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Location of eosinophils in the airway wall is critical for specific features of airway hyperresponsiveness and T2 inflammation in asthma

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Abstract

Eosinophils are implicated as effector cells in asthma but the functional implications of the precise location of eosinophils in the airway wall is poorly understood. We aimed to quantify eosinophils in the different compartments of the airway wall and associate these findings with clinical features of asthma and markers of airway inflammation.

In this cross-sectional study, we utilized design-based stereology to accurately partition the numerical density of eosinophils in both the epithelial compartment and the subepithelial space (airway wall area below the basal lamina including the submucosa) in individuals with and without asthma and related these findings to airway hyperresponsiveness (AHR) and features of airway inflammation.

Intraepithelial eosinophils were linked to the presence of asthma and endogenous AHR, the type of AHR that is most specific for asthma. In contrast, both intraepithelial and subepithelial eosinophils were associated with type-2 (T2) inflammation, with the strongest association between *IL5* expression and intraepithelial eosinophils. Eosinophil infiltration of the airway wall was linked to a specific mast cell phenotype that has been described in asthma. We found that IL-33 and IL-5 additively increased cysteinyl leukotriene (CysLT) production by eosinophils and that the CysLT LTC_4 along with IL-33 increased *IL13* expression in mast cells and altered their protease profile.

We conclude that intraepithelial eosinophils are associated with endogenous AHR and T2 inflammation and may interact with intraepithelial mast cells via CysLTs to regulate airway inflammation.

Key Message: Intraepithelial eosinophils are a specific feature of asthma, associated with endogenous airway hyperresponsiveness and type-2 inflammation and may interact with intraepithelial mast cells via cysteinyl leukotrienes to regulate airway inflammation.

Keywords: eosinophils, airway epithelium, mast cells, asthma, airway hyperresponsiveness

Abbreviations: AHR, airway hyperresponsiveness; CysLT; cysteinyl leukotriene; CPA3, carboxypeptidase A3; CMA1, chymase; EIB, exercise-induced bronchoconstriction; EPX, Eosinophil Peroxidase; LT_1RA, CysLT1 receptor antagonist; LT_2RA CysLT2 receptor antagonist. T2, Type-2; T2GM, T2 gene mean; TPSAB1, tryptase.

Introduction

Eosinophils are implicated in asthma pathogenesis [1-3] and have been associated with type-2 (T2) inflammation based on correlations with immunostaining for eosinophils in the airway tissue, induced sputum, and peripheral blood [4, 5]. However, no prior study has used quantitative morphometry such as design-based stereology to precisely quantify the density of eosinophils within the different compartments of airway wall [6]. Eosinophil location within the airway wall may influence asthma pathogenesis and regulation of T2 inflammation. Intraepithelial eosinophils may be of particular interest since the epithelium produces specific cytokines that serve to modulate innate immune cell function and regulate T2 inflammation [7, 8]. Further, recent studies have demonstrated increased numbers of mast cells within the airway epithelium in asthma [9, 10] but little is known about the relationship between mast cells and eosinophils in the airway epithelium.

Design-based stereology, a form of quantitative morphometry, avoids many biases inherent to 2-dimensional methods and is currently regarded as the gold standard for accurate localization and determination of the numerical density of cells per volume of a reference space [6]. Studies using design-based stereology have revealed differences in the number of airway smooth muscle cells [11], the density of goblet cells [12], thickness of the basement membrane [13], and intraepithelial mast cell density [10].

We recently used design-based stereology to demonstrate a shift in mast cells from the lamina propria and submucosal space (henceforth referred to in this manuscript as subepithelial space) to the epithelium in subjects with asthma and identified associations between this shift in mast cell location, T2 inflammation and airway hyperresponsiveness (AHR) [9]. Here, we use the same cohort of patients and the same methodology to precisely localize and enumerate eosinophils

in the epithelial and subepithelial compartments and relate the numerical density of eosinophils in the different compartments to features of asthma, AHR, and T2 inflammation. This patient cohort is ideal for studying clinical features of asthma and airway inflammation as these individuals were characterized for distinct forms of AHR, including direct AHR, which is sensitive but non-specific for asthma and indirect/endogenous AHR, which is specific for asthma, but less sensitive overall [14, 15]. These patients were also not using controller therapies at the time of sample collection or phenotypic characterization. We also model the T2 microenvironment of the asthmatic epithelium to examine IL-33 and IL-5 effects on eosinophil function and we further explored the influence of mediators derived from the epithelium and eosinophils on mast cell phenotype and T2 gene expression.

Methods

Below is a summary of our methodology. A comprehensive description of the methods is available in the supplementary material. The University of Washington Institutional Review Board approved the study and participants provided written informed consent.

Participants and Study Design

We conducted a cross-sectional study to examine the association between the numerical density of eosinophils in the different airway wall compartments (epithelial and subepithelial spaces) and features of asthma including AHR and T2 airway inflammation. The subepithelial space included the airway wall area extending below the basal lamina including the lamina propria and the submucosal space. We used endobronchial biopsy samples from a repository collected at the University of Washington designed to examine differences between mild to moderate asthmatics with and without EIB, and non-asthmatic controls [16]. Participants underwent assessment of direct AHR via methacholine challenge testing and endogenous AHR in the form of exercise-induced bronchoconstriction (EIB) via dry air exercise challenge. All asthmatics had a positive methacholine challenge. The severity of EIB was quantified by the FEV_1 over 30 minutes after exercise called the area under the FEV_1-time curve (AUC30) and by the maximum fall in FEV_1 after exercise challenge (Max Fall in FEV_1). Healthy controls had negative methacholine and exercise challenge tests. Induced sputum and research bronchoscopy with endobronchial biopsies were conducted on separate days [17]. Subjects with asthma who were receiving controller therapies (e.g., an inhaled corticosteroid) discontinued such therapy for at least 2 weeks before any study procedures or sample collections.

Immunohistochemistry and Design-based Stereology

Murine monoclonal anti-Eosinophil Peroxidase (EPX, clone MM25-82.2) was used to localize eosinophils in endobronchial tissue. The physical disector method (design-based stereology) was conducted as previously described [9, 10]. We used two complementary metrics; the primary metric was the numerical density of eosinophils per reference volume and the secondary metric was the numerical density of eosinophils per surface area of the basal lamina, which enumerates the eosinophils located above or below the basal lamina. The secondary metric aimed to control for potential confounding related to changes in the reference volume associated with the presence of asthma. Supplemental figure E1 provides an overview of the stereology methods.

Quantitative PCR

TaqMan-based PCR of induced sputum cells [9, 18] quantified the expression of selected genes relevant to airway inflammation; *IL4*, *IL5*, *IL13*, *CMA1*, *TPSAB1*, and *CPA3*.

Isolation and assessment of cysteinyl leukotriene formation by peripheral blood eosinophils

Blood samples were obtained from individuals with a physician diagnosis of asthma and/or allergic rhinoconjunctivitis. Granulocytes were isolated from peripheral blood by density gradient centrifugation followed by negative immunomagnetic selection of eosinophils. The eosinophils were treated with human IL-5 and/or IL-33 for 20 minutes followed by immediate measurement of LTC_4 levels by ELISA.

Assessment of the effects of cysteinyl leukotrienes and IL-33 on mast cell T2 gene expression and protease phenotype

Laboratory of Allergic Disease-2 (LAD2) mast cells [19] were treated with human IL-33 and/or LTC_4 for 4 hours and in some conditions, a $CysLT_1$ receptor antagonist (LT_1RA) MK571

(5 uM) and/or a $CysLT_2$ receptor antagonist (LT_2RA) HAMI3379 (5 uM) (Cayman Chemical, Ann Arbor, MI) were added before treatment. TaqMan-based PCR analysis quantified the expression of *IL13, CMA1, TPSAB1,* and *CPA3 genes* relative to the endogenous control gene.

Statistical analysis

Statistical methods are described in figure legends and detailed in the supplementary materials.

Results

Intraepithelial eosinophils are a distinctive feature of asthma and endogenous AHR

Adequate endobronchial biopsy samples for stereology were available from 10 healthy controls, 12 EIB negative asthmatics (EIB-), and 18 EIB positive asthmatics (EIB+). Study participant characteristics are summarized in table 1. Using stereology to evaluate the airway wall overall, we identified a modest increase in the density of eosinophils in the airway wall in subjects with asthma compared to healthy controls that did not reach statistical significance (p=0.08). However, subjects with asthma had a higher eosinophil density in the epithelium than healthy controls (figure 1a). In contrast, there was no significant difference in the subepithelial eosinophil density between individuals with asthma and healthy controls (figure 1c).

Although there were no significant associations between eosinophil location and baseline lung function, individuals with asthma and eosinophil infiltration of their airway epithelium tended to have lower FEV_1 and lower FEV_1/FVC ratio compared to those without eosinophil infiltration of their airway epithelium (p=0.06 and p=0.24 respectively). Further refinement of the asthma group into subjects with and without EIB demonstrated increased intraepithelial eosinophils in EIB+ asthmatics compared to healthy controls but no differences in intraepithelial eosinophils between EIB- asthmatics and healthy controls, or between EIB+ and EIB- groups (figure 1b).

While there was a trend towards higher subepithelial eosinophils in EIB+ asthmatics when compared to healthy controls or EIB- asthmatics, results were not statistically significant (figure 1d). Additional analyses correlating the numerical eosinophil densities in the epithelial and subepithelial compartments and the severity of direct AHR (assessed by the methacholine PC_{20}) and endogenous AHR (assessed by the Max Fall in FEV_1) are presented in table 2. When healthy controls were excluded from the regression analysis, neither intraepithelial nor subepithelial eosinophil densities were associated with the severity of direct AHR; however, we observed a consistent association between intraepithelial eosinophil density and the severity of endogenous AHR in the form of EIB (table 2). The relationship between intraepithelial eosinophil density and severity of EIB was stronger for our secondary metric that assesses the number of eosinophils located within the epithelium above the basal lamina, irrespective of the epithelial volume. Collectively these results indicate that intraepithelial eosinophils are a specific feature of asthma and are related to the presence and severity of endogenous AHR in the form of EIB. Representative 2-dimensional images of eosinophils in the airway wall from each of the groups are shown in figure 2.

T2 inflammation is associated with eosinophils in both the epithelial and subepithelial compartments

To examine the relationship between airway eosinophil location and T2 inflammation, we used the T2 gene mean (T2GM) as a surrogate for T2 inflammation. T2GM combines the expression of *IL4*, *IL5*, and *IL13* in induced sputum cells into a single metric. Subjects were categorized as T2-high if their T2GM was two standard deviations above that of healthy controls within our study population [9, 18]. Across the full study population, T2-high individuals had higher densities of eosinophils in the airway wall overall, the subepithelial, and the epithelial

compartments when compared to T2-low individuals (figure 3a-c). Similar results were obtained when only participants with asthma were analyzed (figure 3d-f).

In relation to the expression of individual T2 genes, subepithelial eosinophil density was associated with *IL4, IL5,* and *IL13* expression in induced sputum cells, while intraepithelial eosinophil density was only significantly associated with *IL5* gene expression (table 3). To further delineate this relationship, we assessed differences between T2 genes and individuals with asthma based on the presence of intraepithelial eosinophils. Asthmatics with intraepithelial eosinophils had higher *IL5* gene expression (figure 2h) in induced sputum when compared to those without intraepithelial eosinophils. The expression of *IL4* and *IL13* were not significantly different among these two groups (figures 3g and 3i).

Induced sputum eosinophils are not associated with intraepithelial eosinophils

Sputum eosinophilia has been considered a surrogate marker for airway tissue eosinophilia in subjects with asthma. As we conducted 3-dimensional quantitative analysis and partitioned eosinophils into their precise location, we were able to refine this relationship. We found that sputum eosinophil concentration was associated with the subepithelial eosinophil density and not with intraepithelial eosinophil density (table 3). This relationship between the subepithelial eosinophil density and sputum eosinophil concentration persisted when the analysis was limited to participants with asthma (table 3).

Airway eosinophilia is associated with expression of specific mast cell genes in the airway epithelium.

Previous studies have identified a unique subpopulation of mast cells residing in the airway epithelium of subjects with asthma that is associated with AHR and T2 inflammation. This subpopulation of mast cells has higher expression of carboxypeptidase A3 (*CPA3*), persistent expression of tryptase (*TPSAB1*), and low expression of chymase (*CMA1*) [9, 20]. Here, we assessed the association between airway eosinophil location and intraepithelial mast cells and sputum mast cell protease expression. We observed no association between the numerical densities of intraepithelial or subepithelial eosinophils and intraepithelial mast cells (table 3); however, we noted a distinctive relationship between both subepithelial and epithelial eosinophils and the expression of mast cell-specific protease genes in induced sputum. The numerical density of subepithelial eosinophils was significantly associated with expression of both *TPSAB1* and *CPA3* but not with *CMA1* (table 3). Intraepithelial eosinophil density was associated with *CPA3* expression and to a lesser extent with *TPSAB1* expression but not with *CMA1* expression (table 3). When individuals with asthma were analyzed separately, a consistent association between subepithelial eosinophil density and the expression of the *TPSAB1* and *CPA3* was observed, and there was less of an association between the density of intraepithelial eosinophils and mast cell-specific protease genes (table 3). However, the median *TPSAB1* and *CPA3* expression was generally higher among asthmatics with intraepithelial eosinophils when compared to those without intraepithelial eosinophils (figure 4a and 4b). This relationship did not hold true for *CMA1* (figure 4c).

IL-33 and IL-5 have an additive effect on eosinophil production of cysteinyl leukotrienes.

We modeled the effects of the asthmatic airway epithelial microenvironment on eosinophil production of LTC_4, representing new CysLT formation. Eosinophils within the airway epithelium are exposed to epithelial-derived cytokines such as IL-33 and to T2 cytokines including IL-5. Consistent with prior work [21, 22], IL-5 caused a modest but significant increase in eosinophil production of LTC_4 ($p=0.02$). IL-33 caused a dose-dependent increase in LTC_4 production (figure 5a). Together, these two cytokines showed an additive effect on LTC_4 production by eosinophils (figure 5b and 5c).

Cysteinyl leukotrienes act in concert with IL-33 to regulate mast cell phenotype and T2 gene expression

Our prior work demonstrated that IL-33 induces a strong T2 signal in mast cells and that T2 gene expression is associated with reduced *CMA1* expression in mast cells [9]. We hypothesized that IL-33 would act along with CysLTs generated by eosinophils and/or mast cells in this local environment to regulate mast cell expression of *IL13*. There was little induction of *IL13* expression in LAD2 mast cells after exposure to a lower concentration of IL-33 (1 ng/mL) in comparison to a higher IL-33 concentration (10 ng/mL) (figure 6a). LTC_4 alone caused a modest induction of *IL13* expression in mast cells (figure 6b). Importantly, a low concentration of IL-33 combined with LTC_4 increased mast cell *IL13* expression (figure 6b). Using receptor antagonists, we demonstrated that the induction of *IL13* expression by treatment with both IL-33 and LTC_4 was largely dependent upon the $CysLT_1$ receptor, but not the $CysLT_2$ receptor (figure 6c).

Next, we examined the effects of IL-33 and CysLTs on expression of mast cell proteases. Either IL-33 or LTC_4 alone reduced mast cell expression of *TPSAB1* and *CPA3* and there was an additive effect on protease expression when IL-33 was combined with LTC_4. This effect was most pronounced for the expression of *CMA1*, which was reduced to undetectable levels in these cells (figure 6d-f) and was partially reversed by a $CysLT_1$ receptor antagonist (LT_1RA) but not by a $CysLT_2$ receptor antagonist (LT_2RA) (figure 6g). Further, the effect of LTC_4 was completely inhibited by the LT_1RA but not by the LT_2RA (figure 6h), while only a portion of the inhibitory effect of IL-33 on *CMA1* expression was inhibited by the LT_1RA (figure 6i). The effects of IL-33 and LTC_4 on *TPSAB1* and *CPA3* expression were less pronounced and have more complex regulation including effects mediated through both CysLT receptors (supplementary figure E2). Collectively, these results indicate that much of the effect of IL-33 and LTC_4 on *CMA1* expression is through LTC_4 and its metabolite LTD_4 binding to $CysLT_1$ receptor, and that IL-33 triggers LTC_4 release from mast cells. However, there is a component of suppression of *CMA1* in mast cells by IL-33 that is independent of LTC_4 generation and autocrine stimulation of the $CysLT_1$ receptor.

Discussion

This is the first study to use a three-dimensional form of quantitative morphometry to precisely enumerate eosinophils in the different airway wall compartments and relate these findings to clinical features of asthma, T2 inflammation, and induced sputum eosinophil concentration using a population of subjects characterized for AHR in the absence of controller therapies. We demonstrate that intraepithelial eosinophils are unique to asthma, associate with the presence of endogenous AHR, *IL5 gene* expression, and contribute to the regulation of mast cell phenotype and T2 inflammation.

Our assessment of the overall eosinophil density in the airway wall identified eosinophils in subjects with asthma and healthy controls with no statistical difference in eosinophil density between these groups. As prior studies have associated the number of eosinophils per area of the airway wall using two-dimensional methods [1, 23, 24], our study suggests that these results might have overestimated differences in the number of subepithelial eosinophils of asthmatics. By partitioning the density of eosinophils per area of reference volume into epithelial and subepithelial spaces, we demonstrated that eosinophils within the epithelium but not the subepithelial space were strongly linked to the presence of asthma. This is consistent with an earlier cross-sectional study that examined the number of eosinophils in bronchial biopsies using two-dimensional histology and found that intraepithelial eosinophils were not present in healthy controls but were present in some asthmatic individuals, most commonly in those with severe asthma [1, 25].

Our finding relating intraepithelial eosinophils specifically to the presence of endogenous AHR in the form of EIB is of particular importance to understanding asthma immunopathogenesis because endogenous AHR results from mediators released from the airways and is a specific but less sensitive clinical feature of asthma [14] and EIB is a generalizable form of endogenous AHR

that does not require allergic sensitization [10]. In contrast, direct AHR represents the response to an exogenously administered bronchoconstrictor and is a less specific feature of asthma [15]. The precise mechanisms underlying endogenous AHR are incompletely understood [14] but our findings implicate an important role for intraepithelial eosinophils in the development of endogenous AHR in asthma. In line with this observation, a prior study found that allergen challenge, which can induce endogenous AHR in individuals with allergic asthma, induced a non-significant increase in intraepithelial but not subepithelial eosinophils [25]. Further, another study revealed an increase in EPX immunostaining among individuals with moderate persistent asthma that was related to the length and branching of airway nerves that reside in the airway epithelium [26]. As eosinophils have been identified in close contact with airway nerves [26, 27], and that CysLTs can activate airway nerves leading to smooth muscle contraction [28-30], the observed close association between intraepithelial eosinophils and endogenous AHR suggests a causal interaction.

Our results diverge from findings of a prior study of subjects with mild-to-moderate asthma that identified an association between endogenous AHR to mannitol and submucosal eosinophils based on two-dimensional histology and immunohistochemistry using immunoreactivity to eosinophil cationic protein as a surrogate for eosinophil density [24]. This finding may be confounded by eosinophil cytolysis whereby these cells release their intact membrane-bound specific granules extracellularly at sites of eosinophil-associated diseases [31, 32] and therefore, may not be as specific as our methodology that counted eosinophil nuclei to identify only intact cells. Nonetheless, we observed a trend for higher subepithelial eosinophils in asthmatics with endogenous AHR and thus such a relationship may exist especially in more severe disease states.

The T2 cytokines IL-4, IL-5, and IL-13 are implicated in the pathogenesis of eosinophilic or T2-high asthma. Although we observed a general association between airway T2 inflammation and eosinophils residing in the epithelial and subepithelial compartments, the strongest association was with *IL5* expression. IL-5 has been linked to direct AHR [33]; however, mepolizumab, a monoclonal antibody against IL-5, failed to completely abrogate direct AHR [34]. Since mepolizumab only resulted in partial depletion of airway tissue eosinophils [35], a potential implication for the success of anti-IL-5 therapies is in their capacity to reduce the activation state of eosinophils, which could be augmented through other therapies that target eosinophil function such as therapies targeting IL-33 that we found had an additive effect with IL-5 in activating eosinophils [36].

In asthma, a shift of mast cells from the subepithelial to the intraepithelial compartment has been linked to an altered pattern of mast cell proteases, T2 inflammation, and endogenous AHR [9]. Further, the expression of T2 genes by mast cells is directly regulated by IL-33, but not other prominent epithelial-derived cytokines including thymic stromal lymphopoietin (TSLP) or IL-25 [9]. The association we found between both intraepithelial and subepithelial eosinophils and the higher expression of mast cell-specific genes in the airway, including *TPSAB1* and *CPA3* suggest that airway eosinophils might alter airway mast cell phenotype. This finding is of particular interest because prior work has demonstrated that both *TPSAB1* and *CPA3* expression in the airways is associated with endogenous AHR and T2 inflammation. By modeling the asthmatic epithelial microenvironment, we examined the potential mechanism for eosinophil-mediated alteration in mast cell protease expression. We demonstrate that IL-33 acts additively with IL-5 on eosinophils to generate LTC_4. We also show that LTC_4, in combination with IL-33, amplifies mast cell T2 gene expression and alters the mast cell protease profile by suppressing chymase

expression. These findings provide insight into the unique population of intraepithelial mast cells that are identified in human asthma. They may also have functional implications as chymase serves to inactivate IL-33 [37, 38]. As this regulation of mast cell function was substantially but not fully mediated through the $CysLT_1$ receptor, these results could explain some of the clinical efficacy of LT_1RAs, including the marked reduction in mast cell degranulation rather than simple antagonism of the end products of mast cell degranulation [39].

As induced sputum eosinophils are often used as a surrogate for T2 airway inflammation [40], our observation that induced sputum eosinophils were associated with eosinophils in the subepithelial space rather than the epithelium suggests that intraepithelial eosinophils may have increased adhesion, while subepithelial eosinophils can transit into the airway lumen [41, 42]. A limitation to this conclusion is the time lag of 2-10 days between the bronchoscopy and induced sputum collection. However, this was done to avoid any potential confounding due to hypertonic saline administration during sputum induction, which can induce the release of mediators of inflammation [43]. Prior studies have shown a modest relationship between the severity of EIB and induced sputum eosinophil concentration [44, 45], and that AHR persists despite near-complete depletion of sputum eosinophilia with anti-IL-5 blockade [46]. Consistent with our findings relating subepithelial eosinophils with T2 inflammation, a marker of steroid responsiveness, prior studies have related sputum eosinophils to the clinical response to inhaled corticosteroids [45]. However, since T2 inflammation persists in many subjects treated with inhaled corticosteroids [47], the relationships identified between eosinophils and mast cell gene expression identify immunopathological alterations in the airway wall that may be responsible for persistent T2 inflammation.

In conclusion, intraepithelial eosinophils are a unique feature of asthma and are related to features of endogenous airway AHR and T2 inflammation. *Ex vivo* modeling of the airway epithelial microenvironment suggests that intraepithelial eosinophils may promote AHR via increased LTC_4 production and regulate mast cell phenotype and function via LTC_4. These findings provide evidence that eosinophils and mast cells interact within the airway epithelial compartment in a cytokine milieu that includes IL-33, IL-5, and CysLTs and may cooperate to regulate features of T2 inflammation and AHR.

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TABLES

Table 1. Baseline characteristics of the study groups*

Characteristic	Control (N=10)	Individuals with Asthma EIB (-) (N=12)	Individuals with Asthma EIB (+) (N=18)	p value 3 groups	p value EIB (+) *vs.* EIB (-)
Age (yrs.)	30.4 ± 12.7	26.4 ± 8.7	24.8 ± 4.95	.34	.64
Gender – Male (%)	2 (20)	3 (25)	6 (33.3)	.62	.63
Ethnicity – White (%)	7 (70)	11 (91.7)	15 (83.3)	.41	.51
BMI, kg/m^2	22 ± 2.5	22.4 ± 2.7	24 ± 3.8	.22	.37
FEV_1 (% Predicted)	96.5 ± 11.3	90.7 ± 9.75	89 ± 11.2	.21	.90
FVC (% Predicted)	95.7 ± 13.5	96.2 ± 8.9	103.5 ± 9.9	.09	.16
FEV_1/FVC ratio	0.87 ± 0.06	0.80 ± 0.09	0.73 ± 0.09	<.001	.03
Methacholine PC_{20}	>8 ± 0	1.8 ± 1.3	0.6 ± 1.5	<.001	.04
Exercise Challenge					
Max Fall in FEV_1 (%)	1.7 ± 2.1	2.3 ± 2.6	27 ± 9.2	<.001	<.001
AUC30	-7.4 ± 58.1	-13.4 ± 69.5	602.5 ± 287.5	<.001	<.001
T2GM	-0.35 ± 0.18	-0.56 ± 0.89	0.49 ± 0.97	.01	.02
Sputum Eosinophil count (#/ml)	16960 ± 19495	9880 ± 7637	24586 ± 33912	.30	.27
Sputum Eosinophil %	5.41 ± 10	1.98 ± 1.77	2.25 ± 2.01	.24	.99

*Values are expressed as means (±SD) for continuous variables and number and relative frequencies for categorical variables.

Table 2. Relationship between the location of eosinophils within the airway wall and features of AHR*

	Methacholine PC_{20}				**Severity of EIB (AUC30)**				**Max Fall in FEV_1**			
	All Subjects		Asthma Group		All Subjects		Asthma Group		All Subjects		Asthma Group	
	r^2	p value	r^2	p value	r^2	p value	r^2	p value	r^2	p value	r^2	p value
Eos/Epith Vol[A]	0.18	**<.01**	0.06	.19	0.17	**.01**	0.10	.09	0.21	**<.01**	0.13	.06
Epith EOS/BL[B]	0.11	**.04**	0.05	.20	0.27	**<.01**	0.21	**.01**	0.28	**<.01**	0.22	**.01**
Eos/SE Vol[A]	0.11	**.03**	0.07	.15	0.06	.11	0.02	.40	0.12	**.03**	0.07	.16
SE Eos/BL[B]	0.08	.06	0.06	.17	0.03	.22	0.01	.52	0.03	.22	0.01	.50

*Relationship is assessed using a simple linear regression with the Methacholine PC_{20}, the severity of EIB over the first 30 minutes after exercise challenge (AUC30), and the Max Fall in FEV_1 representing the dependent variables.

[A] expressed in (cells/mm^3)

[B] expressed in (cells $x10^2/mm^2$)

Abbreviations: AHR, Airway hyperresponsiveness, Eos, Eosinophil; Epith, Epithelium; Vol, Volume; BL, Basal lamina; SE, Subepithelial.

Table 3. Relationship between the location of eosinophils within airway wall and type-2 gene expression in induced sputum cells*

Characteristic	Full Study Population				Individuals with Asthma			
	Eos/Epith Vol.		Eos/SE Vol.		Eos/Epith Vol.		Eos/SE Vol.	
	r^2	p value	r^2	p value	r^2	*P* value	r^2	p value
T2GM	0.13	0.07	0.25	**.008**	0.10	0.17	0.23	**.03**
IL4 gene	0.06	0.21	0.22	**.01**	0.07	0.26	0.25	**.02**
IL5 gene	0.15	**0.05**	0.24	**.01**	0.11	0.15	0.22	**.04**
IL13 gene	0.11	0.09	0.15	**.04**	0.07	0.24	0.13	.13
Mast cell/Epith. Vol (cells/mm^3)	0.09	0.08	0.08	.09	0.05	0.28	0.05	.24
TPSAB1 gene	0.14	0.06	0.21	**.02**	0.17	0.07	0.27	**.02**
CPA3 gene	0.18	**0.03**	0.19	**.02**	0.17	0.07	0.20	**.05**
CMA1 gene	0.007	0.68	0.03	.42	0.008	0.72	0.05	.36
Sputum eosinophils (cells/ml)	0.002	0.75	0.60	**<.001**	0.004	0.73	0.69	**<.001**

*Relationship is assessed using a simple linear regression with the Eos/Epith Vol and Eos/SE Vol representing the independent variables.

Abbreviations: T2GM, T2 gene mean; Eos, Eosinophils; Epith, Epithelium; Vol, Volume; SE., Subepithelial

FIGURE LEGENDS

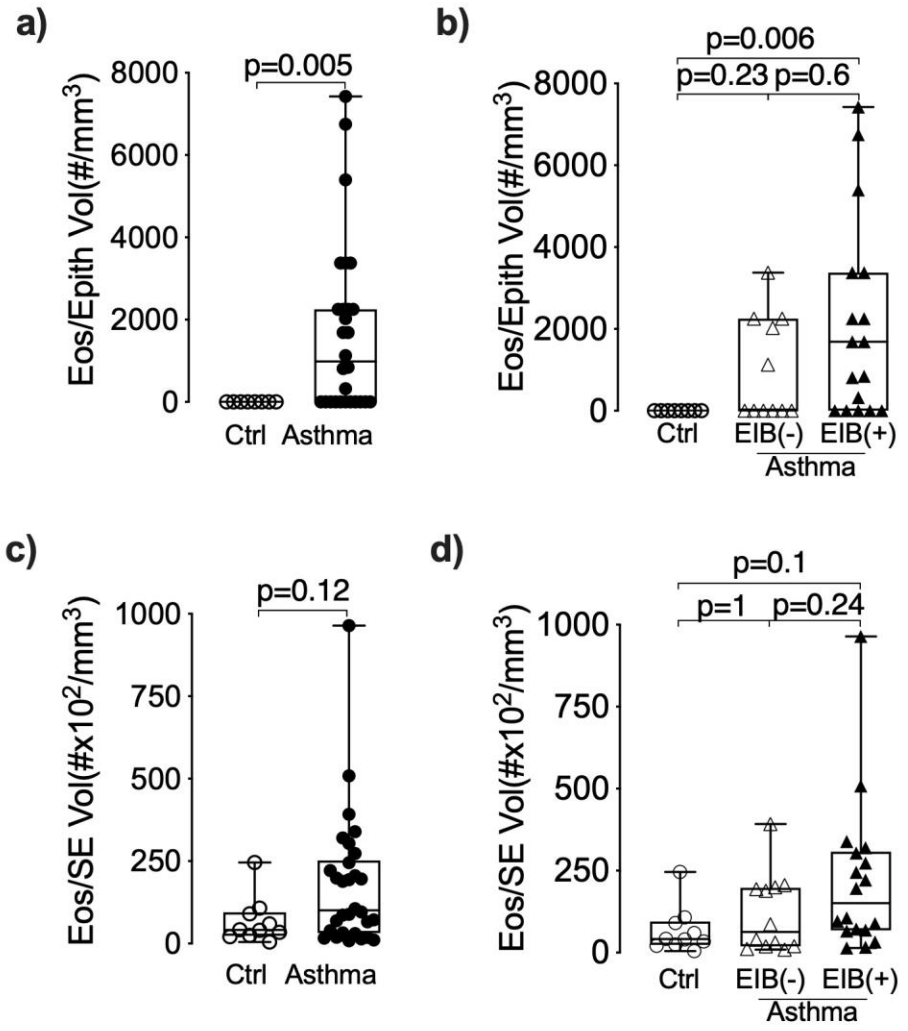

Figure 1. **Intraepithelial eosinophils (Epith), but not subepithelial eosinophils (SE) are increased in individuals with asthma with endogenous airway hyperresponsiveness (AHR).** **a)** Intraepithelial eosinophils were higher in individuals with asthma compared to healthy controls. **b)** Intraepithelial eosinophils are increased in subjects with exercise-induced bronchoconstriction (EIB) compared to healthy controls. **c)** Subepithelial eosinophils were present in healthy controls and there was no statistically significant difference between healthy controls and individuals with asthma**. d)** Subepithelial eosinophils were not different between EIB (+) and healthy controls. Box-

and-whiskers plots are presented. Significance was assessed by the Mann-Whitney *U* test (2-group) or the Kruskal-Wallis test with multiple comparisons (3-group).

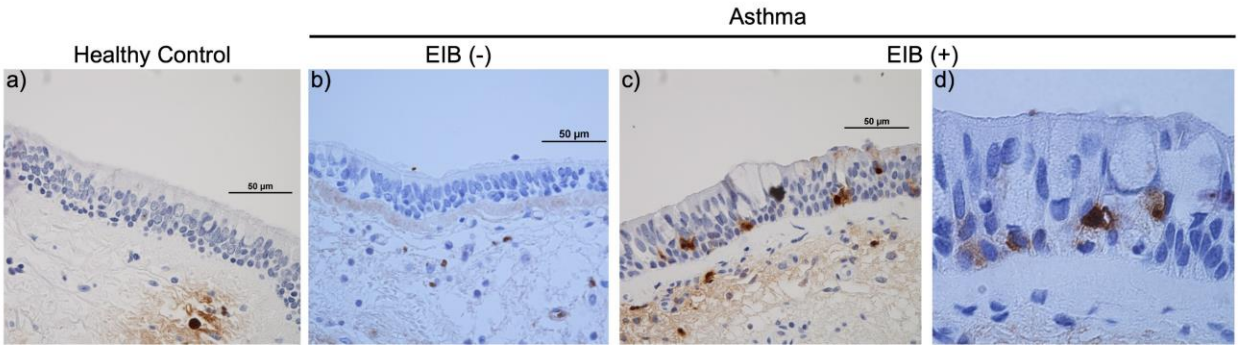

Figure 2. Representative 2-dimensional images of eosinophils within the airway wall. Murine monoclonal anti-Eosinophil Peroxidase (EPX) was used to localize eosinophils in endobronchial tissue from healthy controls **(a)**, EIB(-) asthmatics **(b)**, and EIB(+) asthmatics **(c)**. **d)** A 100X image of an intraepithelial eosinophil.

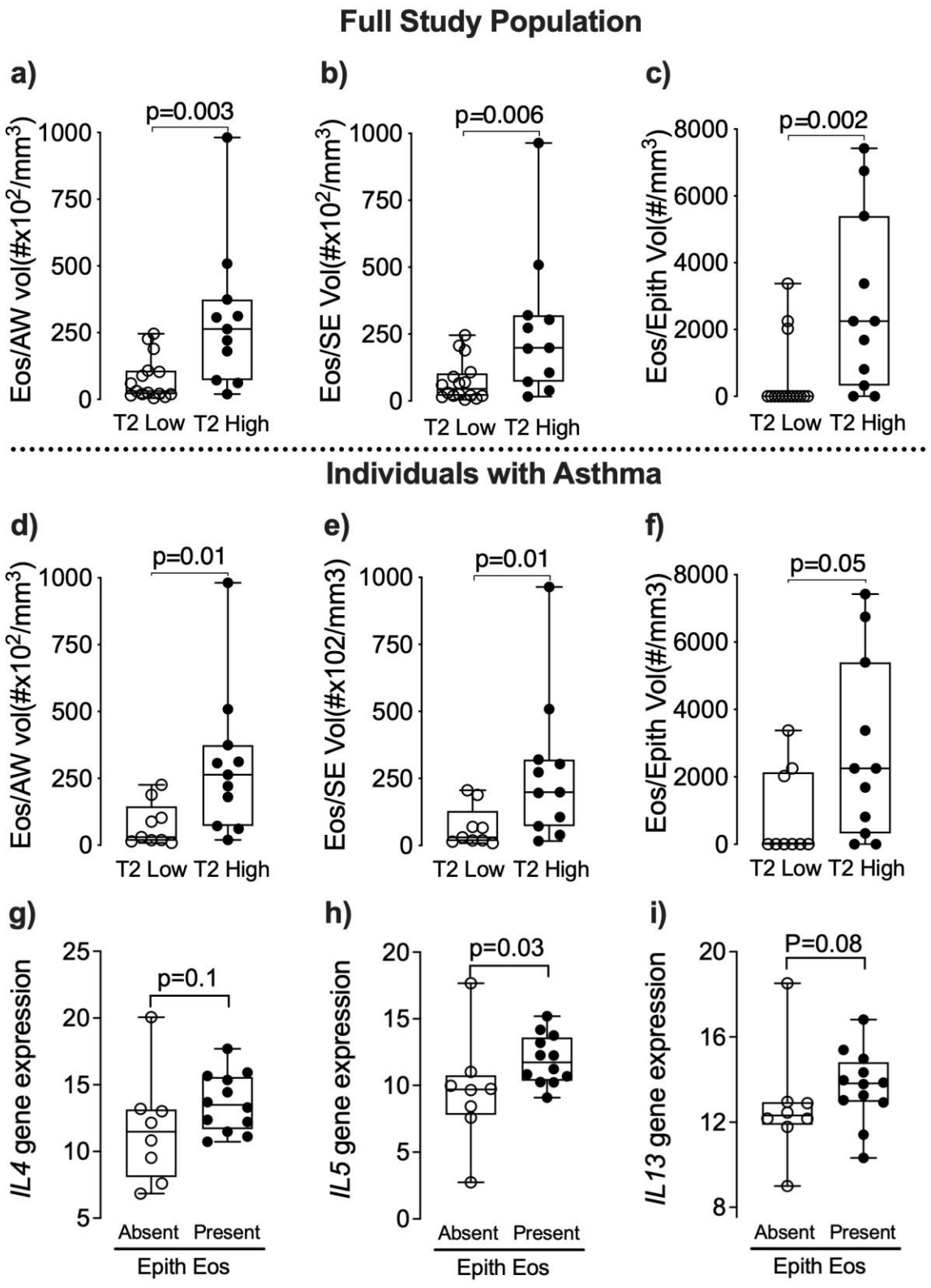
Full Study Population
a)
p=0.003
Eos/AW vol(#x10²/mm³)
1000
750
500
250
0
T2 Low T2 High
b)
p=0.006
Eos/SE Vol(#x10²/mm³)
1000
750
500
250
0
T2 Low T2 High
c)
p=0.002
Eos/Epith Vol(#/mm³)
8000
6000
4000
2000
0
T2 Low T2 High
Individuals with Asthma
d)
p=0.01
Eos/AW vol(#x10²/mm³)
1000
750
500
250
0
T2 Low T2 High
e)
p=0.01
Eos/SE Vol(#x102/mm3)
1000
750
500
250
0
T2 Low T2 High
f)
p=0.05
Eos/Epith Vol(#/mm3)
8000
6000
4000
2000
0
T2 Low T2 High
g)
p=0.1
IL4 gene expression
25
20
15
10
5
Absent Present
Epith Eos
h)
p=0.03
IL5 gene expression
20
15
10
5
0
Absent Present
Epith Eos
i)
P=0.08
IL13 gene expression
20
16
12
8
Absent Present
Epith Eos

Figure 3. Intraepithelial (Epith) and subepithelial (SE) eosinophils are increased among T2 high subjects. a-c) Across the full study population, T2 high individuals had a significantly higher numerical density of eosinophils in the total airway wall, subepithelial space, and the intraepithelial compartment. **d-f)** Among individuals with asthma, T2 high individuals had a significantly higher density of eosinophils in the airway wall, subepithelial space, and intraepithelial compartment. **g-i)**, Among the T2 genes, the expression of the *IL5* gene in the airways was most closely related to the presence of intraepithelial eosinophils, while the differences were not as strong for *IL4* and *IL13* expression. Box-and-whiskers plots are presented. Significance was assessed by the Mann-Whitney *U* test.

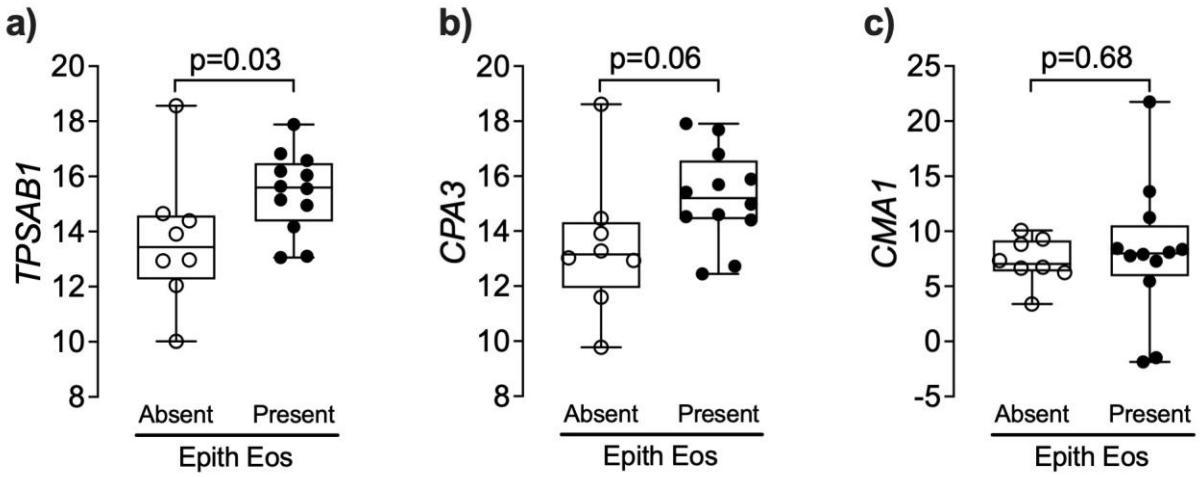

Figure 4. Expression of a specific set of mast cell genes was associated with the presence of intraepithelial eosinophils. a) *TPSAB1* gene expression was significantly elevated in individuals with asthma and intraepithelial eosinophils as compared to those without intraepithelial eosinophils. **b and c)** *CPA3* gene expression was also significantly higher in individuals with asthma and intraepithelial eosinophils, but *CMA1* gene expression was not different between subjects with asthma, with or without intraepithelial eosinophils. Box-and-whiskers plots are shown. Statistical significance was assessed by Mann-Whitney *U* test.

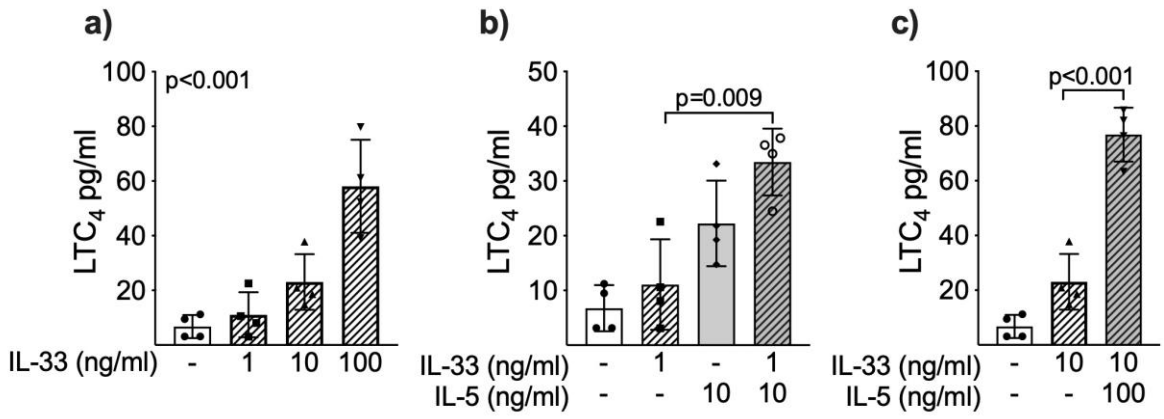

Figure 5. Cysteinyl leukotriene C_4 (LTC_4) production by peripheral blood eosinophils is augmented by the combined effects of IL-33 and IL-5 cytokines. a) IL-33 caused a dose-dependent increase in LTC_4 production by peripheral blood eosinophils leading to a marked increase in the level of LTC_4 at the highest concentration (n = 4 per condition). **b)** IL-5 at a concentration of 10 ng/ml increased the production of LTC_4 from peripheral blood eosinophils pretreated with a low dose (1 ng/ml) of IL-33 (n = 4 per condition). **c)** A higher dose of IL-5 caused a marked increase in the LTC_4 production from peripheral blood eosinophils pretreated with a higher dose of IL-33 (10 ng/ml) (n = 4 per condition). The mean values and SEM bars are shown. Differences between multiple conditions were assessed by 1-way ANOVA with multiple comparisons with P values adjusted to control for false discovery rate using two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli.

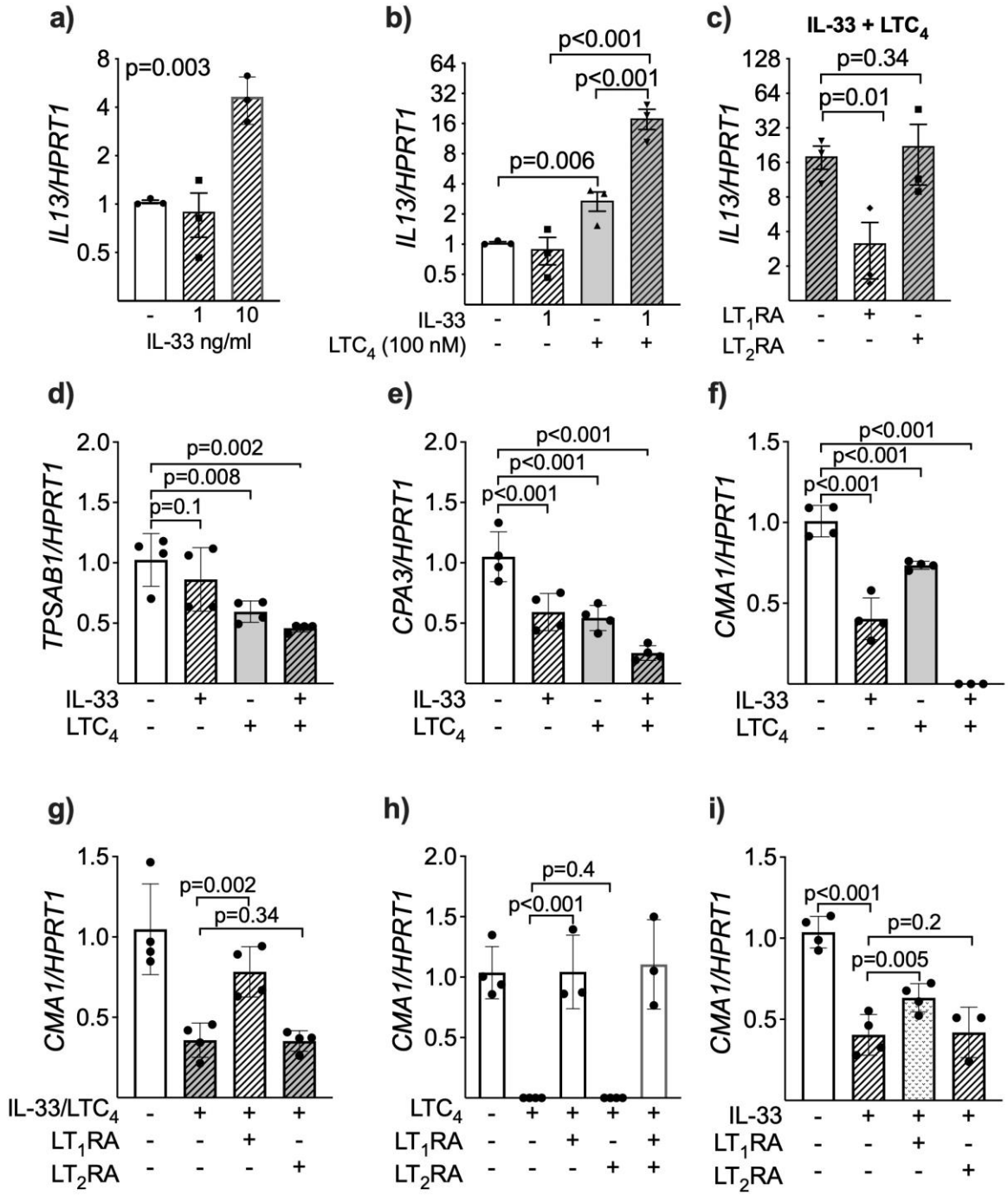

Figure 6. LTC_4 and IL-33 coregulate IL-13 and granular proteases expression in LAD2 mast cells. a) IL-33 resulted in a modest increase in *IL13* gene expression in mast cells at the highest concentration (n = 3 per condition). **b)** LTC_4 alone caused a modest increase in *IL13* gene

expression in mast cells but the combination of LTC_4 with a low concentration of IL-33 resulted in a marked upregulation of *IL13* gene expression in mast cells (n = 3 per condition). **c)** The combined effect of LTC_4 and IL-33 on *IL13* gene expression was abrogated when mast cells were pretreated with a cysteinyl leukotriene 1 receptor antagonist (LT_1RA) but not by a cysteinyl leukotriene 2 receptor antagonist (LT_2RA) (n = *3* per condition). **d-f)** LTC_4 in combination with IL-33 reduced the expression of mast cell granular proteases tryptase (*TPSAB1*), carboxypeptidase A3 (*CPA3*) and chymase (*CMA1*), with the most pronounced effects on *CMA1* where the combination resulted in undetectable levels of this gene (n = 3 to 4 per condition). **g)** The suppressive effect of LTC_4/IL-33 on *CMA1* expression was partially blocked with the LT_1RA, but not the LT_2RA. **h and i)** The effects of LTC_4 on *CMA1* expression were blocked entirely by the LT_1RA, but not the LT_2RA, while the effect of IL-33 was partially blocked by the LT_1RA (n = 3 to 4 per condition). Mean values and SEM bars are shown. Differences between multiple conditions were assessed by 1-way ANOVA with multiple comparisons with P values adjusted to control for false discovery rate using two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli.

Supplementary Materials

Location of eosinophils in the airway wall is critical for specific features of airway hyperresponsiveness and T2 inflammation in asthma

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Figure E1

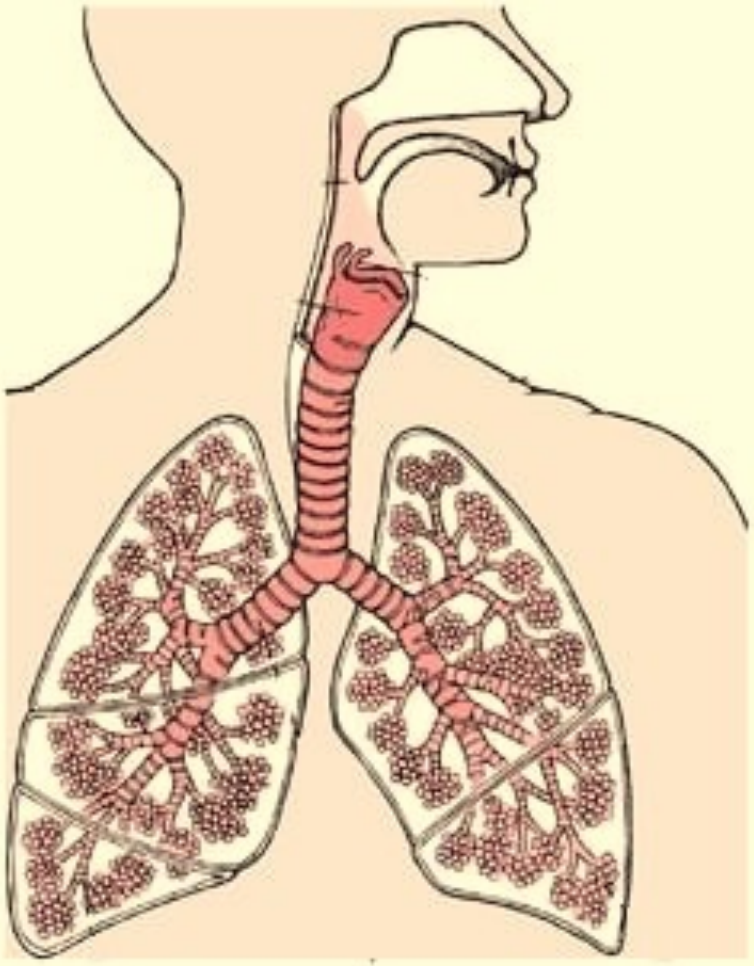
Design-based Stereology

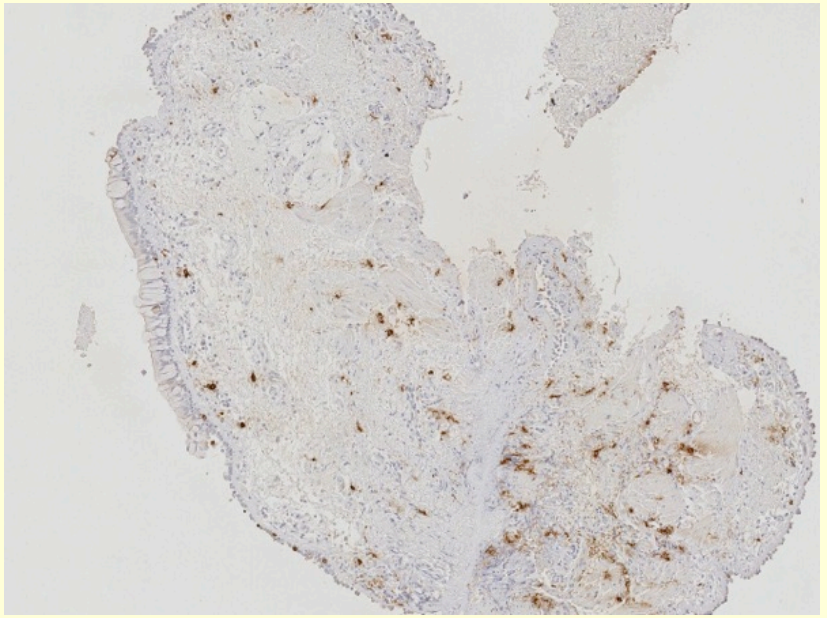
- Stereology - The statistical science of sampling irregular 3D structures in 2D profile using geometric test probes (slabs, sections, lines, points).
- By design, stereological methods make no assumption of the size, shape, orientation, or spatial distribution.
- Estimate a geometrical parameter using samples from the whole:
  - Test points (0D) measure volume (3D).
  - Test lines (1D) measure surface area (2D),
  - Test planes (2D) give length (1D).
  - Test volumes (3D) can measure number (0D)
- Stereological measurements are usually expressed as quantities per unit volume of reference space.

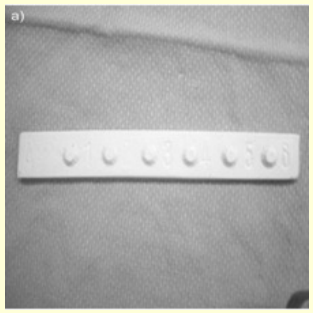
Hsia CCW et al. *AJRCCM*. 2010.

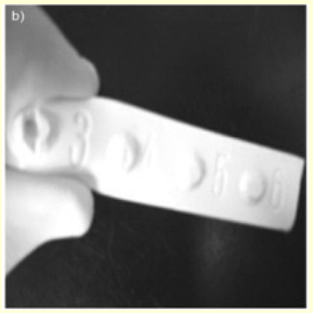
Figure E1 – cont'd

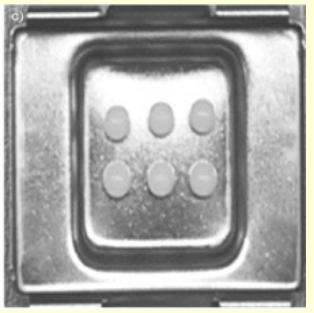
Applying Stereology to Airway Disease in Humans











Woodruff PG et al. *European Respiratory Review*. 2006.

Figure E1 – cont'd

Applying Stereology to Airway Disease in Humans

- The orientation of the endobronchial biopsy must be oriented with isotropic uniform random (IUR) orientation as depicted on the prior slide.
- The samples are then grouped into paraffin blocks and sequential serial sections are placed on the same slide.
- Images of these serial section are taken and loaded into the Visiopharm system, where the images are aligned and systematically sampled.

Figure E1 – cont'd

Applying Stereology to Measure Infiltration of the Airways with Immune Cells

- We present the methods used for mast cells (MC)s as an example but used the granule maker EPX to identify eosinophils in the present study.
- Two different counting frames were used for the analysis as depicted on the subsequent slides.
- Specific cells were identified by counting the nucleus of cells that come uniquely into view between the "reference" and "lookup" sections.
- We then apply two different methods to count the density of eosinophils above or below the basal lamina.

Altman MC et al. *JCI*. 2019.

Figure E1 – cont'd

Measuring Cells of Interest: Two Methods

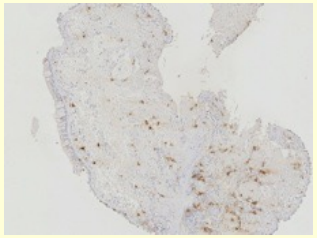
1) Numeric density per reference volume.
   a. Volume of the epithelium.
   b. Volume of the subepithelial space (lamina propria and submucosa).
2) Numeric density per surface area relative to reference volume.
   a. Number of cells above the area of the basal lamina (in the epithelium, irrespective of the epithelial volume).
   b. Number of cells below the area of the basal lamina.

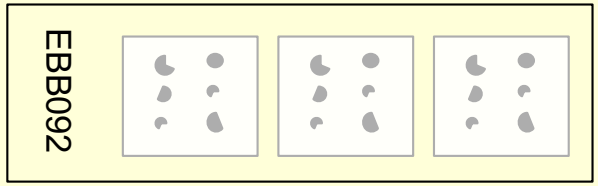
Figure E1 – cont'd

Example of Method 1: Density of Intraepithelial MCs

Numeric Density of MCs per Epithelial Volume

- A physical disector consists of two parallel histological sections generated a known distance apart from the same tissue block.





MC nuclei

$$(1)\ Nv\ MC, epi\ (\#/\mu m^3) = \frac{\sum_{i=1}^{n} Q_{MC}}{(a/f)2h\sum_{i=1}^{n} P_{epi}}$$

Area of the counting frame

Disector height

Points hitting the epithelium

Figure E1 – cont'd

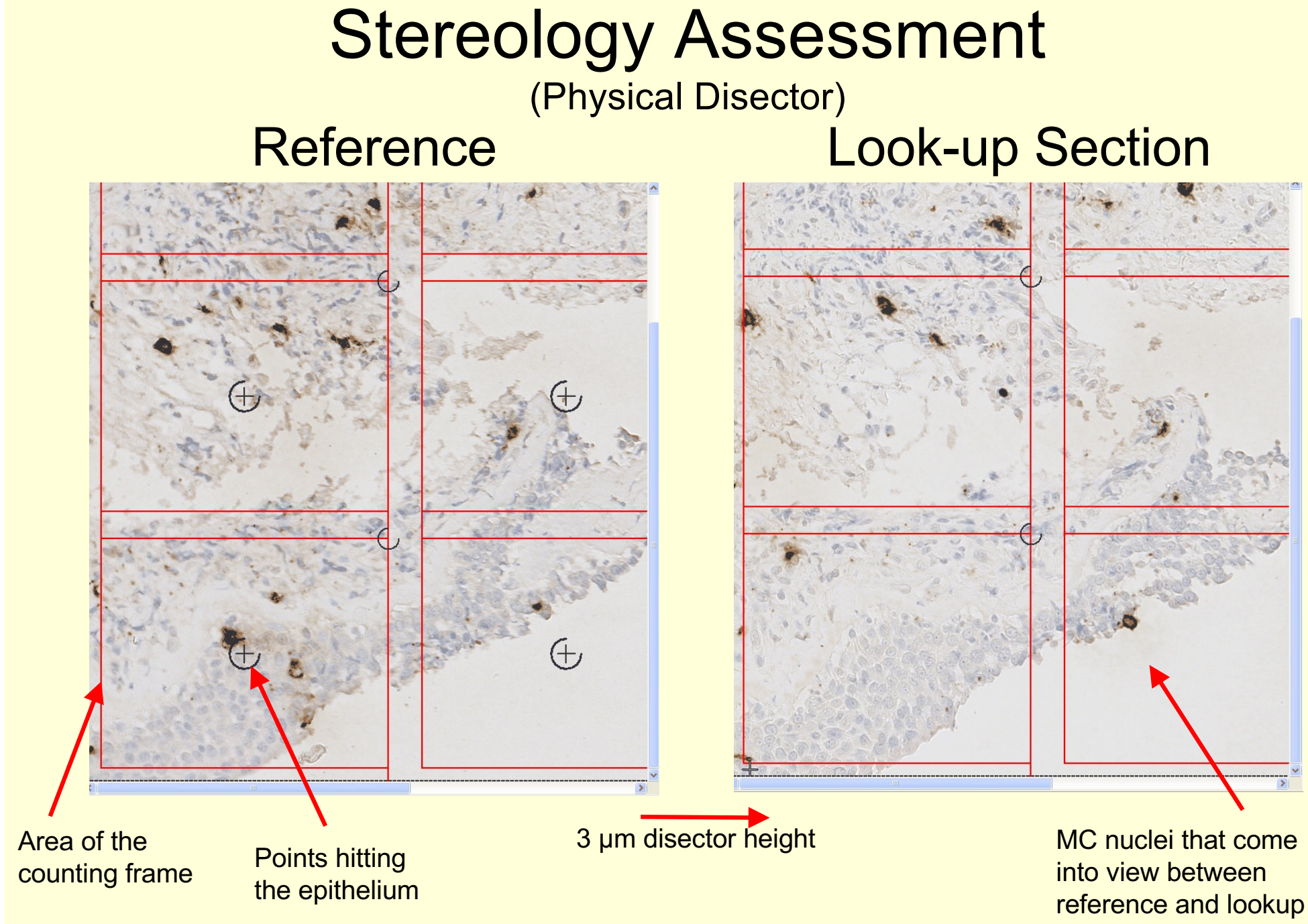


Figure E1 – cont'd

Example of Method 2: Density of Subepithelial MCs

Numeric Density of Subepithelial MCs Relative to Surface Area of the Basal Lamina

- To avoid a "reference trap" due to changes in the reference volume, a secondary method was used to determine the surface area of the basal lamina relative to the reference volume

- Using the same images, we counted points hitting the reference volume (in this example, the subepithelium or P_{sub}) and lines intersecting with the basal lamina (I_{bala}) using a separate line and probe system (see next slide)

Figure E1 – cont'd

Example of Method 2: Numeric Density of Subepithelial MCs Relative to Surface Area of Basal Lamina

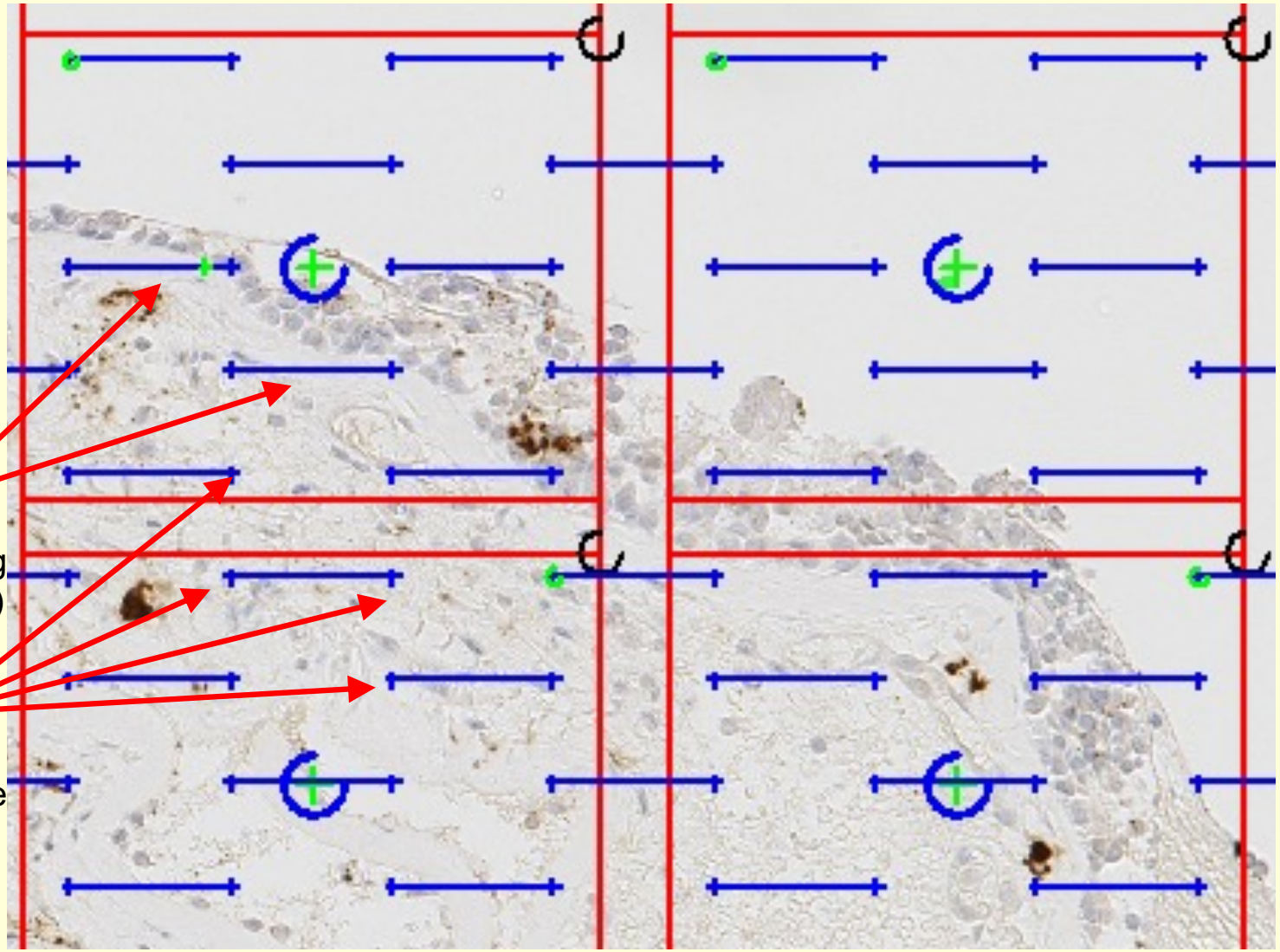


Figure E1 – cont'd

Example of Method 2: Density of Subepithelial MCs

Numeric Density of MCs Relative to Surface Area of the Basal Lamina

- Calculate the number of MCs per reference volume (Formula 1)

$$(1)\ Nv\ MC, sub\ (\#/\mu m^3) = \frac{\sum_{i=1}^{n} Q_{MC}}{(a/f)2h\sum_{i=1}^{n} P_{sub}}$$

- Calculate surface area of basal lamina relative to reference volume (Formula 2)

$$(2)\ Sv\ bala, sub\ (\mu m^2/\mu m^3) = \frac{2\sum_{i=1}^{n} I_{bala}}{l/p\sum_{i=1}^{n} P_{sub}}$$

- Divide Formula 1 by Formula 2 to obtain the numerical density of MCs relative to surface area of the basal lamina

$$(3)\ Ns\ MC, bala\ (\#/\mu m^2) = Nv\ /Sv$$

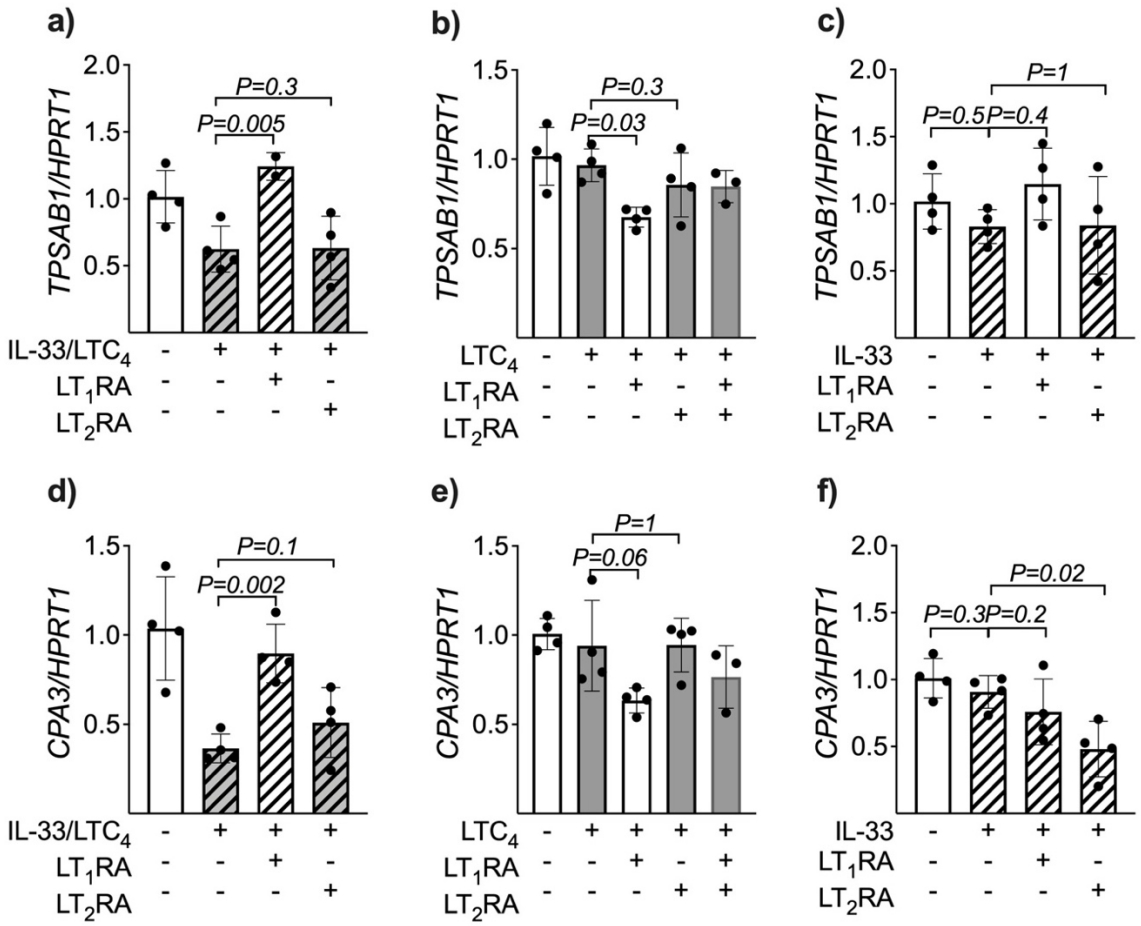


Figure E2. **The effect of LTC_4 and IL-33 on expression of mast cell *TPSAB1* and *CPA3* genes.** **a)** $CysLT_1$ receptor antagonist (LT_1RA) blocked the suppressive effect of IL-33/LTC_4 combined on tryptase (*TPSAB1*) gene expression in LAD2 mast cells. **b and c)** Neither LTC_4 nor IL-33 alone significantly suppressed *TPSAB1* gene expression, but the LT_1RA reduced the expression of *TPSAB1* in the presence of LTC_4, while concurrent treatment with a $CysLT_2$ receptor antagonist (LT_2RA) did not have this effect. **d)** The combined suppressive effect of IL-33/LTC_4 on carboxypeptidase A3 (*CPA3*) gene expression was reversed by treatment with a LT_1RA in LAD2 mast cells. **e)** Neither LTC_4 nor IL-33 alone significantly suppressed *CPA3* gene expression in these experiments, with no significant effects observed for LT_1RA and LT_2RA. **f)** The LT_2RA also decreased the expression of CPA3 in the presence of IL-33. Shown are mean values and SEM bars. Differences between multiple conditions were assessed by 1-way ANOVA with multiple comparisons.

METHODS

Study Subjects and Study Protocol

We used samples from a repository collected at the University of Washington designed to examine differences between mild to moderate asthmatics with and without exercise-induced bronchoconstriction (EIB), which is a form of endogenous AHR, and non-asthmatic controls [1]. Written informed consent was obtained from all participants and the University of Washington Institutional Review Board approved the study protocol and the samples were subsequently placed in a repository. Subjects with asthma had a physician diagnosis of asthma for ≥ 1 year and used only an inhaled β2-agonist for asthma treatment during the study. Some of the subjects in the cohort had used a single daily therapy such as a leukotriene receptor antagonist (LTRA) or low dose ICS that was stopped for at least 2 weeks prior to any of the study procedures or sample collections. None of the subjects with asthma had treatment for acute asthma within the prior month, hospitalization for asthma within the prior 3 months, or history of life-threatening asthma. Subjects were excluded if their FEV_1 was below 65% of predicted, or symptoms of asthma required treatment with a β_2-agonist more frequently than once a day. A methacholine challenge with a PC_{20} < 4 mg/ml was used to confirm the diagnosis of asthma. A dry air exercise challenge was conducted at least 2 days after the methacholine challenge. Based on the results of the exercise challenge, participants with asthma were characterized as EIB+ if they had a ≥ 10% fall in FEV_1 following exercise challenge and EIB- if they had a ≤ 7% fall in FEV_1 following exercise challenge [2, 3]. These cut offs were used to focus on differences in phenotype, those with intermediate values were excluded from the group comparison. Spirometry, exercise, and methacholine challenges were conducted in accordance with American Thoracic Society standards [4, 5].

Control subjects without asthma were enrolled who had a FEV_1 of ≥ 80% predicted, negative methacholine challenge (PC_{20} ≥ 8 mg/ml), negative dry air exercise challenge test (< 7% fall in FEV_1 following exercise), and no more than one positive skin prick test from a panel of 14 aeroallergens. None of the subjects had a history of smoking cigarettes within the prior year or ≥ 7 pack-year total of smoking.

Either epithelial brushings or endobronchial biopsy samples were available from 10 controls, 12 EIB- asthmatics, and 18 EIB+ asthmatics. Endobronchial biopsy tissue was inadequate for stereology assessment in 1 control, 2 EIB- asthmatics, and 1 EIB+ asthmatic. Insufficient RNA was available from the epithelial brushings for the PCR analysis in 1 control, 2 EIB- asthmatics,

and 2 EIB+ asthmatics. Two of the subjects in the EIB- group had been using a daily controller therapy prior to enrollment in the study (a low dose ICS, and a low dose ICS/long-acting beta agonist (LABA) combination), while in the EIB+ group three subjects had been using a daily controller therapy prior to enrollment (a LTRA, a low dose ICS, and a low dose ICS/LABA combination). The remaining subjects with asthma did not use any daily controller therapy for their asthma and none of the subjects were treated with daily controller therapies during the course of the study.

Induced Sputum

Induced sputum was conducted with 3% saline via an ultrasonic nebulizer for 20 min [6]. After the sputum sample was dispersed in 0.1% dithiothreitol, the total cell count was determined with a hemocytometer, and slides for differential cell counts were prepared with a cytocentrifuge. The dispersed induced sputum sample was centrifuged at 250 g for 10 min, the supernatant removed, and the cell pellet treated with caotropic lysis buffer for RNA isolation.

Bronchoscopy

Research bronchoscopy was conducted 2-10 days after the induced sputum in accordance with established guidelines [7]. During bronchoscopy, four to six endobronchial biopsies were obtained from 2nd to 5th generation carina of the right lower and middle lobes using a 1.8 mm forceps. The biopsies were fixed in methyl Carnoy's solution prior to embedding in paraffin.

Quantitative PCR and T2 Gene Mean

Real-time PCR analysis of induced sputum cells was conducted using TaqMan-based quantitative PCR methods [8]. RNA from induced sputum cells from 37 asthmatic participants and 15 healthy control subjects were analyzed for expression of selected genes relevant to airway inflammation: *IL4*, *IL5*, *IL13*, *CMA1*, *TPSAB1*, and *CPA3*. The expression of 4 housekeeping genes, *GAPDH*, *PPIA*, *YWHAZ*, and *PSMB2* were also measured. One sample with housekeeping gene cycle threshold values of greater than 35 was excluded. Some reactions yielded no cycle threshold value, in which case we assigned a gene expression value equal to the minimum gene expression detected in other samples for that gene.

Immunohistochemistry and Design-based Stereology

We previously used the physical dissector method to enumerate the density of mast cells in the airways [9, 10]. Here we apply stereology methods to quantify the density of eosinophils in the epithelium and in the area below the epithelium including the lamina propria and submucosa,

referred to in this manuscript as the subepithelial space. The stereology methods used are illustrated in supplementary figure E1. Endobronchial biopsy specimens from each subject were embedded in isector molds to generate isotropic uniform random (IUR) sampling and then grouped after random orientation into a single paraffin block [11]. Three serial 3 μm sections from each paraffin block were mounted on each slide. Eosinophils in endobronchial tissue were localized using a murine monoclonal anti-Eosinophil Peroxidase (EPX, clone MM25-82.2, provided by Dr. James J. Lee) and a secondary biotinylated antibody (Vector Laboratories, Burlingame, CA) visualized by 3,3'-diaminobenzidine (DAB) with nickel chloride enhancement. Hematoxylin was used as a nuclear counterstain. Slides were scanned in Brightfield at 20X objective using the Nanozoomer Digital Pathology slide scanner (Hamamatsu; Bridgewater, New Jersey). The digital images were then imported into the Visiopharm system using the newCAST Whole Slide Stereology system for quantitative morphometry (Visiopharm, Hoersholm, Denmark).

In the AutoDisector module, regions of interest (ROIs) were outlined and the first and second sections were paired and aligned as the "reference" and "look-up" sections, creating a 3 μm distance for our physical disector. The disector height was based on the size of eosinophil nuclei to provide a discrete reference that can be distinguished from eosinophil granules and fragments of cells. We limited the analysis to samples with at least 3 high-powered fields per biopsy. Counting frames were superimposed on simultaneous images of both the reference and look-up sections, and the number of eosinophil nuclei (Q_{EOS}) coming into view in the disector between the reference and look-up sections was enumerated in both directions. A point associated with each counting frame was used to determine the subepithelial reference volume by enumerating points hitting the subepithelial space (Psub) within the counting frame. The numeric density of subepithelial eosinophils was related to the subepithelial volume of the counting frame (henceforth referred to as the "numerical density of eosinophils" or "eosinophil density") as shown in Formula 1. To avoid the so called "reference trap", resulting from changes in airway wall volume (i.e. changes in reference volume), we also determined the surface area of the basal lamina relative to the subepithelial volume (Sv) on the same set of images by counting points hitting the subepithelial space (Psub) and lines intersecting with the basal lamina (Ibala) using a separate line and point probe system (Formula 2). Dividing Nv by Sv (Formula 3) determined the number of subepithelial eosinophils relative to the surface area of the basal lamina (Ns Eos, bala, henceforth referred to as "surface density of eosinophils"). To minimize artifacts related to inadequate tissue

quality, we excluded tissue samples with 0 counting frame-associated points hitting the subepithelium, and ≤ 1 lines intersecting with the basal lamina.

$$(1)\ Nv\ Eos, sub\ (\#/\mu m^3) = \frac{\sum_{i=1}^{n} Q_{EOS}}{(a/f)2h\sum_{i=1}^{n} P_{sub}}$$

$$(2)\ Sv\ bala, sub\ (\mu m^2/\mu m^3) = \frac{2\sum_{i=1}^{n} I_{bala}}{l/p\sum_{i=1}^{n} P_{sub}}$$

$$(3)\ Ns\ Eos, bala\ (\#/\mu m^2) = Nv/Sv$$

The same principle was applied to estimate the number of eosinophils per reference volume of airway epithelium (i.e., the numerical density of intraepithelial eosinophils per reference volume), and to estimate the numerical density of epithelial eosinophils per surface area of the basal lamina.

This approach based on sampling of isotropic uniform random (IUR) samples that are then aligned and overlayed with counting frames provides a robust and unbiased estimated of the number per density or area but led to the exclusion of some results relating to both eosinophil density in the epithelial and subepithelial compartments. Because resampling of these biopsy specimens could introduce bias, the data points were excluded, and all of the available data were used in the analysis.

Isolation and assessment of cysteinyl leukotriene formation by peripheral blood eosinophils

Peripheral blood eosinophils were obtained from volunteers with a physician diagnosis of asthma and/or allergic rhinoconjunctivitis and ≥ 1.2 x 10^5 eosinophil/ml of peripheral blood. The collection of peripheral blood was approved by the University of Washington Institutional Review Board and written informed consent was obtained from all participants. Granulocytes were isolated from peripheral blood by density gradient centrifugation followed by hypotonic lysis of red blood cells. Eosinophils were removed from the granulocyte fraction by negative immunomagnetic selection (Miltenyi, Bergisch Gladbach, Germany). The purity of eosinophils was determined by differential counts of Romanowski-stained (Diff-Quick) cytospin preparations. Eosinophil viability was assessed by trypan blue exclusion.

Eosinophils were resuspended in HBSS with Ca^{2+} and Mg^{2+} at a concentration of 2 x 10^5 per well in a 5% CO_2 incubator at 37°C. The cells were incubated with human IL-5 (10-100 ng/ml) and/or human IL-33 (1-100 ng/ml) (PeproTech, Rocky Hill, NJ) for 20 min at 37°C. The LTC_4

levels in the supernatant were measured immediately after stimulation with cytokines using an ELISA assay (Cayman Chemical, Ann Arbor, MI).

Assessment of the effects of cysteinyl leukotrienes and IL-33 on mast cell T2 gene expression and protease phenotype

The LAD2 mast cell line was provided by Dr. Arnold Kirshenbaum from the Laboratory of Allergic Diseases, National Institute of Allergy and Infectious Diseases (National Institutes of Health, Bethesda, MD) [12]. LAD2 cells were maintained in STEM Pro-34 SFM Complete Medium (Invitrogen) with L-Glutamine, Penicillin/Streptomycin and human SCF (100 ng/ml). LAD2 cells were propagated by weekly hemi-depletion.

LAD2 cells were resuspended in RPMI medium at a concentration of 5 x 10^5 cells per well in a 5% CO_2 incubator at 37°C and incubated with human IL-33 (1-10 ng/ml) and/or LTC_4 (100nM) for 4 hours. In some conditions, the $CysLT_1$ receptor antagonist (LT_1RA) MK571 (5uM) and/or the $CysLT_2$ receptor antagonist (LTR_2A) HAMI3379 (5uM) (Cayman Chemical, Ann Arbor, MI) were added prior to treatment with IL-33 and/or LTC_4. To assess the effects on the expression of T2 and granule protease genes, total RNA was isolated using a Trizol-based method (Zymo Research, Irvine, CA) and cDNA was prepared prior to quantitative PCR analysis. PCR analysis was conducted using TaqMan primer probe sets with quantification relative to endogenous control gene *HPRT1* using the delta Ct method. Primer-probe sets were obtained from the Applied Biosystems using a FAM probe for *IL13* (Hs00174379_m1), Chymase (*CMA1*) (Hs01095979_g1), Tryptase (*TPSAB1*) (Hs02576518_gH), Carboxypeptidase A3 (*CPA3*) (Hs00157019_m1) and a primer-limited VIC probe for *HPRT1* (4326321E) as an endogenous control (Applied Biosystems, Foster City, CA). Quantitative PCR was performed using a Mastercycler ep realplex system (Eppendorf, Hauppauge, NY).

Statistics

Descriptive analyses were performed to determine the mean, median, standard deviation, and interquartile range for continuous variables and number and relative frequencies of categorical variables. Statistical differences between the different study groups were assessed using a Chi-square for categorical data, Mann-Whitney *U* and Kruskal-Wallis for non-normally distributed continuous variables, and a two-tailed student t-test and ordinary 1-way analysis of variance (ANOVA) with correction for multiple comparisons using the Tukey method for continuous normally distributed variables. A 1-way ANOVA was used to assess differences between the

responses of eosinophils or LAD2 mast cells to the various stimuli. P values were adjusted to control for false discovery rate using Two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli. Associations between continuous variables were assessed by linear regression. A *P* value less than 0.05 was used to establish statistical significance. Statistical analysis was performed using GraphPad Prism version 8 (GraphPad Software, San Diego, California USA)

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