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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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Type 2 inflammation in eosinophilic chronic obstructive pulmonary disease

To the Editor,

Type 2 (T2) inflammation in asthma encompasses the presence of eosinophilic inflammation and/or selected cytokines including interleukin (IL)-13. Some chronic obstructive pulmonary disease (COPD) patients have increased airway eosinophil numbers,[1] which is associated with greater effects of inhaled corticosteroids (ICS).[2] Studies of T2 inflammation in COPD have used hypothesis free, open platform approaches.[3,4] We used these results, plus data from T2 asthma studies,[5] to test a restricted panel of biomarkers (chloride channel accessory 1 [CLCA1], periostin [POSTN], serpin family B member 2 [SERPINB2], C-C motif chemokine ligand 26 [CCL26], IL-13 and cystatin SN [CST1]) to further investigate the nature of T2 inflammation in eosinophilic COPD.

We used two cohorts: bronchial brushings and sputum were obtained from 17 eosinophil^{high} and 20 eosinophil^{low} COPD patients (bronchoscopy cohort previously reported[1]: eosinophil^{high} defined as blood eosinophils > 250 eosinophils μL^{-1} and eosinophil^{low} defined as blood eosinophils < 150 eosinophils μL^{-1}) and sputum only was obtained from 15 eosinophil^{high} and 18 eosinophil^{low} COPD patients (validation cohort: eosinophil^{high} defined as > 150 eosinophils μL^{-1} blood and > 3% sputum eosinophils and eosinophil^{low} defined as < 150 eosinophils μL^{-1} blood and < 3% sputum eosinophils). Full details of inclusion criteria and clinical characteristics are in the Appendix S1 and Table 1. Both cohorts were generally well matched. RNA sequencing examined gene expression in the bronchoscopy cohort, while RT-qPCR was used in the validation cohort. Full details of methods and analysis are in the Appendix S1.

In the bronchoscopy cohort, bronchial epithelial gene expression of CLCA1, CCL26, IL-13 and CST1 was significantly higher ($P < .05$) in eosinophil^{high} versus eosinophil^{low} COPD patients (Figure 1) with fold change differences of 1.9, 2.2, 2.9 and 2.5, respectively. POSTN and SERPINB2 expression trended higher in eosinophil^{high} versus eosinophil^{low} COPD patients but did not achieve statistical significance ($P = .06$ and $P = .07$, respectively; Figure S1).

In the bronchoscopy and validation cohorts, sputum gene expression of CLCA1, CCL26, IL-13 and CST1 was significantly higher ($P < .05$) in eosinophil^{high} versus eosinophil^{low} COPD patients (Figure 1); fold change differences were 3.1, 2.0, 1.8 and 3.5 for the bronchoscopy cohort and 28.2, 9.4, 6.0 and 79.3 for the validation cohort, respectively. Expression of POSTN and SERPINB2 was not different between groups (Figure S1). ICS use and smoking status did not influence gene expression (data not shown). There was a significant positive correlation between a four-gene mean and sputum eosinophil counts in both cohorts (rho 0.6 to 0.7 and $P < .001$; Figure S1), but not other cell types (data not shown).

We report that eosinophilic COPD is associated with a profile of T2 inflammation in both bronchial epithelium and sputum samples; this was consistently observed in sputum samples from two independent cohorts. We tested six genes associated with T2 inflammation in asthma and observed increased expression of four of these genes (CLCA1, CCL26, IL-13 and CST1) in eosinophil^{high} compared to eosinophil^{low} COPD patients. The strengths of this study include a validation cohort, and different lung samples (bronchial epithelium and sputum). The positive signals were > 1.8-fold higher (gene expression) in the eosinophil^{high} group. Different techniques (RNA sequencing and RT-qPCR) were used to analyse sputum samples, but the conclusions and relative fold changes showed the same pattern, adding confidence to our observations. The association between eosinophils and IL-13 expression levels suggests pathways linking eosinophilic inflammation to airway remodelling and mucus secretion,[6] discussed further in the Appendix S1.

TABLE 1 Clinical Characteristics of the Bronchoscopy and Validation cohorts

	Bronchoscopy Cohort			Validation Cohort		
Characteristic	**Eosinophil^{low}**	**Eosinophil^{high}**	***P* value**	**Eosinophil^{low}**	**Eosinophil^{high}**	***P* value**
Number	17	20	N/A	18	15	N/A
Age (y)	62 ± 6	62 ± 4	.7	64 ± 8	67 ± 9	.3
Gender: Male (%)	65	70	.7	44	73	.1
BMI (kg/m^2)	27 ± 5	25 ± 4	.2	25 ± 5	28 ± 4	.06
Current smokers (%)	42	60	.2	61	20	.01
Pack-years history	43 ± 15	38 ± 14	.3	48 ± 19	45 ± 25	.7
ICS use (%)	82	55	.08	39	93	.02
ICS dose	0 (0-2000)	800 (0-2000)	.009	1000 (0-2000)	600 (0-2000)	.35
Post-bronchodilator FEV_1 (L)	1.9 ± 0.4	1.9 ± 0.4	.9	1.7 ± 0.6	1.8 ± 0.5	.6
Postbronchodilator FEV_1 (% predicted)	64 ± 12	66 ± 11	.6	67 ± 17	66 ± 16	.8
Postbronchodilator FVC (L)	3.8 ± 1.3	3.6 ± 0.9	.5	3.2 ± 1	3.3 ± 0.7	.7
Postbronchodilator FEV_1/FVC ratio	50 ± 10	53 ± 7	.3	53 ± 12	55 ± 11	.7
Reversibility (mL)	207 ± 189	217 ± 154	.9	166 ± 142	145 ± 147	.7
$FeNO_{50}$ (ppm)	16 ± 8.4	23 ± 15	.2	9.2 ± 5.2	14.9 ± 8.1	.02
Atopy (% positive)	0	0	N/A	6	13	.4
Total SGRQ	42 ± 17	37 ± 21	.4	56 ± 16	55 ± 16	.9
mMRC	1.8 ± 1.0	1.3 ± 1.2	.2	3.6 ± 0.7	3.7 ± 0.6	.6
CAT	19 ± 9	17 ± 8	.4	22 ± 7	22 ± 6	1
Exacerbation rate, 12 mo prior	0.8 (0-3)	0.7 (0-3)	.6	0.8 (0-4)	1.6 (0-4)	.04
Blood Eosinophils	10 ± 3	432 ± 144	<.0001	9 ± 3	350 ± 240	<.0001
Sputum Cell Counts						
Total (×10^6/g)	5.6 (0.1-26.3)	5.9 (0.5-32.4)	.9	1.9 (0.2-11.4)	2.4 (0.8-12.6)	.2
Neutrophil (×10^6/g)	3.2 (0.1-20.8)	3.6 (0.2-29.6)	.9	3.9 (0.5-24.4)	5.4 (0.4-16.6)	.8
Macrophage (×10^6/g)	1.3 (0.01-4.6)	1.2 (0.2-7.7)	.6	1.4 (0.3-3.5)	1.3 (0.4-4.5)	.5
Eosinophil (×10^6/g)	0.02 (0.0-0.2)	0.3 (0.01-4.2)	<.001	0.04 (0.00 - 0.4)	0.3 (0.1-1.3)	<.001
Lymphocyte (×10^6/g)	0.02 (0.0-0.1)	0.02 (0.0-0.2)	.4	0.04 (0.0-0.6)	0.01 (0.0-0.2)	.4
Epithelial (×10^6/g)	0.04 (0.0-0.7)	0.03 (0.0-1.0)	.7	0.1 (0.02-0.6)	0.2 (0.04-1.6)	.07
Eosinophils (%)	0.5 (0-2.5)	4.5 (0.3-70)	<.0001	0.5 (0-2)	6.5 (2.8-16.5)	<.0001
Neutrophils (%)	81 (17-91)	63 (15-91)	.06	67 ± 20	65 ± 18	.8
Macrophages (%)	17 (5-82)	23 (4-63)	.6	30 ± 19	23 ± 15	.3
Lymphocytes (%)	0.5 (0-2.3)	0.3 (0-2.5)	.3	0.3 (0-2.8)	0.3 (0-3.5)	.8
Epithelial (%)	1 (0-8)	1 (0-8)	.85	2 (0.3-10)	3 (0.3-17)	.08

Note: Data presented as %, mean ± standard deviation, or median (range).

Abbreviations: BMI, body mass index; ICS, inhaled corticosteroids; FEV_1, forced expiratory volume in 1 s; FVC, forced vital capacity; $FeNO_{50}$, fractional exhaled nitric oxide at 50 mL^{-1} s flow rate; PPM, parts per million; SGRQ, St George's Respiratory Questionnaire; mMRC, modified Medical Research Council; CAT, COPD Assessment Test.

These results indicate a wider profile of T2 inflammation in eosinophilic COPD that extends to mechanisms including IL-13 driven pathways. While T2 inflammation is well recognized in asthma, we now provide further evidence of T2 inflammation in a subset of COPD patients. The components of T2 inflammation described here may represent therapeutic targets.

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CONFLICT OF INTEREST

AH has received personal fees from Chiesi. AB, SW, NJ, GL, UK and TS have no conflicts of interest. T-HP, SS, CM and PN are employees of AstraZeneca. DS has received personal fess from AstraZeneca,

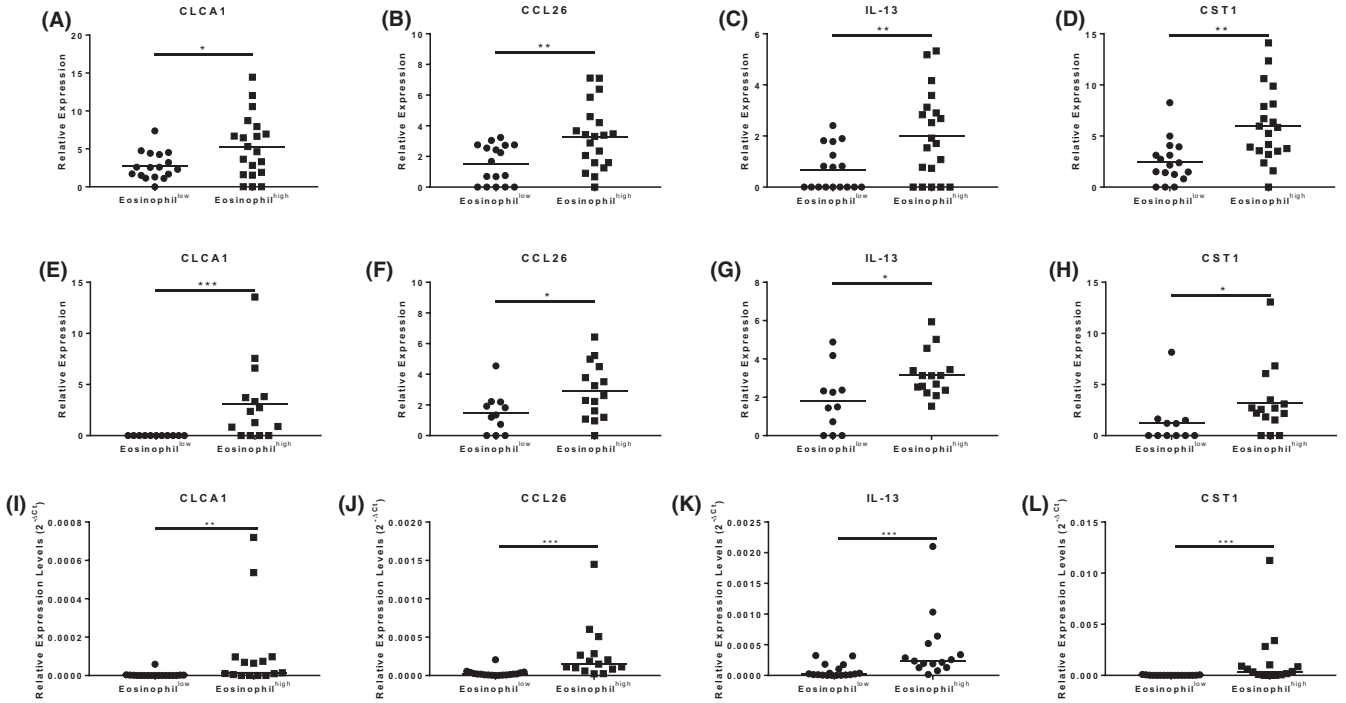

FIGURE 1 T2 gene expression in bronchial brushings and sputum from the bronchoscopy and validation cohorts. RNA sequencing was used to examine the expression of CLCA1 (A, E, and I), CCL26 (B, F and J), IL-13 (C, G and K), CST1 (D, H and L), in RNA isolated from bronchial brushings (A-D) and sputum cells (E-H) from the bronchoscopy cohort and sputum cells from the validation cohort (I-L). Data presented as individual data points with mean (A-H) or median (I-L) where *, ** and *** = significant difference ($P < .05$, $P < .01$ and $P < .001$, respectively). For A-H, gene expression was normalized to the transcript length for each gene. For I-L, gene expression was normalized to GAPDH

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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Inhibition of CpG methylation improves the barrier integrity of bronchial epithelial cells in asthma

To the Editor,

Asthma is a complex and heterogeneous chronic airway inflammatory disease with the involvement of environmental factors through epigenetic mechanisms.[1] Accordingly, repeated injury, repair, and regeneration of the airway epithelium following exposure to environmental factors and inflammation results in histological changes and functional abnormalities in the airway mucosal epithelium, which are associated with the pathophysiology of asthma.[2] Epigenetics is defined by heritable changes in gene expression without changes in the DNA sequence.[3] Regulation of gene expression is mediated by different mechanisms such as DNA methylation, histone modifications, and RNA-associated silencing by small non-coding RNAs. CpG sites are dinucleotides consisting of guanine and cytosine concentrated in clusters referred to CpG islands found at important regulatory sites, such as promoter and enhancer regions.[4] Their de novo methylation occurs in response to various cellular stressors and signals by DNA methyltransferases (DNMT3a and 3b), which add a methyl group to position 5 of cytosine residues at the CpG site. During DNA replication, both of the separated strands of DNA carry one methylated cytosine to be used as a template for duplication. Daughter DNA duplex strands will thus be hemi-methylated, which is recognized by a different DNA methyltransferase isoform (DNMT1). Because DNA methylation is a reversible process, the DNMTs are considered as a therapeutic target. Several DNMT inhibitors have been identified recently, among the non-nucleoside inhibitors, 4-aminoquinoline-based inhibitors, such as SGI-1027 showed potent inhibitory activity. SGI-1027 occupies the binding site of DNMTs resulting in the prevention of access of target DNA to the substrate binding pocket.[5]

We have demonstrated in previous studies from our laboratory that human primary bronchial epithelial cells (HBEC) isolated from patients with asthma showed lower barrier integrity compared to controls.[6] Seeing that important role of DNA methylation in lung cells and asthma pathogenesis by other groups,[7] we investigate the level of global methylation in HBEC of control and asthma samples for the long interspersed nuclear element-1 (LINE-1) methylation levels (Figure 1A). HBEC from asthma patients showed a tendency for higher global methylation levels, together with higher expression of 5-methylcytosine (5-mc) in immunofluorescence staining (Figure 1B). Next, we performed methylation profiling (Illumina Infinium EPIC array) to investigate genes methylated in ALI cultures of HBEC. Interestingly, in a highly methylated group of top 100 genes, we found many genes associated with cell growth, ion transport, and cytoskeletal remodeling (Figure S1). Several pro-inflammatory cytokines had lower methylation levels, whereas regulatory cytokines, such as *IL10RA* and *TGFBR2*, were methylated (Figure S2). In genes involved in the epigenetic process, we found members of histone demethyltransferases, histone deacetylases in both groups, but interestingly ten-eleven translocation enzyme (*TET1*), which can reverse CpG methylation was methylated. On the other side, genes belong to protein arginine methyltransferases (PRMT) were not methylated. Many genes specifically involved in the regulation of bronchial barrier integrity showed higher methylation (eg, TJ family members: *AMOTL1*, *CLDN11*, *CLDN18*, *MAGI1*, *TJP2*, *JAM3*, actin protein: *ACTB*, a component of the cytoskeleton: *TUBA1C*, *ROCK2*, *LLGL1)*. We kept our attention on the methylated epigenetic and tight junction (TJ) genes and further focused on TJs, especially zonula occludens and claudins which showed higher methylation in contrast to occludin, which was not methylated (Figure 1C). As higher methylation levels were observed in HBEC of asthmatic origin, we inhibited the DNA methyltransferase enzyme with a specific inhibitor, SGI-1027, to

Abbreviations: ALI, air-liquid interface; BEGM, bronchial epithelial growth medium; DNMT, DNA methyltransferase; FITC, fluorescein isothiocyanate; HBEC, human bronchial epithelial cells; LINE-1, long interspersed nuclear element-1; 5-mc, 5-methylcytosine; TER, transepithelial electric resistance; ZO, zonula occludens.