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Original Article

Sputum Transcriptomics Reveals FCN1+ Macrophage Activation in Mild Eosinophilic Asthma Compared to Non-Asthmatic Eosinophilic Bronchitis

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ABSTRACT

Purpose: Eosinophilic asthma (EA) and non-asthmatic eosinophilic bronchitis (EB) share similar eosinophilic airway inflammation. Unlike EA, EB did not present airway hyperresponsiveness or airflow obstruction. We aimed to compare the mechanism underlying the different manifestations between EA and EB via sputum transcriptomics analysis.
Methods: Induced-sputum cells from newly physician-diagnosed EA, EB patients, and healthy controls (HCs) were collected for RNA sequencing.
Results: Bulk RNA sequencing was performed using sputum cells from patients with EA (n = 18), EB (n = 15) and HCs (n = 28). Principal component analysis revealed similar gene expression patterns in EA and EB. The most differentially expressed genes in EB compared with HC were also shared by EA, including IL4, IL5 IL13, CLC, CPA3, and DNASE1L3. However, gene set enrichment analysis showed that the signatures regulating macrophage activation were enriched in EA compared to EB. Sputum cells were profiled using single-cell RNA sequencing. FABP4+ macrophages, SPP1+ macrophages, FCN1+ macrophages, dendritic cells, T cells, B cells, mast cells, and epithelial cells were identified based on gene expression profiling. Analysis of cell-cell communication revealed that interactions between FCN1+ macrophages and other cells were higher in EA than in EB. A wealth of transforming growth factor beta (TGF-β) and vascular endothelial growth factor (VEGF) interactions between FCN1+ macrophages and other cells have been shown in EA. The gene expression levels of EREG, TGFBI, and VEGFA in FCN1+ macrophages of EA were significantly higher than those of EB. Furthermore, signatures associated with the response to TGF-β, cellular response to VEGF stimulus and developmental cell growth were enriched in FCN1+ macrophages of EA compared to those of EB.
Conclusions: FCN1+ macrophage activation associated with airway remodeling processes was upregulated in EA compared to that in EB, which may contribute to airway hyperresponsiveness and airflow obstruction.

Keywords: Asthma; sputum; macrophages; RNA-Seq; single-cell gene expression analysis

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Disclosure

There are no financial or other issues that might lead to conflict of interest.

Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request. All sequencing data have been deposited in public, open access repository of NIH's Sequence Read Archive (SRA) (BioProject: PRJNA878650).

INTRODUCTION

Asthma is characterized by airway hyperresponsiveness and variable symptoms, including wheezing, shortness of breath, chest tightness, and coughing.[1] The global prevalence of current asthma was up to 5.4% (95% confidence interval [CI], 3.2%–9.0%), affecting around 357.4 (95% CI, 213.0–590.8) million people worldwide.[2] Eosinophilic airway inflammation is the most common in asthma, referred to eosinophilic asthma (EA).[3,4] Non-asthmatic eosinophilic bronchitis (EB) is a common cause of chronic cough and shares similar eosinophilic airway inflammation with EA, as reflected by similar eosinophil counts in the sputum, infiltration of eosinophils in the submucosa, and expression levels of interleukin (IL)-4, IL-5, and eosinophilic cationic protein in the airway.[5,6] Unlike EA, EB does not present airway hyperresponsiveness or airflow obstruction and does not progress into asthma or chronic obstructive pulmonary disease in most patients.[7] Compared to EA, patients with EB show milder eosinophilic inflammation in the peripheral airway, less infiltration of mast cells in airway smooth muscle, and increased prostaglandin E2 level in sputum, which might contribute to the different manifestations of EB.[8,10] However, the cellular and molecular mechanisms underlying the differential manifestations of EA and EB remain unclear.

Through transcriptomic analysis, particularly bulk RNA sequencing (bulk RNA-seq), gene profiles associated with these phenotypes can be generated by examining genes specifically expressed in certain physiological and pathological status.[11] Recently, single-cell RNA sequencing (scRNA-seq) has provided a better understanding of the cellular features and functions of various cell subtypes in the context of their microenvironments. Induced sputum from the central airways with components at the cellular (*e.g.*, eosinophils and macrophages) and protein (*e.g.*, mucins and cytokines) levels is useful for exploring the mechanism of respiratory disease and therapeutic targets.[12] Transcriptional profiling in induced sputum has uncovered the underlying mechanisms related to severity, exacerbation, progression, phenotypes, and endotypes of asthma, which may identify new therapeutic targets and substantially promote individualized treatment.[13,17] However, no data are available regarding the gene expression profiles of airway inflammatory cells in EA and EB. Since there are similar inflammatory cells in the airways of both EA and EB, it is reasonable to assume that the different activations of specific subtypes of inflammatory cells may contribute to the distinctive manifestation. Since inflammatory cells are plastic and diverse in various microenvironments, scRNA-seq should have special value in uncovering heterogeneity. Therefore, we performed RNA-seq and scRNA-seq of sputum cells to unveil gene expression signatures, immune cell subsets, and potentially distinct immunological molecular mechanisms in EA and EB.

MATERIALS AND METHODS

Details of methods are provided in the **Supplementary Data S1**.

Subjects

Physician-diagnosed adult patients with EA and EB were recruited from outpatient clinics. Healthy control (HC) subjects with no history of chronic airway disease were enrolled. All participants signed an informed consent form prior to enrollment. This study was approved by the Ethics Committee of the First Affiliated Hospital of Guangzhou Medical University (approval No. 201912).

EA was diagnosed on the basis of the criteria below: 1) variable respiratory symptoms such as wheezing, shortness of breath, chest tightness, and cough, 2) evidence of variable expiratory airflow limitation [positive bronchial challenge test (fall in forced expiratory volume in 1 second [FEV1] from baseline of ≥ 20% (PD20-FEV1) with 12.8 μmol of methacholine), or positive bronchodilator reversibility test (increase in FEV1 ≥ 12% and 200 mL from baseline)], and 3) induced sputum eosinophil count ≥ 2%.[1] EB was diagnosed based on the following criteria: 1) chronic cough as a sole symptom lasting more than 8 weeks; 2) unremarkable chest radiographic findings; 3) normal pulmonary ventilation function and negative bronchial challenge test; 4) sputum eosinophil count ≥ 2.5%.[18] We excluded patients who experienced respiratory tract infections or received oral/inhaled corticosteroids within the last 4 weeks.

Clinical and laboratory assessment

The demographic and clinical characteristics of the patients were collected. Cough severity was assessed using the Cough Visual Analogue Scale. Spirometry, bronchial challenge tests and fractional exhaled nitric oxide (FeNO) test were performed.

Sputum induction and processing

Sputum was induced and processed as previously described.[18] Briefly, sputum was induced with 3% saline and the selected sputum plugs was treated with four times its volume of 0.1% dithiothreitol. A portion of the cell pellet was used for cell smear staining with hematoxylin-eosin. The remaining sputum cell pellets were mixed with RNAprotect Cell Reagent (Qiagen, Germany) and stored at −80°C for subsequent bulk RNA-seq.

Bulk-RNA-seq and data analysis

RNA was extracted from sputum cells using AllPrep DNA/RNA Mini Kits (Qiagen, Germany). High-quality RNA (RNA integrity number > 6) was used in the following procedure. Library preparation was performed using the NEBNext Ultra RNA Library Prep Kit for Illumina (New England Biolabs, USA) and NEBNext Multiplex Oligos for Illumina (New England Biolabs, USA) according to the manufacturer's protocol. Libraries were sequenced in a flow cell using the Illumina NovaSeq 6000 platform. Genes with a mean count < 1 were excluded from downstream analyses. The DESeq2 (v1.34.0) package was used for read count normalization and differential expression analyses.[19] Differentially expressed genes (DEGs) between the groups were defined as $|\log_2\text{Foldchange}| > 1$ and *P*.adjust < 0.05. *P*.adjust was attained after the *P*-value was corrected for multiple comparisons using the Benjamini-Hochberg method. Gene set enrichment analysis (GSEA) was performed to examine signaling pathways regulated by the different conditions.[201] Briefly, GSEA aggregates per gene statistics across genes within a gene set. The genes were ranked according to their conditions. Given a prior defined set of genes S (*e.g.*, the gene set from Gene Ontology [GO, http://geneontology.org/] Biological Processes in this study), the goal of GSEA is to determine whether the members of S are randomly distributed throughout the ranked gene list (L) or are primarily found at the top or bottom. Significance scores were computed using a permutation test. Normalized enrichment scores were used to account for differences in gene set size and correlations between gene sets and the expression dataset. When all gene sets were evaluated, the estimated significance level was adjusted with the Benjamini-Hochberg method. GSEA and visualization were performed using the R package clusterProfiler (v4.4.4).[21]

scRNA-seq and data analysis

A single-cell solution was obtained by mixing induced sputum plugs with four times the volume of 0.1% dithiothreitol, and then placed at 37°C for 15 minutes with gentle agitation.

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The single-cell suspension was forced through a 30 μm nylon cell strainer (Miltenyi-Biotec, Bergisch Gladbach, Germany). The suspension was centrifuged at 300 rcf for 5 minutes at 4°C and washed once with cold PBS. Single cell suspensions were manually counted using a hemocytometer and adjusted to a concentration of 800–1,000 cells μL^{-1}. Single Cell 3' libraries were obtained according to the manufacturer's protocol (Chromium™ Single Cell 3' Reagent Kit; 10X Genomics, Pleasanton, CA, USA). The libraries were sequenced on an Illumina NovaSeq 6000 platform. CellRanger Software (v 6.0.1) was used to perform sample demultiplexing, barcode processing, and single-cell 3′ gene counting using standard default parameters. Data analysis was performed using the R package Seurat (v4).[22] The data were excluded based on the following thresholds: less than 1,000 or greater than 6,000 unique expressed genes (nFeature_RNA) and greater than 25% mitochondrial genome content. To minimize batch effects when combining multiple samples for integrated analysis, R package Harmony (v1.0) was used.[23] DEGs among clusters were identified using the FindAllMarkers function (with parameters: only.pos = TRUE; min.pct = 0.20; logfc. threshold = 0.25; Wilcoxon Rank Sum test). The Bonferroni correction was conducted for multiple comparisons of Wilcoxon *P* values. CellPhoneDB (v3) was used to identify cell–cell communication from the combined expression of multi-subunit ligand–receptor complexes.[24] Network visualization was performed using Cytoscape (v3.9.1). The CellChat package (v1.6.1) was also used to infer and visualize cell-cell communication based on the L–R interactions.[25]

Statistical analysis

Statistical analysis was performed using R software (v 4.1.2). Data are expressed as frequency (percentage), mean ± standard deviation or median with an interquartile range [25%, 75%]. Statistical comparisons between groups were performed using a one-way analysis of variance or the Kruskal-Wallis test followed by pairwise comparisons adjusted for multiple testing (Tukey when row variables were normally distributed and Benjamini-Hochberg method otherwise). χ^2 or Fisher's exact tests were used to analyze categorical variables.

RESULTS

Sixty-one induced sputum samples from 18 EA, 15 EB, and 28 HC were processed for bulk RNA-seq. The baseline characteristics of the study participants are presented in **Table**. There were no significant differences in age, sex, body mass index, cough severity, FeNO level, sputum eosinophil count or sputum macrophage count between EA and EB.

EA and EB shared common gene expression signatures enriched in inflammatory response and epithelial cell proliferation

Principal component analysis of bulk RNA-seq data demonstrated a similar pattern between EA and EB (**Fig. 1A**). Most DEGs in EB compared to HC were among the DEGs in EA, including IL4, IL5, IL13, CLC, CPA3, and DNASE1L3 (**Fig. 1B and C**, **Supplementary Tables S1** and **S2**).

In total, 1,011 commonly upregulated and 19 downregulated genes overlapped between EB and EA (**Fig. 1D**). Among the upregulated genes, the major biological processes were enriched in the inflammatory response, innate immune response in the mucosa, and epithelial cell proliferation (**Fig. 1E**, **Supplementary Table S3**).

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Table. Clinical features of participants with bulk RNA sequencing

Characteristics	EA (n = 18)	EB (n = 15)	HC (n = 28)	*P*.overall	*P*.adjust (EA vs. EB)	*P*.adjust (EA vs. HC)	*P*.adjust (EB vs. HC)
Age, years	44.5 ± 11.0	38.1 ± 11.0	41.1 ± 12.9	0.3145	0.2856	0.6161	0.7170
Female	9 (50.0%)	5 (33.3%)	15 (53.6%)	0.4347	0.8119	1.0000	0.8119
BMI	23.2 ± 2.76	22.8 ± 2.87	22.8 ± 2.16	0.8606	0.8941	0.8702	0.9998
Disease duration, months	60.0 [29.8–74.2]	4.00 [3.00–21.0]	-	0.0052	0.0052	-	-
Current smoker	2 (11.1%)	2 (13.3%)	0 (0.00%)	0.1028	1.0000	0.2217	0.2217
Rhinitis	8 (44.4%)	7 (46.7%)	0 (0.00%)	< 0.0001	1.0000	< 0.0001	< 0.0001
Sinusitis	4 (22.2%)	3 (20.0%)	1 (3.57%)	0.0920	1.0000	0.1714	0.1714
Cough VAS	38.1 ± 29.3	31.7 ± 14.7	-	0.4487	0.4487	-	-
Blood-Eos, 10^9	0.35 [0.28–0.42]	0.37 [0.20–0.44]	0.10 [0.10–0.20]	< 0.0001	0.9221	< 0.0001	0.0005
FeNO, ppb	64.0 [37.0–102]	36.5 [34.5–52.8]	-	0.1950	0.1950	-	-
Sputum-Eos	14.2 [4.35–25.9]	5.60 [2.72–8.25]	0.24 [0.00–0.25]	< 0.0001	0.1036	< 0.0001	< 0.0001
Sputum-Neu	55.1 [31.5–81.4]	51.5 [42.0–69.3]	46.1 [38.8–61.8]	0.5069	0.7312	0.5342	0.5342
Sputum-Mac	18.1 [9.75–37.0]	29.0 [20.7–52.2]	53.2 [37.4–60.7]	0.0008	0.1037	0.0011	0.0516
Sputum-Lym	0.50 [0.21–1.00]	0.80 [0.35–1.62]	0.49 [0.23–0.96]	0.3767	0.5458	0.6349	0.5118
Sputum-RIN	8.35 [8.03–8.78]	8.80 [8.45–9.20]	8.75 [8.35–8.93]	0.0597	0.0770	0.1263	0.3371
FEV1, %	82.5 ± 15.8	89.7 ± 14.7	100 ± 10.5	0.0002	0.2718	0.0002	0.0490
FVC, %	102 ± 13.2	93.4 ± 10.8	101 ± 11.8	0.0744	0.1035	0.9799	0.1011
FEV1/FVC	70.2 [65.1–76.1]	82.5 [77.0–87.7]	83.4 [79.0–86.1]	< 0.0001	0.0022	< 0.0001	0.7792
MMEF, %	43.4 ± 18.4	74.2 ± 27.1	75.3 ± 14.2	< 0.0001	0.0001	< 0.0001	0.9819
PD20-FEV1	0.34 [0.17–1.86]	> 12.8	> 12.8	-	-	-	-

EA, eosinophilic asthma; EB, eosinophilic bronchitis; HC, healthy controls; BMI, body mass index; Cough VAS, Cough Visual Analogue Scale; Blood-Eos, blood eosinophil count; FeNO, fractional exhaled nitric oxide; Sputum-Eos, sputum eosinophil count; Sputum-Neu, sputum neutrophil count; Sputum-Mac, sputum macrophage count; Sputum-Lym, sputum lymphocyte count; Sputum-RIN, sputum RNA Integrity Number; FVC, forced vital capacity; FEV1, forced expiratory volume in 1 second; MMEF, maximum mid-expiratory flow; PD20-FEV1, the provocative cumulative dose of methacholine causing a 20% fall in FEV1.

Regulation of macrophage activation signatures was enriched in EA rather than EB

Compared to HC, gene FCN1 (log_2FoldChange = 1.5, *P*.adjust < 0.0001), OSM (log_2FoldChange = 1.8, *P*.adjust < 0.0001), NLRP3 (log_2FoldChange = 1.6, *P*.adjust = 0.0002), THBS1 (log_2FoldChange = 3.1, *P*.adjust < 0.0001), CST7 (log_2FoldChange = 1.9, *P*.adjust < 0.0001), and IL6 (log_2FoldChange = 1.6, *P*.adjust = 0.0002) were upregulated in EA, which were regarded as macrophage activation signatures (**Fig. 2A**). Comparing EA to EB, the upregulated genes also included THBS1 (log_2FoldChange = 2.4, *P*.adjust = 0.0003), CST7 (log_2FoldChange = 1.3, *P*.adjust = 0.0080), and IL6 (log_2FoldChange = 1.6, *P*.adjust = 0.0180) (**Supplementary Table S4**). GSEA showed that the regulation of macrophage activation was significantly enriched in EA compared to that in EB or HC (**Figs. 1C** and **2B**). Signatures of regulation of macrophage activation were not enriched in the EB (**Fig. 2D**). Principal component analysis was performed on the up-regulated genes associated with the regulation of macrophage activation. The first principal component was significantly correlated with PD20-FEV1 (Spearman's $\rho = -0.49$, $P = 0.0001$) and FEV1% (Spearman's $\rho = -0.48$, $P = 0.0001$).

Sputum macrophages were classified into FABP4+, SPP1+ and FCN1 macrophages

scRNA-seq was used to analyze the single-cell resolution transcriptomes of sputum cells from three subjects with EA, two with EB, and three of HC (**Supplementary Table S5**). In total, 22,681 sputum cells (4,185 EA cells, 6,795 EB cells, and 11,701 HC cells) were profiled. Based on known transcriptomic markers, six distinctive sputum cell populations were identified: macrophages (CD163 and AIF1), dendritic cells (CD1C and FLT3), T cells (CD2 and CD3D), B cells (CD19 and CD79A), mast cells (TPSB2 and CPA3) and epithelial cells (KRT8 and KRT19) (**Fig. 3A and C**, **Supplementary Table S6**).

Three discrete subpopulations of sputum macrophages (FABP4+, SPP1+, and FCN1+) were identified in all the subjects. FABP4+ macrophage expressed high levels of FABP4, INHBA,

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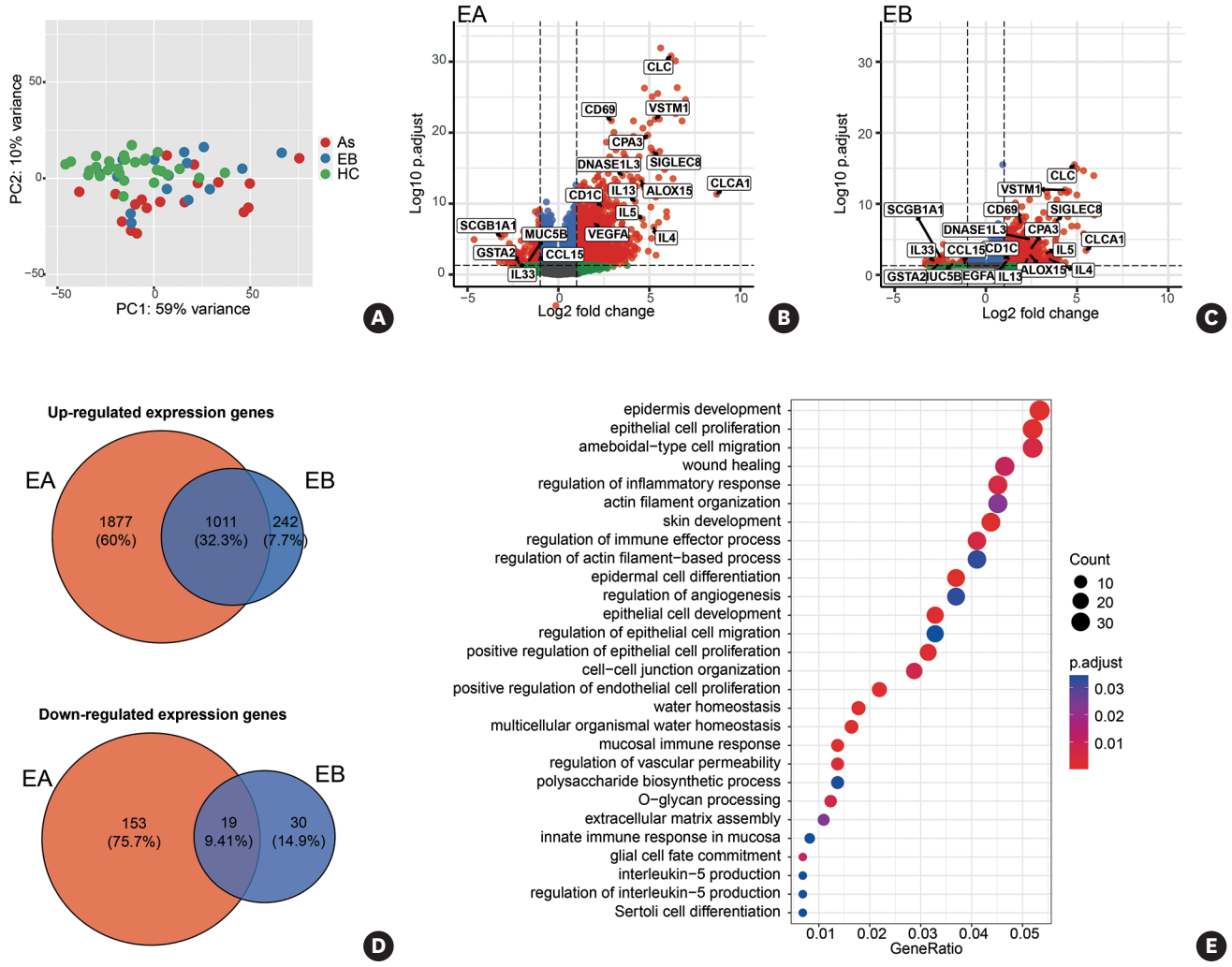

Fig. 1. Transcriptional profiling of bulk RNA sequencing among EA, EB and HCs. (A) Principal component analysis of gene expression profiles for sputum cells from EA, EB and HC, indicating the absence of a clear differentiation between EA and EB. Each point is assigned a location to illustrate potential clusters of neighboring samples, which contain similar gene expression patterns. (B-C) Volcano plots showing differentially expressed genes across EA and EB, in comparison with HC, in which some representative genes were highlighted. (D) Venn diagram showing 1,011 upregulated (top panel) and 19 downregulated genes (down panel) in common across EA and EB. (E) Gene ontology term enrichment of biological processes for common 1,011 upregulated genes.
EA, eosinophilic asthma; EB, eosinophilic bronchitis; HC, healthy control.

SERPHG1, and ATP1B1, and relatively low to undetectable levels of SPP1, FCN1, SDS, and LGMN. Among the upregulated genes (*P*.adjust < 0.05 & average $\log_2$foldchange > 0.25) in FABP4+ macrophage, the major biological processes were related to oxidative phosphorylation, ATP metabolic process, lipid localization, and lipid catabolism (**Fig. 4A and B**). SPP1, LGMN, GSDME, and HAMP were highly expressed in SPP1+ macrophage (**Fig. 4A**). GO biological process analysis showed that the genes upregulated in SPP1+ macrophage were associated with antigen processing and presentation, autophagy, responses to a molecule of bacterial origin, and macrophage activation (**Fig. 4C**). The third macrophage population (FCN1+ macrophages) comparatively highly expressed FCN1, VCAN, and IL1B (**Fig. 4A**). The regulation of T cell activation, cell-cell adhesion, mononuclear cell differentiation, and leukocyte activation involved in the immune response were enriched in FCN1+ macrophage (**Fig. 4D**).

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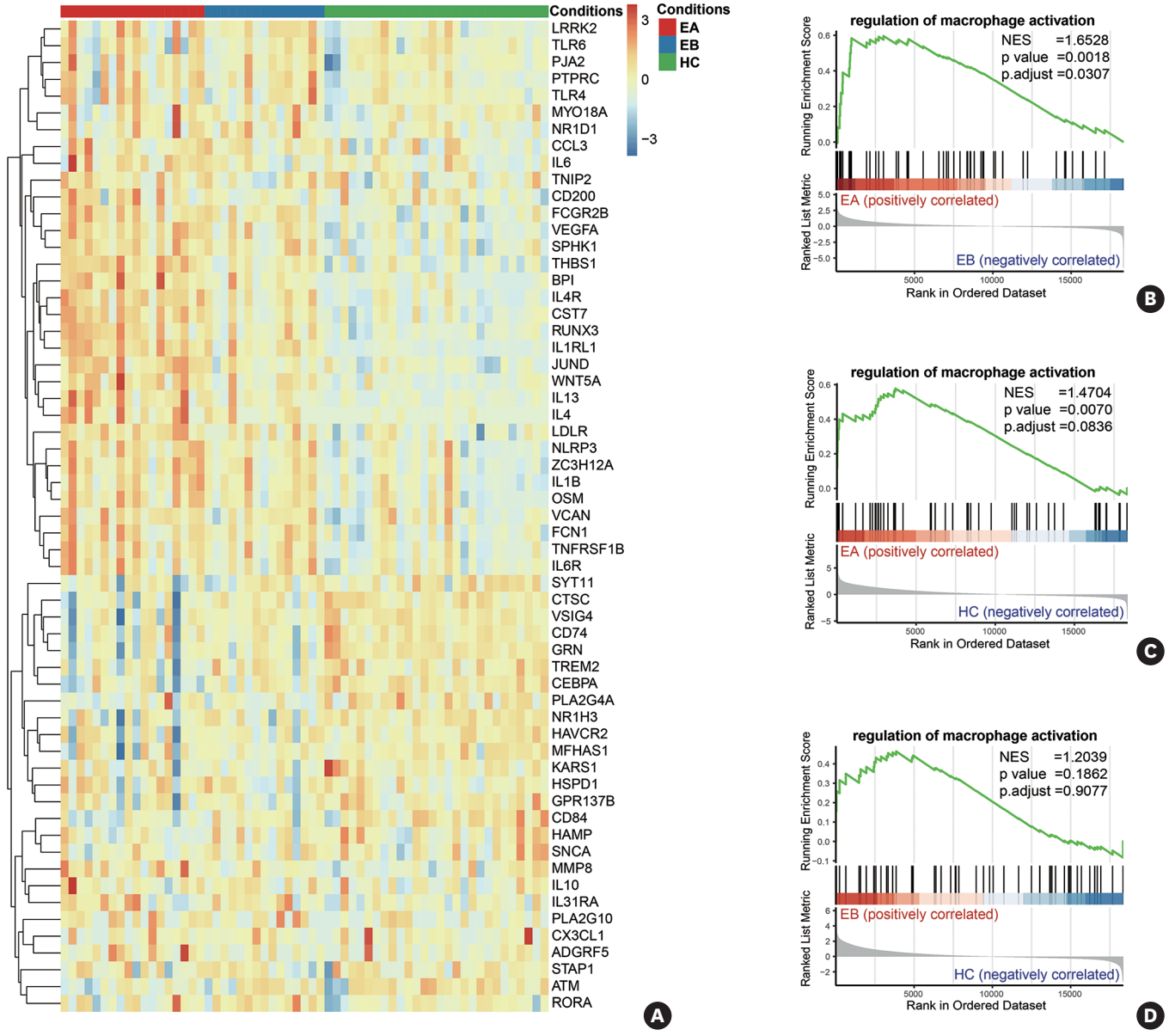

Fig. 2. Macrophage activation across EA, EB, and HCs. (A) Heatmap illustrating gene related to the regulation of macrophage activation. (B-D) Gene Set Enrichment Analysis was performed using the Gene ontology term of biological processes with genes ranked by fold change in gene expression. Enrichment plots together with NES.
EA, eosinophilic asthma; EB, eosinophilic bronchitis; HC, healthy control; NES, normalized enrichment scores.

Cell-cell interactions between FCN1+ macrophage and other cells were increased in EA compared with EB

Potential cell-cell interactions in the sputum were identified using the receptor/ligand database and the statistical inference framework CellPhoneDB (www.cellphonedb.org). The cell-cell interaction landscape of HC was dominated by FABP4+ macrophages communicating with each other (**Fig. 5A and B**). In EA, the number of overall predicted cell-cell interactions, especially between FCN1+ macrophages and other cells, increased. With CellChat analysis, a cell-to-cell communication network was identified. Consistent with the

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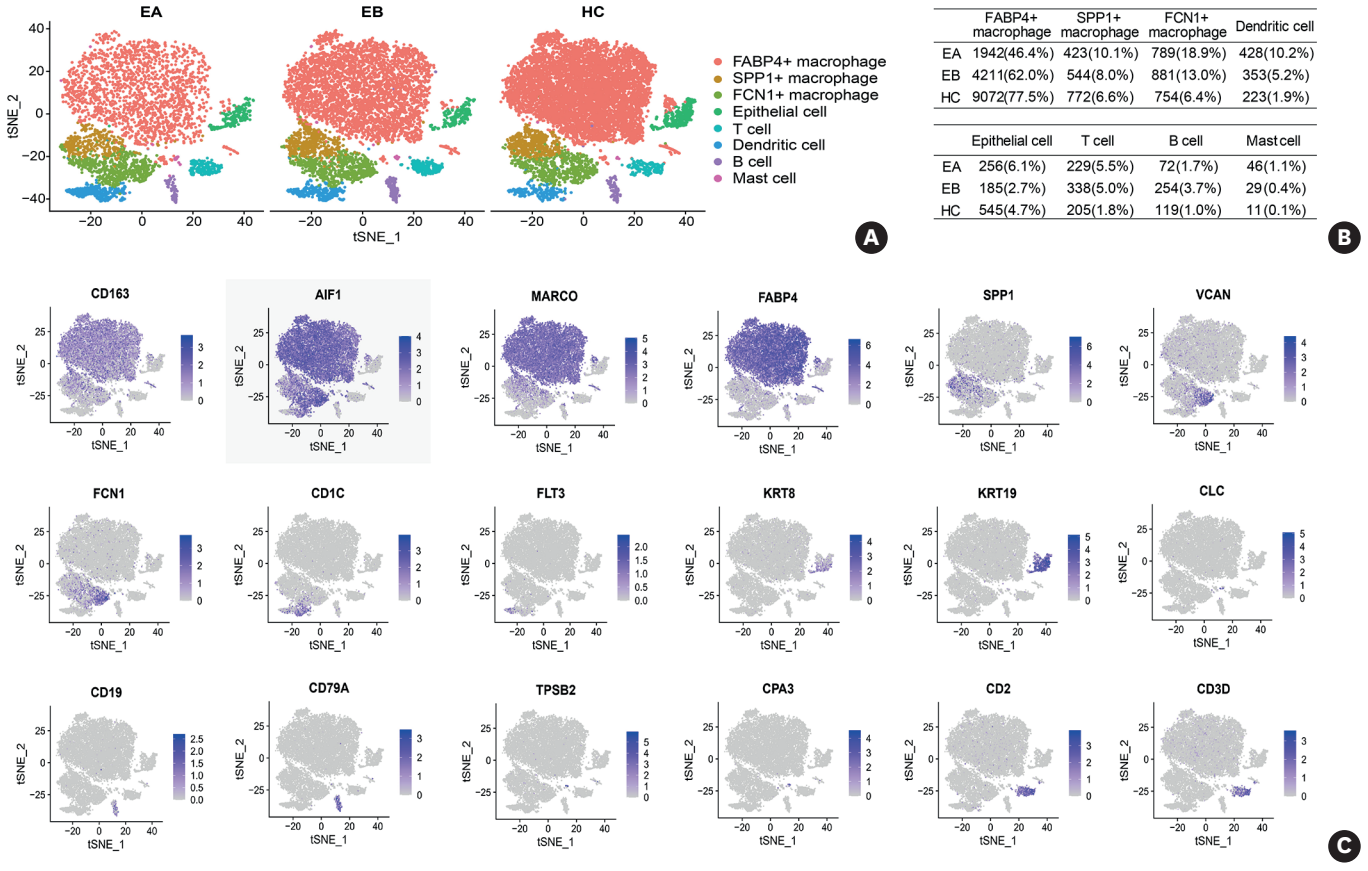

B

	FABP4+ macrophage	SPP1+ macrophage	FCN1+ macrophage	Dendritic cell
EA	1942(46.4%)	423(10.1%)	789(18.9%)	428(10.2%)
EB	4211(62.0%)	544(8.0%)	881(13.0%)	353(5.2%)
HC	9072(77.5%)	772(6.6%)	754(6.4%)	223(1.9%)

	Epithelial cell	T cell	B cell	Mast cell
EA	256(6.1%)	229(5.5%)	72(1.7%)	46(1.1%)
EB	185(2.7%)	338(5.0%)	254(3.7%)	29(0.4%)
HC	545(4.7%)	205(1.8%)	119(1.0%)	11(0.1%)

Fig. 3. Integrated single-cell RNA-sequencing analysis of sputum cells from all participants. Cells were clustered using a graph-based shared nearest neighbor clustering approach and visualized using a tSNE plot. (A) Cellular populations identified on the tSNE plot of all 8 samples originating from 3 EA, 2 EB and 3 HCs. (B) Table with the details of cell numbers (proportion) present in this figure. (C) Canonical cell markers were used to label clusters by cell identity as represented in the tSNE plot.
EA, eosinophilic asthma; EB, eosinophilic bronchitis; HC, healthy control; tSNE, t-distributed Stochastic Neighbor Embedding.

results from CellPhoneDB, CellChat analysis also showed that the number of interactions between FCN1+ macrophages and other cells was increased in EA (**Fig. 6A**).

Compared to EB, EREG (average log_2FoldChange = 1.02, *P*.adjust < 0.0001), TGFBI (average log_2FoldChange = 0.98, *P*.adjust < 0.0001), IL1RN (average log_2FoldChange = 0.65, *P*.adjust < 0.0001), VEGFA (average log_2FoldChange = 0.50, *P*.adjust < 0.0001), and IL6R (average log_2FoldChange = 0.37, *P*.adjust < 0.0001) were significantly up-regulated in FCN1+macrophage of EA (**Supplementary Table S7**). Analysis of the predicted cell-cell interactions between FCN1+ macrophage and other cells in EA revealed a wealth of transforming growth factor beta (TGF-β), vascular endothelial growth factor (VEGF), intercellular cell adhesion molecule-1(ICAM1) and IL-1 pathways (**Figs. 5C** and **6B**). Compared to FCN1+ macrophage of EB, GSEA showed significant enrichment of genes associated with response to TGF-β, cellular response to VEGF stimulus, developmental cell growth, and cell-matrix adhesion in FCN1+ macrophage of EA (**Supplementary Table S8**).

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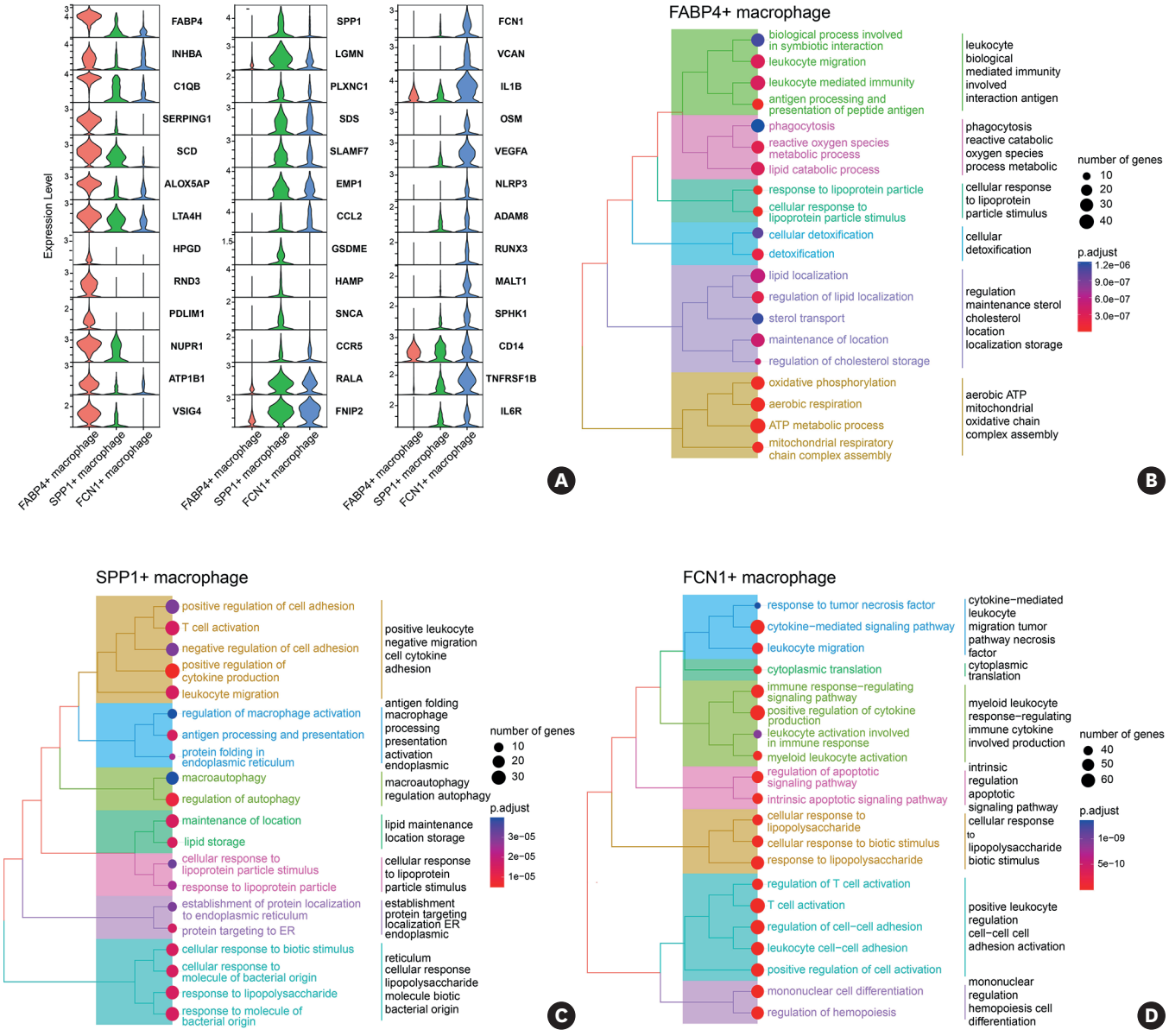

Fig. 4. Differential expression analysis of single-cell RNA-sequencing data from all participants identifies gene characteristics of macrophages. (A) Violin plots of expression for select genes significantly upregulating in the three subpopulations of macrophage. (B-D) Hierarchical clustering of Gene ontology term enrichment of biological processes for upregulated genes in FABP4+macrophage, SPP1+ macrophage and FCN1+ macrophage.

DISCUSSION

In this study, we found that macrophage activation signatures, particularly FCN1+ macrophages, were upregulated in EA compared to that in EB, although EA and EB shared similar patterns in whole transcriptome profiling. Our results provide novel evidence that macrophage activation plays an important role in the different phenotypes and mechanisms of EA and EB. To the best of our knowledge, this is the first study to investigate the genetic differences in the transcriptomes of EA and EB via bulk RNA-seq and scRNA-seq of sputum cells, which improves our understanding of the underlying cellular and molecular mechanisms and may promote individualized treatment of conditions with eosinophilic airway inflammation.

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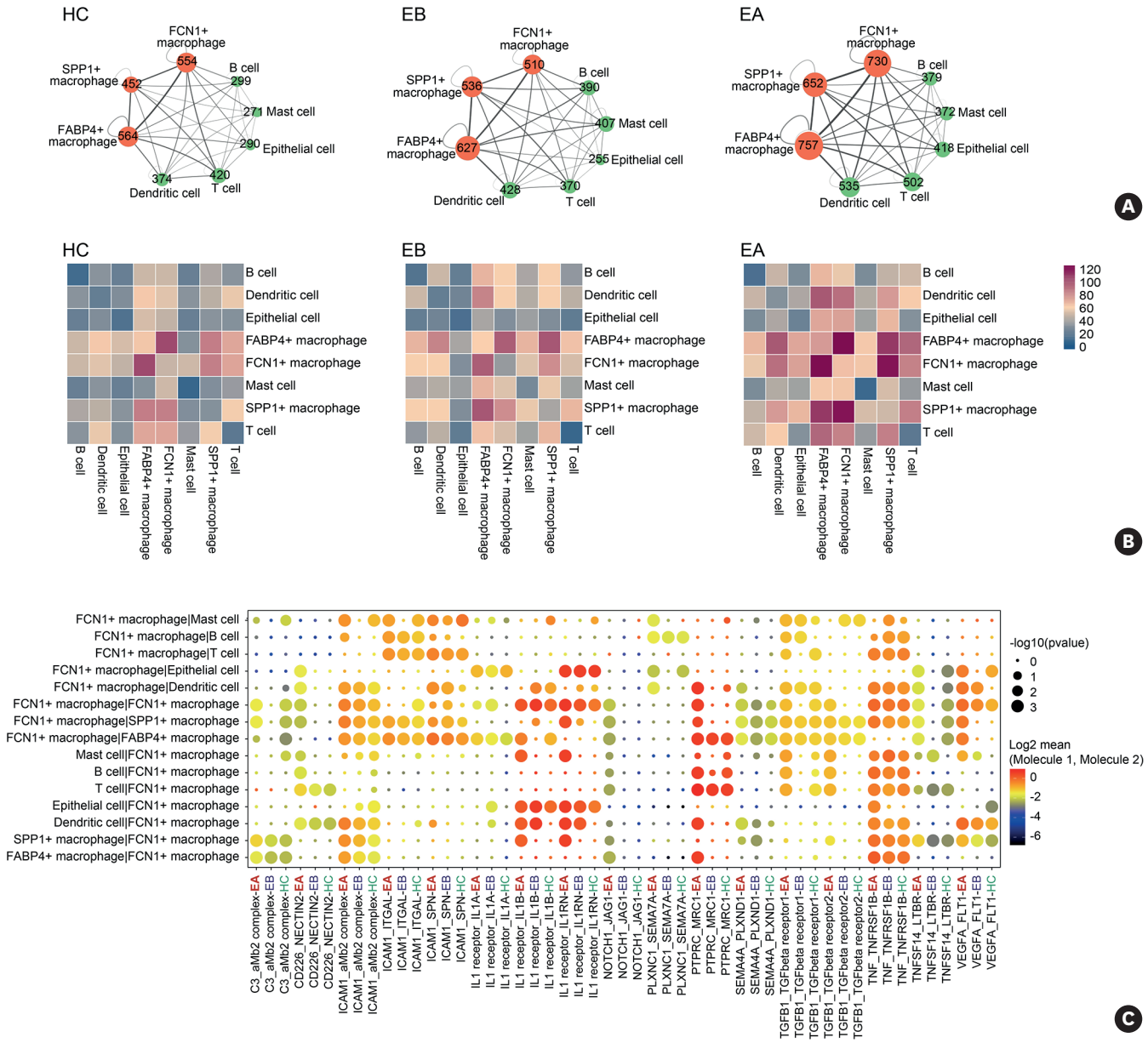

Fig. 5. Cell-to-cell signaling interaction networks across EA, EB and HCs as analyzed by CellPhoneDB (v3). (A) Networks depicting cell types as nodes and interactions as edges. The number within nodes means total number of interactions of each cell, size of cell type is proportional to the total number of interactions of each cell type, and edge thickness is proportional to the number of interactions between the connecting types. (B) Heat map depicting the number of all possible interactions between the cell types analyzed. (C) Dot plot depicting the interactions between selected FCN1+macrophage and other cell types enriched among EA, EB and HC.
HC, healthy control; EB, eosinophilic bronchitis; EA, eosinophilic asthma.

In EA, airway inflammation is thought to be profoundly affected by type 2 cytokines such as IL-4, IL-5, and IL-13, which results in airway eosinophilia, mucus overproduction, immunoglobulin E synthesis, and airway hyperresponsiveness.[26] The current study found that the gene expression of these type 2 cytokine were upregulated in both EB and EA, consistent with previous studies.[9,27,28] In addition, we found that the gene expression of CLC, CPA3, and DNASE1L3 was significantly enhanced in the sputum of EA and EB, which was useful for discriminating EA from other phenotypes and predicting the corticosteroid response.[29]

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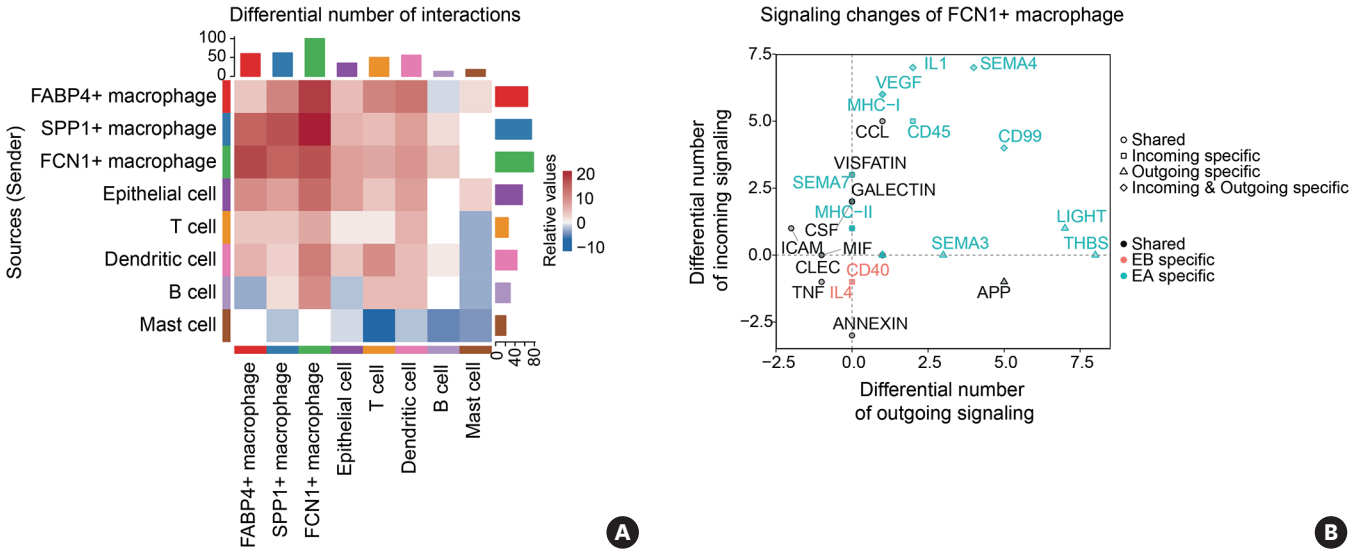

Fig. 6. Differential cell-to-cell signaling interaction between EA and EB as analyzed by CellChat (v1.6.1). (A) Differential number of interactions between EA and EB. The top-colored bar plot represents the sum of the column of values displayed in the heatmap (incoming signaling). The right colored bar plot represents the sum of the row of values (outgoing signaling). In the colored bars, red (or blue) represents increased (or decreased) signaling in EA compared to EB. (B) Specific signaling changes of FCN1+ macrophage between EA and EB. Positive values indicate the increase in the EA, while negative values imply the increase in EB. EA, eosinophilic asthma; EB, eosinophilic bronchitis.

This evidence indicates that both EA and EB have similar type 2 airway inflammation, which cannot explain the distinctive manifestations of EA and EB.

Macrophage number and activation status are reportedly decreased in severe granulocytic asthma.[30] In the present study, we enrolled patients with mild EA or EB. Similar numbers of sputum macrophages were observed in the EA and EB groups. Compared to HC, the regulation of macrophage activation was enriched in EA rather than in EB, indicating that the macrophage activation status differed between EA and EB. Macrophages within the lungs were found to play an important role in the innate and adaptive immune responses.[31] However, the role of macrophages in EA pathogenesis remains unclear. Traditionally, macrophages have been classified as M1 and M2 in an attempt to mirror the Th1/Th2 dichotomy of T cells.[32] Recent published data suggest that increased M2 macrophage polarization in the lungs reflects the severity and development of an allergic inflammatory response in asthma, and the presence of M1 macrophage is associated with high production of pro-inflammatory mediators, intensification of lung injury, and airway remodeling.[31,33] However, in the current study, macrophages were difficult to be simplistically classified as M1/M2 because there were many overlapping M1/M2 genes in one macrophage entity. In addition, more and more data suggested that this dichotomous classification underrepresents the functional and physiological diversity of macrophages.[34,35]

Plasticity and diversity are hallmarks of macrophages. Single-cell analysis of macrophages has added a new dimension for a better understanding of the diversity of macrophage differentiation and activation.[36] Morse *et al.*[37] classified human lung macrophage as FABP4+ macrophage, SPP1+ macrophage, and FCN1+ monocyte/macrophage based on gene expression via scRNA-seq and validated the existence of these subpopulations via immunofluorescent staining. In the current study, FABP4+ macrophage was the largest subset of sputum macrophages in all subjects despite a decrease in the percentage of these cells in asthma. The marker genes of FABP4+ macrophage are associated with phagocytosis,

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lipid localization, lipid catabolic processes, and oxidative phosphorylation, indicating that FABP4+ macrophage is likely traditionally designated alveolar macrophage.[37,38] SPP1+ macrophage is strikingly increased and highly proliferative in idiopathic pulmonary fibrosis, and SPP1 macrophage may contribute significantly to lung fibrosis.[37] In our study, the enhanced expression of genes in SPP1+ macrophage was related to antigen processing and presentation, response to molecules of bacterial origin, and macrophage activation, indicating that SPP1+ macrophage may play a traditional role in the innate immune cell response in eosinophilic airway inflammation.

Integrative sc-RNAseq analysis identified FCN1+ macrophage enriched in severe/critical coronavirus disease 2019 (COVID-19) bronchoalveolar lavage fluid and blood in active tuberculosis,[39] hinting that these types of macrophages might participate in the pro-inflammatory process. It has been reported that FCN1+macrophage from COVID-19 bronchoalveolar lavage fluid express the peripheral monocyte-like markers S100A8, FCN1 and CD14, and its gene expression patterns are similar to those of classic M1-like macrophages.[40] In the current study, FCN1+macrophage highly expressed FCN1, CD14, IL1B, VCAN, and TNFRSF1B, and biological process analysis of gene ontology suggested that these FCN1+macrophages were related to the regulation of T cell activation, mononuclear cell differentiation, and leukocyte activation involved in immune responses, further demonstrating that FCN1+macrophages might play an important role in T cell-driven airway inflammation. In EA, the predicted cell-cell interactions between FCN1+macrophages and other cells consisted of a wealth of TGFβ1, TNF, and VEGFA pathways, resulting in cell proliferation. More importantly, cell-cell interactions between FCN1+ macrophages and epithelial cells were enhanced. Airway epithelium plays a key role in airway remodeling in asthma.[41] On comparing the FCN1+macrophages of EA to those of EB, EREG was significantly upregulated. EREG encodes a secreted protein, 'epiregulin,' a ligand of the epidermal growth factor receptor (EGFR).[42] EREG-EGFR activating signals drive epithelial cell proliferation and viability.[43] Compared to FCN1+ macrophages in EB, signatures associated with the response to TGF-β, cellular responses to VEGF stimulus, developmental cell growth, and cell-matrix adhesion were significantly enriched in FCN1+ macrophages in EA. Our previous study also found that airway remodeling in EB was milder than that in asthma.[8] Airway inflammation, airway remodeling, and airway hyperresponsiveness are closely correlated,[44,45] and macrophage depletion attenuates eosinophilic allergic inflammation and airway remodeling.[46] These results suggest that FCN1+ macrophages may participate in developing those pathologies, which are the key differences between EA and EB. Therefore, FCN1+ macrophages may be a potential therapeutic target for EA. Further studies are needed to investigate the proportion of airway FCN1+ macrophages between EA and EB via flow cytometry and to confirm the relationship between FCN1+ macrophages and airway hyperresponsiveness from *in vivo* and *in vitro* studies. This cell population also expresses high levels of IL1B. Recent evidence suggests that IL1B expression may be driven by intracellular infection of macrophages.[47] Although all subjects in our study were free of upper respiratory tract infection within 4 weeks, whether this change in IL1B expression is a cause of EA or an effect of airway microbiome changes in EA remains to be further addressed.

This study had some limitations. First, few eosinophils expressing CLC were identified in the scRNA-seq data. With single-cell analysis on 10x genomics, capturing eosinophils and other granulocytes generally remains a challenge because of the relatively low RNA content and relatively high levels of RNases in these cells.[48,49] However, genes of eosinophils such as CLC and SIGLEC8 were detected in bulk RNA-seq data, indicating that the transcriptomics

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profiling of sputum eosinophils was taken into account at the whole transcriptome level. Second, induced sputum preferentially samples airway inflammatory cells, including macrophages, granulocytes, and a few lymphocytes and mast cells, and it is likely not a complete representation of inflammatory cells and airway structural cells, which may impact the results. However, this condition is consistent and systemic among all samples. Third, the sputum samples subjected to scRNA-seq were relatively small, and a large number of scRNA-seq and animal studies are required to assess the effects of macrophages on airway remodeling, airway hyperresponsiveness, and airflow obstruction. In this study, sputum samples conducted in bulk RNA-seq or scRNA-seq came from different subjects, and there was no overlap sample between the two types of RNA-seq. The overall transcriptomics profiling of the two types of RNA-seq was consistent, suggesting that the sampling and sequencing methods were robust. Finally, patients with both EA and EB were newly diagnosed in outpatient clinics, and none had received anti-asthmatic medications within the previous 4 weeks. We could not confirm whether these patients, especially EA patients with long disease duration, had received corticosteroids since disease onset. Inhaled corticosteroids can affect sputum eosinophil count and gene expression in sputum cells.[50,51] However, none of the patients had received regular inhaled corticosteroid therapy for longer than a month since the disease onset. Therefore, the effect of inhaled corticosteroids should be minimal, if any.

In summary, the activation of macrophages associated with airway remodeling processes is upregulated in EA compared to that in EB, which may play a key role in the underlying mechanisms of airway hyperresponsiveness and airflow obstruction.

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SUPPLEMENTARY MATERIALS

Supplementary Data S1
Method

Supplementary Table S1
Differentially expressed genes of eosinophilic asthma compared with healthy subjects

Supplementary Table S2
Differentially expressed genes of eosinophilic bronchitis compared with healthy subjects

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Supplementary Table S3
Gene ontology term enrichment of biological processes for common 1011 upregulated genes

Supplementary Table S4
Differentially expressed genes of eosinophilic asthma compared with eosinophilic bronchitis

Supplementary Table S5
Clinical features of participants who completed single cell RNA sequencing

Supplementary Table S6
Gene expression across different celltypes of sputum cell

Supplementary Table S7
Up-regulated genes of FCN1+ macrophage in eosinophilic asthma compared to eosinophilic bronchitis

Supplementary Table S8
Gene set enrichment analysis of FCN1+ macrophage of eosinophilic asthma compared to eosinophilic bronchitis

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