 U/mL penicillin, and 100 μ g/mL streptomycin was layered over the methylcellulose cultures. At 4 weeks, a 1-mL aliquot of Iscove modified Dulbecco medium (IMDM) supplemented with 200 ng/mL SCF, 50 ng/mL IL-6, insulin-transferrin-selenium (Invitrogen), 55 μ mol/L 2-ME, 100 U/mL penicillin, and 100 μ g/mL streptomycin was layered over the methylcellulose cultures. At 6 weeks, all cells were retrieved after dissolving the methylcellulose medium with PBS. The cells were then suspended and cultured in IMDM supplemented with 100 ng/mL SCF, 50 ng/mL IL-6, 0.1% BSA, insulin-transferrin-selenium, 55 μ mol/L 2-ME, 100 U/mL penicillin, and 100 μ g/mL streptomycin, and the culture medium was replaced a week later. After an additional week of culture, the culture medium was switched to IMDM supplemented with 100 ng/mL SCF, 50 ng/mL IL-6, 5% FBS (Invitrogen), 55 μ mol/L 2-ME, 100 U/mL penicillin, and 100 μ g/mL streptomycin. The culture medium was changed weekly thereafter, and the cells were incubated for an additional 5 to 7 weeks. We generated human mast cells from more than 10 donors. The final purity of the mast cells was

determined by measuring Fc ϵ RI expression by using flow cytometry after 12 weeks of culture following 48 hours of stimulation with 1 μ g/mL myeloma IgE and always exceeded 95%. The viability of mast cells exceeded 95% at 72 hours of stimulation, as determined by using trypan blue staining.

NHBE cell culture and infection

Five separate lots of primary NHBE cells were purchased from Lonza. In past studies the viability of control and cytokine-stimulated NHBE cells has been determined based on trypan blue staining and LDH assay. The viability of control and cytokine-stimulated NHBE cells exceeds 90%. The viability of dsRNA alone and dsRNA plus TNF-, IL-1 β -, IL-4-, or IL-17-stimulated cells is 80% to 90%, and the viability of dsRNA plus IFN- γ -stimulated cells is generally 60% to 90% at 72 hours of stimulation.

Influenza A virus strain A/udorn/1972 (H3N2) was a gift from Dr Lamb (Northwestern University, Evanston, Ill). Submerged NHBE cells were infected with IV at an MOI of 0.1 and were stimulated with 10 ng/mL IL-4 at 37°C. Mast cells (5×10^5 cells/mL) were then added to NHBE cells and cultured along with neutralizing anti-IL-1Ra antibody at 20 μ g/mL or 20 μ g/mL control goat IgG for 72 hours.

β -Hexosaminidase assay

Mast cells (5×10^5 cells/mL) were stimulated with IL-4 plus dsRNA-stimulated NHBE cell supernatant (10% volume) or 1 μ mol/L calcium ionophore A23187 (Sigma-Aldrich, Saint Louis, Mo) for 30 minutes in Tyrode buffer containing 0.4% BSA. The contents were then placed on ice for 5 minutes to stop the reaction. All samples were centrifuged at 1800 rpm for 5 minutes at 4°C. For maximum release (content_{total}), mast cells were lysed with 0.5% Triton X-100 (Sigma-Aldrich), incubated for 5 minutes at room temperature, vortexed, and placed on ice until needed. Supernatants from samples or lysates were then incubated with 625 μ mol/L p-nitrophenyl-acetyl-d-glucosamine (Sigma-Aldrich) for 90 minutes at 37°C, and the reaction was quenched with the addition of 0.1 mol/L carbonate buffer. Absorbances were read on a plate reader at 405 and 630 nm. Absorbances from nonstimulated, and stimulated cells were described as release_{spontaneous} and release_{stimulated}, respectively. The percentage of net specific release was calculated by using the following formula:

$$(\text{Release}_{\text{stimulated}} - \text{Release}_{\text{spontaneous}}) / (\text{Content}_{\text{total}} - \text{Release}_{\text{spontaneous}}) \times 100.$$

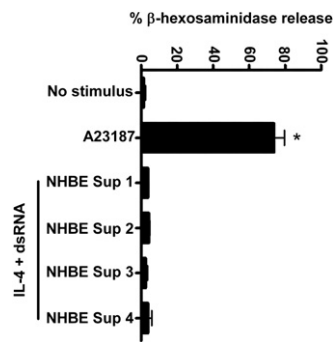


FIG E1. Effect of IL-4 plus dsRNA-stimulated NHBE cells on mast cell degranulation. Mast cells were incubated with NHBE cell supernatants (NHBE Sup; 10% volume) or 1 μ mol/L calcium ionophore A23187 (positive control) for 30 minutes. Cells were centrifuged, and supernatants were incubated with p-nitrophenyl-acetyl-d-glucosamine substrate for 90 minutes. Absorbances were read at 405 and 630 nm, and the percentage of net specific release was calculated. Results shown are means $\pm$ SEMs of 3 independent experiments. * P < .05.

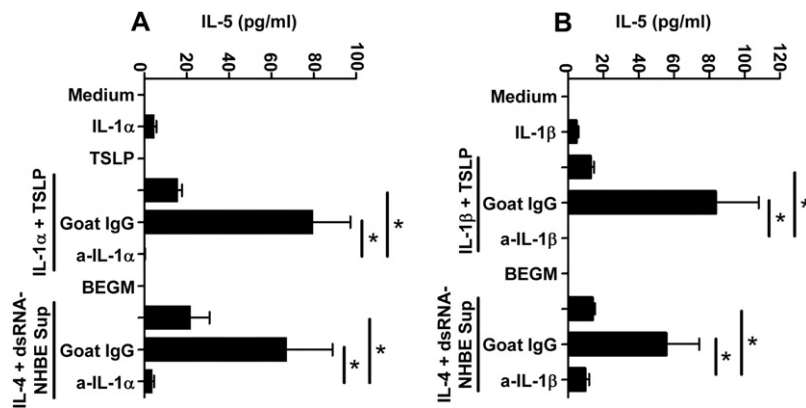


FIG E2. Neutralization of IL-1 suppresses NHBE cell supernatant-induced IL-5 production in mast cells. Mast cells were stimulated with 10 ng/mL IL-1 α , 10 ng/mL IL-1 β , and 10 ng/mL TSLP; epithelial culture medium (bronchial epithelial cell growth medium [BEGM], 10% volume); or IL-4 plus dsRNA-stimulated NHBE supernatants (NHBE Sup; 10% volume) for 48 hours along with neutralizing antibodies against IL-1 α (10 μ g/mL, **A**), IL-1 β (10 μ g/mL, **B**), or respective isotype controls. IL-5 responses were measured by using the cytometric bead array. Results shown are means $\pm$ SEMs of 5 independent experiments. * P < .05, paired Student t test.

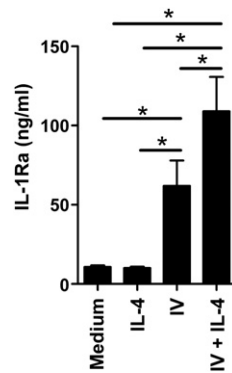


FIG E3. Induction of IL-1Ra by IV infection. NHBE cells cocultured with mast cells were stimulated with IV at an MOI of 0.1 and 10 ng/mL IL-4 for 72 hours. Concentrations of IL-1Ra were measured by using ELISA. Results shown are means $\pm$ SEMs of 6 independent experiments with 4 NHBE cell donors and 3 mast cell donors. * $P < .05$, 1-way ANOVA.