RESEARCH LETTER

Genetic characterization of macrophages from induced sputum of patients with asthma and chronic obstructive pulmonary disease

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Introduction Pulmonary macrophages are important effector immune cells that are involved in recognition of pathogens, ingestion and killing of microbes, clearance of debris, initiation and regulation of inflammatory responses, and adaptive immunity.[1] Some data suggest that dysfunction of macrophages may play a role in the pathogenesis of obstructive lung diseases: asthma and chronic obstructive pulmonary disease (COPD).[2,3] Alveolar macrophages (AMs) from patients with severe asthma produce higher amounts of interleukin 6, CXCL8, and other proinflammatory mediators as compared with AMs from patients with mild asthma.[4] It has been reported that bronchial macrophage (BM) phagocytosis is reduced in both eosinophilic and noneosinophilic asthma.[5] The results of some studies suggest that impaired macrophage phagocytosis of pathogens and apoptotic bodies in COPD promotes local inflammation and tissue damage in COPD.[6]

Most studies on macrophage characteristics involved bronchoalveolar lavage fluid, which contains mainly AMs. Much less is known about BMs and their subpopulations. Induced sputum (IS) is an easily accessible and valuable respiratory sample, which mainly reflects the cellular profile of macrophages located in the trachea and large- and medium-sized bronchi (BMs). Macrophages obtained from IS of patients with asthma are phenotypically and functionally altered compared with healthy individuals.[7]

The aim of the study was to characterize the molecular features of IS macrophages from patients with asthma and COPD.

Patients and methods **Study design** This was a prospective cross-sectional study including 15 patients with COPD, 20 patients with asthma, and 20 controls. Patients were recruited from the pulmonary outpatient department of the Public Central Teaching Clinical Hospital of the Medical University of Warsaw (Warsaw, Poland). In all patients, the diagnosis of asthma and COPD was previously established according to the Global Initiative for Asthma and Global Initiative for Chronic Obstructive Lung Disease guidelines, respectively. The following evaluations were performed after patient enrollment: medical history, physical examination, spirometry with flow-volume curve, airway obstruction reversibility test (when applicable), allergy skin prick tests, and sputum induction. Patient characteristics are shown in Supplementary material (*Table S1*).

The study protocol was approved by the institutional review board (KB/249/2016). An informed consent was obtained from all study participants.

Methods Sputum induction and processing was proceeded as previously described.[8] A FITC Positive Selection Kit (StemCell, Vancouver, Canada) was used for sputum macrophage (SM) separation. The immunomagnetic separation of cells labeled with $CD68^+$ FITC antibody (Thermo Fisher, Waltham, Massachusetts, United States) was performed according to the manufacturer's instruction. The number of macrophages in isolated cells was evaluated on May–Grünwald Giemsa stained smears (Supplementary material, *Table S2*). The threshold for macrophage-dominated material was arbitrarily set as more than 50% of isolated cells. The total RNA was isolated using the Tri reagent/chloroform method (Sigma-Aldrich, Saint Louis, Missouri, United States). RNA quality was measured with the Agilent 2100 bioanalyzer (Agilent Technologies, Santa Clara, Califorinia, United States). Transcriptomic

TABLE 1 Expression of selected genes in macrophage-dominated cells in induced sputum from patients with asthma (without and with inhaled corticosteroid treatment) and chronic obstructive lung disease

Genes	Asthma		COPD	*P* value[a]	*P* value[b]
	No ICS treatment	ICS treatment			
AASDH	1.51 (0.20–27.3)	0.3 (0.2–1.39)	0.35 (0.11–2.36)	0.53	0.55
BTF3	1.87 (0.88–84.1)	1.68 (0.49–21.61)	0.25 (0.16–0.5)	0.90	0.001
CDS2	1.59 (0.45–1.93)	2.14 (1.84–3.38)	0.65 (0.56–0.85)	0.49	0.03
COL6A1	0.65 (0.15–1.81)	2.05 (1.35–2.07)	1.98 (0.31–3.72)	0.33	0.50
CUBN	0.29 (0.18–0.34)	0.17 (0.09–1.01)	1.66 (0.44–5.46)	0.69	0.05
DDX5	2.92 (0.84–45.9)	1.58 (0.69–7.87)	0.4 (0.21–0.64)	0.71	0.005
DNAJC13	1.41 (0.74–3 6)	2.51 (1.59–6.48)	0.53 (0.35–0.81)	0.73	0.005
GNAI2	2.15 (0.8–58.8)	2.07 (1.09–4.12)	0.34 (0.19–0.44)	0.90	<0.001
NRG1	1.27 (0.17–2.37)	0.26 (0.01–2.41)	1.38 (1.28–6.09)	0.80	0.20
RAI14	2.85 (0.45–5.91)	0.72 (0.3–4.39)	1.04 (0.28–3.82)	0.54	0.50
RORB	19.89 (0.8–312)	2.34 (0.10–2.74)	0.93 (0.77–2.10)	0.40	0.28
SCGB1A1	1.43 (0.35–5.89)	0.81 (0.12–2.6)	0.07 (0.03–0.08)	0.43	0.002
SIRPB1	1.43 (0.74–3.61)	2.96 (0.95–6.87)	0.53 (0.31–1.13)	0.71	0.01
TRAF3IP2	4.61 (1.38–0.35)	2.02 (0.69–15.06)	0.76 (0.47–1.20)	0.62	0.009
USP53	1.89 (0.88–4.0)	3.07 (1.27–17.28)	0.46 (0.43–1.86)	0.59	0.04
WDR49	1.52 (0.81–2.89)	1.83 (0.49–7.09)	0.16 (0.08–0.88)	0.94	0.01

Results are presented as median and interquartile range. A *P* value of less than 0.05 was considered significant.

a Asthma (ICS treatment vs no ICS treatment); **b** COPD vs asthma

Abbreviations: COPD, chronic obstructive lung disease; ICS, inhaled corticosteroid

measurements were performed using Affymetrix Human Gene 2.1 ST ArrayStrip (Thermo Fisher).

Statistical analysis Statistical analysis was performed with the use of Statistica 12.0 software package (StatSoft Inc., Oklahoma, United States) or R environment. Continuous data were compared using the Mann–Whitney test. Microarray data were exported by creating.cel files with Gene Atlas Instrument Control Software (Affymetrix, Thermo Fisher) and analyzed with R (version 3.4.1, https://cran.r-project.org/). Data were quantile normalized, background corrected, and summarized using the rma function from oligo package (version 1.40.2, default parameters). The probe sets associated with the X and Y chromosomes were omitted. Differential gene expression was determined with the *t* test (Welch variant) adjusted for multiple comparisons with the Benjamini–Hochberg algorithm. An adjusted *P* value of less than 0.1 was considered significant. Individual genes were mapped to Gene Ontology processes (GeneGo, St. Joseph, MI, http://www.genego.com/metacore.php) and manually by literature searches. The gene array data were uploaded to GEO Omnibus (reference no. GSE112 260).

Results The microarray experiments were performed using 17 samples obtained from 8 patients with asthma (4 untreated, 4 treated with inhaled corticosteroids [ICSs]), 4 patients with COPD (all of them smokers, none treated with ICSs), and 5 controls (4 nonsmokers, 1 smoker). *USP53* was the only gene differentially expressed (adjusted *P* value, 0.09) when comparing ICS-naive and ICS--treated patients with asthma (Supplementary material, *Table S3*). The numbers of differentially expressed genes that did not pass the correction for multiple hypothesis testing ($P < 0.05$) were as follows: 8046 genes (11 210 probe sets) between ICS-naive and ICS-treated asthma, 9294 genes (14 562 probe sets) between ICS-naive asthma and COPD, and 6538 genes (8906 probe sets) between ICS-treated asthma and COPD groups. The most significantly regulated microarray probes are shown in Supplementary material (*Table S3*).

The enrichment of Gene Ontology terms was checked among 5% of genes with the lowest *P* value. The comparison of Gene Ontology pathways between asthma and COPD groups showed 17 upregulated and 26 downregulated biological processes (adjusted *P* value <0.05). The most significantly regulated pathways are listed in Supplementary material (*Table S4*).

Quantitative real-time polymerase chain reaction (PCR) was performed to assess the performance of the 20 selected genes identified by using microarray, 16 of which were amplified in real-time PCR ($2^{-\Delta\Delta CT}$ method). Cells isolated from nonsmoking and smoking subjects were used as controls for asthma and COPD, respectively. The expression of the most regulated genes was evaluated in the group of 17 controls (7 nonsmokers, 10 smokers), 15 patients with asthma, and 11 patients with COPD (**TABLE 1**).

Discussion Our study demonstrated that SMs from asthma, COPD, and control groups are

characterized by a different gene expression profile mainly associated with motility, cilium function, cell junction, and adhesion organization. These results may suggest a potential role of macrophage dysfunction in altered airway pathophysiology in obstructive lung diseases.

Our analysis did not show any differences between SMs of ICS-naive and ICS-treated patients with asthma. It has been suggested that long-term corticosteroid treatment may inhibit macrophage--mediated promotion of adaptive immune responses, but enhance their innate immune functions.[9,10] Although our comparative analysis of microarray results between ICS-naive and ICS--treated patients with asthma revealed *USP53* as a significantly differentiating gene, this result was not confirmed by real-time PCR. The possible explanation for a negative PCR verification of the *USP53* expression was its low signal to noise ratio in microarray measurements. It is also possible that ICS treatment does not affect the SM gene profile but is targeted at AMs localized in the lower airways.

The results of our study suggest that changes in macrophage function in asthma and COPD mainly affect cell movement, motility, and cilium interactions. The recent study showed that the inhibitory effects of cigarette smoke on macrophage phagocytic function in the airways may be associated with disruption of epithelial-macrophage crosstalk via intercellular sphingosine-1-phosphate (S1P) signaling involving spinster homolog 2 (Spns2), a transporter protein for S1P.[11] Spns2 was localized in the cilia in primary bronchial epithelial cells. The results of a different study revealed that downregulation of macrophage motility in COPD is regulated by smoking-related proinflammatory stimulus processes linked to tetraspanins CD9 and CD81.[12] It cannot be excluded that the strong association between the observed gene expression profile and pathways such as cilium organization or cell-substrate adhesion may reflect epithelial dysfunction and may be caused by epithelial composition in analyzed material. However, the median percentage of epithelial cell contamination did not exceed 8% in our study groups (Supplementary material, *Table S2*).

Macrophages have the capacity of efferocytosis, that is, removal of apoptotic cells (mainly epithelial cells and neutrophils) by phagocytosis. We suggest that gene expression profile of SMs may indicate the decreased efferocytic capacity in macrophages from asthma and COPD patients. Firstly, the expressions of the *DNAJC13* and *SIRPB1* genes related to neutrophil degranulation terms according to Gene Ontology were increased in the asthma group. Secondly, the dominating number of pathways associated with the changed expression of genes in SMs was related to the cilium. Mucus overproduction may be a response of secretory epithelial cells to increased concentrations of bacterial material and apoptotic cells in the lungs of patients with asthma and COPD, caused by the dysfunction of efferocytosis in macrophages. We believe that the results of our study may suggest impaired efferocytosis of SMs in obstructive lung diseases due to cilia dysfunction.

We are aware of several limitations of this study. Firstly, the study group was relatively small and only a small number (40%) of samples met the appropriate criteria for microarray measurements. The characterization of the gene expression profile of SMs in study groups revealed only one differentiating gene with a satisfactorily adjusted *P* value. The analyzed subgroups of patients were very small, which significantly limited inferential statistics. We believe that in the face of the limited number of studies using sputum cells for microarray experiments in obstructive lung diseases, our results are important and may be evaluated as a preliminary study. Secondly, the macrophage isolation was not 100% efficient and the analyzed material did not contain pure fraction of macrophages but also a little contamination of the remaining sputum cells. Perhaps, the term "macrophage-dominated material" would therefore be more appropriate in relation to our results.

Conclusions Gene expression profiling of SMs revealed distinct molecular capacity in asthma and COPD. We found a link between the gene expression profile and cell motility, cilium, cell junction, and adhesion organization suggesting an association of macrophage functions with these biological processes in the pathophysiology of obstructive lung diseases.

SUPPLEMENTARY MATERIAL Supplementary material is available with the article at www.pamw.pl.

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