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Allergic Asthma is Distinguished by Sensitivity of Allergen-Specific CD4+ T Cells and Airway Structural Cells to Type 2 Inflammation

Josalyn L. Cho[1,2,†], **Morris F. Ling**[1,†], **David C. Adams**[2], **Lucas Faustino**[1], **Sabina A. Islam**[1], **Roshi Afshar**[1], **Jason W. Griffith**[1,2], **Robert S. Harris**[2], **Aylwin Ng**[3,4], **Giorgia Radicioni**[5], **Amina A. Ford**[5], **Andre K. Han**[1,2], **Ramnik Xavier**[3,4], **William W. Kwok**[6], **Richard Boucher**[5], **James J. Moon**[1,2], **Daniel L. Hamilos**[1], **Mehmet Kesimer**[5], **Melissa J. Suter**[2], **Benjamin D. Medoff**[1,2,†], and **Andrew D. Luster**[1,†,*]

[1]Center for Immunology and Inflammatory Diseases, Division of Rheumatology, Allergy and Immunology, Massachusetts General Hospital, Harvard Medical School, Boston, MA 02114, USA

[2]Division of Pulmonary and Critical Care Medicine, Massachusetts General Hospital, Harvard Medical School, Boston, MA 02114, USA

[3]Center for Computational and Integrative Biology and Gastrointestinal Unit, Massachusetts General Hospital, Harvard Medical School, Boston, MA 02114, USA

[4]Broad Institute of MIT and Harvard University, Cambridge, MA 02142, USA

[5]Marsico Lung Institute, University of North Carolina-Chapel Hill, Chapel Hill, NC 27599, USA

[6]Benaroya Research Institute at Virginia Mason, Seattle, WA 98101, USA

Abstract

Despite systemic sensitization, not all allergic individuals develop asthma symptoms upon airborne allergen exposure. Determination of the factors that lead to the asthma phenotype in allergic individuals could guide treatment and identify novel therapeutic targets. In this study, we utilized segmental allergen challenge (SAC) of allergic asthmatics (AA) and allergic non-asthmatic controls (AC) to determine if there are differences in the airway immune response or airway structural cells that could drive the development of asthma. Both groups developed prominent allergic airway inflammation in response to allergen. However, asthmatic subjects had

*Corresponding Author: Andrew D. Luster, M.D. Ph.D., Massachusetts General Hospital, 149 13th Street, Charlestown, MA 02129, USA, aluster@mgh.harvard.edu, Phone: 617-726-5710, Fax: 617-726-5615.

†These authors contributed equally to the work.

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markedly higher levels of innate type 2 receptors on allergen-specific $CD4^+$ T cells recruited into the airway. There were also increased levels of type 2 cytokines, increased total mucin and increased MUC5AC in response to allergen in the airways of AA subjects. Furthermore, type 2 cytokine levels correlated with the mucin response in AA but not AC subjects, suggesting differences in the airway epithelial response to inflammation. Finally, AA subjects had increased airway smooth muscle mass at baseline measured *in vivo* using novel orientation-registered optical coherence tomography (OR-OCT). Our data demonstrate that the development of allergic asthma is dependent on the responsiveness of allergen-specific $CD4^+$ T cells to innate type 2 mediators as well as increased sensitivity of airway epithelial cells and smooth muscle to type 2 inflammation.

Introduction

Asthma affects more than 300 million people worldwide, and most cases are allergic in origin (1, 2). The cardinal features of allergic asthma are eosinophilic airway inflammation, mucus hypersecretion and airway hyper-responsiveness (AHR) (3). Experimental evidence suggests that allergen-specific T helper 2 (Th2) cells and their cytokines orchestrate allergic airway inflammation, induce mucus production from airway epithelium and promote AHR (3–6). Although airway structural cells, including the epithelium and smooth muscle play an important role in asthma (7, 8), the link between type 2 inflammation and airway structural cell dysfunction is incompletely understood.

Despite systemic sensitization to airborne allergen, not all allergic individuals develop asthma upon allergen exposure. Many of these individuals develop asthma over time (2), suggesting that the pathogenic mechanisms leading to asthma may be incremental and potentially reversible. Thus, identification of differences in the airway response to allergen between allergic asthmatics (AA) and allergic non-asthmatic controls (AC) could provide fundamental insights into asthma pathogenesis and inform therapy. In this study, we performed bronchoscopic segmental allergen challenge (SAC) and characterized changes in airway inflammatory cells, mediators, and T cell subsets, including using class II tetramers to define allergen-specific $CD4^+$ T cells. In addition, we measured mucus and airway smooth muscle (ASM) *in vivo* using optical coherence tomography (OCT) to determine whether structural changes correlated with type 2 inflammation. Our data suggest that allergic asthma results from both the allergen-specific $CD4^+$ T cell response as well as a greater sensitivity of airway structural cells to type 2 inflammation.

Results

Subject characteristics

We enrolled adults with allergy to cat or dust mite to undergo bronchoscopy with SAC (9–11). AA ($n = 36$) had mild asthma and evidence of AHR as defined by a positive methacholine challenge or bronchodilator responsiveness. AC ($n = 48$) had no history of asthma, no lower respiratory tract symptoms in response to inhaled allergen and no evidence of AHR. The baseline characteristics of the subjects in the two groups were well-matched (Supplementary Table 1). Both AA and AC had normal pulmonary function, but AA had lower percent predicted forced expiratory volume in 1 second (FEV_1) and lower ratio of

FEV_1 to forced vital capacity (FVC; FEV_1/FVC). The threshold level of allergen sensitivity was determined by skin prick test titration, and the lowest concentration of extract eliciting a positive skin prick test was used as the allergen concentration for SAC in AA and AC subjects. There were no differences in number of AA and AC receiving house dust mite (*Dermatophagoides pteronyssinus*) or cat hair extract or in the median dose of allergen administered. For comparison, we also enrolled non-allergic healthy control (HC) subjects ($n = 5$).

Airway inflammation after SAC

Bronchoscopic SAC was performed by obtaining a baseline bronchoalveolar lavage (BAL) followed by administration of diluent and allergen in two separate lung subsegments. Twenty-four hours later, BAL was performed in the diluent- and allergen-challenged airways. Cell differential counts in BAL from baseline and 24 hours after SAC revealed eosinophilic inflammation in only the allergen-challenged segment in both AA and AC (Fig. 1a). BAL cell counts and differentials did not differ at baseline or following diluent between AA and AC. In response to allergen, the number of total cells, mononuclear cells, and eosinophils per ml of BAL fluid increased in both AA and AC. Although there was no significant difference in the percentage of eosinophils from the allergen-challenged segment between groups, AA had a higher eosinophil count (0.72×10^6 cells/ml AA vs. 0.48×10^6 cells/ml AC; $P < 0.05$). In contrast, HC did not exhibit a significant increase in total cells in either the diluent or allergen-challenged segment (Fig. 1a).

T cell responses to allergen

The numbers and phenotype of pro-inflammatory allergen-specific T cells and anti-inflammatory regulatory T cells (Tregs) may determine whether allergen exposure results in asthma (9, 13). There were no differences in the number of $CD8^+$ T cells, $CD4^+$ T cells or Tregs at baseline between groups (Fig. 1b). Although the percentage of $CD4^+$ T cells in the BAL did not change, the total number of $CD4^+$ T cells increased similarly in both AA and AC following allergen challenge. The number of Tregs in the BAL fluid after allergen increased in AA and was higher compared to AC (0.74×10^4 cells/ml AA vs. 0.28×10^4 cells/ml AC; $P < 0.01$). There was a small but non-significant increase in the number of Tregs in AC following allergen. There were no significant differences in the percentage of Tregs or in the ratio of Tregs to total $CD4^+$ T cells between AA and AC before or after allergen challenge (Fig. 1b).

Treg phenotype in response to allergen

Although we observed higher numbers of Tregs in the airways of AA compared to AC, we reasoned that Tregs in AC may be more suppressive. Tregs can be subdivided into subpopulations that correlate with their suppressive capacity based on CD45RA and Foxp3 expression, with $CD45RA^-Foxp3^{hi}$ Tregs representing the most suppressive Tregs (Fig. 2a) (14). We therefore assessed the total number and distribution of Treg subpopulations in the BAL before and after allergen challenge in a subgroup of AA ($n = 7$) and AC ($n = 10$). The number of suppressive Tregs ($CD45RA^-Foxp3^{hi}$, group II) and non-suppressive Tregs ($CD45RA^-Foxp3^{int}$, group III) increased in response to allergen in both groups but only achieved statistical significance in AC. There were no significant differences between groups

in either the percentage or number of Treg subpopulations (Fig. 2b) or in the ratio of suppressive Tregs (groups I and II) to T effector and memory cells (Te+m; group IV) (Fig. 2c). $CD4^+CD25^{high}$ Tregs were sorted from the allergen-challenged BAL and genome-wide transcriptome analysis was performed (Fig. S1). There was a trend toward higher levels of IL-9 ($P = 0.05$), CTLA4, and IKZF2 (HELIOS) expression in Tregs sorted from AA after allergen challenge (Fig. 2d) that did not reach statistical significance after correction for multiple comparisons.

Characterization of allergen-specific $CD4^+$ T cells

Although the number of $CD4^+$ T cells was not different between AA and AC at baseline or following SAC, we hypothesized that there could be differences in allergen-specific $CD4^+$ T cells. We used dust mite (Der p 1) and cat (Fel d 1) peptide-specific MHC class II tetramers to identify allergen-specific $CD4^+$ T cells (Fig. 3a, Fig. S2 and Supplementary Table 2). There were very few detectable allergen-specific $CD4^+$ T cells in the airways at baseline and after diluent (Fig. 3b). However, allergen-specific $CD4^+$ T cells markedly increased in the airways of both AA and AC after allergen challenge (Fig. 3b). Surprisingly, there were no significant differences between AA and AC in the percentage or number of allergen-specific $CD4^+$ T cells in the BAL under any condition examined. However, allergen-specific $CD4^+$ T cells from AA had higher expression of the type 2-associated innate immune receptors IL-33R (ST2) (33.6% AA vs. 15.28% AC; $P < 0.05$) and CRTH2 (prostaglandin D_2 (PGD_2) receptor) (26.8% AA vs. 3.24% AC; $P < 0.01$) after allergen challenge (Fig. 3c). Moreover, expression of IL-33R, CRTH2, and IL-25R was enriched on allergen-specific $CD4^+$ T cells compared to bulk $CD4^+$ T cells after allergen only in AA ($P < 0.01$, $P < 0.0001$, and $P < 0.01$, respectively) (Fig. 3d). No significant differences in the expression of the type 2 chemokine receptor CCR4 were observed in either allergen-specific $CD4^+$ T cells compared to bulk $CD4^+$ T cells or between AA and AC.

Cytokine profile after allergen challenge

Inflammatory cytokines play an integral role in the pathogenesis of asthma (3, 15, 16). We therefore measured BAL levels of 44 cytokines and chemokines, many of which have been implicated in asthma pathogenesis (3, 15, 16). At baseline, AA had slightly higher absolute levels of IL-21, IL-33, CXCL1 and CCL21 (Fig. S3). In response to allergen, absolute levels of the type 2 cytokines IL-4, IL-5, IL-9, IL-13, CCL11, and CCL26, as well as the neutrophil-associated cytokines IL-1α, IL-1β, CXCL1 and CXCL8, were increased in AA compared to AC (Fig. 4a and Fig. S3). IFNγ, IL-12p40 and IL-17A were increased after allergen challenge in both AA and AC and were not different between groups (Fig. S3). Given the importance of cytokines in both initiating and propagating allergic airway inflammation (3, 15, 16), we examined the correlation of cytokine levels to cellular inflammation. Levels of IL-4, IL-5, IL-9, IL-13 and CCL26 correlated to the number of eosinophils in the allergen segment in both groups (Fig. 4b) and with IL-33R expression on allergen-specific $CD4^+$ T cells in both groups (Fig. 4c).

We also compared the increase in cytokines after allergen challenge relative to baseline and diluent levels. The increase in IL-1β, IL-4, IL-5, IL-9, IL-13, IL-10, CCL11 and CCL21 in the allergen-challenged segment relative to baseline was higher in AA vs. AC ($P < 0.05$)

(Fig. S4a). Only the increase in IL-4, IL-9, IL-13, and IL-10 in the allergen-challenged segment relative to diluent was greater in AA ($P < 0.05$) (Fig. S4b).

Characterization of the mucin response to allergen

Increased production of mucus in the airway is a phenotypic hallmark of asthma (3). Mucins are the gel-forming components of airway mucus (17), and therefore we used refractometry (18) to measure total mucin in BAL fluid from AA, AC and HC before and after SAC. Baseline levels of mucin were similar in all 3 groups. Both AA and AC had a significant increase in mucin following allergen (7.1 μg/ml baseline vs. 52.5 μg/ml after allergen for AA, $P < 0.0001$ and 7.0 μg/ml baseline vs. 27.4 μg/ml after allergen for AC, $P < 0.01$; Fig. 5a). However, AA had significantly higher levels of mucin after allergen compared to both AC and HC (52.5 μg/ml AA vs. 27.4 μg/ml AC vs. 6.5 μg/ml HC; $P < 0.01$ and $P < 0.001$, respectively). Airway mucus composition has been shown to be altered in asthmatics with increased levels of the gel-forming glycoprotein mucin 5AC (MUC5AC) (17, 19, 20). We therefore measured MUC5AC at baseline and after challenge with a mass spectrometry-based proteomics platform. There were no differences between groups in MUC5AC levels at baseline. However, MUC5AC levels increased in AA BAL after allergen challenge and were significantly higher compared to AC (normalized spectral index 5.6×10^9 vs. 1.1×10^8, $P < 0.05$) (Fig. 5b). We also performed *in vivo* OCT to examine whether there are differences in mucus quality between AA and AC. The average OCT signal intensity (*p*OCT) of mucus within the airway lumen was determined as a surrogate of particulate density (Fig. 5c) (21–23). Mucus *p*OCT intensity correlated to total mucin (Fig. 5d), and at baseline AA, AC and HC had similar *p*OCT intensity (Fig. 5e). However, *p*OCT intensity increased after allergen challenge compared to baseline and diluent only in AA and was greater than *p*OCT intensity in AC and HC (81.6 AA vs 68.1 AC vs. 61.2; $P < 0.05$) (Fig. 5e). Furthermore, *p*OCT intensity correlated with the levels of IL-4 ($R^2 = 0.3802$; $P < 0.05$) and IL-13 ($R^2 = 0.4656$; $P < 0.05$ for both) only in AA (Fig. 5f).

Characterization of airway smooth muscle

Increased ASM mass is an important feature of airway remodeling in moderate to severe asthma, and it has been suggested that ASM mass increases in the setting of type 2 inflammation (24). However, whether ASM mass increases in mild asthmatics or allergic non-asthmatics is unknown. We developed a novel technique to measure ASM *in vivo* using orientation-resolved OCT (OR-OCT) (see Adams, D. *et al.* co-submission). To determine whether differences in ASM mass distinguish AA from AC, we performed OR-OCT at baseline and measured ASM thickness and band width (Fig. 6a). AA subjects had significantly increased ASM thickness (70.3 μm AA vs. 35.4 μm AC vs. 35.9 μm HC; $P < 0.05$ for both) and ASM band width compared to AC and HC (380.3 μm AA vs. 327.3 μm AC vs. 331.7 μm HC; $P < 0.01$ and $P < 0.05$, respectively) (Fig. 6b). There were no significant differences in ASM thickness or band width between AC and HC.

Discussion

In this study, we identified a number of novel differences in the airway immune response to allergen and in airway structural cells between allergic individuals with and without asthma.

AA had slightly higher levels of eosinophils and type 2 cytokines in the airways in response to allergen and a marked increase in expression of the innate type 2 receptors IL-33R and CRTH2 on allergen-specific $CD4^+$ T cells compared to AC. In addition, AA had higher levels of mucin and qualitatively different mucus after allergen challenge. Finally, we measured ASM *in vivo* using OR-OCT and demonstrated that AA had increased ASM mass compared to AC.

Prior studies have documented that allergic non-asthmatics develop prominent type 2 airway inflammation in response to allergens despite a lack of asthma symptoms (25–33). However, these studies were limited by a small number of subjects, different methods for allergen challenge and circumscribed analyses of the immune response. In our comparatively large cohort, we found a greater type 2 inflammatory response to allergen in AA compared to AC, characterized by an increase in airway eosinophils and type 2 cytokines.

We extensively characterized the Treg response to allergen and found that AA had greater accumulation of Tregs in the airways after SAC. We found no differences in the numbers or percentage of suppressive Tregs between groups. Of interest, gene expression analysis suggested that AA Tregs expressed more IL-9 compared to AC Tregs. Although these studies may have been underpowered and require follow up, these data suggest that AA may have more pathogenic “Th2-type” Tregs, which have been described in a mouse model to express Th2-type cytokines and contribute to allergic disease (34).

One of the novel aspects of our analysis is the use of peptide:MHC class II tetramers to define the allergen-specific $CD4^+$ T cell population recruited into the airways in response to allergen (35, 36). Using this technology, we found a marked increase in the numbers of allergen-specific $CD4^+$ T cells in the BAL twenty-four hours following allergen challenge in both groups, suggesting recruitment of these cells into the airway.

More detailed phenotyping of T cells demonstrated enrichment of CRTH2, IL-25R and IL-33R-expression in allergen-specific $CD4^+$ T cells compared to bulk $CD4^+$ T cells only in AA. Importantly, allergen-specific $CD4^+$ T cells from AA expressed higher levels of IL-33R and CRTH2 when compared to AC. These data suggest that AA subjects accumulate more Th2-polarized effector cells in the airways following allergen challenge (37). It is interesting to note that the levels of IL-33 in the airway at baseline were higher in AA compared to AC, suggesting that AA may be primed for more robust type 2 inflammation in response to allergen in the lung. Higher expression of IL-33R and CRTH2 on allergen-specific $CD4^+$ T cells may also enhance responsiveness to type 2 signals from innate immune cells, such as PGD_2 from mast cells and IL-33 from epithelial cells in the lung. In addition, it has recently been shown that IL-33 drives IL-13 expression from Th2 cells independently of the T cell receptor, and thus $IL\text{-}33R^+$ $CD4^+$ T cells may be an important source of IL-13 in allergic asthma (38). Furthermore, allergic asthmatics have been shown to have higher levels of PGD_2 in the airway compared to AC in response to allergen challenge (32). Thus, our data suggest allergen-specific $CD4^+$ T cells drive the development of type 2 inflammation in allergic individuals. Furthermore, differences in the sensitivity of allergen-specific $CD4^+$ T cells to innate type 2 signals may explain the increase in eosinophils, cytokines and mucin in the airways of AA compared to AC.

It is important to note however that there was considerable overlap between groups in measures of inflammation. Moreover, cytokine levels correlated with eosinophils and IL-33R expression on allergen-specific $CD4^+$ T cells in both AA and AC. This suggests that there is a continuum of allergic inflammation in both groups rather than a critical threshold of allergic inflammation that determines the asthma phenotype. Thus, it seems unlikely that the magnitude of the type 2 response by itself can fully explain the dramatic phenotypic differences between AA and AC with respect to asthma symptoms and AHR.

Perhaps the most striking differences we observed were in the response of airway epithelium to allergen and in ASM. Airway epithelial cells alter both the production and composition of mucus in response to IL-4 and IL-13 (17, 19), and mucus hypersecretion is an important determinant of symptoms in asthma (17). However, the mucus response to SAC in AA and AC has not been previously studied. Using multiple modalities, including refractometry, mass spectrometry and a novel OCT-based method to measure mucus *in vivo*, we demonstrated both quantitative and qualitative differences in mucus between AA and AC. Although AC increased mucus production in response to allergen, it was significantly less than AA and not different than HC. Mucus particulate density as determined by OCT (*p*OCT) was increased following allergen challenge and correlated with IL-4 and IL-13 levels only in AA. Furthermore, only AA had a significant increase in MUC5AC levels in the BAL after allergen challenge. MUC5AC is induced in epithelial cells by type-2 cytokines and its levels are elevated in human asthma (17, 39–41). Interestingly, MUC5AC has also recently been shown to be required for AHR in mice (42). These results suggest that the airway epithelium in AA is more responsive to type 2 inflammation than in AC and contributes to the development of the asthma phenotype.

It is also thought that ASM contributes to AHR in asthma (5, 24). Previously, it has not been possible to measure ASM *in vivo*, and studies of ASM mass have relied on histological measurements. We have developed a novel OR-OCT technology (see Adams, D. *et al.* co-submission) that allows for *in vivo* assessment of ASM. Surprisingly, we found increased ASM thickness and band width in AA compared to AC, even though our AA cohort had mild asthma and normal pulmonary function. Interestingly, differences in ASM measurements were observed despite significant overlap in the levels of type 2 inflammation observed in response to allergen in both groups and enrollment of subjects with only mild asthma. Our data could suggest that individuals with increased ASM are predisposed to the development of asthma in the setting of type 2 inflammation. Alternatively, ASM in AA may have increased sensitivity to type 2 inflammatory or other epithelial cell-derived signals that result in AHR and ASM hyperplasia and/or hypertrophy. Further studies are needed to define the mechanistic links between type 2 inflammation and changes in ASM mass and function in AA subjects.

There are several important limitations to our study that should be considered. SAC provides an intense and isolated allergen challenge that does not fully recapitulate aeroallergen exposure to the entire lung, and BAL only samples the airspaces, so cell types and mediators within lung tissue may not be well represented in our analyses. Furthermore, we only studied subjects 24 hours after challenge using just one dose of allergen based on the individual subject's cutaneous "threshold" reaction. Thus, it is possible that we missed

important differences between these groups in the initiation or resolution of inflammation that would be evident with different doses or at different time-points. Finally, we only enrolled subjects with mild asthma. Prior studies have demonstrated that the response to SAC is not different between mild and moderate asthmatics (43), but the response in severe asthmatics may be different (44, 45).

Our results suggest that type 2 airway inflammation is necessary but not sufficient for the allergic asthma phenotype, and that increased sensitivity of airway structural cells to type 2 inflammation is also required. Whether structural cells in asthmatics are intrinsically more sensitive to type 2 inflammation or develop altered responses in the setting of chronic inflammation remains to be determined. Future investigations into mechanisms that determine responsiveness of airway structural cells to allergen and type 2 inflammation may reveal important new therapeutic targets for asthma that can alter the course of this chronic disease.

Materials and Methods

Study Design

The primary aims of this study were to compare the immune and airway response to segmental allergen challenge (SAC) in aeroallergen allergic subjects with asthma (AA) and without asthma (AC). A group of healthy controls (HC) was also included. Participants were recruited via advertisement in the Massachusetts General Hospital outpatient clinics and around the Boston metropolitan area. Interested volunteers were screened for eligibility with a full medical history, baseline spirometry and methacholine challenge and allergen skin testing. In order to determine eligibility for tetramer studies, HLA subtyping was performed on peripheral blood samples by the American Red Cross reference laboratories. Detailed inclusion and exclusion criteria as well as the study protocol are provided in the Supplementary Materials. Subjects gave their written informed voluntary consent before testing and sample collection. Eligible subjects underwent SAC with optical coherence tomography (OCT) imaging and collection of BAL fluid before and after allergen challenge for analysis. The study was approved by the Partners Healthcare Institutional Review Board.

Allergen Skin Testing

Standardized allergen extract for cat hair and *Dermatophagoides pteronyssinus* were purchased from Greer Laboratories. AA and AC had a positive skin prick test to either cat hair or dust mite extract while HC had negative skin tests to allergens. The threshold level of allergen sensitivity was determined by skin prick test titration using serial 3-fold dilutions of extract (12). The lowest concentration of extract eliciting a positive skin prick test (3 mm wheal diameter) was used as the allergen concentration for SAC in AA and AC subjects; for HC, 500 AU/ml of cat hair extract was administered.

Segmental Allergen Challenge

SAC was performed as previously described (9–11). Briefly, a pre-challenge bronchoalveolar lavage (BAL) was obtained from the lingula using 4 × 30 l aliquots of normal saline. Diluent (2 ml) was then administered to the anterior segment of the right

upper lobe followed by administration of allergen (2 ml) to the lateral segment of the right middle lob. Twenty-four hours later, BAL samples were obtained from the diluent and allergen-challenged lung segments.

Cytospins

Cell differential counts for BAL were determined by enumerating mononuclear cells, neutrophils, and eosinophils on cytocentrifuge preparations (9, 10).

Flow cytometry

BAL cells were stained with fluorescent-conjugated antibodies against CD3, CD4, CD8, CD25, CD45RA, CCR4, CRTH2, IL-33R, and IL-25R (Biolegend). Some cells were stained for Foxp3 (eBioscience). Phycoerythrin (PE)-conjugated MHC class II tetramers containing either house dust mite (Der p 1) or cat hair (Fel d 1) peptides (Supplementary Table 2) were prepared in our laboratory as previously described (35, 46, 47). Baseline and diluent samples underwent enrichment for tetramer positive cells with anti-PE micromeads and magnetic column selection (Miltenyi Biotec) as previously described (35, 46, 47). Samples were run on an LSRFortessa flow cytometer (BD Biosciences) and analyzed with FlowJo software (Tree Star).

Cytokine and chemokine analysis—Cytokines and chemokines were analyzed by Milliplex MAP kit (EMD Millipore) using 30-fold concentrated BAL supernatant. BAL was concentrated in Amicon Ultra-15 centrifugal filter units (EMD Millipore) and used in the kit according to the manufacturer's instructions. Cytokines and chemokines were measured on a Luminex 200 and analyzed using xPONENT 3.1 software (Luminex Corp.).

Transcriptome analysis—BAL cells from the allergen-challenged segment were enriched for $CD4^+$ T cells by negative selection using magnetic beads (StemCell Technologies). Cells were stained with CD4 and CD25, and $CD4^+CD25^{high}$ Tregs were sorted to > 97% purity with a FACSAria II (BD Biosciences). RNA was purified with the RNeasy Plus Mini Kit (Qiagen). Global gene expression was profiled using a Human HT-12 V4 Expression BeadChip according to manufacturer's instructions (Illumina). Image analysis, bead-level processing and quantile normalization of array data were performed using the Illumina LIMS platform, BeadStudio. A gene-wise linear model was fitted to the data and was performed in R programming language (http://www.r-project.org/), utilizing functions from Linear Models for Microarray Data (48). For each probe, a moderated t-statistic (with standard errors moderated across genes) was computed using a Bayesian model. For each transcript, log-transformed ratios were computed for the allergen segment relative to the baseline or diluent segment.

Mucin measurement—Total mucin concentration was measured in BAL samples using differential refractometry (tREX, Wyatt Technology) coupled with size exclusion chromatography as described previously (18).

MUC5AC Assessment

BAL samples were prepared for analysis as previously described (49). Briefly, 250 μl BAL samples were denatured, reduced and alkylated. Samples were then buffer-exchanged into a pH 8.0 digestion buffer (50 mM ammonium bicarbonate) using a HiTrap Desalting column (GE Healthcare). Samples were digested with trypsin at 37°C overnight. The final digest was vacuum dried to remove bicarbonate salts. The digest peptides were re-solubilized in 20 μL 0.1 % formic acid water or were stored −30 °C until liquid chromatography-tandem mass spectrometry (LC-MS/MS) was run. The LC-MS/MS was performed with a Dionex UltiMate 3000 RSLCnano system coupled to a hybrid quadrupole orbitrap mass spectrometer with a Nano spray source (Q Exactive, Thermo Fisher Scientific). Peptides were analyzed by a data-dependent top 10 method dynamically choosing the most abundant precursor ions from the survey scan (300–1650 Th) for HCD fragmentation. Proteins identified from the BAL were quantified using a label-free method termed the normalized spectral index (SI(N)). SI(N) is defined as the cumulative fragment ion intensities for all spectra counted for a protein (50).

OCT Mucus Assessment

Mucus was automatically detected using custom image segmentation algorithms. The suite of algorithms used a two-step identification process. In the first step, the sheath and tissue lumen were identified. The second step looked for mucus in the region between sheath and tissue lumen using a combination of thresholding and intelligent exclusion of image artifact. All of the data was checked for accuracy and frames in which the mucus content was ambiguous were excluded. Mucus particulate content was assumed to be proportional to the scattering intensity of the mucus (21), and the average quantity of particulate content per dataset was equated to the mean pixel intensity for all mucus-identified pixels.

ASM measurement—ASM band width and thickness was assessed using OR-OCT as described (see Adams, D. *et al.* co-submission). Regions 6mm in length and of comparable radius (+/− 0.1 mm) were analyzed for both ASM thickness and band width. To compare the measured ASM thickness between datasets, the measured ASM area was normalized to the local basement membrane perimeter.

Statistical analysis—Data are expressed as mean ± SEM unless otherwise noted. Chi squared test or Fisher's exact test was used to evaluate categorical values and the non-parametric t test was used for continuous data. Median allergen doses were not normally distributed and thus, comparison was performed with the use of the Mann-Whitney test. Two-way ANOVA was used to compare mean differences between groups. Tukey's or Sidak's test was used to correct for multiple comparisons. Since the distribution was skewed, cytokine data were log transformed for analysis. Pearson correlation coefficients were used for assessing the strength of association between pairs of predefined variables. In all cases, differences in results were considered to be statistically significant when P was less than 0.05 based on a two-sided test.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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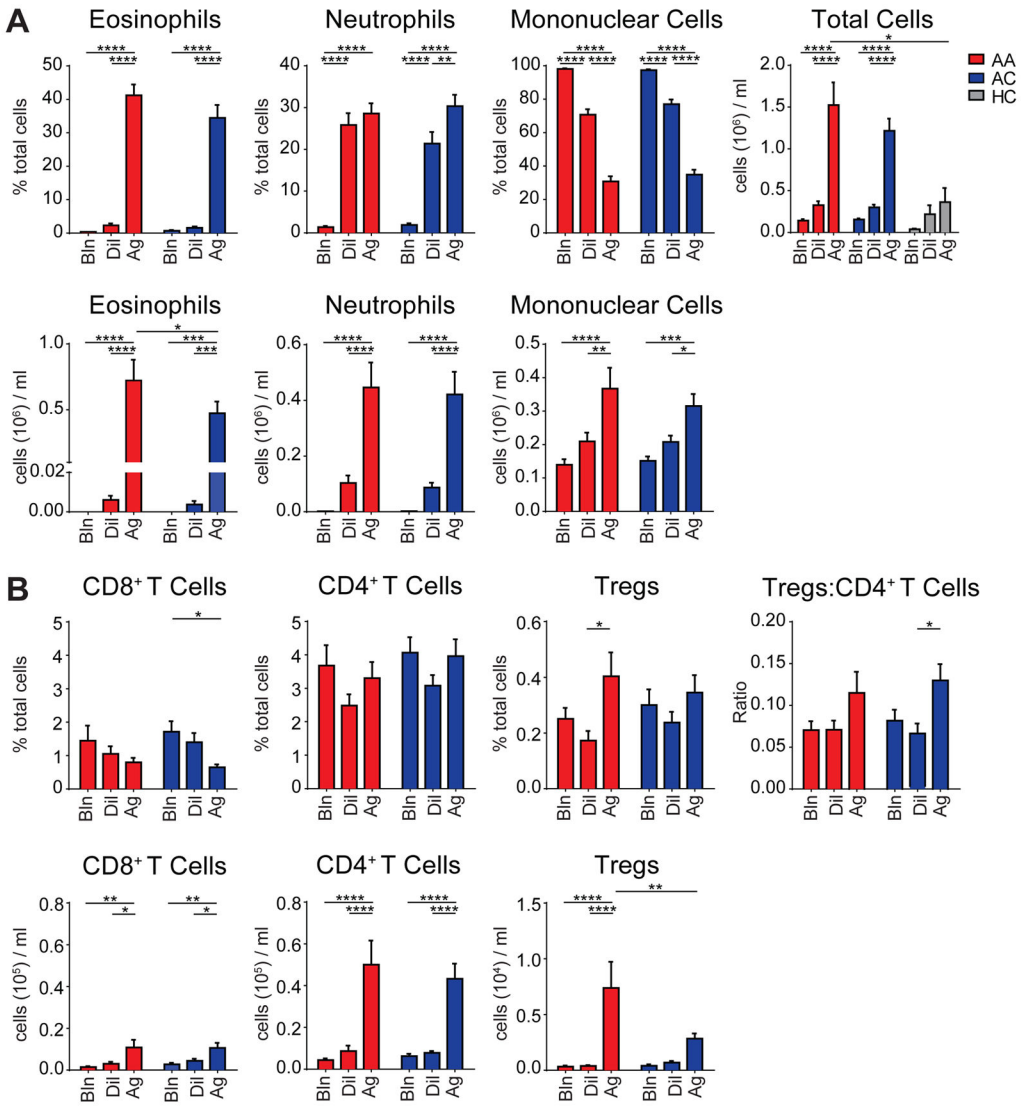

Fig. 1. Airway cellular response after segmental allergen challenge (SAC)

(**a**) Percentages and numbers of eosinophils, neutrophils and mononuclear cells in BAL fluid at baseline and 24 hours following diluent or allergen exposure in AA (red) and AC (blue) subjects ($n = 36$ AA, $n = 48$ AC). Total cell counts are also shown (healthy controls (HC) shown in gray ($n = 4$)). (**b**) Percentage and number of $CD8^+$ T cells and $CD4^+$ T cells in the BAL ($n = 31$ AA, $n = 44$ AC). Percentage and number of Tregs in the BAL and ratio of Tregs to total $CD4^+$ T cells ($n = 22$ AA, $n = 23$ AC). Bln, baseline; Dil, diluent, Ag, antigen. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$ by two-way ANOVA.

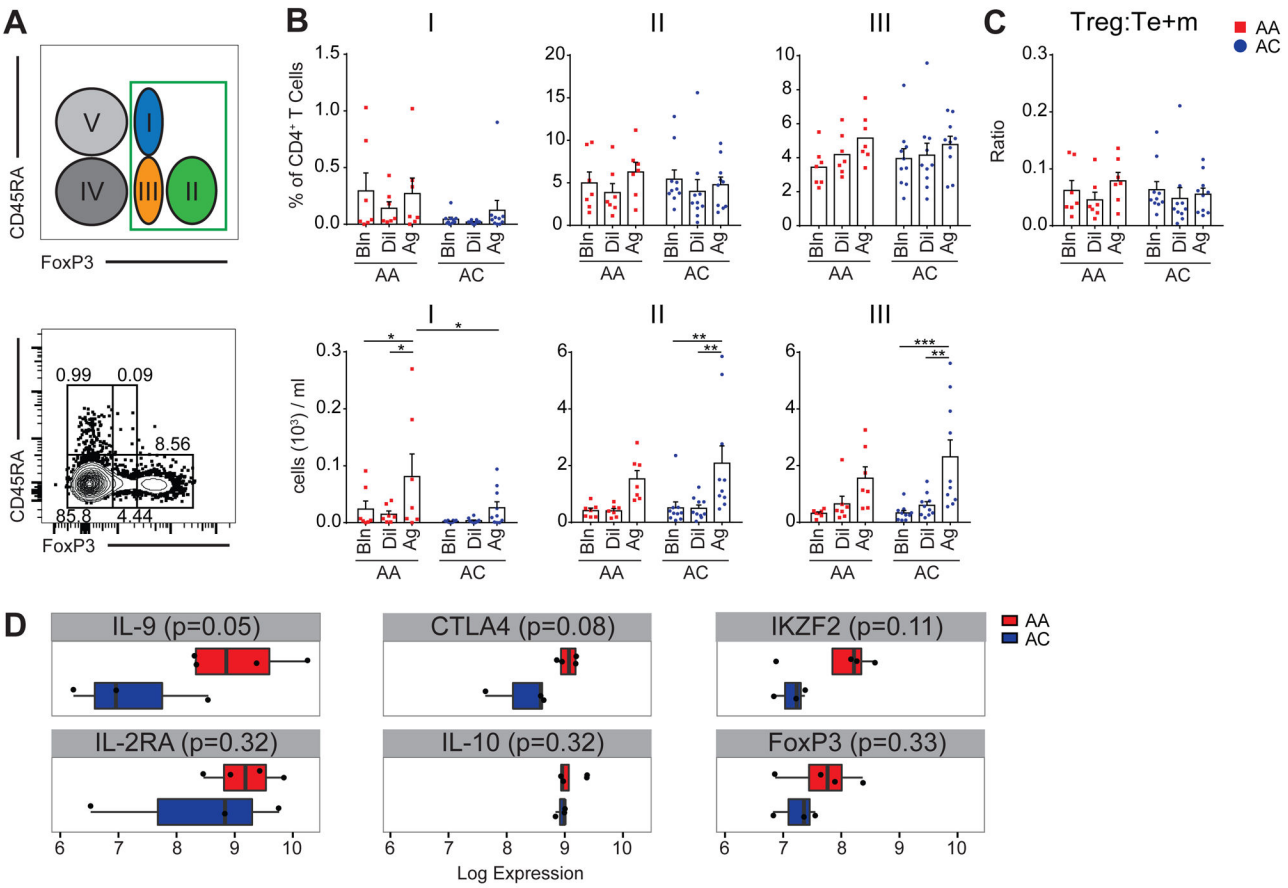

Fig. 2. Phenotyping of Tregs

(**a**) Functional Treg subpopulations (green box) and a representative flow plot. I – naïve Tregs, II – suppressive Tregs, III – non-suppressive Tregs, IV effector and memory $CD4^+$ T cells (Te+m) and V – naïve $CD4^+$ T cells. (**b**) Percent and numbers of Treg subpopulations in AA (red squares) and AC (blue circles) subjects ($n = 7$ AA, $n = 10$ AC). $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ by two-way ANOVA. (**c**) Ratio of suppressive Tregs (I and II) to Te+m cells (IV) ($n = 7$ AA, $n = 10$ AC). (**d**) Expression levels of 6 genes relevant for Treg function are shown from a genome-wide transcriptome microarray analysis performed on sorted cells obtained after SAC from AA (red bars) and AC (blue bars) subjects. Data are displayed as medians (bar), interquartile range (box; 25^{th} to 75^{th} percentiles), and minimum and maximum values (whiskers). Nominal P values were determined utilizing linear models for microarray (LIMMA).

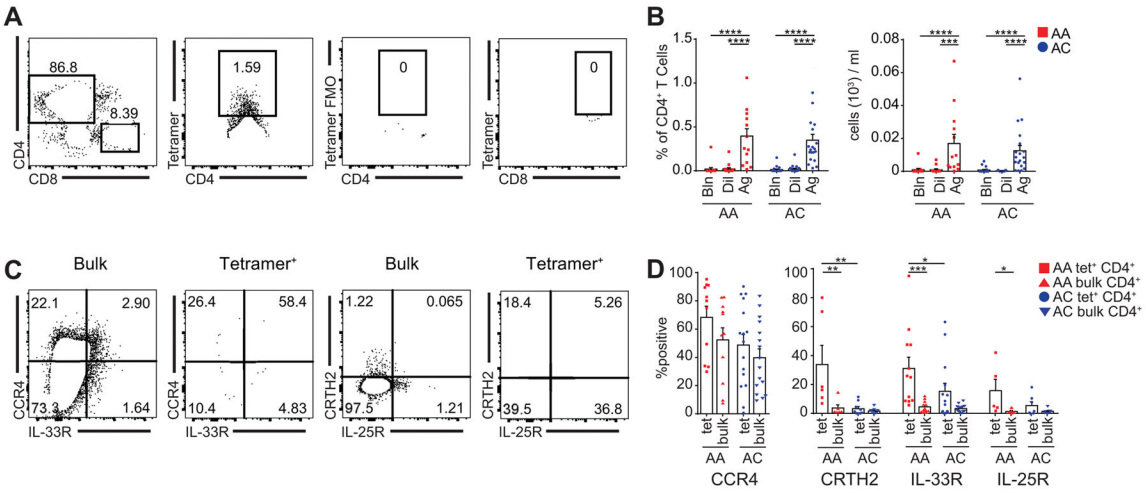

Fig 3. Phenotyping of allergen-specific CD4$^+$ T cells

(**a**) Representative gating for tetramer$^+$CD4$^+$ T cells. (**b**) The percentage and number of tetramer$^+$CD4$^+$ T cells in the BAL for AA (red squares) and AC (blue circles) subjects (n = 13 AA, n = 19 AC). (**c**) Representative gating for Th2 markers on bulk and tetramer$^+$CD4$^+$ T cells from an AA subject. (**d**) Percentage of tetramer$^+$CD4$^+$ (AA red squares, AC blue circles) or bulk CD4$^+$ (AA red triangles, AC blue triangles) T cells expressing the Th2 markers CCR4 (n = 11 AA, n = 16 AC), CRTH2 (n = 6 AA, n = 9 AC), IL-33R (n = 12 AA, n = 14 AC), and IL-25R (n = 5 AA, n = 7 AC). *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 by two-way ANOVA.

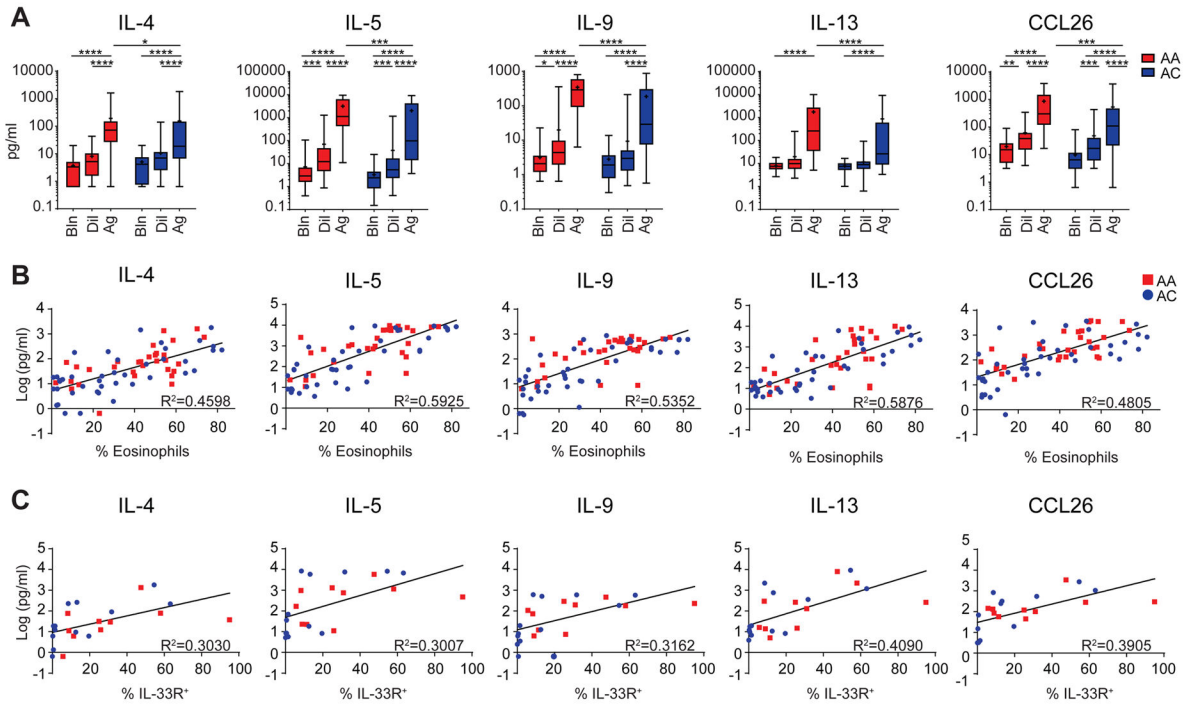

Fig. 4. Th2 cytokine levels and correlations

(**a**) Protein levels of IL-4, IL-5, IL-9, IL-13 and CCL26 in BAL fluid ($n = 32$ AA, $n = 41$ AC). Data are presented as median (bar), interquartile range (box; 25th to 75th percentiles) and minimum and maximum values (whiskers); cross denotes the mean. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$ by two-way ANOVA. (**b**) Correlation between IL-4, IL-5, IL-9, IL-13 and CCL26 levels in BAL fluid and the percentage of eosinophils in the allergen segment ($n = 32$ AA, $n = 41$ AC). Pearson's correlation, $P < 0.0001$ for all. AA shown in red squares, AC shown in blue circles). (**c**) Correlation between IL-4, IL-5, IL-9, IL-13 and CCL26 and percent IL-33R$^+$ allergen-specific CD4$^+$ T cells in the allergen segment ($n = 10$ AA, $n = 13$ AC). Pearson's correlation, $P < 0.001$ for IL-13, $P < 0.01$ for all others.

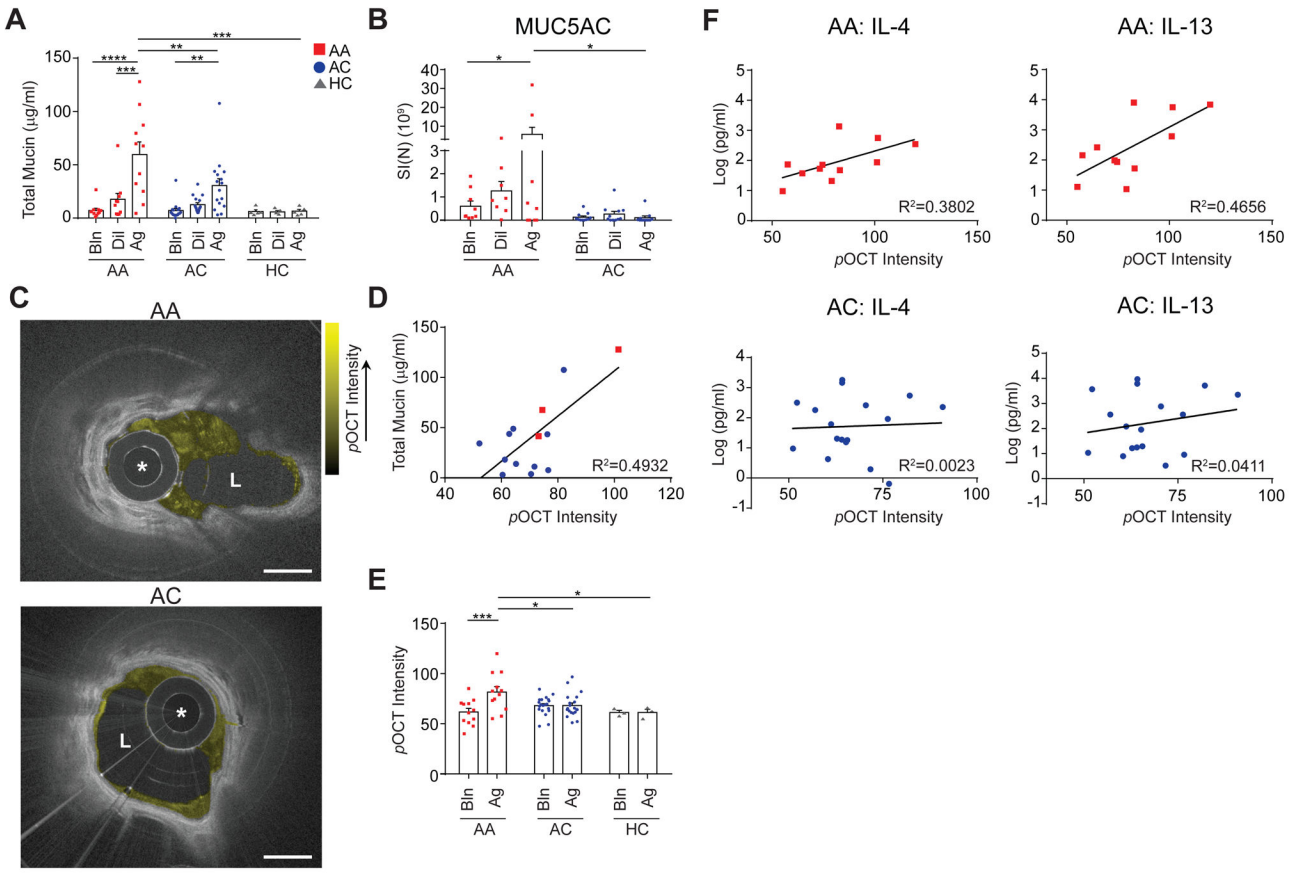

Fig. 5. Characterization of airway mucus after SAC

(**a**) Total mucin in BAL fluid at baseline and after diluent and allergen exposure (n = 11 AA, n = 16 AC, n = 5 HC). (**b**) MUC5AC levels in BAL fluid at baseline and after diluent and allergen exposure (n = 9 AA, n = 11 AC). SI(N) = normalized spectral index. (**c**) Representative images of airway mucus using optical coherence tomography (OCT). Mucus is represented in yellow. *, catheter; L, airway lumen; scale bar 1 mm. pOCT = mean OCT intensity. (**d**) Correlation between pOCT intensity and total mucin measured by refractometry (n = 3 AA, n = 11 AC). Pearson's correlation, $P < 0.001$). (**e**) pOCT intensity of airway mucus before and after SAC (n = 12 AA, n = 20 AC, n = 3 HC). (**f**) Correlation between pOCT intensity and levels of IL-4 and IL-13 in BAL fluid after allergen (n = 11 AA, n = 17 AC). Pearson's correlation, $P < 0.05$ for AA IL-4 and AA IL-13. For 5a–b and 5d–f, AA shown in red squares, AC shown in blue circles, HC shown in gray triangles. For 5a, 5b, and 5e *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$ by two-way ANOVA.

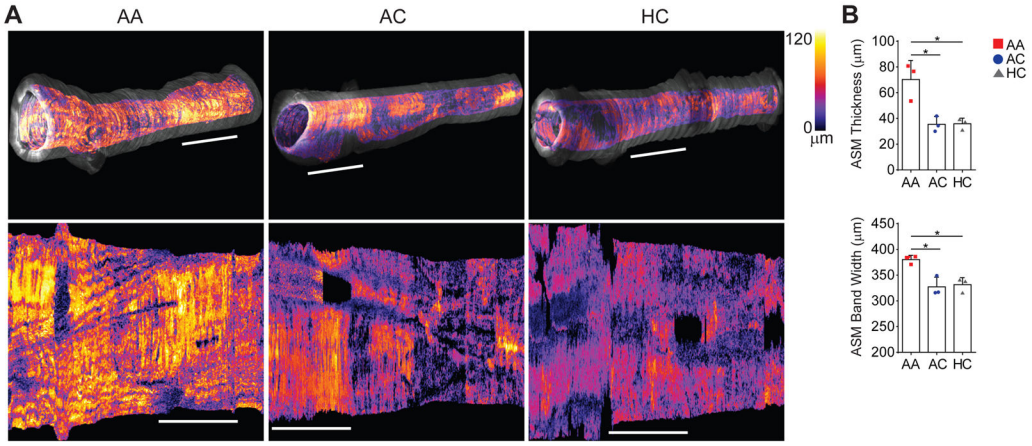

Fig. 6. Characterization of airway smooth muscle

ASM measurements obtained using orientation-resolved OCT (OR-OCT). (**a**) Top panels are volumetric reconstructions from circularized data with ASM represented in terms of thickness (color) overlaid on airway structure (grayscale). Bottom panels are the same ASM data unrolled circumferentially and "flattened" for ASM quantification. Scale bars are corresponding 6 mm regions of comparable airway perimeter from each dataset where the ASM distribution was quantified. (**b**) Quantification of ASM thickness and band width (n = 3 AA, n =3 AC, n= 3 HC). AA shown in red squares, AC shown in blue circles, HC shown in gray triangles. *$P < 0.05$ by two-way ANOVA.