Alteration of immune profiles is associated with pulmonary function and symptoms in patients with chronic obstructive pulmonary disease

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Abstract. Inflammation serves a key role in chronic obstructive pulmonary disease (COPD). However, changes in the immune profiles of patients with COPD remain unclear. The present prospective observational study aimed to determine the expression profiles of immune cells and inflammatory factors of patients with COPD and healthy controls, and to analyze the relationship between immune profiles and smoking history. A total of 140 subjects were enrolled in the present study between September 2018 and April 2019 at West China Hospital of Sichuan University (Chengdu, China). These included 87 patients with stable COPD and 24 patients with acute exacerbated COPD, as well as 29 healthy controls. Baseline characteristics were recorded during the screening period, and levels of immune cells were examined using flow cytometry. In addition, levels of inflammatory factors were measured using ELISAs. The results revealed increased expression of the CD64$^+$/CD14$^+$ mononuclear phagocyte system (MPS) and CD16$^+$CD66$^+$ neutrophils, and decreased expression of CD3$^+$CD4$^+$ T cells and CD3$^+$ CD8$^+$ T cells (all P<0.05) in the peripheral blood of patients with COPD and smokers relative to non-smoking controls. In addition, significant differences were observed in protein levels of IL-6, IL-1β, TNF-α, TGF-α, IFN-γ, IL-8, IL-17A and CRP among the three groups (all P<0.05). Furthermore, the IL-17A, TNF and NF-κB signaling pathways were found to serve a key role in the inflammatory network of COPD. Pearson's correlation analysis revealed a positive correlation between CD3$^+$T lymphocyte counts and pulmonary function, and a negative correlation with smoking and exacerbations. Furthermore, neutrophils and MPS were negatively associated with pulmonary function, and IL-8 was positively associated with cough. There was also a negative association between CRP and IL-17A with pulmonary function but a positive correlation with symptoms and exacerbation. Club cell secretory protein was also negatively associated with emphysema parameters. In conclusion, the present findings revealed significant differences in profiles of immune factors among patients with COPD, smokers and non-smoking controls and their association with clinical characteristics. The clinical trial registration number of the present study is: ChiCTR1800015700 (registered with http://www.chictr.org.cn/index.aspx, 2018/04/16).

Introduction

Chronic obstructive pulmonary disease (COPD) ranks fourth among disease-related causes of death worldwide (1). COPD, regarded as a chronic systemic inflammatory disease, is characterized by persistent limitation of airflow due to airway and/or alveolar abnormalities (2). Cigarette smoking (CS) is the main cause of COPD, although other factors have also been identified (3). Furthermore, its pathogenesis is based on genetic determinants, lung growth, as well as environmental stimuli, such as oxidative stress, inflammation, extracellular matrix proteolysis, and apoptotic and autophagic cell death (4). In addition, bacterial and/or viral infections have been reported to exacerbate COPD (3). Airway inflammation is a key driver of COPD progression, resulting in several phenotypes with different patterns (5). Attempts have been made to segregate patients with COPD into different subtypes based on immune profiles, such as neutrophil- or eosinophil-associated COPD (6), with evidence indicating corticosteroids to be the most effective in patients with COPD with eosinophilic inflammation (5,7). In addition, novel biological therapies targeting specific inflammatory biomarkers have been explored. For instance, anti-IL5 therapy in COPD reduces exacerbations in patients with a high blood eosinophil count by ~20% (8).

Recently, potential biomarkers such as IL-5 and TNF-α have been considered effective in targeted anti-inflammatory therapy (9). Multiscale models of CS-induced COPD have

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enabled identification of several positive feedback loops, such as M1 and M2 transformation and balance, as well as network elements, and these have served a determinant role in CS-induced immune response and COPD progression (10,11). An increased proportion of infiltrating macrophages, as well as a reduction of $CD4^+$ and $CD8^+T$ cells, are both independently associated with smoking status or the level of airflow limitation (10,11). CRP is associated with elevation of acute exacerbated COPD (AECOPD), while CRP-guided prescription of antibiotics for AECOPD results in less use of antibiotics with no evidence of harm (12). Levels of IL-6 are associated with the degree of airflow limitation (13), and both IL-6 and IL-8 are associated with emphysema severity (14). Similarly, IL-1β, IL-17A, TNF-α and IFN-γ have also been linked to persistent airway inflammation and greater exacerbation (15-17). In addition, pulmonary surfactant proteins might predict relapse of patients with AECOPD (18), while lack of club cell secretory protein (CC16) in the lungs results in enhanced airway remodeling in COPD (19). Members of the extracellular MMP family of proteins are associated with emphysema pathogenesis (20). Consequently, MMP inhibitors have been developed as novel therapeutics, with doxycycline approved by the Food and Drug Administration (20). It is possible that more biomarkers could promote the understanding of COPD pathogenesis and development of targeted therapies. However, the complexity of these biomarkers poses challenges in distinguishing between COPD phenotypes and endotypes. It is, therefore, imperative to focus on biomarkers that have a clear distinction of specific clinical phenotypes or endotypes (21).

The present study analyzed the peripheral blood expression of immune cells and serum levels of 20 inflammatory factors with the aim to elucidate the complex network of immune profiles in COPD and airway inflammatory patterns. The present findings may provide insight to guide the future development of novel therapies.

Materials and methods

Study patients and ethics. A total of 140 subjects with mean age of 65.13 were enrolled in the study between September 2018 and June 2019. In total, 87 patients with stable COPD (62 male, 25 female, age: Mean ± SEM 64.94±0.97) were recruited at the Outpatient Department of West China Hospital, Sichuan University (Chengdu, China). Inclusion criteria used for the recruitment included: i) Diagnosed with COPD by respiratory physician; ii) performed pulmonary function test [forced expiratory volume in 1 sec (FEV1)/forced vital capacity (FVC) ≤70%] and chest CT scan; iii) no malignant tumor or autoimmune disease; iv) no liver and kidney failure or special infections such as HIV; v) no exacerbation in 3 months; vi) no other chronic and acute severe respiratory diseases; and vii) no chronic or acute systemic infections. A total of 24 patients with AECOPD (17 male, 7 female, age: Mean ± SEM 67.00±2.13) admitted at West China Hospital were enrolled using the following inclusion criteria: i) Main diagnosis for admission was AECOPD; ii) pulmonary function test (FEV1/FVC ≤70%) and chest CT scan were performed; iii) no malignant tumor or autoimmune diseases; iv) no liver and kidney failure or special infections such as HIV; and v) no other severe chronic or acute

respiratory disease. For those patients for whom pulmonary function test and chest CT scan were not performed, medical reports for the past year were required. COPD was defined according to international guidelines (2), while severity of airflow obstruction was graded using current Global Initiative for Chronic Obstructive Lung Disease criteria (2). A total of 29 healthy controls (18 non-smokers and 11 smokers, 18 male, 11 female, age: Mean ± SEM 64.15±1.34) were recruited at the Physical Examination Center of West China Hospital (Chengdu, China) using the following inclusion criteria: i) Pulmonary function test (FEV1/FVC <70%) and chest CT scan were performed, indicating normal pulmonary function; ii) no malignant tumor or autoimmune disease; iii) no heart, liver and kidney failure or special infections; iv) no other chronic or acute respiratory disease; and v) no chronic or acute systemic infections. Spirometry parameters were measured according to the recommendations of the European Respiratory Society, and then expressed as a percentage of the predicted (22). The modified Medical Research Council dyspnea scale (mMRC) was adopted as the classification criterion for symptom assessment (23), detailed description of baseline characteristics was recorded by the respiratory physician. According to smoking history, all the subjects were divided into four groups: Smoking (73) and nonsmoking (24) COPD patients and smoking (8) and nonsmoking (17) controls. The present study was approved by The Ethics Committee of West China Hospital, Sichuan University (Chengdu, China) in accordance with the Declaration of Helsinki (24). All patients provided written informed consent before participating in the study.

Flow cytometry. Fresh peripheral blood (2 ml) anticoagulated in EDTA was collected from each patient and processed within 24 h. Peripheral blood was divided into 2 tubes (1 ml). The corresponding antibody (2 µl) was added to each tube (tube 1: CD45,PE-cy5.5;CD14, FITC;CD64, PE;CD66, APC;CD16, PE-cy7; tube 2: CD45,PE-cy5.5;CD3, PE; CD8, APC-cy7;CD4, FITC;CD56, PE-cy7;CD19, APC) and incubated for 30 min in the dark at room temperature, then mixed with red blood cell lysis solution (FCM Lysing solution for BC, Hangzhou Multi Sciences (Lianke) Biotech Co.,Ltd.) with the ratio of 1:3, lysing for ~10 min until the solution was clear. Following centrifugation (500 x g for 5 min at 4°C), the supernatant was removed and washed by 500 µl-1 ml PBS for three times, centrifuged at 400 x g for 5 min at 4°C, and then resuspended in 200 µl PBS. The cells were stored on ice in the dark, or in a refrigerator at 4°C until use. Samples were analyzed using multicolor flow cytometry (Navios EX flow cytometer; Beckman Coulter, Inc.) based on $CD45^+$ human leukocytes. $CD64^+/CD14^+$ represented the monocyte-macrophage system (MPS), $CD3^+$ T lymphocytes were divided into $CD4^+$ and $CD8^+$ subsets, while $CD3^+CD4^-CD8^-$ was selected as the marker for γδT cells. In addition, $CD3^+CD56^+$ marked natural killer T lymphocytes (NKTs), while $CD3^-CD56^+$ marked natural killer (NK) cells. Antibodies ready to use were acquired from eBioscience (Thermo Fisher Scientific, Inc.; CD14 Monoclonal Antibody, FITC, human, cat. no. 11-0149-42; CD64 Monoclonal Antibody, PE, human, cat. no. 12-0649-42; CD66 Monoclonal Antibody, APC, human, cat. no. 17-0668-42; CD16 Monoclonal Antibody, PE-cy7, human, cat. no. 25-0168-42;

CD3 Monoclonal Antibody, PE, human, cat. no. 12-0038-42; CD8 Monoclonal Antibody, APC-cy7, human, cat. no. A15448; CD4 Monoclonal Antibody, FITC, human, cat. no. 11-0049-42; CD56 Monoclonal Antibody, PE-cy7, human, cat. no. 25-0567-42; CD19 Monoclonal Antibody, APC, human, cat. no. 17-0199-42; CD45 Monoclonal Antibody, PE-cy 5.5, human, cat. no. 35-0459-42), and cells were stained according to the manufacturer's recommendations. All the antigens were human source and stored at 4°C in the dark. Monocyte-macrophage-granulocyte included antigens of CD45, CD14, CD64, CD66 and CD16, while lymphocyte-NK-NK included CD45, CD3, CD8, CD4, CD56 and CD19. FlowJo version 10.0.7 software (FlowJo LLC) was used for analysis.

Analysis of inflammatory factors. A total of 5 ml peripheral EDTA-anticoagulated blood was collected from each patient, 2 ml used for FCM, the rest (3 ml) centrifuged at 1,600 x g for 15 min at 4°C. The resulting plasma was stored at -79°C for subsequent ELISA tests and multi-plex panel tests.

ELISA. The levels of five biomarkers were measured using ELISAs. The plasma stored at -79°C was defrosted in a constant temperature water bath to 37°C. CRP (Human C-Reactive Protein/CRP Immunoassay kit; cat. no. DCRP00), CC16 (Human Uteroglobin Quantikine ELISA kit; cat. no. DUGB00) and TGF-β (Human TGF-beta Quantikine ELISA kit; cat. no. DB100B) levels were assessed using Quantikine ELISA kits (all R&D Systems, Inc.), while fibrinogen(Human Fibrinogen ELISA kit; ab208036) and neutrophil elastase (Human PMN Elastase ELISA kit; ab119553) were analyzed using Simple Step ELISA kits (Abcam) according to the to the instructions of the respective manufacturer's instructions.

Multi-plex panel. Two multi-factor panels, including 15 biomarkers, were tested using the Multiplexed kit (Magnetic Luminex® Assay, Human Premixed Multi-Analyte kit; LXSAHM, R&D Systems, Inc.) based on the Luminex 200 system with xPONENT 3.1 (Luminex Corporation). One panel tested the levels of 13 analytes, including IL-6, TNF-α, IL-1β, IFN-γ, IL-8, IL-33, IL-17A, IL-4, IL-5, IL-13, TGF-α, Human growth-regulated oncogene α (GRO-α) and pulmonary surfactant-associated protein D (SP-D), while the other was used to assess myeloperoxidase (MPO) and MMP-9 (Supplementary Materials).

Bioinformatics. Proteins (IL-6, IL-1β, TNF-α, IFN-γ, IL-8, IL-33, IL-17A, TGF-α, GRO-α, CRP) and their functional interactions were analyzed using the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database version 11.0 (25), and enriched Kyoto Encyclopedia of Genes and Genomes pathways were determined using STRING [10 items (human)-STRING interaction network (string-db.org)].

Statistical analysis. All samples were tested 3 times. Continuous variables are presented as the mean ± standard error of the mean and were analyzed using SPSS 22.0 (IBM Corp.). A normality test was performed using a P-P diagram in SPSS 22.0. One-way ANOVA and Tukey's post hoc test were used for comparisons of normally distributed continuous variables among different groups. Kruskal Wallis and Dunn's post hoc test were used for comparisons of non-normally distributed continuous variables among different groups. In addition, χ^2 and Bonferroni's correction were used for categorical variables. Pearson's correlation was used to assess the relationship among continuous variables, while Spearman's correlation test was used for categorical data. Figures were generated using FlowJo version 10.0.7 software (FlowJo LLC) and GraphPad Prism 8 (GraphPad Software, Inc.). P<0.05 was considered to indicate a statistically significant difference.

Results

Clinical characteristics of the patients. A total of 87 patients with stable COPD and 24 patients with AECOPD, as well as 29 healthy controls were recruited in the study. There were no significant differences in terms of age, sex and BMI among the three groups (Table I). As expected, the proportion of smokers was higher among patients with COPD compared with the controls (COPD vs. AECOPD vs. controls; 75.9 vs. 62.5 vs. 37.9%, respectively), and the lung function (FEV1/FVC, FEV1% and maximal mid expiratory flow (MMEF)75/25*) of patients with COPD was worse compared with controls (Table I). AECOPD had more group D patients with severe airflow limitation (stage III, 38.89 vs. 22.50%; stage IV, 50.00 vs. 28.75%) compared with COPD (Table I). The score of mMRC assessment was higher in patients with AECOPD compared with patients with stable COPD (3.61 vs. 2.24). The most common drug therapy for patients with COPD and AECOPD was long-acting muscarinic antagonist (LAMA) + long-acting beta-2 agonist (LABA) + inhaled corticosteroid (ICS; 63.29 and 45%, respectively), while 17.86% of patients with stable COPD did not receive drug therapy. In conclusion, patients with COPD who smoked had worse lung function compared with healthy controls, patients with AECOPD were severe and needed more medication than stable patients with COPD.

Immune cells expression profiles. The flow cytometry results and workflow are shown in Figs. 1 and 2. Following staining, living cells were imaged and the CD45+ subset were gated as human leukocyte There were significant differences in CD3+ T lymphocytes (P<0.001), including the CD4+ T (P<0.001) and CD8+ T (P=0.004) subsets, CD14+ (P=0.027) and CD64+ (P=0.026) MON and CD16+CD66+ neutrophil (P=0.011) proportions across the study groups (Table II; Fig. 3). CD66+CD16+ neutrophils and MPS (CD14+ and CD64+ subsets) were highly expressed, while CD4+ and CD8+ T lymphocytes were expressed at lower levels in patients with stable COPD and patients with AECOPD compared with healthy controls (Table II). CD4+ T lymphocyte counts were decreased in patients with stable COPD compared with controls, and in AECOPD compared with stable COPD (AECOPD vs. COPD vs. controls, 6.50 vs. 9.04 vs. 12.36%, respectively). CD64+ and CD14+ MON tended to be increased in AECOPD compared with stable COPD and controls (CD64+ MON: AECOPD vs. COPD vs. controls, 4.53 vs. 3.94 vs. 2.85%; CD14+ MON: AECOPD vs. COPD vs. controls, 4.57 vs. 4.19 vs. 3.04%, respectively). CD14+CD64+ MON levels were increased in AECOPD compared with controls (3.82 vs. 2.53%). Furthermore, CD19+ B lymphocytes, CD3+CD56+ NKTs and

Table I. Clinical baseline characteristics of patients with COPD, patients with AECOPD and healthy controls.

Baseline features	COPD (n=87)	AECOPD (n=24)	Healthy controls (n=29)	P-value
Age, years (mean ± SEM)	64.94±0.97	67.00±2.13	64.15±1.34	0.519
Male, n (%)	62 (76.7)	17 (75.0)	18 (62.7)	0.444
BMI (mean ± SEM)	22.05±0.58	19.70±1.75	21.23±1.48	0.299
Smoking history, n (%)	66 (75.9)	15 (62.5)	11 (37.9)	0.001[a]
Smoking amount, pack-year (mean ± SEM)	31.59±3.26	22.38±4.23	9.71±3.57	0.001[a]
Exacerbation, n (%)	51 (58.62)	24 (100)		0.001[a]
Pulmonary function				
No. of patients analyzed (n)	78	16	22	
FEV1/FVC (mean ± SEM)	50.94±1.24	46.74±2.32	78.91±1.37	0.001[a]
FEV1% (mean ± SEM)	51.83±2.44	40.22±4.47	96.23±2.27	0.001[a]
RV/TLC (mean ± SEM)	142.14±8.61	145.06±14.72	110.58±4.34	0.281
MMEF75/25 (mean ± SEM)	22.45±1.78	13.49±1.87	71.52±3.42	0.001[a]
GOLD stage[b]				
No. of patients analyzed	84	22		
A, n (%)	23 (27.38)	0 (0.00)		0.001[a]
B, n (%)	22 (26.19)	3 (13.64)		0.001[a]
C, n (%)	8 (9.52)	1 (4.54)		0.001[a]
D, n (%)	31 (36.9)	18 (81.82)		0.001[a]
Stage of Airflow limitation[b]				
No. of patients analyzed	80	18		
I (mild), n (%)	12 (15)	1 (5.56)		0.001[a]
II (moderate), n (%)	27 (33.75)	1 (5.26)		0.001[a]
III (severe), n (%)	18 (22.5)	7 (38.89)		0.001[a]
IV (very severe), n (%)	23 (28.75)	9 (50)		0.001[a]
Assessments				
No. of patients analyzed	84	20		
mMRC (mean ± SEM)	2.24±0.11	3.61±0.14		0.001[a]
Cough (mean ± SEM)	1.88±0.14	2.09±0.45		0.528
Sputum (mean ± SEM)	5.22±0.33	5.64±0.28		0.724
Drug therapy				
No. of patients analyzed	84	20		
0 (none), n (%)	15 (17.86)	0 (0)		
1 (LAMA/LABA), n (%)	8 (9.52)	0 (0)		
2 (LABA+ICS), n (%)	13 (15.48)	3 (15)		
3 (LAMA+LABA+ICS), n (%)	53 (63.29)	9 (45)		
4 (LAMA+LABA), n (%)	3 (3.57)	8 (40)		

[a]P<0.001. [b]GOLD stage and airflow limitation severity in accordance with GOLD 2018 report, severity of airflow limitation: FEV1%, ≥80, stage I; FEV1%, 50-79, stage II; FEV1%, 30-49, stage III; and FEV1%, <30, stage IV. COPD, chronic obstructive pulmonary disorder; AE, acute exacerbated; FEV1, forced expiratory volume in 1 sec; FVC, forced vital capacity; RV, residual volume; TLC, total lung compacity; MMEF75/25, maximal mid expiratory flow; GOLD, Global Initiative for Chronic Obstructive Lung Disease; mMRC, modified Medical Research Council dyspnea scale; LAMA, long-acting muscarinic antagonist; LABA, long-acting beta-2 agonist; ICS, inhaled corticosteroid. Some patients were unable to finish complete lung function test, so patients able to complete the test were analyzed.

CD3⁻CD56⁺ NKs levels were not significantly different among the three groups.

There were 73 smoking patients with COPD and 24 non-smoking patients with COPD, as well as 8 smoking controls and 17 non-smoking controls. The majority of smokers were male, and their age was comparable among the four groups. There were also significant differences in CD3⁺CD8⁺ T lymphocyte (P<0.001), CD14⁺ (P=0.014) and CD64⁺ (P=0.020) MON, and CD16⁺CD66⁺ neutrophil (P=0.002) proportions only between smokers and non-smokers (Table III). CD4⁺ T lymphocytes levels were sharply decreased in patients with COPD compared with non-smoking controls (smoking COPD vs. non-smoking COPD vs. non-smoking controls: 8.16 vs. 9.42 vs. 12.75%). Levels of CD8⁺ T lymphocytes were decreased in both patients
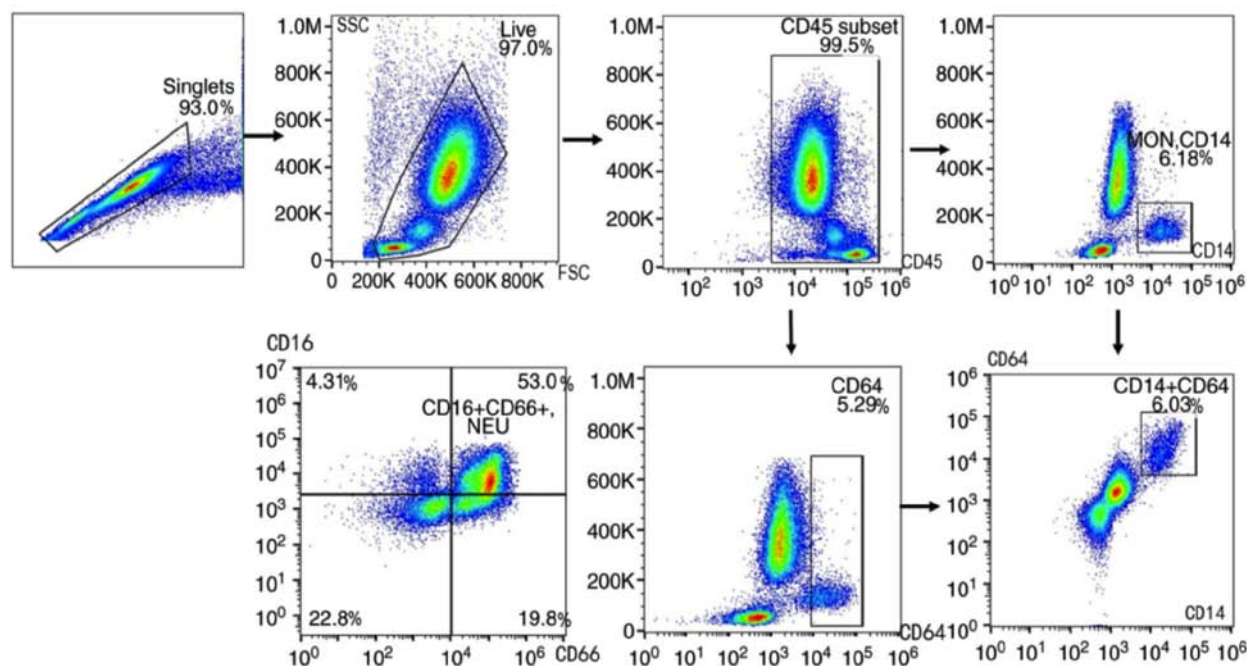
Figure 1. Flowchart of mononuclear phagocyte system antigen staining of flow cytometry. Ratio of CD14$^+$, CD64$^+$ monocyte and CD16$^+$CD66$^+$ neutrophils among CD45$^+$ leukocytes (%). FSC, forward scatter; SSC, side scatter; MON, monocyte; NEU, neutrophil.
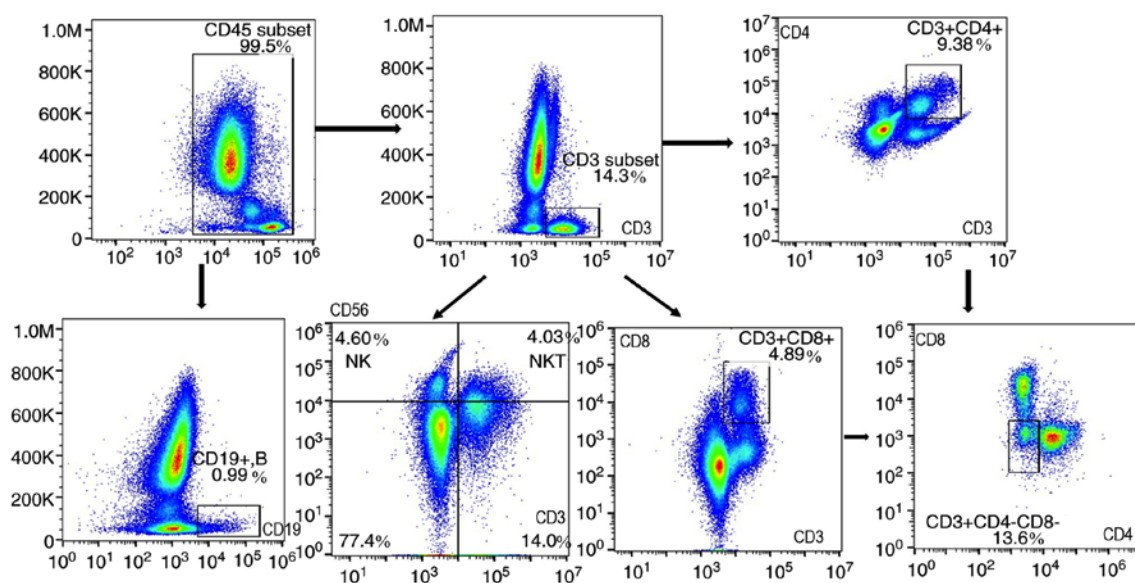

Figure 2. Flowchart of lymphocytes antigen staining of flow cytometry. Ratio of CD3$^+$CD4$^+$ T cells, CD3$^+$CD8$^+$T cells, CD3$^+$CD4$^-$CD8$^-$T cells (γδT cells), CD3$^+$CD56$^+$ NKT cells, CD3$^-$CD56$^+$NK cells and CD19$^+$ B cells among CD45$^+$ leukocytes (%). NKT, natural killer T; NK, natural killer.

with COPD and smoking controls compared with non-smoking controls (smoking COPD vs. non-smoking COPD vs. smoking controls vs. non-smoking controls: 5.15 vs. 5.98 vs. 5.33 vs. 9.11%). While CD66$^+$CD16$^+$ neutrophils (smoking COPD vs. non-smoking COPD vs. smoking controls vs. non-smoking controls: 68.83 vs. 64.99 vs. 66.41% vs. 56.04%) MON (CD14$^+$ and CD64$^+$ subsets) were more highly expressed in patients with COPD and smoking controls compared with non-smoking controls (Table III). CD14$^+$CD64$^+$ MON levels were increased in smokers compared with non-smokers (smoking COPD vs. non-smoking COPD, 3.49 vs. 2.86%; smoking control vs.

non-smoking control, 3.58 vs. 2.03%). Furthermore, the levels of CD19$^+$ B lymphocytes, CD3$^+$CD56$^+$ NKTs and CD3$^-$CD56$^+$ NKs were not significantly different among the four groups.

Inflammatory biomarkers. Higher IL-6 (COPD vs. AECOPD vs. controls, 6.95 vs. 20.51 vs. 3.82), IL-1β (COPD vs. AECOPD vs. controls, 6.69 vs. 7.24 vs. 4.09) and TGF-α (COPD vs. AECOPD vs. controls, 2.15 vs. 2.20 vs. 1.46) levels were observed in the COPD and AECOPD groups compared with healthy controls. The levels of TNF-α (AECOPD vs. COPD vs. controls, 21.71 vs. 11.23 vs. 8.56), IFN-γ (AECOPD vs. COPD vs. controls,

Table II. Peripheral blood immune profiles of patients with COPD, patients with AECOPD and healthy controls.

Immune profiles	COPD (mean ± SEM)	AECOPD (mean ± SEM)	Healthy control (mean ± SEM)	P-value
Immune cells[a], %				
CD3^{+} T cell	14.01±1.08	11.90±0.9	20.11±2.04[b,c]	0.001[e]
CD3^{+}CD4^{+} T cell	9.04±0.53[c]	6.50±0.89[b]	12.36±1.06[b,c]	0.001[e]
CD3^{+}CD8^{+} T cell	5.55±0.36	4.70±0.47	7.85±0.97[b,c]	0.004
CD4/CD8	1.97±0.13[c]	1.47±0.21[b]	1.89±0.17	0.160
CD3^{+}CD4^{-}CD8^{-}γδT cell	1.13±0.17	1.46±0.45	1.40±0.23	0.586
CD3^{+}CD56^{+} NKT	2.88±0.24	3.01±0.32	2.93±0.48	0.976
CD3^{-}CD56^{+} NK	5.85±0.42	5.90±0.71	6.27±0.76	0.877
CD19^{+} B cell	2.11±0.25[c]	1.69±0.20[b]	2.01±0.34	0.723
CD66^{+}CD16^{+} NEU	67.82±1.29	68.21±2.6	59.36±1.18[b,c]	0.011
CD14^{+} MON	4.19±0.26	4.57±0.33	3.04±0.41[b,c]	0.027
CD64^{+} MON	3.94±0.26	4.53±0.53	2.85±0.37[b,c]	0.026
CD14^{+}CD64^{+}	3.19±0.23	3.82±0.38	2.53±0.37[c]	0.070
Inflammatory factors, pg/ml (by multi-plex panel)				
IL-6	6.95±1.16	20.51±3.59[b,d]	3.82±1.14[b,c]	0.001[e]
TNF-α	11.23±1.79	21.71±3.31[b,d]	8.56±2.09[c]	0.023
IL-1β	6.69±1.33	7.24±1.20	4.09±1.00[c]	0.044
IFN-γ	11.91±1.80	29.1±10.79[b,d]	10.32±1.45[c]	0.032
IL-8	17.58±3.71	53.39±14.61[b,d]	12.87±3.11[c]	0.008[e]
IL-33	11.10±2.27	25.56±11.17[d]	6.11±1.98[c]	0.029
IL-17A	4.57±0.60	9.56±0.89[b,d]	4.15±1.02[c]	0.047
IL-4	18.58±3.29	12.23±1.28	17.08±2.55	0.595
IL-5	12.18±1.04	12.71±1.13	11.5±0.6	0.843
IL-13	19.27±0.87	20.27±1.71	17.15±1.92	0.296
TGF-α	2.15±0.57	2.20±0.73	1.46±0.25[b,c]	0.006
GRO-α	223.3±33.5[c]	382.7±135.4[b]	323.1±78.1	0.065
SP-D	6671±926.7	7122±2273	7767±1948	0.800
MPO	8856±843.8	7201±1084	8911±1156	0.405
MMP-9	10627±1901	6852.9±1367	7019.7±905	0.377
Inflammatory factors, pg/ml (by ELISA)				
CC16	18.77±1.31[c]	34.03±12.43[b]	19.43±3.76	0.342
Fibrinogen	841.2±55.65	716.4±76.07	778.7±71.67	0.211
NE	499.3±80.34	742.5±267.1	634.8±178.6	0.432
CRP	1882±412.4	4395±915.9[b,d]	2020±958.6[c]	0.010
TGF-β	40.61±3.8	39.51±7.53	55.44±10.00	0.193

[a]P<0.05 vs. human leucocytes (CD45^{+}). [b]P<0.05 vs. COPD; [c]P<0.05 vs. AECOPD; [d]P<0.05 vs. control. [e]P<0.001. COPD, chronic obstructive pulmonary disorder; AE, acute exacerbated; NKT, natural killer T; NK, natural killer; NEU, neutrophil; MON, monocyte; GRO-α, human growth-regulated oncogene α; SP-D, pulmonary surfactant-associated protein D; MPO, myeloperoxidase; CC16, club cell secretory protein; NE, neutrophil elastase.

29.10 vs. 11.91 vs. 10.32), IL-8 (AECOPD vs. COPD vs. controls, 53.39 vs. 17.58 vs. 12.87), IL-17A (AECOPD vs. COPD vs. controls, 9.56 vs. 4.57 vs. 4.15) and CRP (AECOPD vs. COPD vs. controls, 4395 vs. 1882 vs. 2020) were markedly increased in the AECOPD group compared with the COPD group and healthy controls. IL-33 levels were higher in the AECOPD group compared with healthy controls (AECOPD vs. controls, 25.56 vs. 6.11), while GRO-α was higher in the AECOPD group compared with the COPD group (AECOPD vs. COPD, 382.7 vs.

223.3). The other parameters exhibited no significant differences among the groups (Table II; Fig. 4).

Bioinformatics analysis. Proteomic interaction analysis of key biomarkers was performed by inputting 10 significant biomarkers (IL-6, IL-1β, TNF-α, IFN-γ, IL-8, IL-33, IL-17A, TGF-α, GRO-α and CRP) into the STRING database. The results indicated that there were three core biomarkers of COPD: IL-6, TNF-α and IL-8. In addition, IFN-γ, CRP, GRO-α, IL-33

Table III. Peripheral blood immune cells of smoking and non-smoking subjects.

Characteristics	Smoking COPD (n=73)	Non-smoking COPD (n=24)	Smoking control (n=8)	Non-smoking control (n=17)	P-value
Age, years (mean ± SEM)	65.81±0.96	64.27±2.04	63.18±2.94	65.15±1.23	0.715
Male, n (%)	68 (93.15)	8 (33.33)	8 (100.00)	7 (41.18)	0.001[a]
Pack-years (mean ± SEM)	40.78±2.88		31.56±7.02		0.327
CD3^{+} T cell (mean ± SEM)	12.88±0.65	15.43±1.67	15.82±2.57	22.25±2.66[b-d]	0.001[a]
CD3^{+}CD4^{+} T cell (mean ± SEM)	8.16±0.48	9.42±1.24	11.58±1.95	12.75±1.29[b,c]	0.003
CD3^{+}CD8^{+} T cell (mean ± SEM)	5.15±0.31	5.98±0.74	5.33±1.01	9.11±1.30[b-d]	0.001[a]
CD4/CD8 (mean ± SEM)	1.89±0.12	1.82±0.16	2.45±0.40	1.61±0.13	0.296
CD3^{+}CD4^{-}CD8^{-} (mean ± SEM)	1.14±0.19	1.41±0.33	1.06±0.31	1.57±0.30	0.675
CD3^{+}CD56^{+} NKT (mean ± SEM)	2.91±0.24	2.87±0.39	2.41±0.49	3.19±0.68	0.854
CD3^{-}CD56^{+} NK (mean ± SEM)	6.26±0.42	4.71±0.64	5.82±1.41	6.50±0.93	0.268
CD19^{+} B cell (mean ± SEM)	1.74±0.13[c]	2.41±0.34[b]	1.58±0.63	2.23±0.41	0.125
CD66^{+}CD16^{+} NEU (mean ± SEM)	68.83±1.24	64.99±2.73	66.41±2.85	56.04±4.29[b-d]	0.002
CD14^{+} MON (mean ± SEM)	4.42±0.27	3.85±0.25	4.05±0.69	2.56±0.47[b-d]	0.014
CD64^{+} MON (mean ± SEM)	4.18±0.27	3.75±0.48	3.96±0.73	2.32±0.37[b-d]	0.020
CD14^{+}CD64^{+} (mean ± SEM)	3.49±0.24	2.86±0.32[b]	3.58±0.66	2.03±0.40[b,d]	0.029

[a]P<0.001. [b]P<0.05 vs. smoking COPD group; [c]P<0.05 vs. non-Smoking COPD; [d]P<0.05 vs. smoking control group. COPD, chronic obstructive pulmonary disorder; NKT, natural killer T; NK, natural killer; NEU, neutrophil; MON, monocyte.
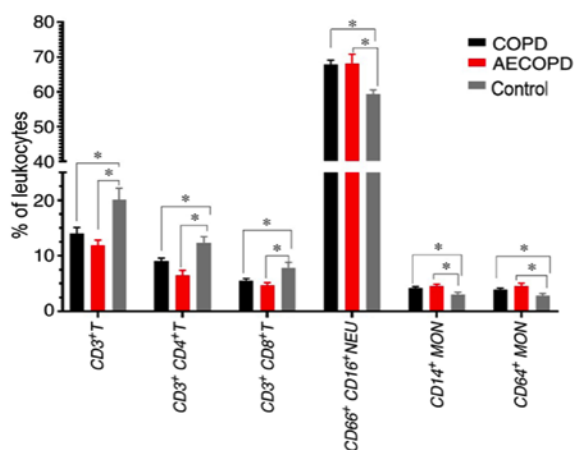

Figure 3. Proportions (%) of immune cell expression in patients with COPD, patients with AECOPD and healthy controls. *P<0.05. COPD, chronic obstructive pulmonary disorder; AE, acute exacerbated; MON, monocyte; NEU, neutrophil.

and IL-17A were also essential in the complex network (Fig. 5). The present study used network expansion function through STRING and found some potential genes closely associated with these proteins, including inhibitor of NF-κB kinase regulatory subunit γ (IKBKG), inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase β (IKBKB), receptor-interacting serine/threonine kinase 1 (RIPK1), TNF receptor superfamily member 1A associated via death domain (TRADD) and TNF receptor-associated factor 2 (TRAF2; Fig. 6).

Correlation analysis. Pearson's correlation analysis revealed a mild positive correlation between CD3^{+} T lymphocytes (mainly CD4 subset) with FEV1/FVC (r=0.33), FEV1% (r=0.27) and maximal mid expiratory flow (MMEF)75/25 (r=0.34) parameters; however, Spearman's correlation analysis revealed a mild or weak negative correlation with presence of smoking and exacerbation history. Myeloid-derived immune cells, such as CD16^{+}CD66^{+} neutrophil (r=-0.26, r=-0.25) and CD14^{+} (r=-0.22, r=-0.24)/CD64^{+} (r=-0.14, r=-0.22) MON exhibited a weak negative correlation with pulmonary function (FEV1/FVC, MMEF75/25). In addition, MMP-9 exhibited a mild negative correlation with cough (r=-0.33) but was not correlated with spirometry measurements. Furthermore, IL-8 (r=0.33) and IL-17A (r=0.31) exhibited a mild positive correlation with cough, while CRP was mildly negatively correlated with FEV1/FVC (r=-0.21), FEV1% (r=-0.24) and MMEF75/25 (r=-0.26), smoking history (r=-0.62) but mild positively correlated with exacerbations (r=0.26), mMRC (r=0.26) and cough (r=0.28). Additionally, CC16 exhibited a moderate negative correlation with TLC (total lung compacity) (r=-0.44) and RV (residual volume)/TLC (r=-0.41; Tables IV and V; all P<0.05; correlation strength: r=0.6-0.8 strong; r=0.4-0.6 moderate; r=0.2-0.4 mild and r=0.0-0.2 weak or uncorrelated).

Discussion

Inflammation serves a central role in the activation and alteration of immune profiles in COPD (26). In the present study, significant differences in the expression profiles of immune factors in peripheral blood of patients with COPD were observed, based on results from flow cytometry and analysis of multiple biomarker panels. Specifically, there was a decrease in CD3^{+}T lymphocytes and an increase in CD14^{+}/CD64^{+} MPS and CD16^{+}CD66^{+} neutrophils in both the COPD/AECOPD groups and smoking controls compared with non-smoking controls. In addition, alterations of systemic inflammatory markers, such as IL-6, TNF-α, IFN-γ, IL-17A and CRP, were reported, suggesting
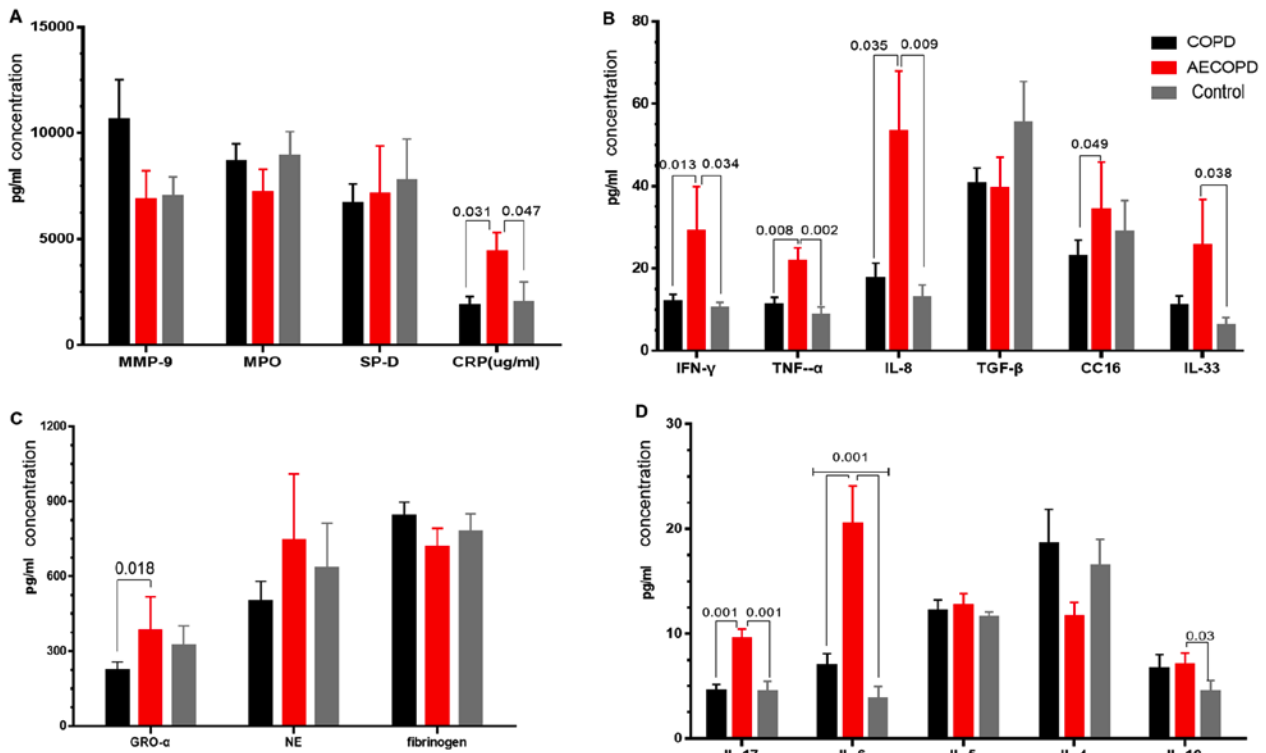
Figure 4. Levels of inflammatory factors in patients with COPD, patients with AECOPD and healthy controls. (A) Levels of CRP were increased in the AECOPD group. (B) Levels of TNF-α, IFN-γ and IL-8 were increased in the AECOPD group. (C) Levels of GRO-α were increased in the AECOPD group compared with COPD. (D) Levels of IL-6 and IL-17A were increased in the AECOPD group. Data were analyzed using the Kruskal Wallis test and Dunn's post hoc test. COPD, chronic obstructive pulmonary disorder; AE, acute exacerbated; MPO, myeloperoxidase; SP-D, pulmonary surfactant-associated protein D; CC16, club cell secretory protein; GRO-α, human growth-regulated oncogene α; NE, neutrophil elastase.
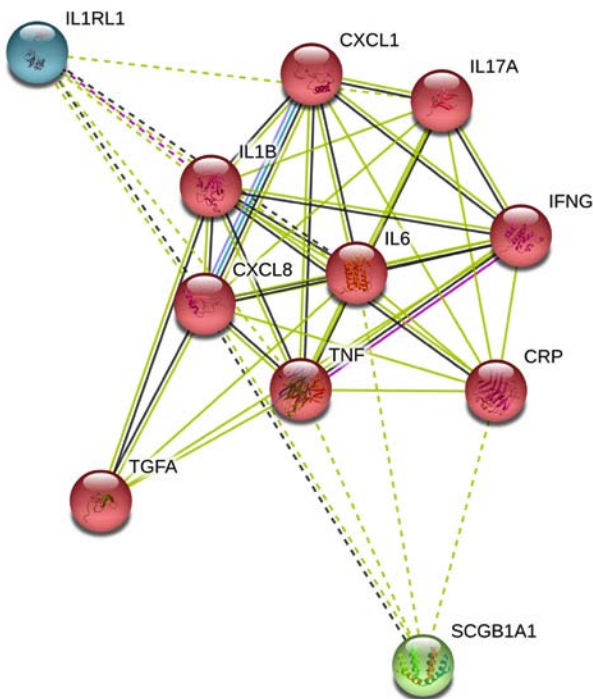

Figure 5. Protein-protein interaction network of significant inflammatory factors (IL-6, IL-1β, TNF-α, IFN-γ, IL-8, IL-33, IL-17A, TGF-α, GRO-α and CRP). Network nodes represent proteins (colored nodes: Query proteins and first shell of interactors), while edges represent protein-protein associations [from STRING website, 10 items (human)-STRING interaction network (string-db.org)]. IL-6, TNF-α and IL-8 were predominant in the network of significantly different inflammatory factors (triangle).
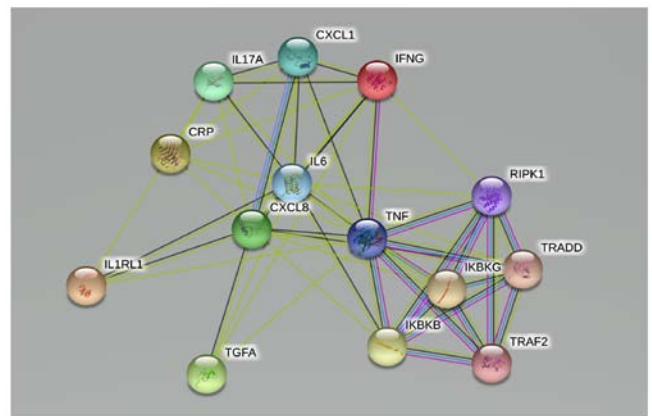

Figure 6. Expansion of protein-protein interaction network of significant inflammatory factors. Network-associated proteins IKBKG, IKBKB, RIPK1, TRADD and TRAF2 (expanded search) were closely associated with other factors. IKBKG, including inhibitor of NF-κB kinase regulatory subunit γ; IKBKB, inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase β; RIPK1, receptor-interacting serine/threonine kinase 1; TRADD, TNF receptor superfamily member 1A associated via death domain; TRAF2, TNF receptor-associated factor 2.

that immune profiles were different among patients with COPD, patients with AECOPD and healthy controls, CRP and IL-17A were associated with smoking status.

A previous study reported an increase in the proportion of macrophages (M1-M2) and a decrease in T-lymphocytes (mainly CD4$^+$T) in lung tissues of current smokers with COPD

Table IV. Pearson correlation coefficient between immune profiles and clinical features.

Correlation (r)	FEV1/FVC	FEV1	FVC	RV/TLC	TLC	MM75/25
CD3^{+} T cell	0.33	0.27	0.14	0.13	-0.10	0.34
CD4^{+} T cell	0.35	0.28	0.24	-0.01	-0.15	0.38
CD8^{+} T cell	0.24	0.13	0.05	0.19	0.02	0.23
CD14^{+} MPS	-0.22	-0.24	-0.19	-0.07	-0.01	-0.24
CD64^{+} MPS	-0.14	-0.12	-0.10	-0.12	-0.47	-0.22
CD16^{+}CD66^{+}	-0.26	-0.20	-0.01	-0.17	0.06	-0.25
IL-6	-0.19	-0.15	0.20	0.02	0.11	-0.14
CRP	-0.21	-0.24	-0.23	-0.04	0.02	-0.26
CC16	-0.03	-0.02	-0.07	-0.41	-0.44	-0.05
IL-8	-0.09	-0.08	-0.18	-0.17	0.21	-0.07
IL-17A	-0.24	-0.31	-0.28	-0.44	0.41	-0.26
Fibrinogen	-0.05	-0.04	-0.01	-0.13	-0.13	-0.11

MPS, mononuclear phagocyte system; CC16, club cell secretory protein; FEV1, forced expiratory volume in 1 sec; FVC, forced vital capacity; RV, residual volume; TLC, total lung compacity; MMEF75/25, maximal mid expiratory flow.

Table V. Spearman's correlation coefficient between immune profiles and clinical features.

Correlation coefficient	Smoking	Exacerbation	mMRC	Cough
CD3^{+} T cell	-0.29[a]	-0.24[a]	-0.04	0.06
CD4^{+} T cell	-0.20[a]	-0.21	-0.17	0.03
CD8^{+} T cell	-0.23[a]	-0.15	0.20	-0.05
CD14^{+} MPS	0.27[a]	0.16	0.13	-0.04
CD64^{+} MPS	0.31[a]	0.10	0.10	-0.07
CD16^{+}CD66^{+}	0.24[a]	-0.09	-0.06	-0.07
IL-6	0.15	0.22	0.16	0.24[a]
CRP	-0.62	0.26[a]	0.26[a]	0.28[a]
CC16	-0.04	0.16	0.18	-0.13
IL-8	0.12	0.14	0.20	0.33[a]
IL-17A	-0.24	0.30[a]	0.14	0.31[a]
Fibrinogen	0.07	-0.24	-0.21	-0.20
MMP-9	0.07	0.02	-0.06	-0.33[a]

[a]P<0.005. MPS, mononuclear phagocyte system; CC16, club cell secretory protein; mMRC, modified Medical Research Council dyspnea scale.

compared with non-smokers and smokers without COPD (27). Furthermore, the proportion of monocytes was markedly different between non-smokers and smokers in the blood only (27). The present study reported an increase in the proportion of CD16^{+}CD66^{+} neutrophils and CD14^{+}/CD64^{+} MPS, a decrease in the proportion of CD3^{+}T lymphocytes (both CD4^{+} and CD8^{+} T Cell) in patients with COPD and smokers compared with non-smoking controls in peripheral blood. This indicated the predominant effects of smoking and cumulative immune composition changes of COPD.

MPS is defined as a cell lineage, including promonocytes and their precursors, in the bone marrow, monocytes in circulation and macrophages in tissues (28). Once in the blood, monocytes undergo transformation into tissue macrophages

with phagocytic function, and serve a central role in the immune regulation by generating antigens to T lymphocytes and secreting proinflammatory factors such as TNFα, IL-1β and IL-6 that are involved in host defense and inflammation (28,29). Although the present study did not assess macrophages in lung tissues and sputum, the increased ratio of CD14^{+}/CD64^{+} monocytes and CD16^{+}CD66^{+} neutrophils in blood revealed the prominent systemic inflammation of smokers and patients with COPD, and the decreased ratio of CD3^{+} T lymphocytes (CD4^{+} and CD8^{+} subsets) in the blood revealed the cumulative immune deficiency of smokers and patients with COPD. Lower proportions of lymphocytes (CD3^{+} T cells) indicated poorer lung function and a higher risk of exacerbation in COPD. On the other hand, higher levels of myeloid cells (CD16^{+}CD66^{+} neutrophils and

CD14^{+}/CD64^{+} MPS) indicated poorer lung function in smokers with COPD compared with non-smoking controls. However, the trigger of immune composition changes in non-smoking patients with COPD remains unclear.

Blood biomarkers have a significant value in diagnosis and prognosis of COPD (14). A study on COPD Gene and SPIROMCIS measured 114 candidate plasma and serum biomarkers using the 13 panel Luminex assays (30). A total of nine cytokines and chemokines were selected for further analysis using a Meso Scale Discovery platform. These MSD multiplex panels were used to measure Single nucleotide polymorphism in 2,123 subjects from COPD Gene and 1,117 subjects from SPIROMICS. The results indicated a strong association between eotaxin and IL-6 with airflow obstruction (13). In the present multiplex panel involving 20 biomarkers, it was identified that IL-6, TNF-α and IL-8 (CXCL-8) served key roles in the inflammation response network of COPD. Pearson's correlation analysis revealed a close relationship between CRP and airway airflow obstruction, while CC16 could be a novel marker of emphysema. Furthermore, higher levels of IL-6, CRP, IL-8 and IL-17A indicated more cough, sputum and dyspnea. Although no biomarker has been demonstrated to be useful in the diagnosis of COPD to date, to the best of our knowledge, blood-based biomarkers for predicting progression of COPD remain relevant (31). The present findings may provide novel insights into biomarkers that have potential for prediction and evaluation of COPD pulmonary function, as well as its symptoms, and are expected to be beneficial for development of novel therapies for COPD phenotypes.

The present study analyzed the interaction among biomarkers, identified the importance of IKBKG, RIPK1, TRADD and TRAF2 and also investigated IL-17, TNF and NF-κB pathway, which are associated with inflammatory cell chemotaxis and immune response (25). The IL-17A, TNF and NF-κB signaling pathways have been identified to be important in the inflammatory response of COPD (32). Fisetin, which inhibits the TNF-α and NF-κB signaling pathways, could be a good candidate drug for improving lung function in patients with COPD (33). In addition, IL-17A-driven type-2 inflammation is another endotype of COPD, which could be an indicator of steroid-unresponsive subgroup of COPD (34).

Previous studies have found T helper 17 (Th17) cells, a subset of CD4^{+} T cells, to be positively correlated with IL-17A levels, which are increased in patients with COPD (35). The present study reported elevated levels of IL-17A, although the proportion of CD4^{+} T cells was decreased in patients with COPD compared with healthy controls. A possible reason for this paradox is that Th17 cells could be inhibiting the expansion of CD4^{+} regulatory T (Treg) cells (36). An imbalance in Th17/Treg has been reported to serve a pivotal role in COPD development and progression (37). Studies have also demonstrated a negative correlation between Th17 cell and Treg cells in patients with COPD (34,38). It is possible that the lower proportion of Treg cells could have contributed to the decrease of CD4^{+} T cells in COPD in the present study. Furthermore, a positive correlation between Treg proportion and TGF-β level has been reported (38). However, the present study did not observe a significant decrease of TGF-β in COPD. Further functional experiments investigating the subset of immune cells are required to investigate this.

The present study had several limitations. The wide range of immune cells should be classified into more detailed subgroups.

The functional and dynamic evolution of various immune components at different stages will need to be explored in future studies. The biomarker panel used was selected from available assays, based on possible mechanisms in COPD. This panel was heavily weighted towards systemic inflammatory markers, not lung-specific biomarkers. In addition, a larger population of participants would be helpful in identifying subgroups to help decrease the heterogeneity. Despite these limitations, the present study identified COPD and smoking associated immune profiles, and indicated those relevant to pulmonary functions and symptoms. Potential pathways and genes of the inflammatory molecular network were also identified.

Overall, the present study revealed changes in the immune profiles in patients with COPD and smokers. A decrease in CD3^{+} T cells and an increase in neutrophils and MPS were also reported. In addition, levels of IL-6, TNF-α, IFN-γ, IL-8 IL-17A and CRP were higher in patients with AECOPD compared with patients with COPD and healthy controls. Furthermore, IL-6, TNF-α and IL-8 (CXCL8) were identified as core biomarkers in COPD pathogenesis, while immune profiles were also relevant to pulmonary functions and symptoms. Future studies will need to recruit more patients with COPD in order to validate and expand the results. Particular focus should be paid on investigating lung-specific biomarkers to identify more COPD subgroups and help advance novel therapies for COPD.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

SL and SZ conceived the present study, enrolled the subjects, and recorded all the clinical data. ZW assisted in the testing of inflammatory factors. FW performed the flow cytometry. WL organized the study, supervised the study and was involved in data aquisition, analysis and interpretation. SL and SZ confirm the authenticity of all the raw data. All authors reviewed and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by The Ethics Committee of West China Hospital, Sichuan University (Chengdu, China) and all patients provided written informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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