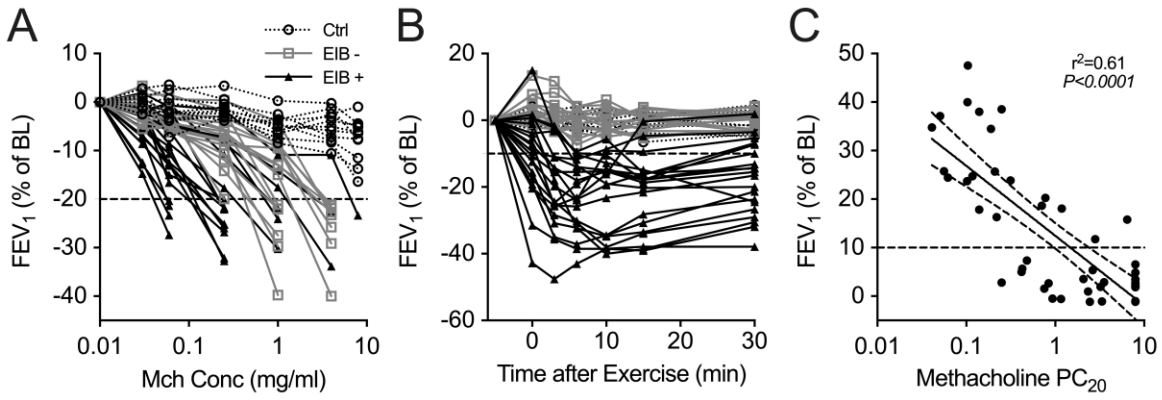
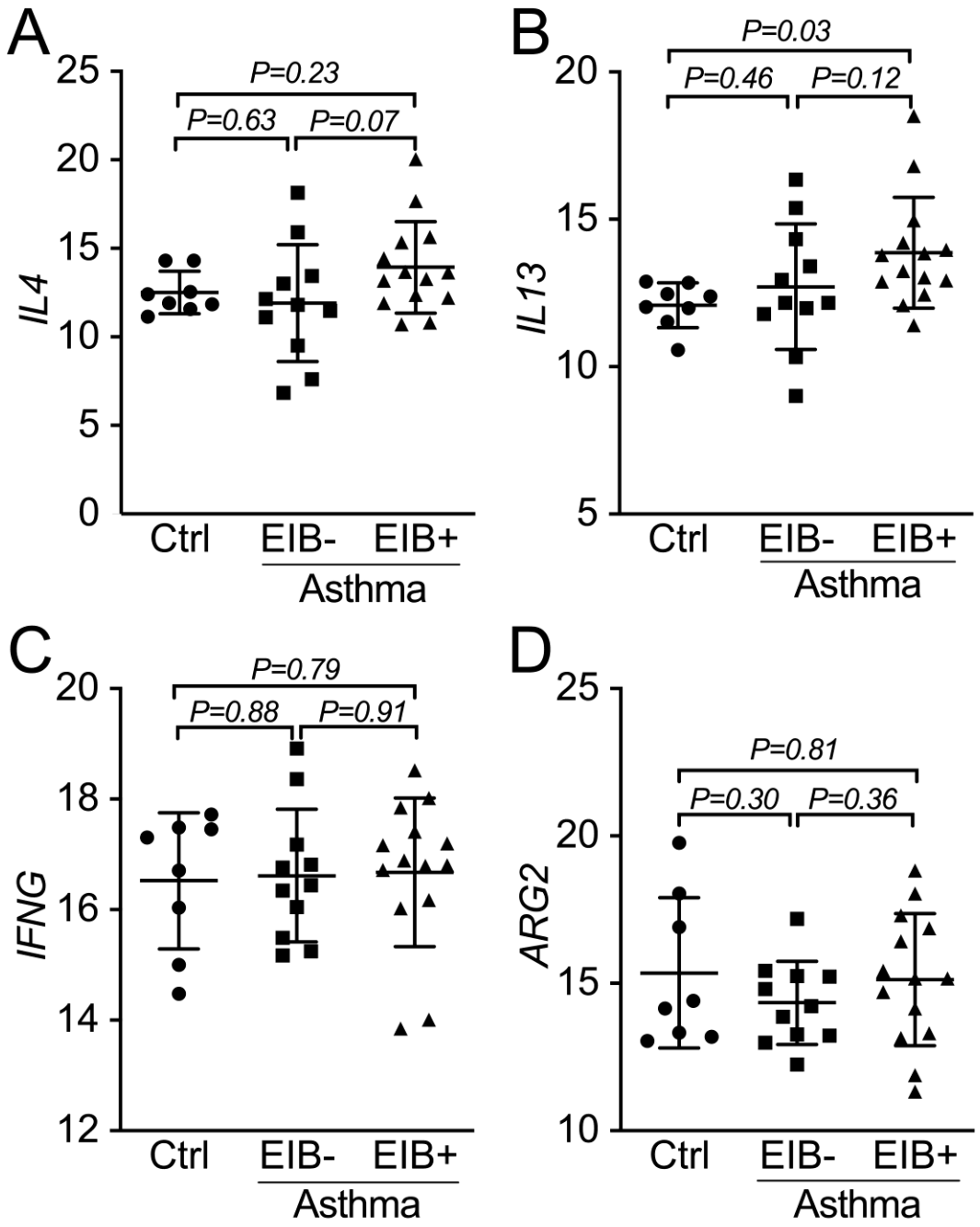
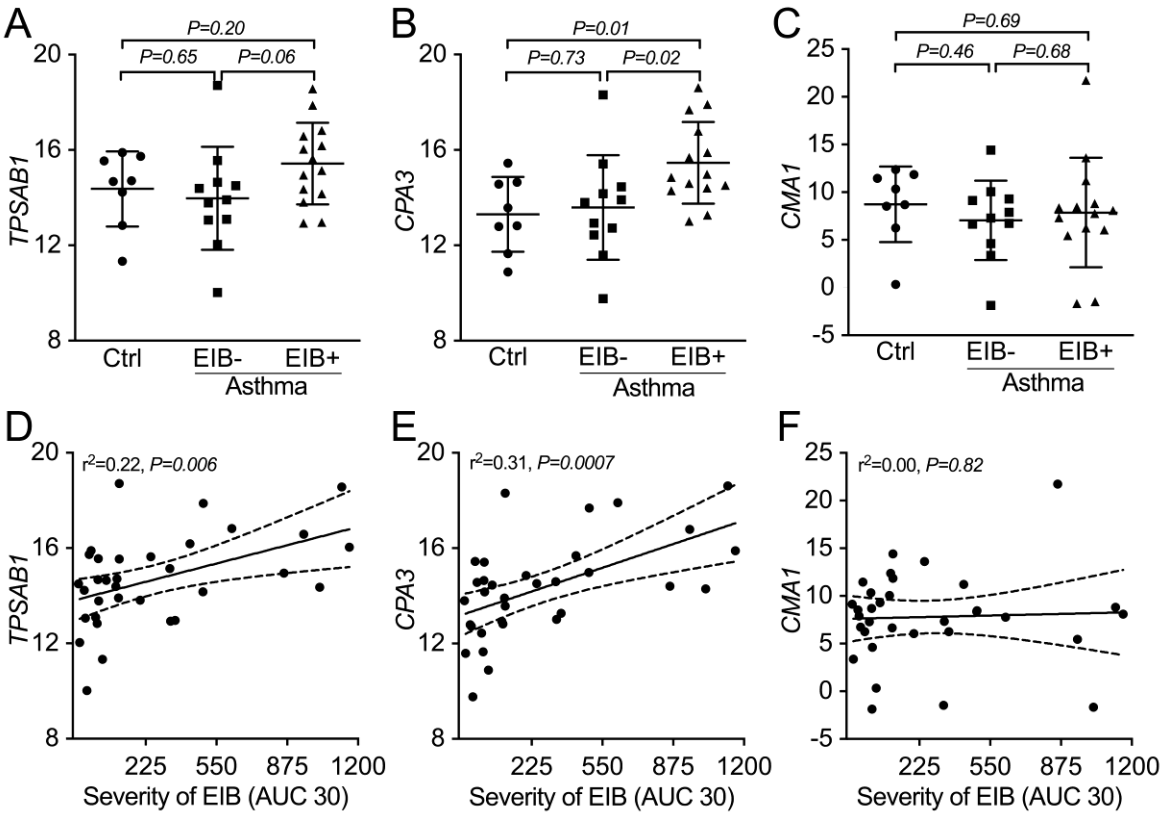


Supplemental Figure 1. (A) Direct AHR to methacholine was present in all subjects with asthma and not in the healthy controls. (B) EIB+ subjects demonstrated indirect airway hyperresponsiveness measured by the response to dry air exercise challenge resulting in >10% drop in FEV_1 (dotted line). (C) Severity of direct AHR and indirect AHR were correlated with one another. The association was tested by linear regression, shown is the regression line and 95% confidence bounds.



Supplemental Figure 2. (A) *IL4* expression trended towards higher in the EIB+ group relative to the EIB- group. (B) *IL13* was significantly higher in the EIB+ group compared to the healthy controls. (C) *IFNG* expression was not different among the 3 groups. (D) *ARG2* expression was

not different among the 3 groups. Shown are means and standard deviations. Analyses are by one-way ANOVA with correction for multiple comparisons.



Supplemental Figure 3. (A) Sputum *TPSAB1* expression was tended to be higher in the EIB+ group relative to the other two groups. (B) Sputum *CPA3* expression was significantly higher in the EIB+ group relative to the other two groups. (C) Sputum *CMA1* expression was equivalent in the 3 groups. Shown are means and standard deviations. Analyses are by one-way ANOVA with correction for multiple comparisons. (D) Sputum *TPSAB1* expression was correlated with EIB severity as measured by the AUC30. (E) Sputum *CPA3* expression was correlated with EIB severity as measured by the AUC30. (F) Sputum *CMA1* expression was not correlated with EIB severity. Associations were tested by linear regression, shown is the regression line and 95% confidence bounds.

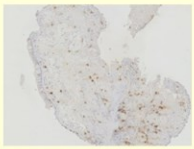
Design-based Stereology

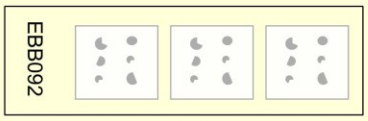
- Stereology - The statistical science of sampling irregular 3D structures in 2D profile using geometric test probes (slabs, sections, lines, points).
- By design, stereological methods make no assumption of the size, shape, orientation, or spatial distribution.
- Estimate a geometrical parameter using 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.

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.
- Mast cell nuclei that come into view between the two sampled sections are counted.





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

MC nuclei

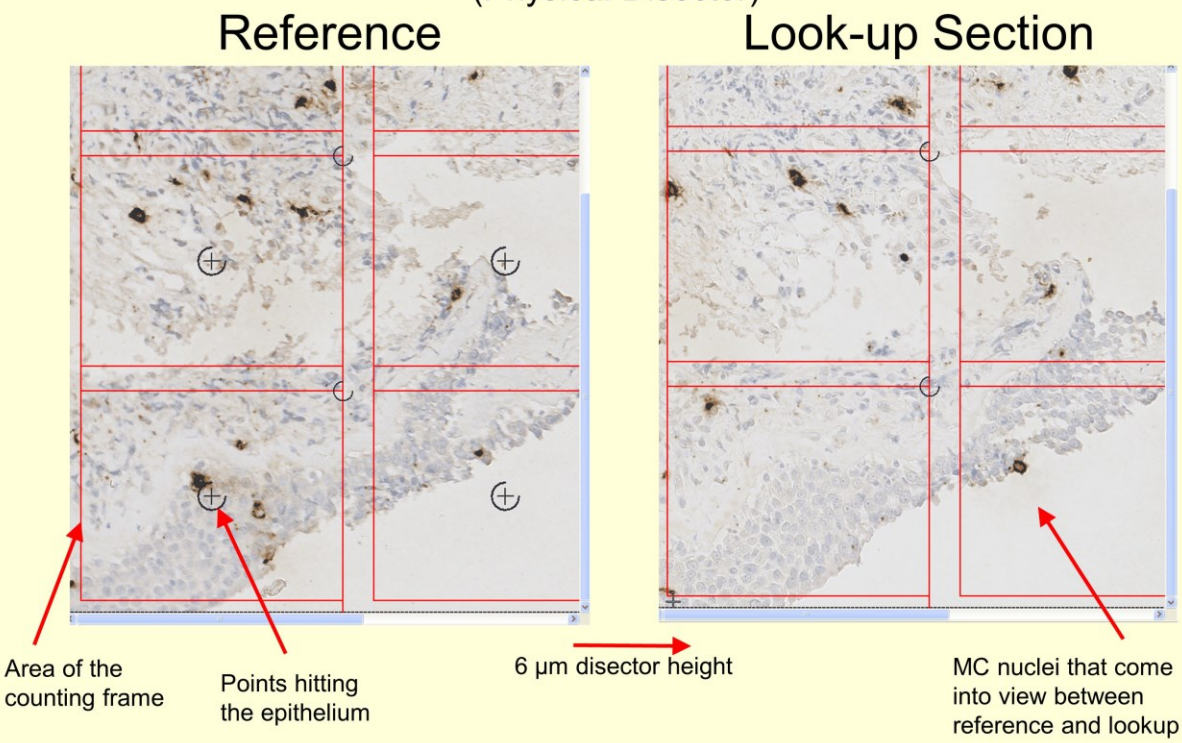
Area of the counting frame

Disector height

Points hitting the epithelium

Stereology Assessment

(Physical Disector)



Density of Submucosal MCs

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

MC nuclei

Area of the counting frame

Disector height

Points hitting the submucosa

Estimation of the numerical density per surface area (surface density)

- To determine the surface density, you must first determine the surface area of the basal lamina per reference submucosal or epithelial volume (Sv).
- We used a linear probe - as illustrated in the next slide and using the following formula.

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

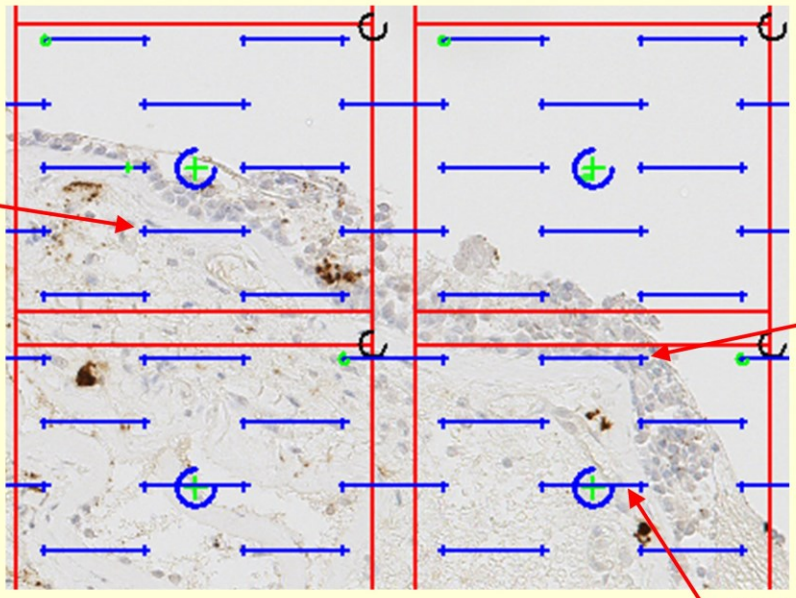
Line intersecting with the basal lamina. More intersections means larger surface area

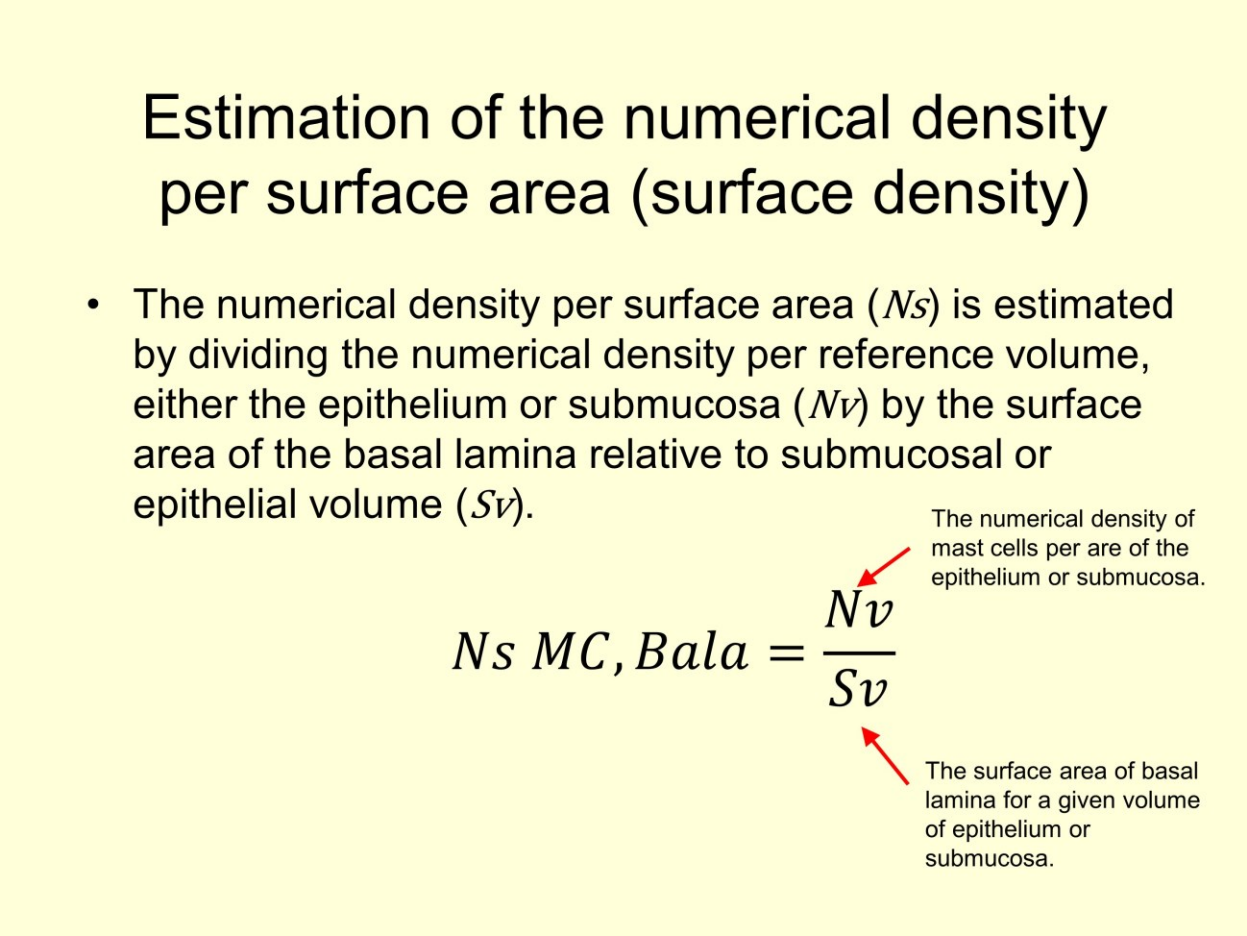
$$Sv\ bala, sub\ (\mu m^2/\mu m^3) = \frac{2\sum_{i=1}^{n} I_{bala}}{l/p\sum_{i=1}^{n} P_{sub}}$$

Points hitting the reference space (epithelium or submucosa)

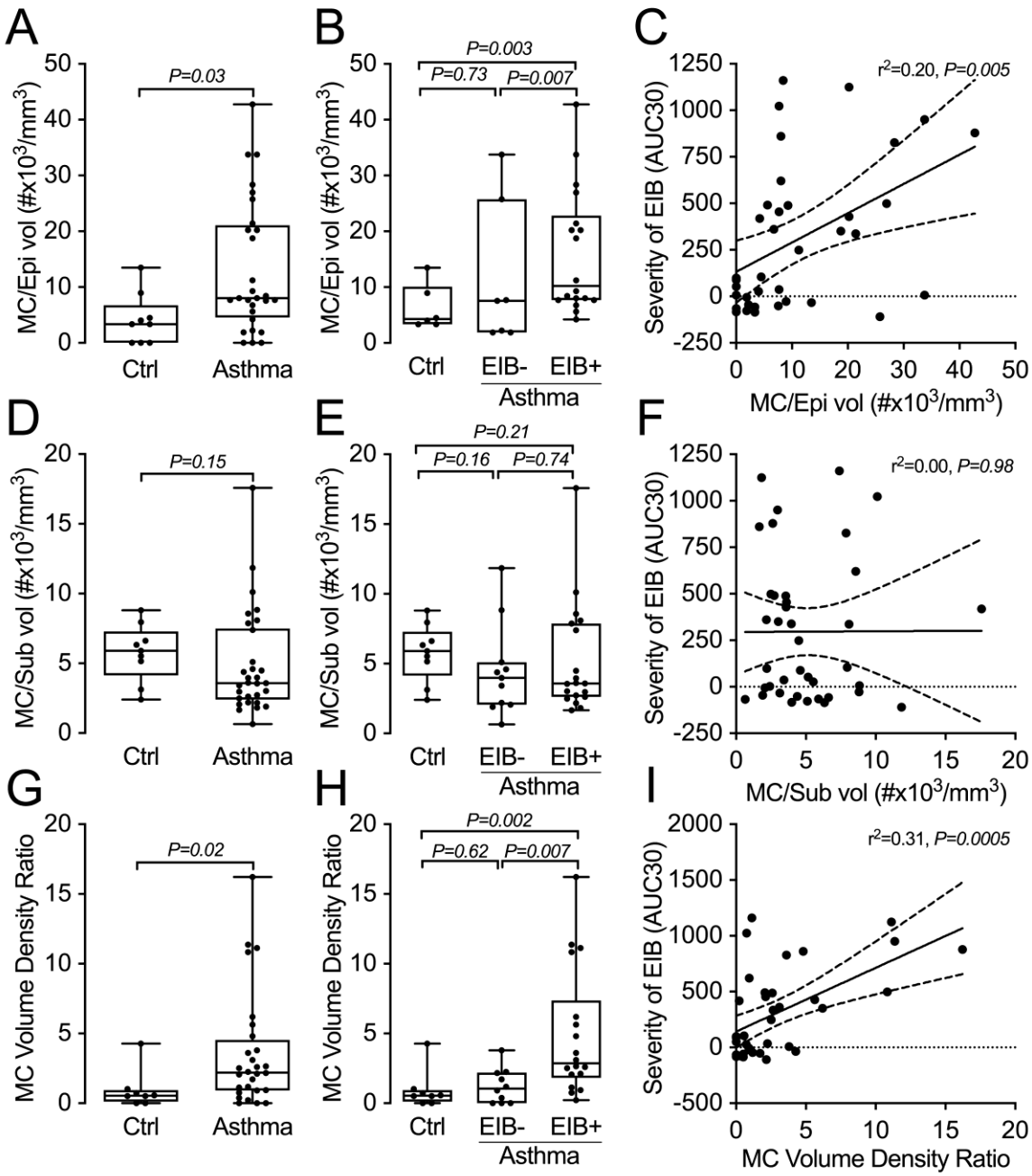
Length of the test line

Stereology Assessment of Surface Area Per Reference Volume



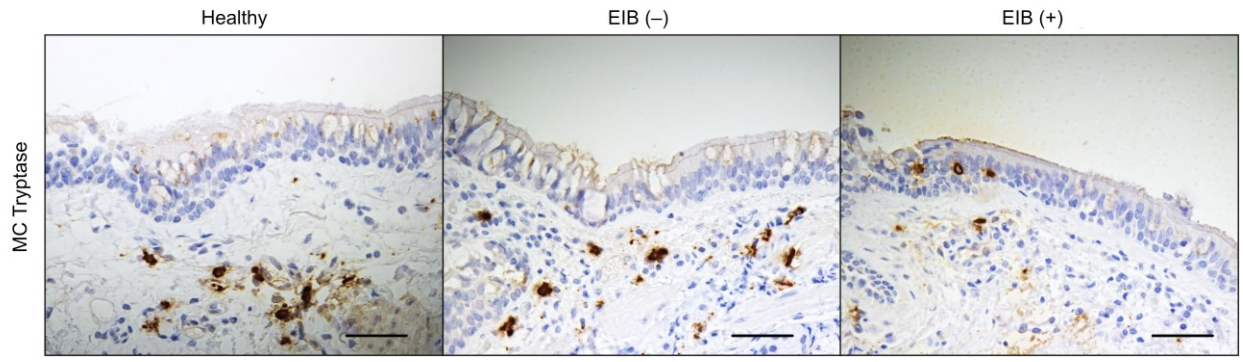


Supplemental Figure 4. Overview of the concepts and the specific methods that were used to quantify the density of mast cells in the epithelium and submucosal spaces by design-based stereology. The density of mast cells per epithelial volume was assessed by the physical disector (i.e. two sections) method using counting frames that were aligned on samples of subsequent sections. The assessment of surface density utilized the linear probe to determine the surface area of the basal lamina relative to the area of the epithelium or submucosa. This metric was combined with the density of mast cells per volume of the epithelium or submucosa to determine the number of mast cells either above or below an associated area of the basal lamina.

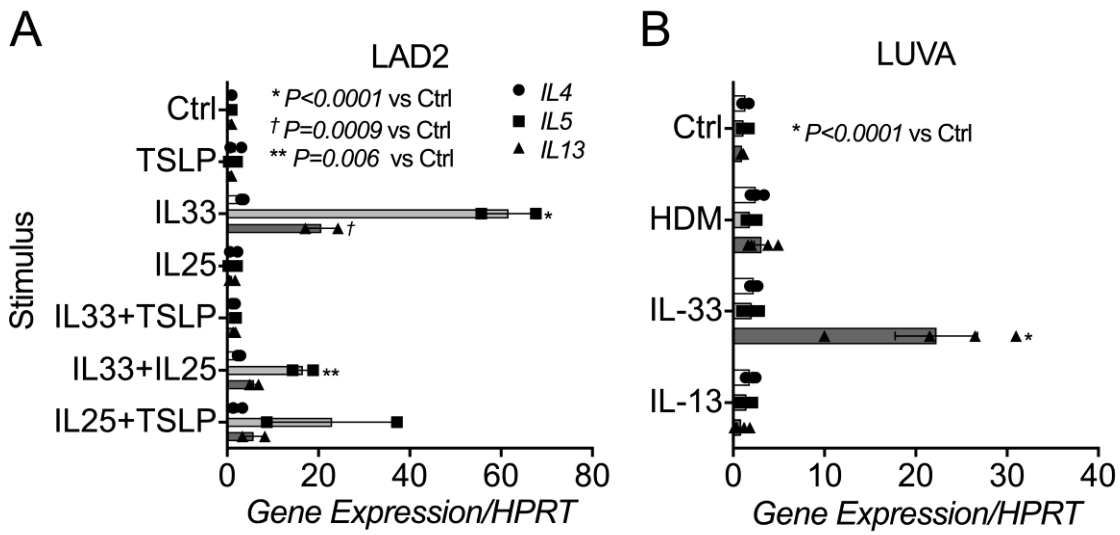


Supplemental Figure 5. (A) Intraepithelial mast cell density relative to the epithelial volume is increased in asthma compared to healthy controls. (B) Intraepithelial mast cell density relative to the epithelial volume is highest in EIB+ subjects compared to EIB- subjects and healthy controls. (C) Intraepithelial mast cell density relative to the epithelial volume is significantly correlated

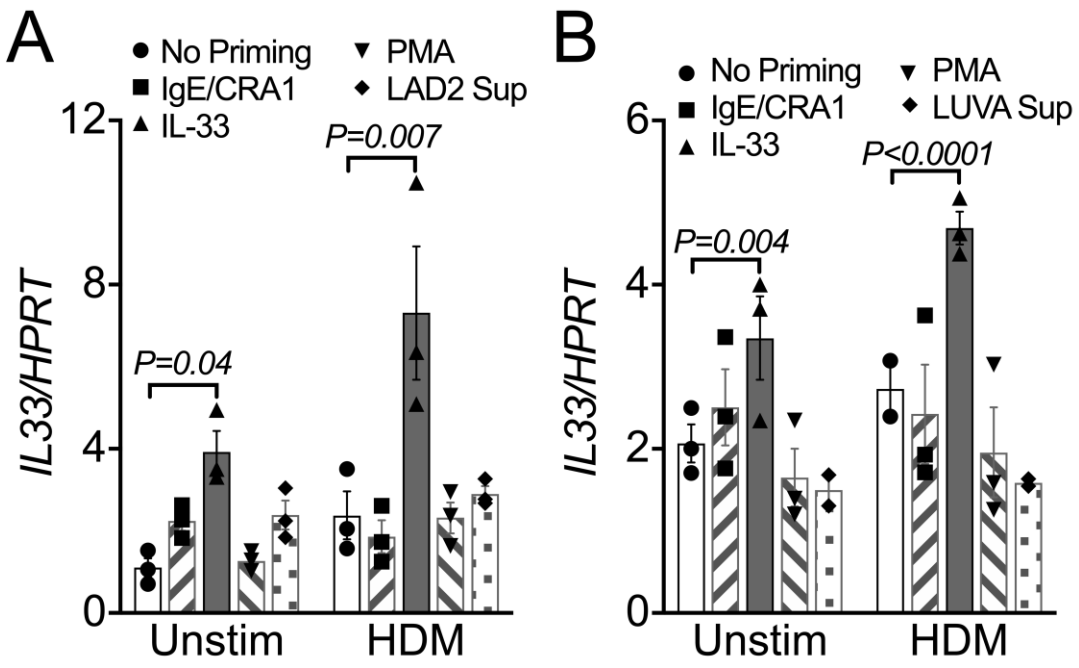
with severity of indirect AHR measured by AUC30. (D) Submucosal mast cell density relative to the surface area of the basal lamina trends towards lower in asthma compared to healthy controls but is not significantly different. (E) Submucosal mast cell density relative to the surface area of the basal lamina is not different comparing EIB+ asthma subjects, EIB- asthma subjects and healthy controls. (F) Submucosal mast cell density relative to the surface area of the basal lamina is not associated with severity of indirect AHR measured by AUC30. (G) The volume density ratio of mast cells in the epithelium relative to the submucosa is increased in asthma. (H) It is highest in the EIB+ asthma subjects relative to the EIB- subjects and healthy controls. (I) Additionally, it is significantly associated with the severity of indirect AHR measured by AUC30. Group comparisons are shown as boxplots with median, interquartile range, minimum, and maximum. Significance was assessed by the Mann-Whitney *U* test (two group) or the Kruskal-Wallis test with Dunn post-hoc test for multiple comparisons (three group). Associations are by linear regression, shown are regression lines and 95% confidence bounds.



Supplemental Figure 6. Representative images that illustrate the shift in mast cell populations identified by design-based stereology. Images show granule staining of mast cells (dark brown DAB staining of tryptase) in the presence of Hematoxylin to counterstain the nucleus. Mast cells were identified in the epithelium above the basal lamina predominantly in EIB (+) asthmatics, while submucosal mast cells below the basal lamina were identified in all of the groups and were particularly prominent in the healthy controls. Images were taken using a 40x objective. The bar represents a 5 μm distance.



Supplemental Figure 7. (A) IL-33 induced gene expression of the type-2 cytokines *IL5* and *IL13*, but not *IL4*, in LAD2 cells, whereas IL-25, TSLP, HDM extract, or IL-13 did not. In the LAD2 cell line, the addition of IL-25 or TSLP to IL-33 during stimulation partially attenuated expression of *IL5* and *IL13* (n=2/condition). (B) IL-33 induced gene expression of the type-2 cytokine *IL13*, but not *IL4* or *IL5*, in LUVA cells, whereas IL-25, TSLP, HDM extract, or IL-13 did not (n=4/condition). Differences between multiple groups were assessed by one-way ANOVA with correction for multiple comparisons. Shown are mean values and standard error bars.



Supplemental Figure 8. (A) IL-33 primed LAD2 mast cells amplified epithelial *IL33* expression either with or without HDM treatment of the epithelial surface. In contrast priming mast cells by IgE-mediated degranulation, PMA stimulation, or using LAD2 supernatant (sup) did not alter epithelial *IL33* expression (n=3/condition). (B) The same relationship was observed with LUVA mast cells (n=3/condition). Differences between multiple conditions were assessed by one-way ANOVA with correction for multiple comparisons. Shown are mean values and standard error bars.

Supplemental Table 1. Shown are the clinical and demographic characteristics of the 46 subjects. Values are mean ± standard deviation or count (percentage). P-values are from appropriate test for data – two-tailed Student's t-test for continuous data or Fisher's exact tests for categorical data.

	Healthy controls N=11	EIB- asthma N=15	EIB+ asthma N=20	*P-value*
Age (yrs)	31.5 ± 12.6	24.4 ± 4.5	26.6 ± 8.5	*0.13*
Gender: male	2 (18%)	4 (27%)	6 (30%)	*0.84*
Race: White Asian	8 (73%) 3 (27%)	14 (93%) 1 (7%)	17 (85%) 3 (15%)	*0.36*
Ethnicity: Non-hispanic	11 (100%)	15 (100%)	18 (90%)	*0.41*
T2GM sample: Yes	10 (91%)	9 (60%)	14 (70%)	*0.23*
Stereology sample: Yes	10 (91%)	12 (80%)	19 (95%)	*0.38*
Baseline FEV_1 (L)	3.4 ± 0.8	3.5 ± 0.5	3.3 ± 0.7	*0.63*
Baseline FEV_1 % Predicted	97.2 ± 11	91.7 ± 9.2	88.4 ± 10.7	*0.09*
Baseline FVC (L)	4 ± 1	4.4 ± 0.9	4.5 ± 1.1	*0.35*
Baseline FVC % Predicted	96.1 ± 12.5	96.7 ± 8.6	102.5 ± 10	*0.15*
Baseline FEV_1/FVC	0.9 ± 0.1	0.8 ± 0.1	0.7 ± 0.1	*<0.001*
PC_{20} (mg/ml)	8 ± 0	1.7 ± 1.2	0.7 ± 1.5	*<0.001*
Severity of EIB (Max % Fall in FEV_1)	2.2 ± 2.5	2.4 ± 2.6	26.9 ± 9.9	*<0.001*
Severity of EIB (AUC30)	1.8 ± 63	-8.6 ± 71.7	603.5 ± 303	*<0.001*

P-values are from appropriate test for data – t-test for continuous data or Fisher's exact tests for categorical data.

Supplemental Table 2. Associations between individual physiological variables and the individual type-2 cytokines as well as the T2GM in the full study population and in the group of individuals with asthma.

Full Population (N=33)								
	IL4		*IL5*		*IL13*		T2GM	
	r^2	*P value*	r^2	*P value*	r^2	*P value*	r^2	*P value*
FEV_1 (%)	0.04	*0.26*	0.1	*0.08*	0.06	*0.17*	0.08	*0.12*
FEV_1/FVC	0.13	*0.04*	0.18	*0.01*	0.23	*0.005*	0.22	*0.006*
PC_{20}	0.04	*0.28*	0.1	*0.07*	0.13	*0.04*	0.1	*0.07*
AUC30	0.2	*0.008*	0.26	*0.002*	0.23	*0.005*	0.28	*0.002*
Asthma (N=25)								
	IL4		*IL5*		*IL13*		T2GM	
	r^2	*P value*	r^2	*P value*	r^2	*P value*	r^2	*P value*
FEV_1 (%)	0.01	*0.59*	0.12	*0.09*	0.05	*0.27*	0.06	*0.23*
FEV_1/FVC	0.14	*0.07*	0.15	*0.06*	0.22	*0.02*	0.2	*0.03*
PC_{20}	0.08	*0.17*	0.04	*0.33*	0.05	*0.26*	0.07	*0.21*
AUC30	0.21	*0.02*	0.23	*0.02*	0.19	*0.03*	0.25	*0.01*

Values for the T2GM correspond graphically with Figure 2. Associations were assessed by linear regression.

Supplemental Table 3. Shown are the clinical and demographic characteristics of the pediatric subjects.

	Healthy controls N=3	Asthma N=3
Age (yrs)	10.8 ± 4.9	11.7 ± 4.4
Gender: male	2 (67%)	2 (67%)
Total IgE (IU/mL)	10.7 ± 8	582 ± 399
# Sensitizations	0	3 ± 2.6
FeNO (ppb)	10.3 ± 4.5	32 ± 31.9
FEV_1 % Predicted	102.3 ± 8.5	101 ± 7.5
FVC % Predicted	106.0 ± 14	111 ± 3
FEF25-75 % Predicted	92.7 ± 1.5	83.3 ± 31.1

Values are mean ± standard deviation or count (percentage).

Supplemental Materials and Methods

Adult 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 EIB and non-asthmatic controls (1). Subjects with asthma had a physician diagnosis of asthma for ≥ 1 year, and used only an inhaled β_2-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 forced expiratory volume in the first second (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 extremes of 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 ($PC_{20} \geq 8$ 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 19 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.

Skin Prick Testing

Skin prick testing was conducted to 14 aeroallergens (*Alternaria tenius*, *Hormodendrum*, *Penicillium* mix, Grass mix, *Dermatophagoides farinae, Dermatophagoides pteronyssinus*, Cockroach mix, Cat dander, Dog dander, Alder, Birch (white), Cottonwood, English plantain, Ragweed mix) in addition to positive (histamine) and negative (saline) controls. Antihistamines and tricyclic antidepressants were restricted for 48 hours prior to testing. The results of the skin prick testing were quantified as follows: 0, no reaction; 1+, erythema ≤ 15 mm; 2+ erythema ≥ 15 mm with wheal < 3 mm; 3+, wheal 3-6 mm; 4+, wheal > 6 mm or pseudopod.

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 chaotropic 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, 4 epithelial brushings were obtained from 2^{nd} to 5^{th} generation airways of the left lower lobe and lingua using a 3 mm nylon cytology brush. Four to 6 endobronchial biopsies were obtained from 2^{nd} to 5^{th} 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. Two of the brushings were used to establish primary airway epithelial cell cultures, and two brushings were used to make cell lysates for RNA isolation.

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 8 genes relevant to airway inflammation; *IL4*, *IL5*, *IL13*, *IFNG*, *CMA1*, *TPSAB1*, *CPA3* and *ARG2*. 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.

Real-time PCR analysis of epithelial brushings was conducted using TaqMan primer probe sets with quantification relative to a standard curve of the gene copy number (9). First strand cDNA synthesis was conducted using oligo (dT) primers (SuperScript III, Invitrogen, Carlsbad, CA). Primer-probe sets were obtained from Applied Biosystems with FAM probes for *TPSAB1* (Hs02576518_gH), *CPA3* (Hs00157019_m1), and *CMA1* (Hs01095979_g1). Real-time PCR was performed using a Mastercycler ep realplex system (Eppendorf, Hauppauge, New York). Quantities of the specific transcripts of *TPSAB1*, *CPA3*, and *CMA1* were determined by comparing Ct values observed in each sample with Ct values obtained from standard curve generated using a dilution series of plasmid DNA containing a single copy of the gene of interest.

Cytokine Measurement by ELISA

Human IL-5, IL-13 and IL-33 protein content from cell culture supernatants was determined by ELISA per manufacturer's instructions (For IL-5 and IL-13, R&D, Minneapolis, MN; for IL-33, eBioscience, San Diego, CA). ELISA plates were read using a Biotek Synergy 4 or EL800 platereader (Winooski, VT).

Immunohistochemistry and Design-based Stereology

We previously used the physical dissector method to enumerate the density of mast cells in the epithelial layer (1). Here we apply stereology methods to quantify the density of mast cells in the epithelium and submucosa. 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 (10). Three serial 3 μm sections

from each paraffin block were mounted on each slide. Mast cells in endobronchial tissue were localized using a murine monoclonal anti-tryptase antibody (clone AA1, Invitrogen, Carlsbad, CA) 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 mast cell nuclei to provide a discrete reference that can be distinguished from mast cell granules and fragments of cells. We limited the analysis to samples with at least 2 biopsy specimens, and 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 mast cell nuclei (Q_{MC}) 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 reference volume in the submucosa and epithelium by enumerating points hitting the submucosa (P_{sub}) and epithelium (P_{epi}) within the counting frame. The numeric density of submucosal mast cells was related to the submucosal volume of the counting frame as shown in Formula 1. We similarly assessed the numeric density per epithelial volume. We also determined the surface area of the basal lamina relative to the submucosal volume and relative to the epithelial volume (*Sv*) on the same set of images by counting points hitting the submucosa (P_{sub}) and point hitting the

epithelium (P_{sub}) and associated lines intersecting with the basal lamina (I_{bala}) using a separate line and point probe system (Formula 2). Dividing *Nv* by *Sv* (Formula 3) determined the number of submucosal mast cells relative to the surface area of the basal lamina (*Ns MC, bala*). We used a similar approach to identify the number of mast cells relative to the surface area of the basal lamina (*Ns MC, epi*). To minimize artifacts to related inadequate tissue quality, we excluded tissue samples with ≤ 3 counting frame-associated points hitting the submucosa, and ≤ 4 lines intersecting with the basal lamina based on the frequency distribution of sampling in the study (5th percentile).

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

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

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

Human Mast Cell Culture

Human umbilical cord blood was obtained from anonymous donors to Bloodworks Northwest in accordance with established institutional guidelines. Heparin-treated cord blood was sedimented with 3% dextran to remove nucleated red blood cells, and the plasma layer transferred to a Ficoll gradient. CD34+ cells were isolated from the mononuclear cell layer by immunomagnetic selection and placed in RPMI 1640 medium containing 10% FCS and 2 mM L-Glutamine. During the first week of culture the CD34-selected cord blood cells were treated with IL-3 (30 ng/ml), IL-6 (100 ng/ml), and SCF (100 ng/ml) (11). During subsequent weeks, nonadherent cells were transferred to a new flask containing IL-6 (100 ng/ml), and SCF (100 ng/ml) (11). Cytospin preparations were monitored weekly using toluidine blue staining to

monitor the percentage of metachromatic cells. Mature CBMCs were defined by >95% toluidine blue positivity, typically at about 8 weeks of cell culture.

The LUVA cell line was provided by Dr. John W. Steinke from the University of Virginia, Charlottesville, VA (12). The LAD2 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 (13). LUVA and LAD2 human mast cell lines 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.

Primary Bronchial Epithelial Cell Culture

Primary bronchial epithelial cell (BEC)s were isolated from tracheal segments that were collected from a discarded segment of donor airway at the time of lung transplant. The procedure for isolation of epithelial cells was approved by the University of Washington Human Subjects Review Committee. After removal of connective tissue and adherent lymph nodes, the tissue was divided and placed in a protease and DNAse digestion solution for 24hr prior to neutralization and isolation of BECs (14). The isolated epithelial cells were re-suspended in bronchial epithelial basal media (BEBM, Lonza, Allendale, NJ) supplemented with bovine pituitary extract, insulin, hydrocortisone, gentamicin, amphotericin B, fluconazole, retinoic acid (RA), transferrin, triiodothyronine, epinephrine, and human recombinant epidermal growth factor (EGF) (serum-free BEGM) and seeded into culture vessels pre-coated with type 1 collagen and maintained at 37°C in a humidified incubator. Cells were cryopreserved and/or subcultured at 90-95% confluence. Cryopreserved primary epithelial cells at passage 1 or 2 were used for differentiated air-liquid-interface (ALI) organotypic cultures. Epithelial cells were sub-cultured at a density of

1.0×10^5 cells/well in type I collagen-coated transwell membranes (12 mm diameter, 0.4 μm pore size). The cells were initially proliferated submerged in medium composed of equal amounts of BEBM and DMEM supplemented as before but with 30 ng/ml RA (100 nM), 10 ng/ml EGF, ethanolamine (80 μM), $MgCl_2$ (0.3 mM), and $MgSO_4$ (0.4 mM) for approximately 7 days (14). After the cells were fully confluent, the apical medium was removed, and the cells were culture in ALI for an additional 21 days with the same medium except that the EGF concentration was lowered to 0.5 ng/ml.

Epithelial-Mast Cell Co-culture Model System

In initial experiments with mast cells alone, CBMCs, LUVA cells or LAD2 cells were treated with IL-33, IL-25, TSLP (each 10 ng/ml, alone or in combination), HDM and IL-13 (10 ng/ml), and RNA was isolated after 4 hours of culture, or the supernatant was collected for protein analysis after 48 hours. Details of the individual experiments are provided in the figure legends.

We extended these results to a model of primary human BECs co-cultured with LAD2 cells, LUVA cells or CBMCs that were primed with different stimuli such as IL-33 for 4h or unprimed in a separate dish, washed and then resuspended in ALI media and transferred to the basolateral compartment of the transwell-containing culture dish for co-culture with primary BECs in organotypic culture. HDM (100 μg/ml) or PBS was then added to the epithelial cells in the apical compartment of the transwell and allowed to incubate at 37ºC for 4h. The PBS or HDM was then removed from the apical compartment and the epithelial cells were washed with PBS and allowed to incubate for a total of 48h. RNA was isolated from the epithelial cells after 48 hours, and in some cases the basolateral fluid was collected for cytokine analysis. In our primary model, we compared IL-33 (10 ng/ml) priming to no stimulus prior to transfer into co-

culture. In additional experiments, we compared priming with IL-33 for 4 hours, to treatment of mast cells on the plate prior to transfer with HDM (100 μg/ml), PMA (10 ng/ml), LAD2 supernatant, LUVA supernatant, and passive sensitization with human polyclonal IgE (Catalog # 31-AI01, Fitzgerald, Concord, MA) followed by IgE crosslinking with the CRA1 antibody ("IgE/CRA1", CRA1, clone AER-37, 100 ng/ml, Invitrogen, Carlsbad, CA) (1).

Pediatric Study Subjects and Study Protocol

Children ages 6-18 years who were undergoing an elective surgical procedure requiring endotracheal intubation and general anesthesia were recruited for this study. A detailed medical history was obtained at enrollment. Inclusion and exclusion criteria for children with atopic asthma included a one year history of physician-diagnosed asthma, use of a short-acting beta-agonist ≥ twice a month or daily use of an inhaled corticosteroid or leukotriene receptor antagonist, birth at ≥ 36 weeks gestation, and one or more of the following atopic features: history of positive skin prick test or positive serum specific IgE for a common aeroallergen, elevated serum total IgE, history of physician-diagnosed allergic rhinitis, or history of physician-diagnosed atopic dermatitis. Inclusion and exclusion criteria for healthy children included birth at ≥ 36 weeks gestation, lack of atopy and asthma by the above definitions, lack of any other clinical diagnosis of lung disease, and lack of a family history of asthma.

From each child, a blood sample at the time of elective surgery was used to measure total serum IgE and allergen-specific IgE to dust mites (*Dermatophagoides farinae* and *Dermatophagoides pteronyssinus*), cat epithelium, dog epithelium, *Alternaria alternata*, *Aspergillus fumigatus*, and timothy grass. A clinical follow-up visit was completed within 2 months of elective surgery and collection of BECs from subjects, at which time lung function measurements were performed. Subjects with asthma were at their clinical baseline during

clinical follow-up visits. The fraction of exhaled nitric oxide (FeNO) was measured according to American Thoracic Society (ATS)/ European Respiratory Society (ERS) guidelines using a NIOX MINO nitric oxide analyzer (Aerocrine®, Sweden) (15). Spirometry, including the forced vital capacity (FVC), FEV_1, and forced expiratory flow between 25% and 75% of FVC (FEF25-75) were measured according to ATS guidelines using a VMAX® series 2130 spirometer (VIASYS Healthcare, Hong Kong). Among children with asthma, spirometry measurements were obtained when subjects were at their clinical baseline and were repeated 15 minutes following administration of 4 puffs of albuterol from a metered dose inhaler via a spacer to determine if a bronchodilator response was present.

Pediatric Bronchial Epithelial Cell Isolation, Proliferation, and Differentiation

Immediately after the endotracheal tube was secured three bronchial BEC samples were obtained from subjects while under general anesthesia using 4mm Harrell® unsheathed bronchoscope cytology brushes (CONMED® Corporation, Utica, NY). As described by Lane et al., the unprotected brush was inserted through an endotracheal tube, advanced until resistance was felt, and rubbed against the airway surface for 2 seconds (16). Cells were seeded onto T-25 cell culture flasks pre-coated with type I collagen and proliferated under submerged culture conditions. Using passage 2 or 3 cells, epithelial cells were differentiated in ALI organotypic culture for 3 weeks using methods previously described by our lab (17).

Phenotyped Primary Epithelial Cell-Mast Cell Co-culture Model

Differentiated pediatric BEC ALI organotypic cultures were co-cultured with unprimed LUVA cells or with LUVA cells that were first primed with IL-33 at a concentration of 10ng/ml for 4 hours at 37°C. Following IL-33 priming, LUVA cells were washed in PBS, centrifuged at 1200 RPM for 5 minutes then re-suspended in PneumaCult™-ALI Medium (Stemcell™

Technologies). Either IL-33 primed or unprimed LUVA cells were suspended in 1ml of medium at a cell density of 2.5 x10^5 cells/ml then added to the basolateral chamber under differentiated BECs growing on 12 mm 0.4 μm permeable polyester membrane transwell inserts (Corning®). Either 100 μl of HDM suspended in HBSS (100 μg/ml) or 100μl HBSS (vehicle control) were added to the apical surface of BECs for 4 hours then removed. Following co-culture of BECs and LUVA cells for 48 hours, RNA was extracted from the BECs. RNA was isolated from BECs cells using a RNAqueous™-Micro Total RNA Isolation Kit (ThermoFisher/Life Technologies). RNA concentration was determined using a NanoDrop™ 2000 analyzer (Thermo Scientific) and *IL33* expression was determined as described above.

Statistics

For comparisons among groups, P-values were calculated as appropriate for the data – Fisher's exact test for categorical data, a two-tailed Student's t-test for two group comparisons for normally distributed continuous data, and a Mann-Whitney *U* test for two group comparisons for non-normally distributed continuous data. A one-way ANOVA with correction for multiple comparisons using the two-stage step up method of Benjamini, Krieger and Yekutieli was used for comparisons of normally distributed continuous data among multiple groups, and a Kruskal-Wallis test with Dunn's post-hoc tests for multiple group comparisons of non-normally distributed continuous data. A two-way ANOVA was used to assess differences between the response of epithelial cells from the different groups to co-culture with mast cells. Associations between continuous variables were assessed by linear regression. A *P* value less than 0.05 was considered significant.

Supplemental References

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