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Bronchial Allergen Challenge of Patients with Atopic Asthma Triggers an Alarmin (IL-33, TSLP, and IL-25) Response in the Airways Epithelium and Submucosa

Wei Wang,* Yan Li,* Zhe Lv,* Yan Chen,* Yun Li,* Kewu Huang,† Chris J. Corrigan,‡ and Sun Ying*,‡

The alarmin cytokines IL-25, IL-33, and thymic stromal lymphopoietin (TSLP) play a critical role in asthma pathogenesis by inducing mucosal Th2-type cytokine production. Although environmental exposure to aeroallergens has been proposed as an alarmin trigger in asthma, there has been no systematic parallel study of the effects of allergen exposure on the expression of these cytokines in the airways of human asthmatics. Using single and sequential double immunohistochemistry, we evaluated the numbers and phenotypes of IL-25–, IL-33–, and TSLP-immunoreactive cells in sections of bronchial biopsies from mild atopic asthmatics (n = 16) before and 24 h after allergen inhalational challenge. Allergen challenge highly increased expression of baseline immunoreactivity for IL-25, IL-33, and TSLP, both in the bronchial epithelium and submucosa ($p < 0.001$), to a degree that correlated with the extent of the late phase of airway obstruction. Aside from epithelial cells, the principal source of immunoreactivity for all three alarmins, TSLP, and IL-33 immunoreactivity colocalized principally with endothelial cells and mast cells, neutrophils, and fibroblasts, whereas IL-25 immunoreactivity colocalized principally with eosinophils as well as endothelial cells, mast cells, and fibroblasts. The data implicate that allergen challenge directly increases airway alarmin expression in atopic asthmatics to a degree correlating with increase late-phase airway obstruction, affirming these molecules as potential molecular targets for the inhibition of allergen-induced airway inflammation and obstruction. ***The Journal of Immunology*****, 2018, 201: 2221–2231.**

Asthma is a disease characterized by airway smooth muscle hyperresponsiveness causing reversible airway obstruction and chronic inflammation of the airways typically associated with elevated expression of Th2-type cytokines, the prevalence of which continues to increase (1, 2). Although this inflammation has conventionally been attributed to the activities of Ag-activated, Th2-type T cells, increasing attention has been paid recently to the putatively more prominent role of group 2 innate lymphoid (ILC2) cells because of their propensity to produce relatively large amounts of the Th2-type cytokines IL-4, IL-5, and IL-13 independently of exposure to particular Ags (3–5). Further, it is now clear that the principal alarmin cytokines IL-33, IL-25, and thymic stromal lymphopoietin (TSLP) play a critical role in the genesis of Th2-type inflammation in the asthmatic airway mucosa by directly activating ILC2 cells as well as inducing Th2-type T cell differentiation (6). These largely epithelial-derived cytokines have already been implicated in the initiation and regulation of adaptive and innate immune responses associated with Th2 cytokine–mediated inflammatory diseases such as asthma and atopic dermatitis (3–8). There is emerging evidence that allergens alone, or possibly in combination with other environmental stimuli, can act directly on the airway epithelium to initiate alarmin responses in the respiratory mucosal microenvironment that promote innate, Th2-type inflammation (9). Furthermore, alarmin cytokines such as TSLP, IL-33, and IL-25 may act on other inflammatory and structural cells within the airway mucosa, which contribute to the airway inflammation and associated structural changes, such as fibrosis and neoangiogenesis, observed in the airways of patients with asthma (3–9). Airway administration, or lung-specific transgenic expression of TSLP, IL-25, or IL-33, alone is sufficient to induce such changes in the airways of experimental animals including inflammation, infiltration by eosinophils, goblet cell hyperplasia, and airway hyperresponsiveness (10–12). Elevated expression of these cytokines and their receptors has also been observed in bronchial biopsies and bronchoalveolar lavage fluid of human asthmatic patients compared with controls (13–16), whereas targeting these cytokines or their receptors inhibits asthma-associated pathologic conditions in animal surrogates (17–19) and subjects with severe asthma (20).

The understanding that exposure to conventional aeroallergens may propagate Th2-type, eosinophilic inflammation in the airway mucosa of patients with asthma through the generation of alarmin responses is clearly of enormous significance because it detracts from the current dogma that this inflammation is necessarily

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Abbreviations used in this article: FEV_1, forced expiratory volume in the first second; HPMEC, human pulmonary microvascular endothelial cell; IHC, immunohistochemistry, immunohistochemical; ILC2, group 2 innate lymphoid cell; MBP, major basic protein; TSLP, thymic stromal lymphopoietin.

generated through mechanisms dependent on the presence of allergen-specific, Th2-type T cells and allergen-specific, IgE-mediating mast cell and basophil degranulation upon allergen exposure, so-called "allergic inflammation." Nevertheless, there have been few studies to date addressing the effects of inhalational allergen exposure on the expression of alarmins in human asthmatic airways, although we have previously shown that such exposure ncreases the expression of IL-25 immunoreactivity in the bronchial mucosa (21). The purpose of the current study was to extend this observation by quantifying the expression of immunoreactivity in parallel for all three alarmin cytokines, TSLP, IL-33, and IL-25, and to document their cellular provenance in the bronchial mucosa of a group of sensitized, atopic asthmatics before and 24 h after allergen inhalational challenge. We hypothesized that allergen challenge provokes elevated mucosal expression of all three alarmins in the 24-h period following allergen exposure to an extent that can be correlated with the degree of the late phase of airway obstruction induced by this challenge. We also hypothesized that, in addition to epithelial cells, these alarmins are produced by a variety of additional airway inflammatory and structural cells both before and after allergen exposure.

Materials and Methods

Subjects and clinical protocol

Subjects ($n = 16$) with mild atopic asthma were recruited in the Department of Asthma, Allergy and Respiratory Science, King's College London School of Medicine, U.K. The study was approved by the Ethics Committee of King's College Hospital, and each participant provided written, informed consent. The asthmatics had a clear history of mild symptoms requiring inhaled β_2-agonist therapy only, a forced expiratory volume in the first second (FEV_1) $\geq$70% of the predicted value, and a histamine provocation concentration causing a 20% fall in FEV_1 of $<$8 mg/ml measured within a 2-wk period prior to the study. None had ever smoked, and there was no history of other respiratory disease. All subjects were clinically free of respiratory infection and systemic glucocorticoid therapy for at least 2 mo prior to the study. Atopy was defined as a positive skin prick test (wheal at 15 min $>$3 mm in diameter in the presence of positive histamine and negative diluent controls) to one or more extracts of common local aeroallergens. Exclusion criteria were symptoms of an upper respiratory tract infection in the previous 2 wk, allergy to pollens in subjects recruited during the pollen season, and a history of chronic illness other than asthma. Subjects underwent fiberoptic bronchoscopy with bronchial biopsy prior to allergen bronchial challenge using a well-established protocol with an allergen that they were clinically sensitized to and then repeat bronchoscopy with biopsy 24 h later as previously described (21).

Processing of bronchial biopsies

Bronchial biopsies were fixed in 4% paraformaldehyde in 0.1 M of PBS, pH 7.4 (Sigma-Aldrich, Gillingham, U.K.), for 2 h, then rinsed twice for 1 h in 15% sucrose in 0.1 M of PBS, embedded in tissue-freezing medium, then snap frozen in precooled isopentane (Sigma-Aldrich) and stored at −80°C until processed.

Single immunohistochemistry

Frozen sections (6-μm thickness) were cut and mounted on poly-L-lysine–precoated slides (Sigma-Aldrich). mAbs against human IL-33 (1:20, clone 12B3C4; Invitrogen), IL-25 (1:20, clone 68C1039.2; Novus Biologicals), and TSLP (1:10, clone: 55N1D10; Novus Biologicals) were purchased from Fisher Scientific UK (Loughborough, U.K.). Single immunohistochemistry (IHC) was performed using the alkaline phosphatase/antialkaline phosphatase technique using a well-established protocol that we have previously described (21, 22). Briefly, sections were incubated with the primary Abs in PBS containing 5% normal human serum overnight at room temperature, washed, then incubated with the secondary Ab (donkey anti-mouse, 1:50; Jackson ImmunoResearch Laboratories, West Grove, PA), washed again, and then incubated with the third layer alkaline phosphatase/antialkaline phosphatase complex (1:30; Dako, Ely, U.K.) (30 min each). Positively stained cells were detected using Fast Red (Sigma-Aldrich). Phenotypes of inflammatory and structural cells (endothelial cells and fibroblasts) in the sections were identified using the same technique, employing primary mAbs purchased from Dako (mouse anti-human endothelial cells, CD31, 1:30; macrophages, CD68, 1:50; mast cell tryptase, 1:50; and neutrophil elastase, 1:50), Becton Dickinson (Oxford, U.K.) (mouse anti-human T cells, CD3, 1:10), and Abcam (Cambridge, U.K.) (mouse anti-human fibroblasts, CD90, ab181469, 1:50). Mouse antieosinophil major basic protein (MBP) was a kind gift from Prof. A.B. Kay (21, 22). Omission or substitution of the primary Ab with an irrelevant Ab of the same species and isotype were used as negative controls.

The sections were imaged by an operator ignorant of their provenance using an Olympus BX40 microscope connected with a Zeiss Vision KS300 imaging system (Zeiss), and the total numbers of cells expressing immunoreactivity for IL-25, IL-33, and TSLP and cellular phenotypic markers enumerated in the entire biopsy sections. Numbers of immunoreactive cells were expressed per unit area of the sections in the bronchial submucosa and per unit length of the epithelial basement membrane within the epithelium. The mean $\pm$ SD of entire cross-sectional areas (including epithelium and submucosa) of the bronchial biopsy sections before and after allergen challenge were 2.9 $\pm$ 0.5 and 2.8 $\pm$ 0.4 mm^2, respectively, with coefficients of variation from 8.12% (allergen challenge) to 9.87% (baseline). The between observer coefficients of variation for duplicate counts of all markers examined for bronchial biopsies were 4.2–6.7%.

We previously reported the cellular sources of TSLP in the bronchial mucosa of asthmatic patient at baseline and those of IL-25 before and after allergen challenge (13, 14, 21). To further investigate whether pulmonary endothelial cells, epithelial cells, and fibroblasts express IL-33, human pulmonary microvascular endothelial cells (HPMEC; C-12281, PromoCell, Heidelberg, Germany), human tracheal epithelial cells (C-12644; PromoCell) (22), and human pulmonary fibroblasts (C-12360; PromoCell) were cultured in chamber slides containing complete medium [media kits for culturing endothelial cells (C-22-20), epithelial cells (C-21060), and fibroblasts (C-23020); PromoCell] for 24 h, then fixed in 4% paraformaldehyde/PBS for 10 min. After rinsing in PBS for 10 min, the cells in the chamber slides were stained for IL-33 immunoreactivity exactly as described above.

Double sequential IHC

To identify the cellular sources of immunoreactivity for IL-25, IL-33, and TSLP, double IHC was employed as previously described with some modifications (21, 23). Briefly, sections were first incubated with rabbit polyclonal Abs against human IL-25 (LS-C165200, 1:30), IL-33 (LS-C104697, 1:50), and TSLP (LS-B614, 1:30) purchased from LifeSpan BioSciences (Nottingham, U.K.). After washing the sections, immunoreactivity was detected using a second layer, alkaline phosphatase-conjugated donkey anti-rabbit IgG (code: 711-055-152, 1:50; Jackson ImmunoResearch) and developing with Fast Red. Sections were then incubated with murine mAbs against cellular phenotypic markers as described above, then washed, then counter-stained with a second layer Ab (1:50, peroxidase-conjugated AffiniPure Donkey Anti-Mouse IgG, code: 715-035-151; Jackson ImmunoResearch), followed by SIGMAFAST 3,3'-diaminobenzidine (Sigma-Aldrich). Double-positive cells were stained red/brown.

Statistical analysis

Data were analyzed using a statistical package (Minitab Release 7; Minitab, State College, PA). ANOVA was performed using Friedman test, followed by paired comparisons between pre- and postallergen challenge using the Wilcoxon signed rank test with Bonferroni correction. Correlation coefficients were obtained by Spearman rank-order method with correction for tied values. For all tests, p values <0.05 were considered significant.

Results

Allergen inhalation challenge induced airways obstruction and infiltration of inflammatory cells

Allergen challenge induced bronchoconstriction at both the early phase (within 2 h) and late phase (4–24 h after challenge) in all of the asthmatic subjects who participated in the current study. The details are summarized in Table I.

Allergen inhalation challenge significantly increased the median numbers of $CD3^+$ T lymphocytes, MBP^+ eosinophils, and $tryptase^+$ mast cells, both in the epithelium and the submucosa of the bronchial biopsy sections, compared with those prior to allergen challenge ($p < 0.01$) (Fig. 1). In addition, the median numbers of $CD31^+$ vascular endothelial cells, $CD90^+$ fibroblasts, and $elastase^+$ neutrophils, but not $CD68^+$ macrophages, were also significantly

Table I. Clinical characteristics of the atopic asthmatics and their responses to allergen challenge

Data Element	Values/Range
Asthma	16
Gender (F:M)	8:8
Age (y)	35 (20–57)
Allergen Used	*n/N*
HDM	8/16
Cat dander	3/16
Grass pollen	5/16
Baseline FEV_1 predicted (%)	99.7 (80.3–107.1)
Maximum FEV_1 fall during LPR (%)	−37.15 (−13.6 to −58.2)

F:M, female:male; HDM, house dust mite; LPR, late phase response.

elevated in the submucosa of the bronchial biopsy sections taken 24 h after allergen challenge compared with those before challenge ($p < 0.05$) (Fig. 1).

Allergen inhalation challenge increased expression of TSLP in the bronchial mucosa

Typical exemplars of expression of immunoreactivity for TSLP, IL-33, and IL-25 in the bronchial biopsy sections before and after allergen challenge are shown in Fig. 2. Allergen challenge was accompanied by a highly significant increase in the median numbers of cells expressing TSLP immunoreactivity in both the epithelium and the submucosa of the bronchial biopsy sections compared with those prior to allergen challenge ($p \leq 0.0001$) (Fig. 3). The total TSLP-immunoreactive cells in the entire biopsy sections correlated inversely with the percentage of predicted FEV_1 of the asthmatic patients both at baseline prior to challenge (Fig. 4; $r = -0.575$, $p = 0.02$) and with the lowest FEV_1 recorded in the 4–24-h period following allergen challenge (Fig. 4; $r = -0.740$, $p = 0.001$).

Allergen inhalation challenge increased expression of IL-25 in the bronchial mucosa

Allergen challenge of the atopic asthmatic patients was also associated with a highly significant increase in the median numbers of cells expressing IL-25 immunoreactivity both in the epithelium ($p = 0.008$) and the submucosa ($p = 0.0001$) of the bronchial biopsy sections (Figs. 2, 3). In contrast to TSLP, the total numbers of IL-25–immunoreactive cells correlated inversely with the lowest FEV_1 recorded in the 4–24-h period following allergen challenge (Fig. 4; $r = -0.536$, $p = 0.032$), but not at baseline (prior to allergen challenge).

Allergen inhalation challenge increased expression of IL-33 in the bronchial mucosa

Again, allergen challenge of the atopic asthmatic patients was also associated with a highly significant increase in the median numbers of cells expressing IL-33 immunoreactivity both in the epithelium and the submucosa of the bronchial biopsy sections compared with those prior to challenge ($p < 0.0001$ in each case) (Figs. 2, 3). The total numbers of IL-33–immunoreactive cells in the bronchial biopsy sections also correlated inversely with the FEV_1 of the patients both at baseline prior to challenge (Fig. 4; $r = -0.559$, $p = 0.024$) and with the lowest FEV_1 reported in the 4–24-h period following allergen challenge ($r = -0.649$, $p = 0.006$).

Cellular sources of TSLP, IL-33, and IL-25 in the bronchial mucosa

Clearly, as is apparent from Fig. 2, airway epithelial cells formed the majority of cells expressing immunoreactivity for TSLP, IL-33, and IL-25 in the bronchial biopsy sections from these patients. Interestingly, some specific immunoreactivity for all three cytokines was also expressed by some of the bronchial smooth muscle cells within the sections (data not shown). To further characterize other cellular sources of TSLP, IL-25, and IL-33 in the bronchial biopsy sections before and after allergen challenge, double sequential IHC was employed (Fig. 5). After allergen challenge, epithelial cells, $CD31^+$ endothelial cells, $tryptase^+$ mast cells, $elastase^+$ neutrophils, and $CD90^+$ fibroblasts were the major cellular sources of TSLP immunoreactivity, accounting for (baseline versus postallergen challenge) 45.8 ± 2.09% versus 45.5 ± 1.5%, 19.3 ± 0.8% versus 20.4 ± 0.6%, 16.9 ± 0.9% versus 17.6 ± 0.9%, and 11.6 ± 0.6 versus 12.6 ± 0.7% (mean ± SEM)

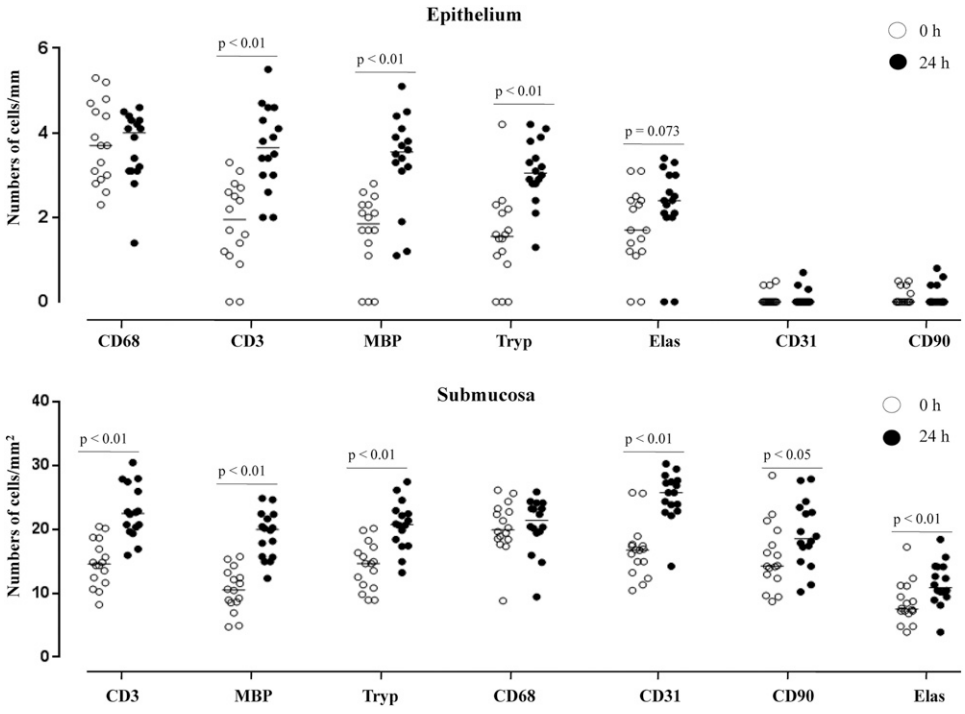

FIGURE 1. Numbers of inflammatory, endothelial cells and fibroblasts in the epithelium (top; cells per millimeter of basement membrane) and submucosa (bottom; cells per square millimeter) of sections of bronchial biopsies from atopic asthmatic patients before and 24 h after allergen inhalation challenge ($n = 16$). Bars show the medians of $CD68^+$ macrophages, $CD3^+$ T cells, MBP^+ eosinophils, $tryptase^+$ (Tryp) mast cells, $Elastase^+$ (Elas) neutrophils, $CD31^+$ endothelial cells, and $CD90^+$ fibroblasts. Wilcoxon signed-rank test.

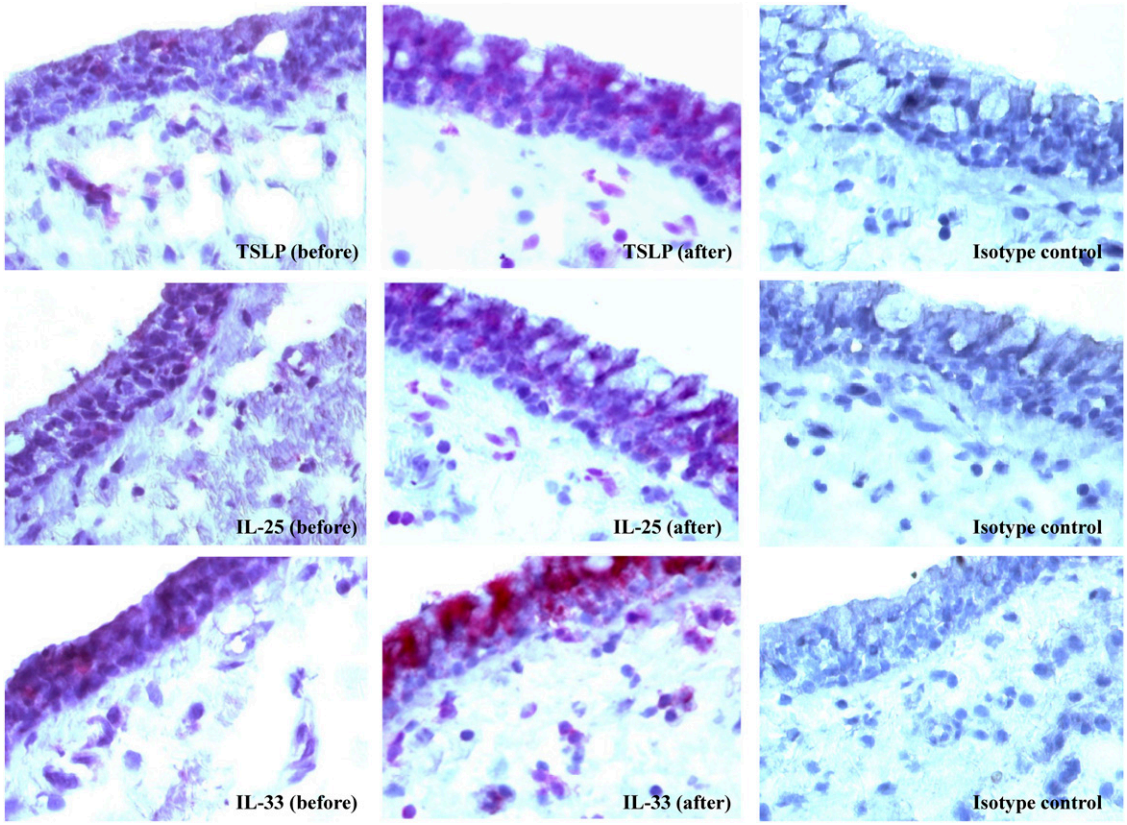

FIGURE 2. Typical photomicrographs showing TSLP, IL-33, and IL-25 immunoreactivity in sections of bronchial biopsies stained with anti-TSLP, anti–IL-25, and anti–IL-33 Abs before (left panels) and 24 h after allergen challenge (middle panels) and negative, isotype-matched control Abs (right panels). Immunoreactive cells stain red. Original magnification ×40.

of total, nonepithelial TSLP$^+$ cells in the bronchial mucosal sections, respectively (Fig. 5, top). In general, the phenotypic profile of the cells expressing TSLP immunoreactivity was not obviously altered by allergen challenge. In contrast, only subsets of these cells, ranging from ~20 to 60% of the total both before and after allergen challenge expressed detectable TSLP (Fig. 5, bottom).

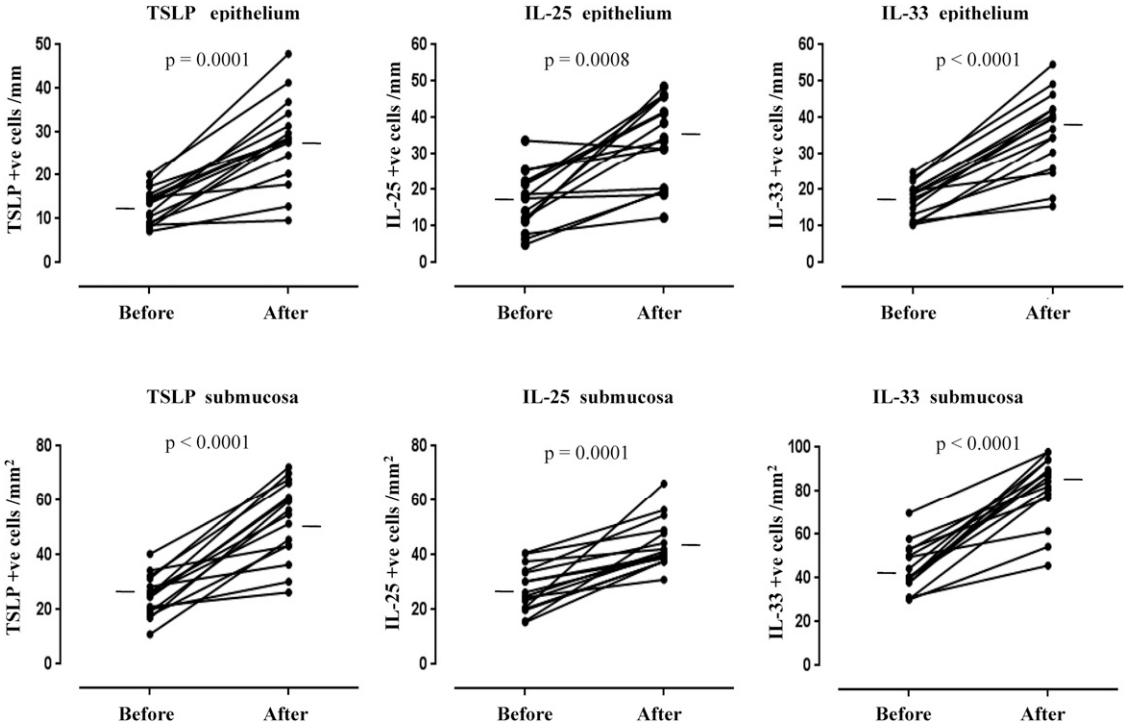

FIGURE 3. Numbers of TSLP- (left panel), IL-25– (middle panel), and IL-33– (right panel) immunoreactive cells in the epithelium (top; cells per millimeter of basement membrane) and submucosa (bottom; cells per square millimeter) of the atopic asthmatic bronchial submucosa before and 24 h after allergen inhalation challenge (n = 16). Wilcoxon signed-rank test.

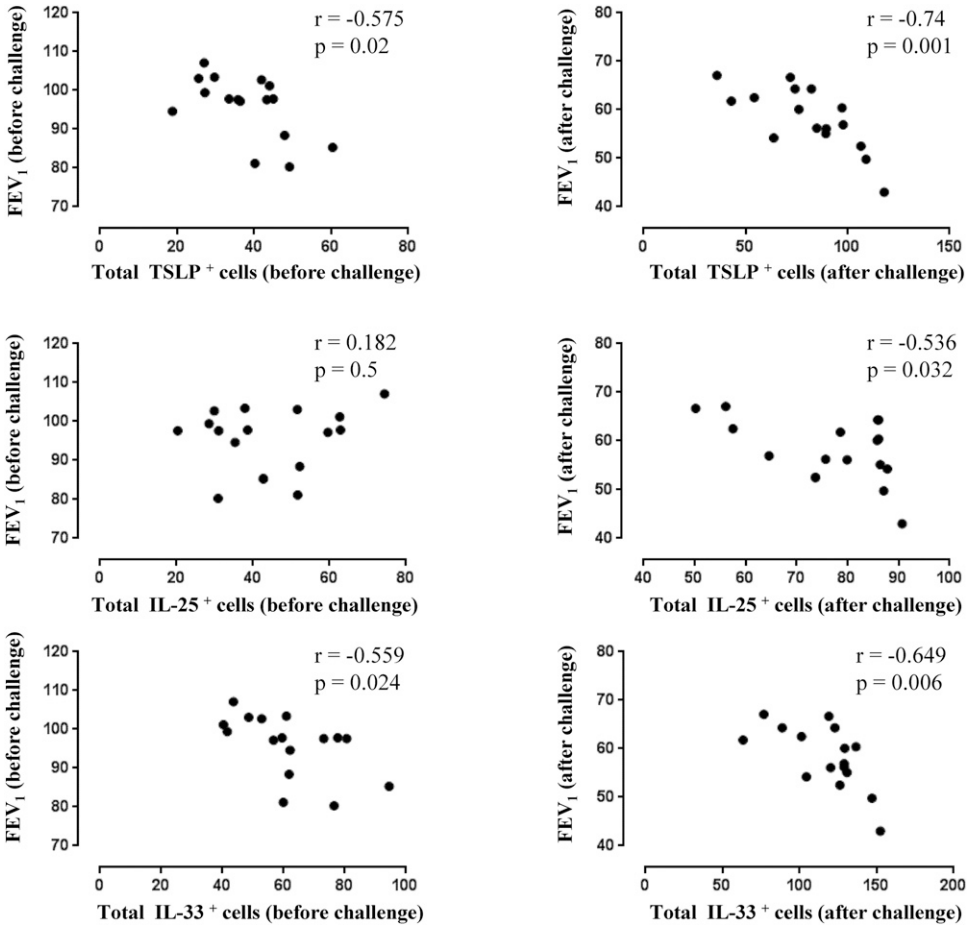

FIGURE 4. Correlations between FEV_1 and total numbers (cells per square millimeter of the entire biopsy sections) of $TSLP^+$, IL-25^+, and IL-33^+ cells before and after allergen challenge (FEV_1 after challenge is the lowest FEV_1 recorded in the 4–24-h period following challenge) (n = 16).

As with TSLP (aside from bronchial epithelial cells), $CD31^+$ endothelial cells, $tryptase^+$ mast cells, $CD90^+$ fibroblasts, and $elastase^+$ neutrophils were the major cellular sources of IL-33 immunoreactivity, accounting for (baseline versus allergen challenge) 29.6 ± 1.1% versus 30.2 ± 1.9%, 24.1 ± 1.3% versus 24.9 ± 1.4%, 15.3 ± 1.7% versus 18.4 ± 2.0%, and 9.2 ± 0.6% versus 10.3 ± 1.0% (mean ± SEM) of the total, nonepithelial IL-33^+ cells, respectively (Fig. 6, top; Fig. 7). Again, the phenotypic profile of the cells expressing IL-33 immunoreactivity was not significantly altered following allergen challenge. As with TSLP, only subsets of these cells, ranging from ~32 to 60% of the total both before and after allergen challenge expressed detectable immunoreactivity for IL-33 (Fig. 6, bottom). We verified the ability of unstimulated HPMEC, bronchial epithelial cells, and pulmonary fibroblasts to express IL-33 immunoreactivity in primary cultures of these cells (Fig. 8).

Slightly discordantly with the situation with TSLP and IL-33 (aside from bronchial epithelial cells), MBP^+ eosinophils, $CD31^+$ endothelial cells, $tryptase^+$ mast cells, and $CD90^+$ fibroblasts were the major cellular sources of IL-25 immunoreactivity in the bronchial biopsy sections, accounting for (baseline versus allergen challenge) 34.5 ± 2.2% versus 38.8 ± 2.6%, 20.1 ± 1.4% versus 20.2 ± 1.5%, 21.4 ± 1.4% versus 24.6 ± 1.7%, and 14.6 ± 2.2% versus 11.3 ± 1.0% (mean ± SEM) of the total nonepithelial IL-25^+ cells, respectively (Fig. 9, top). As with TSLP and IL-33, these percentages were little influenced by allergen challenge, and only subsets of these cells (ranging from ~22 to 63% of the total) expressed detectable IL-25 immunoreactivity both before and after allergen challenge (Fig. 9, bottom).

Associations between numbers of cells expressing TSLP, IL-33, and IL-25 immunoreactivity

Analysis showed that the numbers of cells expressing TSLP immunoreactivity in the submucosa of the bronchial biopsy sections of the atopic asthmatic patients significantly correlated with those expressing IL-33, but not IL-25, immunoreactivity both before (r = 0.711, p = 0.002) and after (r = 0.809, $p < 0.001$) allergen challenge (Fig. 10). In contrast, there was no significant correlation between the numbers of IL-25–immunoreactive cells and those of the TSLP- or IL-33–immunoreactive cells in the submucosa, either before or after allergen challenge.

In addition, the numbers of cells showing immunoreactivity for IL-33 and TSLP in the entire bronchial biopsy sections and within the epithelium alone significantly correlated with the total numbers of MBP^+ eosinophils in the entire sections (total IL-33^+ cells versus total MBP^+ cells: r = 0.737, p = 0.001; total $TSLP^+$ cells versus total MBP^+ cells: r = 0.624; p = 0.010; epithelial IL-33^+ cells versus total MBP^+ cells: r = 0.655, p = 0.006; epithelial $TSLP^+$ cells versus total MBP^+ cells: r = 0.575, p = 0.02).

Discussion

Despite evolving evidence for critical roles for the Th2-promoting cytokines IL-33, IL-25, and TSLP (the principal alarmin cytokines) in the pathogenesis of the airway inflammation characteristic of asthma (24), there is a paucity of studies addressing their contemporaneous expression and cellular provenance in human asthma, particularly in the context of allergen exposure. In the current study, we have quantified, to the best of our knowledge, for

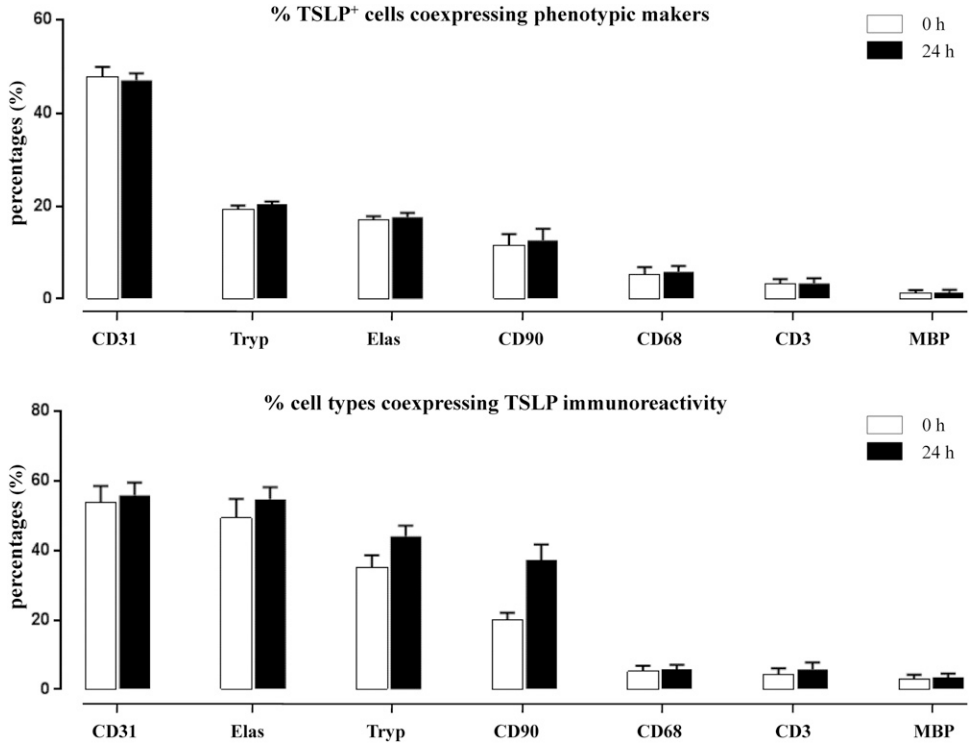

FIGURE 5. Mean (± SEM) percentages of the total nonepithelial cells expressing TSLP immunoreactivity accounted for by cells of the stated phenotypes (top) and percentages of total cells of each phenotype expressing TSLP immunoreactivity (bottom) in sections of bronchial biopsies before and 24 h after allergen challenge (n = 16). CD3, CD3$^+$ T cells; CD31, CD31$^+$ endothelial cells; CD68, CD68$^+$ macrophages; CD90, CD90$^+$ fibroblasts; Elas, elastase$^+$ neutrophils; MBP, MBP$^+$ eosinophils; Tryp, tryptase$^+$ mast cells.

the first time, the expression and cellular provenance of all three cytokines in the bronchial mucosa of atopic asthmatics before and 24 h after allergen inhalational challenge. Allergen challenge was associated with markedly elevated expression of immunoreactivity for all three cytokines, both in the epithelium and submucosa, to a degree that closely correlated with the degree of increased

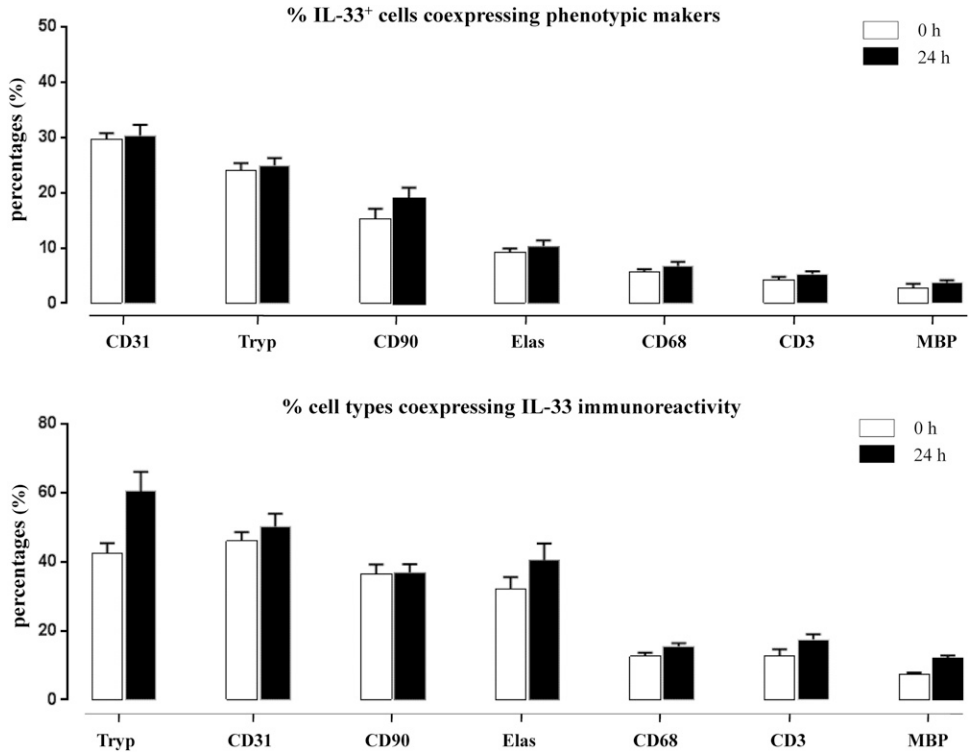

FIGURE 6. Mean (± SEM) percentages of the total nonepithelial cells expressing IL-33 immunoreactivity accounted for by cells of the stated phenotypes (top) and percentages of total cells of each phenotype expressing IL-33 immunoreactivity (bottom) in sections of bronchial biopsies before and 24 h after allergen challenge (n = 16). CD3, CD3$^+$ T cells; CD31, CD31$^+$ endothelial cells; CD68, CD68$^+$ macrophages; CD90, CD90$^+$ fibroblasts; Elas, elastase$^+$ neutrophils; MBP, MBP$^+$ eosinophils; Tryp, tryptase$^+$ mast cells.

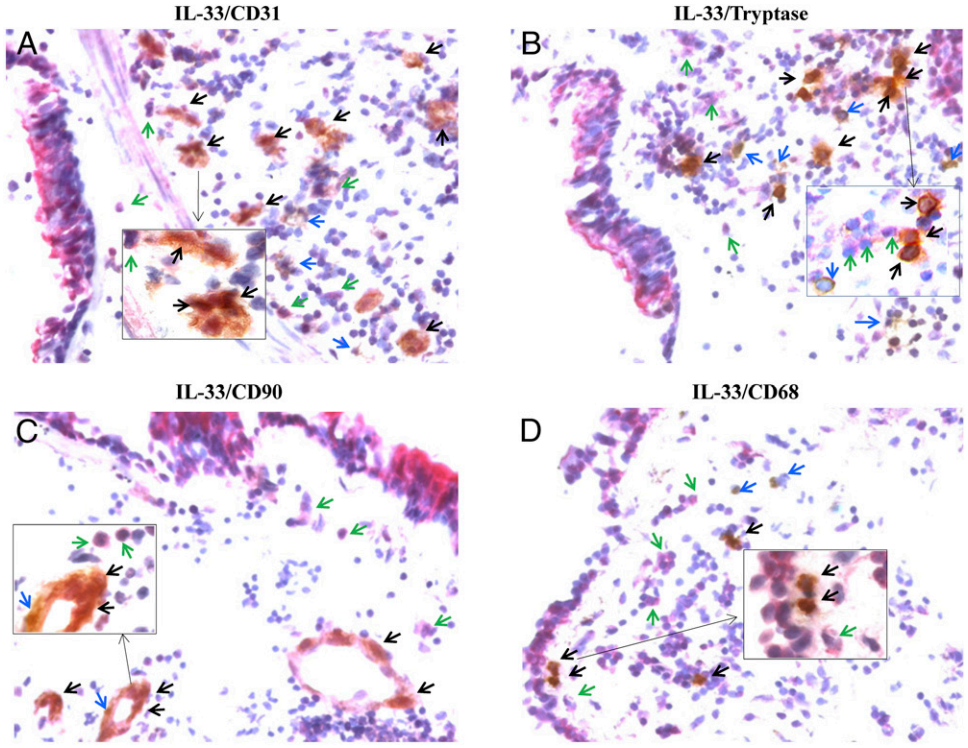

FIGURE 7. Double sequential IHC staining showing IL-33$^+$/CD31$^+$ endothelial cells [(**A**) green arrows: IL33$^+$/CD31$^-$; black arrows: IL33$^+$/CD31$^+$; blue arrows: IL33$^-$/CD31$^+$], IL-33$^+$/tryptase$^+$ mast cells [(**B**) green arrows: IL33$^+$/tryptase$^-$; black arrows: IL33$^+$/tryptase$^+$; blue arrows: IL33$^-$/tryptase$^+$], IL-33$^+$/CD90$^+$ fibroblasts [(**C**) green arrows: IL33$^+$/CD90$^-$; black arrows: IL33$^+$/CD90$^+$; blue arrows: IL-33$^-$/CD90$^+$], and IL-33$^+$/CD68$^+$ macrophages [(**D**) green arrows: IL33$^+$/CD68$^-$; black arrows: IL33$^+$/CD68$^+$; blue arrows: IL33$^-$/CD68$^+$] in sections of bronchial biopsies 24 h after allergen challenge. IL-33–immunoreactive cells are red and cellular markers are brown, whereas double-positive cells are red/brown. Original magnification ×20; inset ×100.

obstruction of the airways as measured by depression of the FEV_1 in the period between 4 and 24 h after allergen exposure. In the case of TSLP and IL-33, such a relationship was, in addition, observable prior to allergen exposure. A caveat is that we were not in a position to formally compare the possible induction of alarmin expression in the same patients on a separate occasion following diluent challenge, which would have allowed us to estimate the theoretical effects of nonspecific airway perturbation.

We find our observed correlation between alarmin expression in the airways and the magnitude of the late-phase bronchoconstrictor response to allergen challenge of particular interest because it is compatible with the hypothesis that alarmins play a pivotal role in the delayed, so-called "late-phase" of obstruction to airflow in the airways, demonstrated in this study by reduction in the FEV_1, which typically follows allergen challenge of sensitized, atopic asthmatics. We speculate that this obstruction reflects reduction of the internal diameter of the airways engendered by the alarmin-induced inflammatory response that we demonstrate in the current study. Inflammatory cells recruited and activated by alarmins may, in addition, be the source of mediators such as leukotrienes, which are conventionally regarded as contributing to bronchoconstriction following allergen challenge (25). In such a scenario, we would speculate that not all atopic asthmatics show a detectable late-phase response to bronchial challenge with an allergen to which they are sensitized because of insufficient exposure to the allergen, which is limited in individual patients by the magnitude of their IgE-mediated, early-phase response. In the current study, all of our patients by definition were capable of being exposed to sufficient allergen to generate a late-phase, as well as an early-phase, response. Alternatively, or in addition, allergens may vary in their capacity to induce alarmin production in different individuals for reasons that remain to be fully characterized. In addition to the effects of local release of bronchoconstricting mediators and local edema, we have recently shown (26) that polycationic ligands of the calcium-sensing receptor (CaSR), including eosinophil basic proteins, may increase the intrinsic hyperresponsiveness of airway smooth muscle, which may also contribute to exacerbation of obstruction of airflow in the airways in this scenario. Our data also suggest that TSLP and IL-33 may play a significant role in regulating airflow in the airways in chronic asthma at baseline, whereas IL-25 may be more involved in the acute exacerbations of airflow obstruction arising from environmental perturbations brought about, for example, by allergen exposure. It is finally intriguing to speculate as to how far these phenomena are dependent on IgE-dependent mechanisms in contrast to the early-phase reduction of airflow in the airways of sensitized, atopic asthmatics following allergen challenge, which is conventionally attributed to the release of bronchoconstricting mediators such as histamine from IgE-sensitized tissue mast cells. We have previously shown (27, 28) that both IL-25 and IL-33 applied directly to the airways of experimental animals can reproduce many of the key pathophysiological changes in the airways associated with asthma in an IgE-independent fashion. Certain allergens such as house dust mite have been shown to act directly on human airway epithelial cells to induce alarmin expression (29). Furthermore, such proteases may themselves further increase the potency of locally released IL-33 by proteolytic cleavage (30).

The data in the current study confirm and considerably expand our previous studies. We previously reported elevated expression of TSLP mRNA and IL-33 immunoreactivity in the bronchial mucosa of patients with asthma at the baseline; in the case of TSLP, to a degree that correlated with the degree of airway obstruction (13–15). We also previously reported elevated expression of IL-25

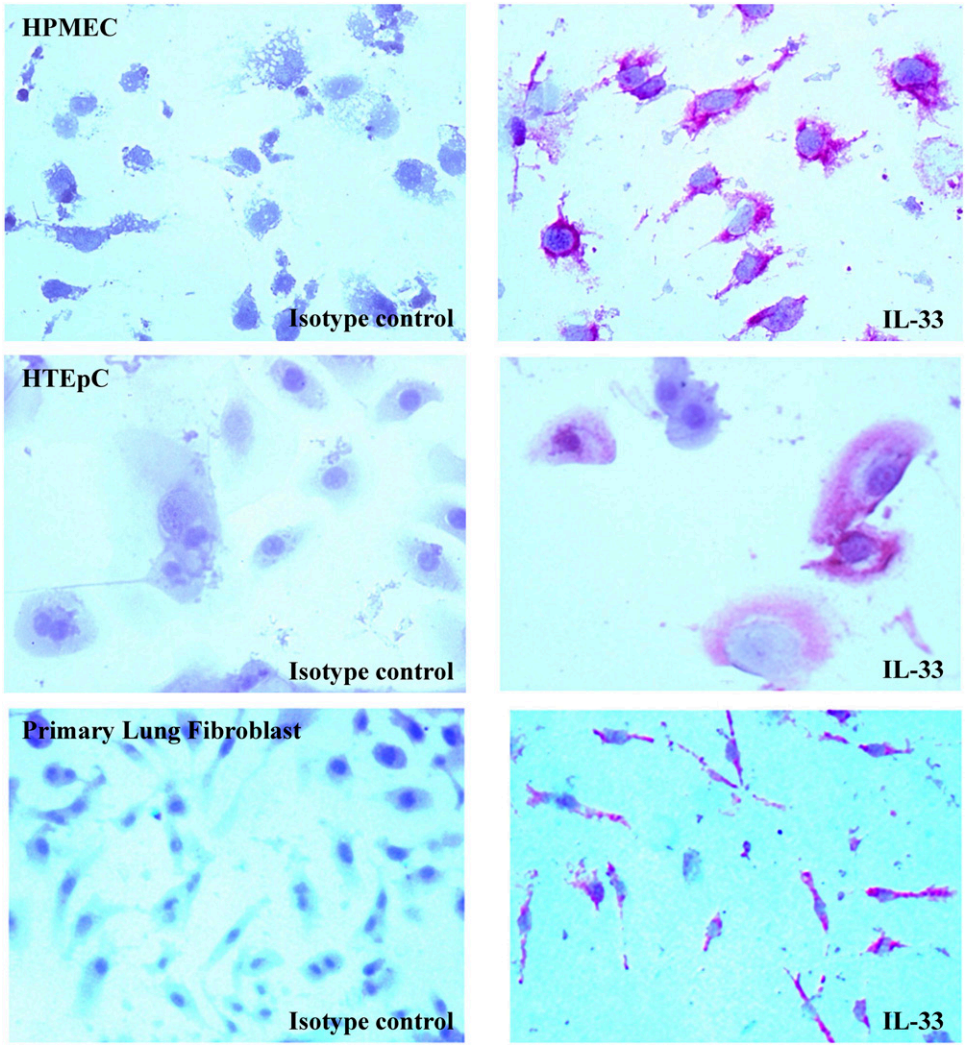

FIGURE 8. Typical photomicrographs of immunocytochemical staining of HPMEC, human tracheal epithelial cells (HTEpC), and human pulmonary fibroblasts with anti–IL-33 Abs. Immunoreactive cells are red. Original magnification ×40.

immunoreactivity in the bronchial mucosa of atopic asthmatics following allergen challenge (16).

Aside from epithelial cells, it is known that alarmins can be expressed by many other cell types including immune cells (T cells, NK cells, eosinophils, neutrophils, macrophages, dendritic cells, mast cells, and basophils) and structural cells (endothelial cells, fibroblasts, and smooth muscle cells) (13, 14, 16, 21, 23, 24, 31). In the current study, we have documented, in parallel, the cellular provenance of the TSLP, IL-33, and IL-25 immunoreactivity expressed in the airway mucosa of our atopic asthmatic patients both before and after allergen challenge. It is noteworthy that, aside from epithelial cells, alarmin expression was not clearly confined to one or another of these cellular types, either before or after their expression was augmented by allergen exposure. Although this observation might be taken as consistent with the possibility that allergen exposure triggers similar pathways for production of these cytokines regardless of their cells of origin, other mechanisms must be invoked to explain why this occurred apparently only in subsets of these cells. It is also worthy of note that although allergen challenge did not appreciably alter the profile of cellular sources of these cytokines (aside from epithelial cells $CD31^+$ endothelial cells), $tryptase^+$ mast cells, $elastase^+$ neutrophils, and $CD90^+$ fibroblasts were the major cellular sources of both TSLP and IL-33 immunoreactivity, whereas MBP^+ eosinophils were the major source of IL-25 immunoreactivity; although endothelial cells, mast cells, and fibroblasts also contributed. These similarities of the cellular sources of TSLP and IL-33 may at least partly account for the close correlation between the numbers of TSLP- and IL-33–immunoreactive cells that we observed in the submucosa of the bronchial biopsies both before and after allergen challenge. The distinct cellular profiles of origin of TSLP and IL-33 on the one hand and IL-25 on the other may also partly account for reports of discordant upregulation of these molecules, depending on the clinical context and the airway inflammatory cells sampled to quantify their expression. For example, previous studies have described elevated expression of TSLP and IL-33, but not IL-25, by epithelial and dendritic cells isolated from induced sputum (32) and exhaled breath condensate and serum (33) from patients with asthma compared with controls, whereas others (34) have, in contrast, reported elevated epithelial expression of mRNA encoding IL-25, but not TSLP or IL-33, in a subset of relatively mild, recently diagnosed asthmatics characterized as demonstrating high corticosteroid responsiveness. It is also worth noting, of course, that these studies addressed baseline expression of these alarmins in asthmatic patients of various severity taking various medications and in the absence of any acute environmental perturbation such as allergen challenge.

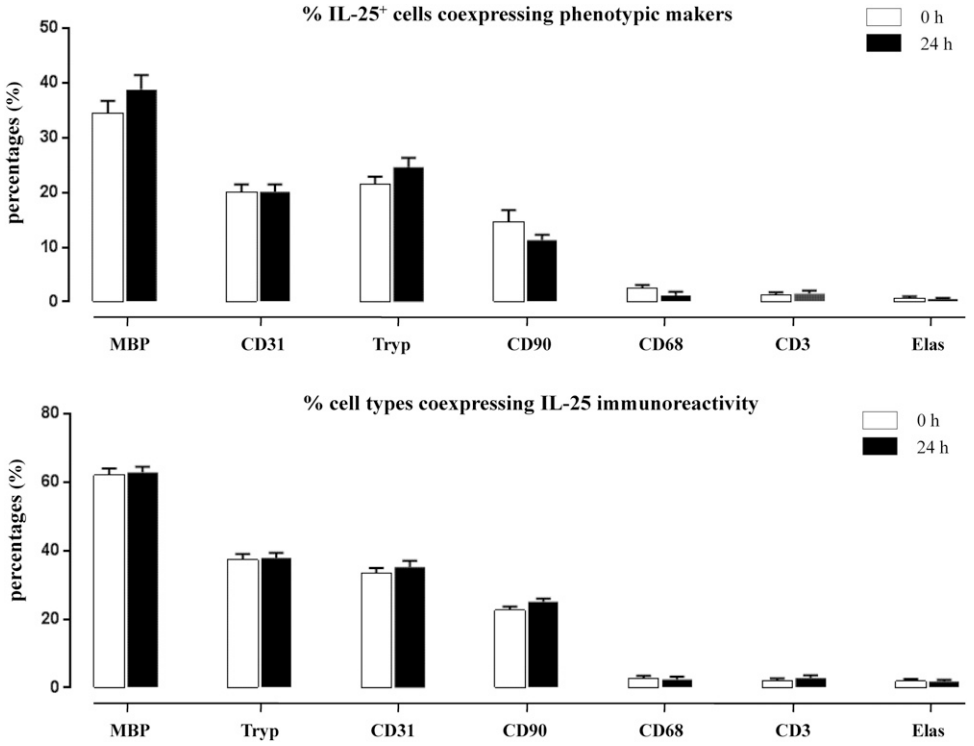

FIGURE 9. Mean (± SEM) percentages of the total cells expressing IL-25 immunoreactivity accounted for by cells of the stated phenotypes (top) and percentages of total cells of each phenotype expressing IL-25 immunoreactivity (bottom) in sections of bronchial biopsies before and 24 h after allergen challenge (n = 16). CD3, CD3$^+$ T cells; CD31, CD31$^+$ endothelial cells; CD68, CD68$^+$ macrophages; CD90, CD90$^+$ fibroblasts; Elas, elastase$^+$ neutrophils; MBP, MBP$^+$ eosinophils; Tryp, tryptase$^+$ mast cells.

In the past 10 y, the limelight has been directed away from Th2-type T cells and increasingly toward bronchial mucosal ILC2 cells as the key source of the Th2-type cytokines, such as IL-5 and IL-13, that appear to generate the airway inflammation typically associated with asthma and, consequently, the alarmin cytokines TSLP, IL-33, and IL25, which directly stimulate these ILC2 cells

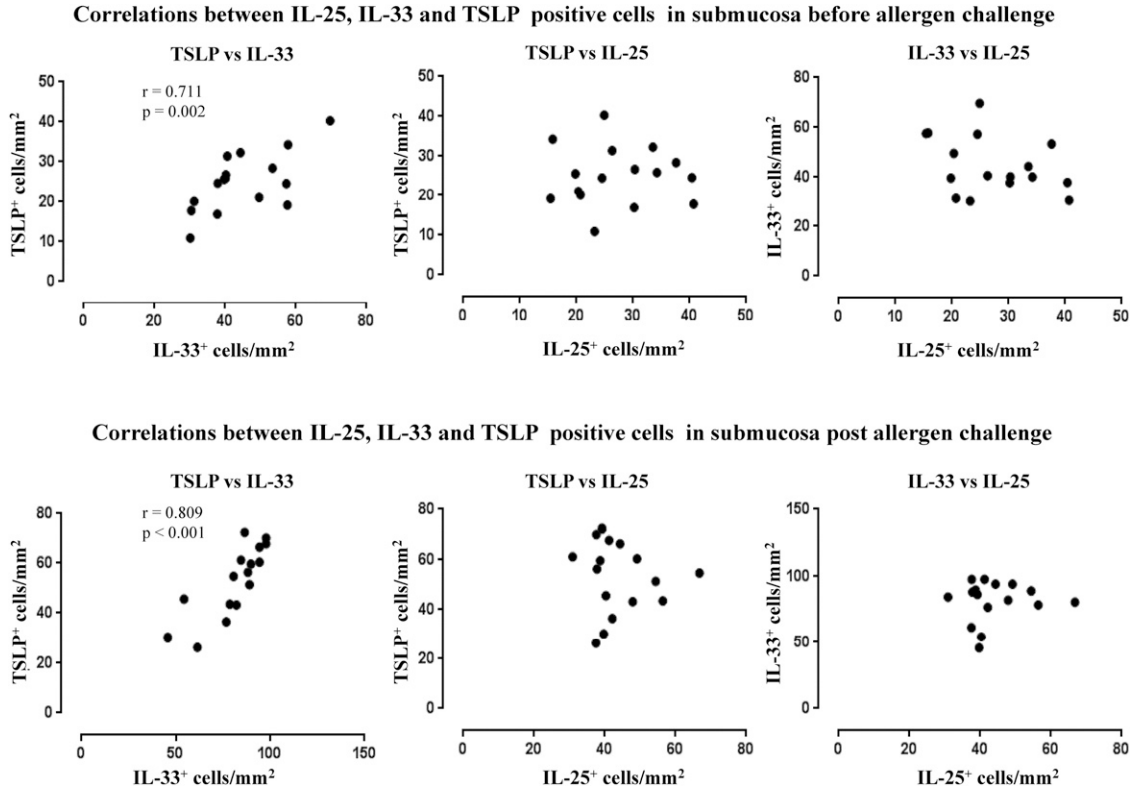

FIGURE 10. Correlations between the total numbers of cells showing immunoreactivity for TSLP, IL-33, and IL-25 in the submucosa of sections of bronchial biopsies before and 24 h after allergen challenge (n = 16).

to produce these cytokines by acting on their own receptors (3–9, 34, 35). Chen and colleagues (36) have shown that allergen exposure increases the numbers of ILC2 cells in the sputum of subjects with atopic asthma. In addition, TSLP and IL-33 may also contribute to migration of bone marrow dendritic cells and $CD34^+$ hematopoietic progenitor cells into the human airways following allergen exposure (37, 38). Finally, a very recent report from P. O'Byrne's group (39) has also shown significant elevation of cells expressing immunoreactivity for IL-33 and its receptor ST2 in the epithelium and submucosa of atopic asthmatics 24 h after allergen bronchial challenge, producing a late-phase response; there was a trend for an increase in TSLP-immunoreactive cells, perhaps reflecting the fact that considerably fewer patients were included in this study. These data are consistent with our findings in the current study.

In summary, we have shown that allergen exposure markedly increased the expression of immunoreactivity for all three alarmin cytokines, TSLP, IL-33, and IL-25, in the bronchial mucosa of asthmatics, a phenomenon that was closely related to the degree of increased airway obstruction following exposure. Although characterization of the precise cellular sources and mechanisms of regulation of these alarmin cytokines will require further research, it is attractive to hypothesize that targeting of these molecules will provide clinical benefit in asthma by reducing the inflammatory response to a range of environmental insults, including allergens. Indeed, recent clinical studies have shown that treatment with AMG 157, a human anti-TSLP mAb, reduced allergen-induced bronchoconstriction and indices of airway inflammation before and after allergen challenge in atopic asthmatics (40) as well as disease control in more severe, therapy-resistant asthmatics (20), whereas evaluation of the targeting of IL-33 and IL-25 is still ongoing (24).

Disclosures

The authors have no financial conflicts of interest.

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