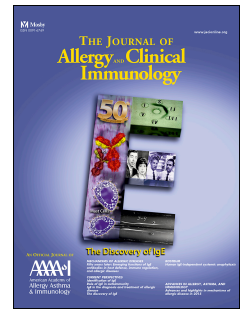


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AUTHORS' CONTRIBUTIONS:

PGW designed the overall bronchoscopy sub-study; CMF, NEA, & JLC designed the Immunophenotyping protocol; MKH, CBC, NNH, NP, & MZ assisted with sample collection; SXL, AJH, VRS & CMF performed experiments; EEC, DJC, &WKO assisted with collection and dissemination of participant data; CHM, JLC, CMF analyzed data; RGB, ERB, SAC, CBC, CMD, ATH, EAH, RJK, FJM, DAM, RP, & SIR assisted with data analysis and interpretation; CHM & CMF wrote the first draft, and all Authors participated in revisions and approved the final version.

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ABBREVIATIONS

BAL, bronchoalveolar lavage

CI, confidence interval

COPD, Chronic obstructive pulmonary disease

ICS, inhaled corticosteroids

SPIROMICS, Subpopulations and Intermediate Outcomes in COPD Study

NS, never-smoker(s)

CS-NAO current smoker(s) with no airflow obstruction

FS-NAO, former smoker(s) with no airflow obstruction

CS-COPD current smoker(s) with COPD

FS-COPD former smoker(s) with COPD

KEYWORDS

BAL

Cigarette smoke

COPD

Eosinophil

Human

SUMMARY

1 Active smoking in stable COPD subjects significantly increased eosinophil accumulation
2 in the distal airspaces, but not in sputum or peripheral blood. Our findings support the need to
3 investigate this cell-type as a potential driver of COPD symptomatology and progression.

4

To the Editor:

Novel therapies for chronic obstructive pulmonary disease (COPD) are urgently needed. Eosinophilic inflammation is an appealing target, as blood or sputum eosinophils in stable COPD may predict responses to systemic or inhaled corticosteroid (ICS) therapy¹. Titrating steroid therapy in the stable state based on sputum eosinophils reduced severe exacerbations² and has been recommended for clinical practice³. However, the prevalence of eosinophilic inflammation in COPD and its uniformity between systemic and lung compartments remain incompletely defined. Controversy exists on whether sputum analysis (reflecting large airway events) is required, or if blood eosinophilia can suffice, based on the strong correlation between the two found by one group⁴. Thus, better understanding of eosinophils in COPD is needed.

The Subpopulations and Intermediate Outcomes in COPD Study (SPIROMICS) (ClinicalTrials.gov NCT01969344T4), which recently completed enrolling 2,981 participants, provides a unique opportunity to address these controversies⁵. This analysis was performed on a subset ($n = 139$) of SPIROMICS participants who agreed to a bronchoscopy sub-study, which used multicolor flow cytometry to identify leukocyte subsets and to define their activation states. All investigations were conducted according to principles of the Declaration of Helsinki. The protocol was approved by the institutional review boards of the eight participating clinical centers. Briefly, participants underwent sputum induction, then 2-4 weeks later, returned for a bronchoscopy visit at which we collected peripheral blood and bronchoalveolar lavage (BAL). Sputum, blood, and BAL samples were stained on the day of collection at the clinical centers, then fixed and shipped to a central laboratory for flow cytometry analysis. Additional details can be found in the Online Supplement. We categorized participants as never-smokers (NS); current

smokers (CS-NAO) and former smokers (FS-NAO) with no airflow obstruction; and current smokers (CS-COPD) and former smokers (FS-COPD) with COPD (**Table 1**).

Using our described staining protocol and flow cytometric gating⁶, we identified eosinophils as CD45⁺, CCR3⁺, CD16⁻ cells with high FSC and SSC (**Supplemental Fig. 1**). When grouping subject by smoking status and COPD disease status, we found no differences between eosinophil percentages in peripheral blood (**Fig. 1A**) or sputum (**Fig. 1B**). By contrast, BAL eosinophils were significantly increased as a percentage of all CD45⁺ cells in current smokers with COPD, relative to other groups (**Fig. 1C**). However, percentage of BAL eosinophils did not correlate significantly in any sample type with FEV₁% predicted or imaging variables (percent emphysema or Pi10) (not shown). IgE levels were largely within the normal range [geometric mean 46.7 IU/mL (95% CI 35.3, 60.2)]. Log₁₀-transformed IgE levels neither differed between groups nor correlated with eosinophil percentages in any of the three sample types, whether among all subjects or only the CS-COPD group (not shown). We found no correlation of BAL eosinophil percentages with plasma levels of the CCR3 ligands CCL5, CCL8 and CCL24 (not shown), and did not measure CCL11 or IL-5.

To better understand the correlation between current smoking and elevated BAL eosinophil percentages in COPD, we performed multivariate modeling. We adjusted for demographics (age, sex, African-American race), smoking intensity (per 10 pack-year exposure), obstruction and smoking status (never vs. former vs. current), chronic bronchitis, histories of asthma or gastroesophageal reflux, and ICS use. We also controlled for the presence of self-reported eye/nose allergies (defined as both any diagnosis and current diagnosis, with positive response [presence of allergy symptoms] reported by 18.5% of participants) and self-reported seasonal allergies (positive response [presence of allergy symptoms] reported by 13.3% of

participants). Results showed a significant association between eosinophil percentage in BAL with current smoking plus COPD, with a 2.5 fold increase in eosinophil percentage in smokers with COPD ($\beta = 2.5$; 95% CI 1.0, 3.9).

To gain insights into eosinophil activation states in various compartments, we examined cell-surface expression of the adhesion molecules CD11b (Clone CBRM1/5, which recognizes an activation-specific epitope), CD34, and CD49d; the activation receptor CD69; and CD125, the IL-5 receptor alpha chain. Within a given subject group, every receptor showed significant differences between sample types in percentages of positive eosinophils (**Supplemental Table 1, column p values**). By contrast, within sample types, only CD125 in BAL differed between groups (significantly lower in CS-COPD) (**Supplemental Table 1, row p values**).

Accordingly, we analyzed eosinophil receptor-positivity in the three sample types regardless of subject groups (**Supplemental Fig. 2**). Relative to blood, significantly more eosinophils were positive for CD11b, CD34 and CD69 in BAL and for CD34 and CD69 in sputum. CD69 may contribute to intrapulmonary eosinophil retention as shown for lung-resident T memory cells⁷, although for both cell types, CD69 might be up-regulated by the lung environment but not causing retention.

Finally, we examined the magnitude of receptor expression by mean fluorescence intensity (MFI) (**Supplemental Table 2 & Supplemental Fig. 3A**), which, as with the percent of eosinophils expressing a receptor, differed significantly between sample types for every receptor. In terms of subject comparisons, there were no significant differences in expression of CD34, CD69 or CD125 by BAL eosinophils, but expression of CD11b was significantly greater in FS-NAO relative to FS-COPD (**Supplemental Fig. 3B**). Moreover, expression of CD49d was

significantly higher in BAL eosinophils of CS-COPD than in groups other than CS-NAO, which was also elevated relative to FS-COPD (but not to other groups) (**Supplemental Fig. 3B**).

This analysis demonstrates that active smoking increases steady-state localization of eosinophils to the distal lung in COPD, relative to smokers (current or former) without airflow obstruction and to never-smokers. This interaction was not observed in FS-COPD, implying that smoking reversibly impacts eosinophil recruitment or retention (or both). Eosinophilic inflammation was compartmentalized, as eosinophils as a percentage of all leukocytes in BAL showed no correlation with results in blood or in sputum (data not shown). These data extend two previous studies that used cytopsin differential counts to enumerate BAL eosinophils. Both found, as we did, that induced sputum results neither differed between groups nor correlated with BAL percentages^{8,9}. Our results are also congruent with those of Wen and colleagues, who found increased BAL eosinophils in current smokers with COPD, relative to ex-smokers with COPD⁹.

Strengths of our study include its size, inclusion of extensively phenotyped subjects at multiple clinical centers, and rigorous analytic plan, using a single flow cytometry instrument and analysis by a limited number of scientists blinded to clinical data. Limitations of our entire immunophenotyping study include the absence of viability staining at the time of flow acquisition and the duration (usually 2-4 weeks) between sputum and bronchoscopy visits. Smoking status was determined by self-report, albeit in temporal proximity to the bronchoscopy visit, but was not verified by objective measurements. Finally, subjects who agreed to in the bronchoscopy sub-study were self-selected and may not be representative of the general population.

Table 1: Subject characteristics

Group	NS	FS-NAO	CS-NAO	FS-COPD	CS-COPD	<i>p</i> value
Subjects, n	21	29	30	37	22	
Sex ratio, M/F	8/13	13/16	16/14	24/13	12/10	0.32
Age, years (SD)	52 (8)	63 (8)	51 (6)	65 (7)	60 (7)	<0.0001
Smoking, pack-years (SD)	0 (0)	39 (16)	35 (12)	51 (24)	53 (20)	0.0007 *
FEV1, % pred. (SD)	100 (0)	102 (12)	97 (13)	79 (19)	78 (17)	<0.0001
FEV1/FVC (SD)	0.82 (0.06)	0.76 (0.06)	0.76 (0.05)	0.58 (0.09)	0.58 (0.10)	<0.0001
ICS ³ use (yes/no)	1/20	1/28	3/26 **	15/22	6/16	0.0002

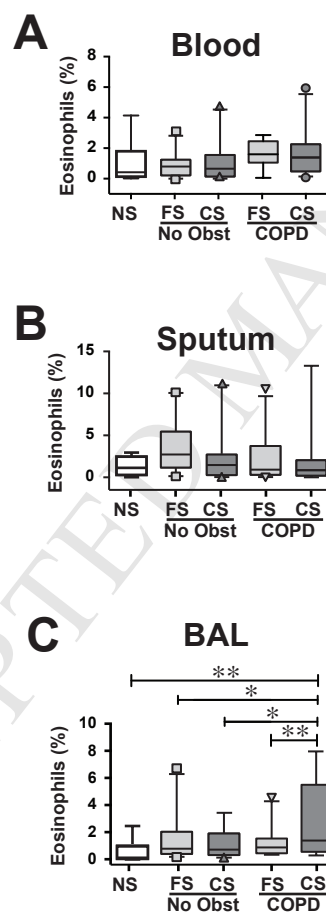
¹, Data are presented as mean (SD) except for sex ratios and ICS use; M, male; F, female; ², former smoker defined as having quit for more than six months; ³, ICS, inhaled corticosteroids. NS, never-smokers; FS-NAO, former smokers, no airflow obstruction; CS-NAO, current smokers, no airflow obstruction; FS-COPD, former smokers with COPD; CS-COPD, current smokers with COPD. One-way ANOVA with Holm-Sidak post-hoc testing was used to determine significant differences between groups. *, NS group omitted, no single group statistically different by post-hoc testing. **, data missing from one subject.

Figure Legends**Figure 1. Active smoking significantly increases BAL eosinophil percentages in COPD.**

Flow cytometric analysis of eosinophils in (A) blood ($n=97$), (B) sputum ($n=94$) and (C) BAL ($n=91$). NS, never-smokers; FS-NAO, former smokers, no airflow obstruction; CS-NAO, current smokers, no airflow obstruction; FS-COPD, former smokers with COPD; CS-COPD, current smokers with COPD. Box and whiskers plot showing median $\pm$ interquartile range, 5th and 95th percentiles, with outliers shown as symbols. *, $p<0.05$; **, $p<0.01$; ANOVA with Holm-Sidak post-hoc testing.

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ONLINE SUPPLEMENTAL MATERIALS

**Alveolar eosinophilia in current smokers with chronic obstructive pulmonary disease
in the SPIROMICS cohort**

MATERIALS AND METHODS*Subject population & Specimen Collection*

SPIROMICS recruitment was designed to achieve a balance of COPD severities and of smokers without airflow obstruction, based on fixed-ratio, post-bronchodilator spirometry¹, plus additional never-smokers (<100 lifetime cigarettes). The bronchoscopy sub-study deviated slightly by excluding subjects with FEV₁ <30% predicted, and by deliberately over-sampling never-smokers and smokers without obstruction. Subjects were consented at eight different clinical centers. Self-reported smoking history was collected by standardized questionnaire during an in-person interview; former smoking was defined as no tobacco products within six months of the bronchoscopy study visit. All participants understood the study's purpose and provided written informed consent before undergoing any research activities. Each subject underwent bronchoscopy only once.

For this analysis, we used samples collected between February 2012 and April 2016. Participants underwent a sputum induction during a period of clinical stability, while taking usual medications including ICS; then 2-4 weeks later, returned for a bronchoscopy visit, at which we collected peripheral blood and BAL from the right middle lobe and lingula². Both BAL returns were pooled for immunophenotyping. Not all sample types were collected successfully from every participant.

Immunophenotyping

To minimize variability, antibodies for immunophenotyping were aliquoted at the Ann Arbor VA and then shipped to the clinical centers prior to a subject visit. Each of the eight clinical

centers was responsible for collecting and staining specimens, which were then fixed in 2% paraformaldehyde and shipped back to the Ann Arbor VA for analysis on a single LSR II flow cytometer (BD Bioscience, San Jose, CA)². Data were collected using FACSDiva software (BD Biosciences) with automatic compensation and were analyzed using FlowJo software v.9.6.2 (Tree Star, Ashland, OR) on Macintosh Quad-Core Intel Xeon computers running OSX 10.11.5 (Apple; Cupertino, CA).

A limited number of scientists (CMF, SXL, AJH, VRS), blinded to subject identity, analyzed primary data using a standardized workflow. Data were reviewed by at least two individuals. We identified eosinophils as CD45+, CCR3+, CD16- cells with characteristic light-scatter (FSC, SSC) properties. To determine the percentage of eosinophils expressing a given receptor (CD11b, CD34, CD49d, CD69, or CD125), we used a 5% probability contour plot to select positive events, as distinguished using the appropriate isotype control. To obtain sufficient numbers of eosinophils for analysis, we collected an average of 122,028 CD45+ events in BAL (SD = 76,290; Median = 114,857) and 112,126 CD45+ events in sputum (SD = 132,110; Median = 65,380). In situations where there were fewer than 100 events in the eosinophil positive gate, MFI values were not included. Analysis of neutrophils from a subset of the same subjects was performed by gating on CD45+ cells with a high side-scatter profile and low auto-fluorescence, and then selecting cells that were double-positive for CD16b and CD66b. Our data, as a percent of leukocytes, show a mean $\pm$ SD of $41.1 \pm 14.4\%$ in peripheral blood, $9.0 \pm 14.9\%$ in sputum, and $1.2 \pm 3.4\%$ in BAL. Antibodies used have been described².

Potential eosinophil chemotactic factors

Samples of serum and plasma collected at the baseline SPIROMICS visit were analyzed using RBM Myriad technology (Salt Lake City, UT); relevant to this study, analytes included the

chemokines CCL5, CCL8 and CCL24³. IgE was measured in the same samples using ELISA by the Advanced Diagnostic Laboratories (National Jewish Health, Denver, CO).

Statistical analyses

We tested for normal distribution of flow cytometry and IgE results using the D'Agostino & Pearson test. We related eosinophil percentages in different sample types using the non-parametric Spearman's correlation coefficient (r_s). We used one-way ANOVA or Kruskal-Wallis test, as appropriate, to analyze eosinophil percentages and their receptor expression between sample types or between groups defined by airflow obstruction and smoking. We tested the contribution of smoking and airflow obstruction to eosinophil percentages in BAL using linear regression and in multivariable models adjusted for demographics and for variables known or suspected to be associated with airway disease (reported chronic bronchitis, physician-diagnosed asthma or gastroesophageal reflux disease, ICS use and total pack-years). We used Prism 6.0f (GraphPad Software, Inc; La Jolla, CA) and Stata v.12 (Stata Corp., College Station, TX). A p-value of <0.05 was considered significant.

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Supplemental Table 1. Eosinophil receptor expression as percent of positive cells in specimen types by subject group and sample type.

No Obstruction												COPD							
NS				FS				CS				FS				CS			
<i>p values across subject group</i>																			
CD11b	Blood	31.3	±	8.6	41.5	±	7.6	37.6	±	7.3	38.5	±	6.8	28.9	±	9.0	<i>n.s</i>		
	Sputum	50.3	±	10.1	40.3	±	9.1	40.1	±	7.7	40.8	±	7.6	28.3	±	9.9	<i>n.s</i>		
	BAL	58.0	±	10.2	71.4	±	8.1	42.9	±	10.4	46.1	±	8.7	31.3	±	10.5	<i>n.s</i>		
	<i>p values across specimen types</i>	<i>n.s</i>			<i>p<0.02</i>			<i>n.s</i>			<i>n.s</i>			<i>n.s</i>					
CD34	Blood	7.1	±	1.9	12.0	±	4.3	23.9	±	8.4	15.7	±	4.2	14.6	±	4.4	<i>n.s</i>		
	Sputum	34.5	±	10.0	25.9	±	5.7	17.3	±	4.3	22.7	±	5.1	21.5	±	8.7	<i>n.s</i>		
	BAL	45.0	±	9.1	39.9	±	7.1	29.3	±	6.8	39.8	±	7.9	23.2	±	8.5	<i>n.s</i>		
	<i>p values across specimen types</i>	<i>n.s</i>			<i>p<0.006</i>			<i>n.s</i>			<i>p<0.02</i>			<i>n.s</i>					
CD49d	Blood	40.7	±	10.8	31.6	±	7.7	46.3	±	9.0	44.1	±	7.6	32.1	±	8.9	<i>n.s</i>		
	Sputum	31.2	±	10.4	16.2	±	6.7	14.9	±	6.0	21.7	±	5.7	21.2	±	9.7	<i>n.s</i>		
	BAL	51.3	±	10.4	51.3	±	8.4	34.5	±	8.4	34.5	±	8.2	33.4	±	10.8	<i>n.s</i>		
	<i>p values across specimen types</i>	<i>n.s</i>			<i>p<0.02</i>			<i>p<0.04</i>			<i>p<0.007</i>			<i>n.s</i>					
CD69	Blood	38.6	±	8.4	46.9	±	6.6	42.3	±	5.6	39.4	±	4.9	25.3	±	6.9	<i>n.s</i>		
	Sputum	74.6	±	8.6	62.6	±	8.0	60.3	±	9.0	62.3	±	6.8	40.0	±	10.3	<i>n.s</i>		
	BAL	76.6	±	9.8	80.4	±	8.2	51.3	±	11.4	73.6	±	8.8	56.2	±	15.0	<i>n.s</i>		
	<i>p values across specimen types</i>	<i>p<0.01</i>			<i>p<0.02</i>			<i>n.s</i>			<i>p<0.002</i>			<i>n.s</i>					
CD125	Blood	55.9	±	9.7	41.5	±	7.7	50.5	±	7.9	44.0	±	6.6	49.2	±	8.9	<i>n.s</i>		
	Sputum	19.5	±	6.2	19.9	±	6.8	13.9	±	6.0	9.8	±	4.3	16.4	±	8.7	<i>n.s</i>		
	BAL	59.1	±	11.4	68.9	±	8.4	38.6	±	9.0	63.4	±	9.1	25.6	±	10.1	<i>p<0.03</i>		
	<i>p values across specimen types</i>	<i>p<0.03</i>			<i>p<0.0001</i>			<i>p<0.004</i>			<i>p<0.0001</i>			<i>p<0.04</i>					

Data are mean $\pm$ SD of the percentage of eosinophils expressing each receptor. Subject groups are abbreviated as in the Legend to Figure 2; *n.s.*, not significant; *p* values by Kruskal-Wallis non-parametric ANOVA with Dunn's post-hoc testing. *n* for NS = 13 blood, 11 sputum, and 16 BAL samples; *n* for FS-NAO = 20 blood, 19 sputum, and 21 BAL samples; *n* for CS-NAO = 23 blood, 22 sputum, and 19 BAL samples; *n* for FS-COPD = 27 blood, 24 sputum, and 22 BAL samples; *n* for CS-COPD = 12 blood, 15 sputum, and 10 BAL samples.

Supplemental Table 2. Median and IQR of receptor MFI in specimen types by subject group and sample type.

		No Obstruction			COPD		<i>p</i> values across subject group
		NS	FS	CS	FS	CS	
CD11b	Blood	6.2 (2.2, 18)	9.7 (4.4, 22)	9.7 (5.8, 21)	6.6 (2.8, 13)	10 (5.8, 22)	<i>n.s</i>
	Sputum	266 (72, 716)	112 (72, 498)	241 (143, 628)	108 (71, 276)	108 (62, 250)	<i>n.s</i>
	BAL	229 (19, 621)	712 (168, 872)	392 (19, 1100)	203 (5, 381)	571 (26, 801)	<i>p<0.04</i>
	<i>p</i> values across specimen types	<i>p<0.006</i>	<i>p<0.0001</i>	<i>p<0.0001</i>	<i>p<0.0001</i>	<i>p<0.001</i>	
CD34	Blood	2.9 (1.8, 4.7)	2.7 (1.6, 5.6)	3.3 (1.9, 4.6)	2.3 (1.2, 3.3)	3.0 (2.2, 9.0)	<i>n.s</i>
	Sputum	362 (252, 897)	293 (227, 525)	206 (149, 313)	154 (74, 284)	176 (79, 421)	<i>n.s</i>
	BAL	466 (58, 641)	249 (96, 551)	249 (96, 551)	214 (58, 784)	280 (17, 453)	<i>n.s</i>
	<i>p</i> values across specimen types	<i>p<0.002</i>	<i>p<0.0001</i>	<i>p<0.0001</i>	<i>p<0.0001</i>	<i>p<0.03</i>	
CD49d	Blood	4.7 (3.0, 9.1)	6.0 (3.5, 20)	5.2 (2.7, 1.6)	4.2 (2.0, 9.3)	9.2 (2.4, 18)	<i>n.s</i>
	Sputum	66 (16, 135)	65 (24, 132)	75 (33, 140)	44 (12, 77)	34 (16, 143)	<i>n.s</i>
	BAL	145 (7.9, 248)	179 (68, 540)	476 (63, 942)	100 (22, 191)	953 (27, 1720)	<i>p=0.0003</i>
	<i>p</i> values across specimen types	<i>p<0.04</i>	<i>p<0.0008</i>	<i>p<0.0001</i>	<i>p<0.0001</i>	<i>p<0.0001</i>	
CD69	Blood	7.1 (3.9, 14)	8.6 (5.3, 19)	7.2 (2.6, 1.2)	5.2 (1.4, 11)	6.9 (4.2, 13)	<i>n.s</i>
	Sputum	973 (195, 1420)	643 (344, 1860)	1390 (209, 2020)	262 (63, 1177)	404 (108, 1880)	<i>n.s</i>
	BAL	1855 (58, 2340)	1980 (639, 2450)	820 (18, 2140)	1960 (62, 2377)	1925 (67, 2040)	<i>n.s</i>
	<i>p</i> values across specimen types	<i>p<0.009</i>	<i>p<0.0001</i>	<i>p<0.0001</i>	<i>p<0.0001</i>	<i>p<0.002</i>	
CD125	Blood	13 (5.0, 17)	20 (12, 26)	10 (6.0, 25)	12 (5.8, 18)	18 (5.3, 25)	<i>n.s</i>
	Sputum	183 (57, 511)	173 (104, 243)	244 (91, 383)	131 (50, 328)	(80, 41, 279)	<i>n.s</i>
	BAL	422 (10, 776)	491 (305, 689)	627 (23, 762)	540 (140, 691)	735 (22, 931)	<i>n.s</i>
	<i>p</i> values across specimen types	<i>p<0.004</i>	<i>p<0.0001</i>	<i>p<0.0001</i>	<i>p<0.0001</i>	<i>p<0.002</i>	

Data are median x 10² (25th, 75th quartile) for receptor MFI on eosinophils. *n.s*, not significant; *p* values by Kruskal-Wallis non-parametric ANOVA with Dunn's post-hoc testing. Number of samples per group shown in the footnote to Supplemental Table 1.

Supplemental Figure Legends.

Supplemental Figure 1. Gating strategy to identify eosinophils in sputum and BAL. Flow cytometry was performed on samples of peripheral blood (A, D, G, J); sputum (B, E, H, K) and BAL (C, F, I, L). Samples were first sequentially gated using SSC-A vs. FSC-A (A-C); SSC-A vs. CD45+ cells with high SSC (D-F); and then with isotype controls (G-I) or monoclonal antibodies against CCR3 and CD16 (J-L). Representative examples from NS subjects.

Supplemental Figure 2. Expression of various eosinophil receptors (as percentage positive) by sample type in all subjects. A, CD11b; B, CD34; C, CD49d; D, CD69; E, CD125. Data are median $\pm$ interquartile (IQR) range, 95th & 5th percentiles, with outliers shown as symbols. Note that the median for BAL cells expressing CD34 is 99%. Samples with fewer than 100 events in the eosinophil positive gate were excluded. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.000$; ****, $p < 0.00001$; ANOVA with Holm-Sidak post-hoc testing. We interpret the reductions in sputum eosinophil expression of CD49d and CD125 to be artefactual and due to the effects of dithiothreitol, as previously shown⁴.

Supplemental Figure 3. Eosinophil expression of adhesion and activation receptors (as MFI) by BAL eosinophils, divided by subject group. A, Representative histograms from never-smokers are shown for CD11b, CD34, CD49d, CD69, and CD125 for blood, sputum, and BAL. Red histogram, isotype control; Blue histogram, mAb-specific staining. B, Cumulative expression by BAL eosinophils of CD11b, CD49d. NS, never-smokers; FS-NAO, former smokers, no airflow obstruction; CS-NAO, current smokers, no airflow obstruction; FS-COPD, former smokers with COPD; CS-COPD, current smokers with COPD. Data are median, IQR, 95th & 5th percentiles, with outliers shown as symbols. Samples with fewer than 100 events in

the eosinophil positive gate were excluded. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.000$; ANOVA with Holm-Sidak post-hoc testing.

