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RESEARCH ARTICLE

Assessment of Treg/Th17 axis role in immunopathogenesis of chronic injuries of mustard lung disease

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Abstract

Purpose: Sulfur mustard (SM) lung is a heterogeneous disease associated with abnormal inflammatory immune responses. The Th17/Treg axis imbalance is associated with the pathogenesis of chronic inflammatory pulmonary disease. We aimed to determine the distribution of different Th17 and Treg cells in patients with SM lung and chronic obstructive pulmonary disease (COPD) and evaluate the clinical implications in this homeostasis. *Methods*: In this analytical cross-sectional study, $CD4^+Foxp3^+$ Treg and $CD4^+IL-17^+$ Th17 cells were measured in peripheral blood mononuclear cells (PBMCs) and transbronchial biopsy (TBB) samples of 15 SM-exposed patients, 12 COPD and 13 healthy controls (HCs). The potential correlation between the ratio of Th17/Tregs and lung function was evaluated with multivariate logistic regression (MLR) analysis. *Results*: The frequency of $CD4^+FoxP3^+$ Tregs and $CD4^+IL-17^+$ Th17 was increased ∼1.7-fold (8.71/4.95) and ∼2.7-fold (1.028/0.371) respectively, in the PBMC of SM patients compared with the health controls ($p < 0.001$). The results indicated that there were increases in the frequency of Th17 and Tregs cells in the patients with COPD versus the HC, that is, ∼2.6-fold (0.987/0.371) and ∼1.4-fold (7.12/4.95), respectively; but they did not reach to SM level ($p \geq 0.05$). Moreover, in the TBB samples, the $CD4^+IL-17^+$ Th17 and $CD4^+FoxP3^+$ Tregs numbers were significantly higher in SM and COPD patients than HC ($p < 0.05$). The Th17 and Treg cells were inversely correlated with forced expiratory volume in 1s (FEV1%) ($r = -0.351$, $p = 0.001$; $r = -0.344$, $p = 0.021$) and FEV1/FVC ($r = -0.44$, $p = 0.001$; $r = -0.302$, $p = 0.011$), respectively. Instead, positive correlations were found between Treg/Th17 ratios and forced FEV1%pred ($r = 0.156$, $p = 0.007$), as well as FEV1/FVC ratio ($r = 0.334$, $p = 0.006$). *Conclusions*: The imbalance of Th17/Treg has a key role in immunopathogenesis of chronic phase of mustard lung disease.

Abbreviations: SM: sulfur mustard; **BO**: bronchiolitis obliterans; **COPD**: chronic obstructive pulmonary disease; **Tregs**: T regulatory cells; **Foxp3**: forkhead box protein 3-positive; **IPF**: idiopathic pulmonary fibrosis; **TNFα**: tumor necrosis factor α; **TGF-β**: transforming growth factor β; **HCs**: healthy controls; **T1DM**: type 1 diabetes mellitus; **GOLD**: chronic obstructive lung disease; **CAT**: COPD assessment test; **SGRQ**: St. George respiratory questionnaire; **FEV1**: forced expiratory volume in 1s; **FVC**: forced vital capacity; **VC**: vital capacity; **RV**: residual volume; **MEF25-75**: maximal expiratory flow between 25% and 75% of VC; **PBMCs**: peripheral blood mononuclear cells; **TBB**: transbronchial biopsy; **H&E**: hematoxylin and eosin; **HRP**: horseradish peroxidase; **HRP**: horseradish peroxidase; **SD**: standard deviation; **BMI**: body mass index; **MMP-9**: matrix metalloproteinase 9; **MUC5A**: mucin 5A; **MUC5B**: mucin 5B; **BMSU**: Baqiyatallah University of Medical Science

Introduction

Sulfur mustard, bis (2-chloroethyl) sulfide (SM), is a high alkylating chemical warfare agent most widely used against Iranian soldiers and civilians in Iraq–Iran-imposed war (1980–1988) (1,2). One of the most significant delayed toxic complications of SM is pulmonary disorder with the main underlying pathology of bronchiolitis obliterans (BO)

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and chronic obstructive pulmonary disease (COPD) (3). About 80% of exposed patients show chronic clinical symptoms, such as cough, sputum production, shortness of breath, hemoptysis, chest pain, nocturnal dyspnea, after 20 years of exposure to SM gas (3–5). Oxidative stress, protease-anti-protease imbalance (6), and chronic inflammation (6–8) mechanisms in SM lung may lead to tissue damage, immune cells recruitment and finally airway remodeling. This, in turn, results in an increased expression of pro-inflammatory cytokines (9–11), profibrotic cytokines (12,13) and some alterations in the innate and adaptive immune cells, including NK cells, $CD8^+$ and $CD4^+$ T cells, at the site of injury in chronic phase of mustard lung (14–18). Nevertheless, immunopathogenesis of SM lung is unclear (19).

Two subsets of $CD4^+$ T cells are known to have a key role in the immunopathogenesis of inflammatory diseases: $CD4^+CD25^+$ forkhead box protein 3-positive ($Foxp3^+$) T regulatory cells (Tregs), acting as the effector cells in peripheral tolerance and down-regulation of persistent inflammation and $CD3^+CD4^+$ IL-17-producing T cells (Th17), working as pro-inflammatory effectors T cell in chronic inflammation (20,21). Therefore, these cells may have opposite functions in the pathogenesis of several inflammatory diseases, such as COPD, asthma and idiopathic pulmonary fibrosis (IPF) (22–25). Furthermore, some studies have indicated that the Th17/Treg imbalance may be an important feature of pathologic chronic inflammatory disorders (9,26–31).

With respect to the increased production of pro-inflammatory and profibrotic cytokines, such as IL-1β, IL-6, IL-21, IL-22, IL-23, transforming growth factor β (TGF-β) (9–11), and IL-4, IL-13 and TNF-α (tumor necrosis factor α) (12,13) in SM lung microenvironments, we think that T cells may differentiate and proliferate to Th17 cells in SM lung. We compared the Th17/Treg balance in peripheral blood mononuclear cells (PBMCs) and lung tissue of patients with SM and COPD and with healthy controls (HCs). Here, we found a significant increase in $CD4^+$ IL-17$^+$ Th17 and $CD4^+$ FoxP3$^+$ Tregs cell in SM and COPD patients. We also provided compelling evidence that this imbalance is positively correlated with airflow limitations.

Materials and methods

Subjects and study design

The study was performed from June 2013 to January 2014 in the pulmonary department of Baqiyatallah Hospital, Tehran, Iran. This hospital provides medical care for SM-exposed Iraq–Iran war veterans. This study was approved by a local ethical committee of Baqiyatallah Hospital, according to the Declaration of Helsinki. Patients with COPD were diagnosed according to the definition supplied by the Global Initiative for Chronic Obstructive Lung Disease (GOLD) guidelines (32). Totally, 40 patients were selected according to the inclusion criteria and subdivided into three groups: 13 patients with COPD diagnosed more than 2 years prior to the study, 15 patients exposed to SM and 12 HCs with normal pulmonary function.

The exclusion criteria were acute bronchiolitis and/or pneumonia, history of pulmonary tuberculosis or resection of one or more lobes, history of smoking and participating in simultaneous clinical trials. All patients with COPD were clinically stable and had not experienced any exacerbations for ≥3 months preceding inclusion in the study. Participants with some autoimmune component, such as type 1 diabetes mellitus (T1DM), interstitial lung disease, rheumatoid arthritis, acute upper respiratory tract infections or other immune-related diseases were excluded as well. The HC group had normal lung function tests. Informed consent was obtained from all subjects. The COPD assessment test (CAT) and St. George Respiratory Questionnaire (SGRQ) were used for all participants during the study, after history taking and physical examination for the assessment of the health status.

Lung function tests

Pulmonary function tests were performed by Spirometry according to the American Thoracic Society criteria (33). The forced vital capacity (FVC) and forced expiratory volume within the first second (FEV1) were measured using a standard spirometer (Jaeger, Hochberg, Germany). All subjects were in a sitting position on a comfortable chair with a nose clip in place and were asked to perform at least three forced expiratory maneuvers. Recordings of the maneuver with the largest FVC were used for evaluations, and all indices were from the same maneuver. A constant volume body plethysmograph was used to determine residual volume (RV) and total lung capacity (TLC) (34).

Flow cytometry analysis

Fresh PBMC were isolated by Ficoll–Hypaque (Lymphoprep; Nycomed Pharma, Oslo, Norway) density centrifugation (400 g, at room temperature (RT), 20 min). In order to identify $CD4^+$ Foxp3$^+$ Treg and $CD4^+$ IL-17$^+$ Th17 cells, PBMC (2×10^6/mL) were stimulated with 50 ng/mL of phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich, St. Louis, MO) and 1 μg/mL of ionomycin (Sigma-Aldrich, St. Louis, MO) in the presence of GolgiStop (1.7 μg/mL of monensin) (Sigma-Aldrich, St. Louis, MO) in 10% fetal calf serum (Roswell Park Memorial Institute 1640) at 37 °C for 3.5 h. The harvested cells were surface labeled with PerCP/Cy 5.5-labeled anti-human CD4 (BD Biosciences, CA) at 4 °C for 30 min, fixed and permeabilized, followed by intracellular staining with PE-labeled anti-human IL-17A and Alexa Fluor 647 anti-human FoxP3 (BD bioscience, CA). The cells were gated on living lymphocytes and then on $CD4^{++}$ cells and the percentages of $CD4^+$ IL-17$^+$ Th17 cells and $CD4^+$ Foxp3$^+$ Treg cells were determined by flow cytometry analysis. The frequencies of $CD4^+$ IL17A$^+$ Th17 and $CD4^+$ FoxP3$^+$ Treg cells were analyzed by FACS Canto II flow cytometer (BD Biosciences, CA); 500 000–1 000 000 events were assessed in each tube. Data were analyzed with FlowJo software (version 10.0). Data are representative charts or the percentages of individual subjects. The lines indicate median values for each group. Isotype-matched control antibodies were all purchased from BD (BD bioscience, CA) and used at the same concentration as test antibodies. Fluorescence minus one (FMO) controls was used for determining the percentage of positive cells.

Lung tissue sampling

All lung tissue samples were obtained by transbronchial biopsy (TBB) in the bronchoscopy section. Bronchial biopsies were taken through the bronchoscope with standard forceps from the subcarina of a basal segment bronchus. All subjects were premedicated intramuscularly with atropine (0.5 mg) and diazepam (10 mg) and orally with dihydrocodein (10 mg). Nares and oropharynx were topically anesthetized with 10% lidocaine before bronchoscopy. Bronchoscopy was performed with a flexible fiberoptic bronchoscope (Pentax FB-18P; Asahi Optical Co. LTD, Tokyo, Japan) in all participants. All bronchoscopy tissue samples were fixed and embedded in Tissue Tek II OCT (Miles Scientific, Naperville, IL), frozen 15 min in isopentane, precooled in liquid nitrogen and then stored at $-80\,^{\circ}$C. Next, the best frozen sample was orientated and cut as 5-mm-thick cryostat sections for hematoxylin and eosin (H&E) light microscopy and immunohistochemical analysis. Microscopic analysis of all the slides was performed by a light microscopy (Olympus Cor., Tokyo, Japan) linked to computerized image system (Image-Pro Plus V6.0, Silver Spring, MD). Cells were quantified by counting the number of immunolabeled cells in 10 randomized high-power fields with a square grid area of 0.0645×0.2 mm. (10 fields per subject because this number of fields was sufficient to obtain a mean value per subject). The cases were coded, and measurements were made in a blinded fashion by two pathologists. Bronchial biopsies were processed according to the local ethics committee guidelines by the bronchoscopy laboratory, division of clinical pulmonology, Baqiyatallah Hospital, Tehran, Iran.

Immunohistochemistry

Foxp3 staining

Foxp3 staining was performed using streptavidin–biotin alkaline phosphatase complex method by Vectastain ABC-AP standard kit (Vector Laboratories; Burlingame, CA). Antigen retrieval was achieved by treatment in a high-temperature pressure cooker heated for 90 s in citrate buffer, pH 6.0. Nonspecific reactions were blocked using 2% normal mouse serum 30 min at RT (Santa Cruz Biotechnology, Santa Cruz, CA). Also sections were incubated in levamisole (Vector Laboratories, Burlingame, CA) to quench endogenous alkaline phosphatase. The slides were then incubated with primary monoclonal mouse anti-human Foxp3 (2 μL 1:400, eBioscience, San Diego, CA). The resulting slides were incubated in a humid chamber for 10 min at RT with biotinylated secondary antibody (3 μL; 1:400). Then, an avidin–biotin alkaline phosphatase complex was added for 30 min at RT (3 μL 1:400 Promega, Madison, WI). The color was developed with a Vector Red alkaline phosphatase substrate kit (Vector Laboratories). Normal mouse IgG, normal preimmune rabbit IgG or Tris-buffered saline (TBS) was used as negative controls.

IL-17 staining

IL-17 expression (Th17 marker) was identified as cytoplasm immunolocalization. Indirect immunohistochemistry staining was performed by horseradish peroxidase (HRP) method. Briefly, after retrieving the antigen in citrate buffer for 90 s, nonspecific reactions were blocked using 2% normal mouse serum for 30 min at RT (Santa Cruz Biotechnology, Santa Cruz, CA). The endogenous peroxidase activity was quenched in 0.5% H_2O_2 for 10 min and the slides were incubated with primary monoclonal mouse anti-human IL-17 for 75 min (2 μL; 1:50, Abcam, Cambridge, UK). The resulting slides were incubated for 45 min in a humidity chamber with anti-IgG-conjugated HRP secondary antibody (3 μL; 1:50, Promega, Madison, WI) and subsequent rinses with PBS three times for 7 min. 3′3-diaminobenzidine–tetrahydrochloride (40 mg/mL DAB containing 0.3% H_2O_2, Sigma-Aldrich, St. Louis, MO). The sections were counterstained in nuclear fast red for 10 min. All reactions were performed with positive and also negative controls (isotype controls and omission of primary antibody).

Statistical analysis

All quantitative data were transferred to Excel and the statistical analyses were computed with SPSS software for Windows (Version 21, SPSS Inc., Chicago, IL). Data are presented as mean ± standard deviation (SD) or median (range) and IQR when appropriate. For data not distributed normally, comparisons between three groups were made using one-way Kruskal–Wallis test ($p < 0.05$ was considered statistically significant). If this test indicated significance, the Mann–Whitney test was used for *post hoc* analysis for comparison between two groups, with corrections of p values according to Bonferroni ($p < 0.05$ was considered statistically significant). The Spearman's rank correlation coefficient test was employed to determine the association between two variables. Here, $p < 0.05$ were considered as statistically significant. All charts were performed with Prism 5.0 (GraphPad, La Jolla, CA).

Results

Clinical findings

The demographic and clinical characteristics of the subjects were compared in Table 1. There was no difference between the groups in terms of age, gender and body mass index (BMI). The unequal sex ratio was mostly due to the much higher prevalence of COPD in men than in women in Iran (35). There was a significant difference in the smoking history of patients with SM and COPD ($p < 0.05$). Also, the patients exposed to SM were hospitalized much longer than patients with COPD ($p < 0.001$). As expected, spirometry test results showed considerably lower values for FEV1, predicted FEV1%, predicted FVC %, FEV1/FVC%, MEF25–75 and RV/TLC in patients exposed to SM compared with the control group ($p < 0.001$). The patients exposed to SM had significantly lower values for FEV1 and MEF25–75 than those with COPD ($p < 0.05$). Other spirometry factors were not significantly different between patients with SM disease and COPD. However, the values of RV %, predicted TLC%, RV/TLC, SGRQ and CAT were significantly higher in patients with SM disease and COPD than HCs group ($p < 0.001$). Moreover, predicted TLC%, SGRQ, CAT values

Table 1. Demographic and baseline clinical characteristics of the patients.

Measure	HC	COPD	SM
Demographic variables			
Subjects	13	12	15
Age (yr)	49.14 ± 1.32	49.26 ± 7.83	50.46 ± 3.92
Gender (M/F)	9/6	12/3	15/0
BMI (kg/m^2)	24.90 ± 1.45	25.99 ± 2.29	25.80 ± 1.82
Smoking history, pack years	NA	15 ± 9	9 ± 3
Number of admissions (time/year)	NA	3.3 ± 0.60	4.10 ± 0.70[d]
Spirometry variables			
FEV1 (L)	3.51 ± 0.72	1.91 ± 0.47[b]	1.98 ± 1.01 [b]
FEV1% pred	89.94 ± 13.98	56.20 ± 6.39 [b]	59.21 ± 21.54 [b]
FVC (L)	3.98 ± 0.31	2.70 ± 0.31 [a]	2.94 ± 0.67 [b]
FVC % pred	91.07 ± 9.07	72.54 ± 9.40 [b]	76.27 ± 1.32 [b]
FEV1/FVC %	89.78 ± 9.075	63.14 ± 7.27 [b]	64.73 ± 13.08 [b]
MEF 25–75	91.78 ± 6.21	59.93 ± 9.54 [b]	48.83 ± 13.08 bc
RV/TLC	30.06 ± 8.64	40.60 ± 4.30 [b]	41.70 ± 5.29 [b]
Quality of life variables			
SGRQ	20.47 ± 9.03	45.81 ± 1.50 [b]	62.40 ± 1.14 bd
CAT	12.94 ± 3.01	22.34 ± 3.57 [b]	27.04 ± 4.86 bd

Data are presented as median (IQR) for smoking history, mean $\pm$ SD for all others. Patients with COPD had not received any treatment within the preceding month. HC, healthy controls with normal lung ventilation function; COPD, patients with COPD; SM, sulfur mustard lung; BMI, body mass index; FEV1, forced expiratory volume in 1s, FVC, forced vital capacity; MEF25–75, maximal expiratory flow between 25% and 75% of VC; RV, residual volume; TLC, total lung capacity; SGRQ, St-George respiratory questionnaire; CAT, COPD assessment test.
[a]$p < 0.05$.
[b]$p < 0.001$ versus the HC group.
[c]$p < 0.05$.
[d]$p < 0.001$ versus the COPD group.

were significantly higher in patients exposed to SM than those with COPD ($p < 0.001$). These data suggest that quality of life in patients exposed to SM is critically lower than healthy individuals.

Flow cytometry results

The frequency CD4$^+$ Foxp3$^+$ Treg and CD4$^+$ IL-17$^+$ Th17 cells in SM, COPD and HC groups

We first investigated the frequencies of Treg and Th17 cells in peripheral blood (PB) of different groups using the classic definition. The plot of flow cytometry analysis for CD4$^+$ IL-17$^+$ Th17 and CD4$^+$ Foxp3$^+$ Treg subpopulations in SM, COPD and HC groups are shown in Figure 1(A, B), respectively. Considerable differences of CD4$^+$ IL-17$^+$ Th17 were found in patients with SM disease (1.028 ± 0.12) and COPD (0.987 ± 0.136) compared with the HC group [(0.371 ± 0.062) $p < 0.0001$] (Figure 2A). We found that IL-17A expression was increased by CD4$^+$ high T cells in the SM ($\sim$2.7-fold increase) and COPD ($\sim$2.6-fold increase) groups compared with the HC group. Also, CD4$^+$ Foxp3$^+$ Treg cells frequency was significantly increased in SM (8.71 ± 2.46) group compared with the HC group [(4.95 ± 1.21) ($p \leq 0.001$)]; nearly 1.7-fold increases (Figure 2B). In line with previous studies (36,37), Treg identification using CD4$^+$ Foxp3$^+$ showed no difference in the circulating Treg number in patients with COPD (7.12 ± 1.62) compared with HC (4.95 ± 1.21) [$\sim$1.4-fold increased, $p = 0.103$]. Varied subpopulation frequencies suggested that the balance between immunosuppressive and inflammatory subsets in CD4$^+$ T cells shifted towards inflammation in patients with SM disease and COPD.

Disturbed homeostasis among circulations Th17 and Treg populations in SM and COPD patients

Th17/Treg ratio is one of the important indexes in diseases development showing the balance between the suppressive and pro-inflammatory subpopulations of CD4$^+$ T cells. To determine the ratio of Th17/Treg cells, 13 HC, 12 COPD and 15 SM patients were recruited (Figure 1C). Interestingly, patients with COPD had the highest Th17/Treg imbalance ratios ($\sim$2-fold increase). Compared with the HC group (0.071 ± 0.012), the Th17/Treg ratio was also higher in patients with SM disease (0.115 ± 0.01; $p = 0.042$) and COPD (0.152 ± 0.013; $p = 0.001$) (Figure 2C). These results indicate that Th17/Treg ratio is dramatically worsened in patients with COPD and SM.

Histological findings

Pathological changes of transbronchial sections

To evaluate airway inflammation, TBB lung sections were stained with H&E and analyzed using light microscopy. Representative H&E staining of TBB sections for all groups are shown in Figure 3. In response to SM exposure, infiltrating inflammatory cells were subjected to disorientation and structural depolarization in lung epithelial cells. A significant increase was evident in connective tissues and cilia cell layer in both SM and COPD groups. However, there was no significant difference in the basal membrane thickness between the three groups. There were high infiltration of alveolar macrophages in TBB of patients with SM disease and COPD, indicating higher levels of inflammation (Figure 3B, C). Next, we performed immunohistochemistry
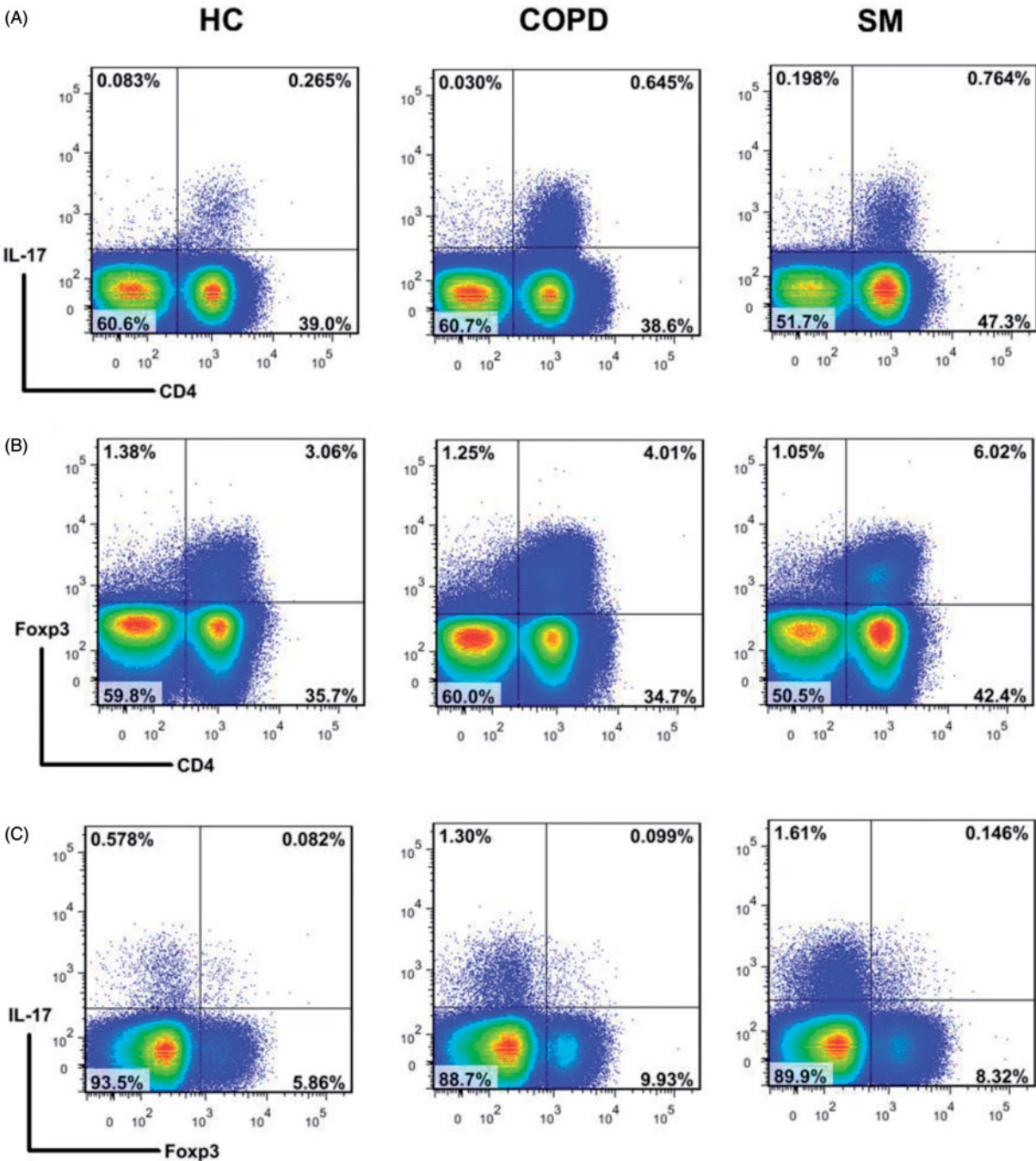
Figure 1. The frequencies of Th17 and Treg cells relative to CD4^{+}T cells in the puerperal blood samples of patients with sulfur mustard disease, chronic obstructive pulmonary disease and healthy controls. (A): Representative dot plots of Treg cells (CD4^{+}Foxp3^{+}Treg cells). (B): Representative dot plots of Th17 cells (CD4^{+}IL-17^{+}Th17 cells). (C): Treg/Th17 ratio. HC, health controls; COPD, chronic obstructive pulmonary disease; SM, sulfur mustard.

for CD4^{+}Foxp3^{+}Treg and CD4^{+}IL-17^{+}Th17 cells in the TBB sections.

The frequency of CD4^{+} Foxp3^{+} Treg and CD4^{+} IL-17^{+} Th17 cells in SM, COPD and HC lung tissues

Comparison of Th17 and Treg subsets cells in lung tissues between the SM, COPD and HC group are shown in Table 2. The numbers of CD4^{+}IL-17^{+}Th17 and CD4^{+}Foxp3^{+}Treg cells were markedly increased in the SM group compared with the HC group ($p < 0.05$). Also, the CD4^{+}IL-17^{+} numbers were increased in COPD group compared with the

HC group ($p < 0.001$). We also observed significant differences in CD4^{+}IL-17^{+}Th17 between the SM and COPD groups ($p = 0.018$) (Table 2). Comparative results of PB and TBB samples show no significant difference between the COPD and HC groups for CD4^{+}Foxp3^{+}Treg cells.

Imbalance of local Th17/Treg ratios in patients with COPD and SM disease

We stained the Th17 and Treg in the TBB samples of each group using double immunohistochemistry staining methods to illustrate the local balance between the suppressive and
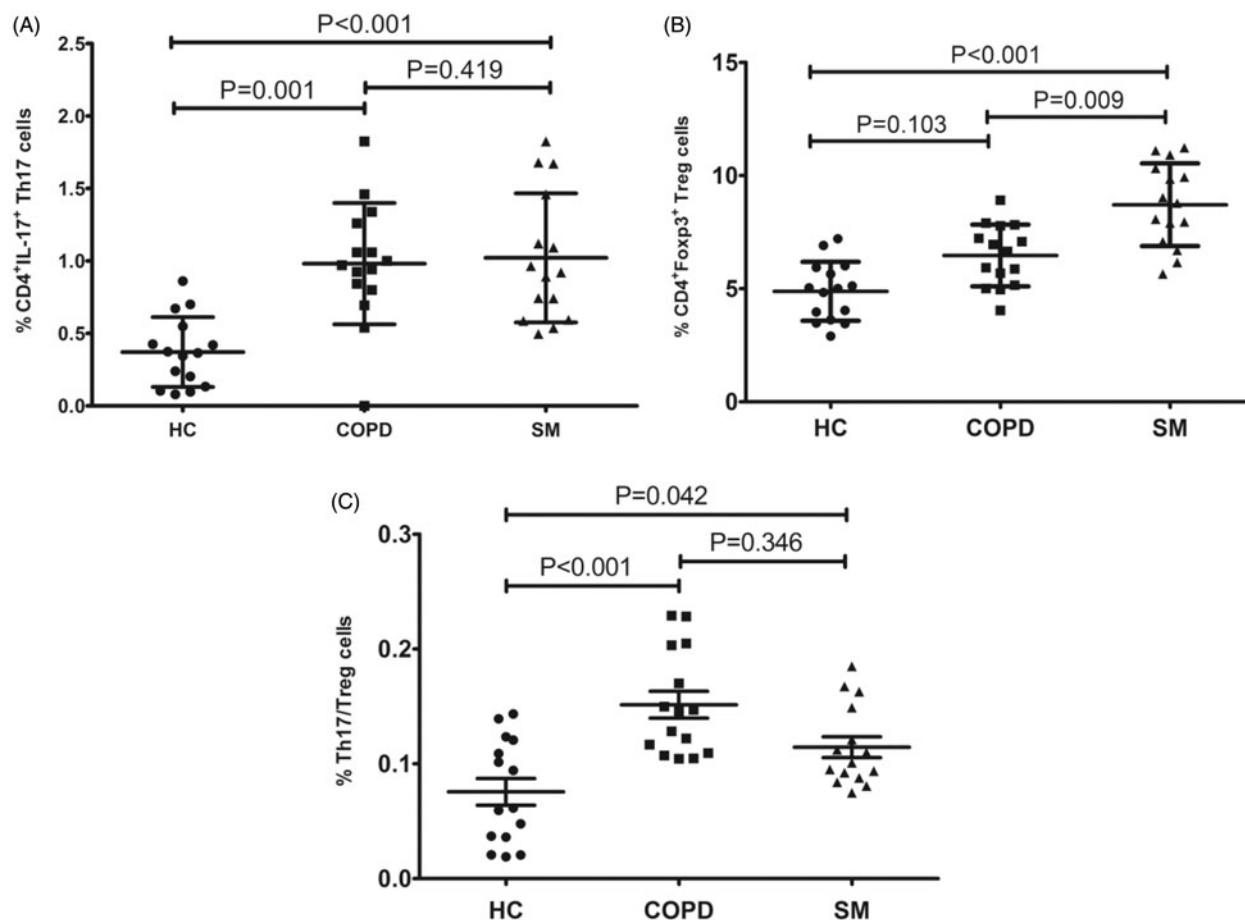
Figure 2. Comparison of the Th17 and Treg subpopulations between sulfur mustard, chronic obstructive pulmonary patients and healthy patients in puerperal blood sample. (A); $CD4^+Foxp3^+$ Treg cells for HC, COPD and SM patients (B); and Th17/Treg cells for three groups. (C). In general, higher percentages of Th17, Treg subsets, were observed in PB of SM and COPD patients. Horizontal bars show mean ± SD; HC, health controls; COPD, chronic obstructive pulmonary disease; SM, sulfur mustard.

pro-inflammatory subpopulations of $CD4^+$ T cells (Figure 4A). Compared with the HC group, the ratio of Th17/Treg cells was also higher in patients exposed to SM [(mean 1.52 (range 3.17) $p = 0.039$)] and those with COPD [(mean 1.68 (range 2.38) $p = 0.0012$)] (Figure 4B). Similar to the results from circulating Th17/Treg ratios, we found that Th17/Treg ratios were significantly higher in TBB of patients with SM and COPD compared with HC, as well as there is no significant difference between the SM and COPD groups (Figures 2C & 4B). Also, the Treg level was increased in PB and lung tissue of patients with SM disease and COPD. Totally, the imbalance of local Th17/Treg between SM and COPD patients is significant.

Correlations

By considering both local and circular Th17 and Treg frequency simultaneously, Th17 frequency was inversely correlated with FEV1%pred ($r = -0.351$, $p = 0.001$) and FEV1/FVC ratio ($r = -0.44$, $p = 0.001$) (Figure 5A, B). Strikingly, the Treg frequency was inversely correlated with FEV1%pred ($r = -0.344$, $p = 0.021$) and FEV1/FVC ratio ($r = -0.302$, $p = 0.011$) (Figures 5C, D). In general, higher Th17 and Treg cell frequencies are correlated with disease severity and lung dysfunction in patients exposed to SM.

It has been previously demonstrated that patients with SM and COPD exhibited enhanced CD4 T-lymphocyte activation,

which was correlated with disease severity as measured by FEV1 (16,38). Moreover, it has been shown that CD4 and CD8 T-cell activation could be suppressed by Tregs (37,39) and promoted by IL-17 (40). In this regards, we found that the Treg frequency were inversely correlated with frequencies of $CD4^+$ cells ($r = -0.399$, $p < 0.001$; $r = -0.416$, $p < 0.001$; data not shown).

Figure 5(E and F) show positive correlation of Treg/Th17 ratios with forced FEV1%pred ($r = 0.156$, $p = 0.007$) (Figure 5C) and FEV1/FVC ratio ($r = 0.334$, $p = 0.006$) (Figure 5F), respectively. Totally, imbalance of Th17/Treg ratios was associated with limitations in pulmonary function in the SM and COPD group.

Discussion

New insights about immunopathogenesis of inflammatory diseases, such as COPD (9) and IPF (22), have emphasized on the role of Th17/Treg axis. Similar to previous reports, all our patients exposed to SM showed lung function impairment in spirometry indices, especially in FEV/FVC, that correlates with disease severity (41,42). Also, CAT and SGRQ indices were worse in SM-exposed patients than patients with COPD.

Immunohistochemistry results for Th17 level in lung tissue indicate that patients with SM disease and COPD had a significant increase in Th17 cells. Th17 cells and its signature cytokine, IL-17, directly induce epithelial cells, airway
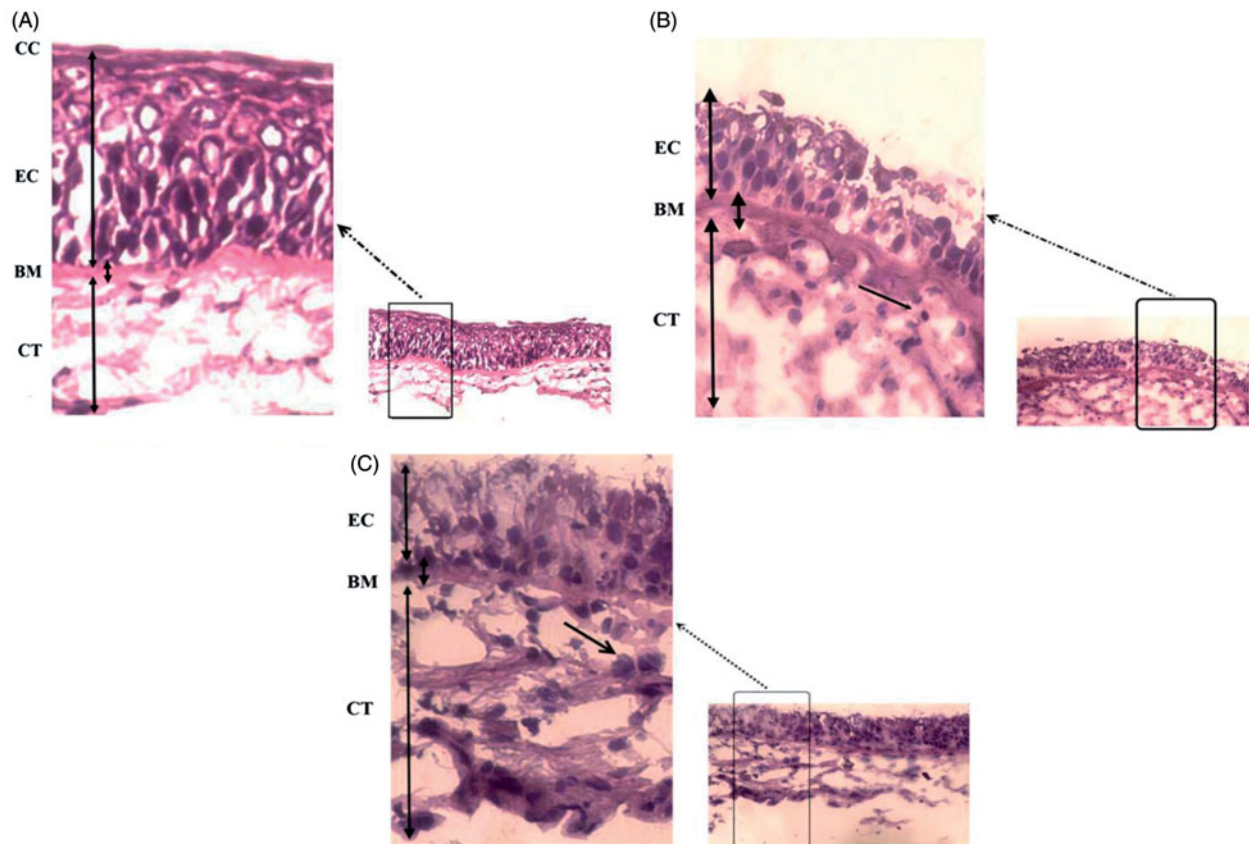
Figure 3. Comparison of representative H&E-stained in TBB lung sections between HC (A), COPD (B) and SM (C) groups (original magnification: ×400). HC, health controls; COPD, chronic obstructive pulmonary disease; SM, sulfur mustard; CC, Cilia Cells; EC, Epithelial Cells; BM, Basal membrane; CT, Connective tissue.

Table 2. Comparison of CD4$^+$T cells subsets in lung tissues between sulfur mustard, chronic obstructive pulmonary patients and healthy controls.

Subpopulations	HC	COPD	SM
CD4$^+$	9 ± 2	15 ± 2[b]	15 ± 6[b]
IL-17$^+$	6 ± 2	15 ± 2[b]	17 ± 2bc
Foxp3$^+$	8 ± 2	9 ± 2	10 ± 2[a]

CD4$^+$IL-17$^+$T Th17; CD4$^+$Foxp3$^+$ Treg cells are presented for HC controls ($n = 15$), and COPD ($n = 12$) and SM patients ($n = 15$). the frequencies of CD4$^+$IL-17$^+$Th17 cells were found to be more frequently detected in SM and COPD than HC group.

All data expressed as mean ± SD (range). Patients with COPD had not received any treatment within the preceding month. HC, healthy controls, with normal lung ventilation function; COPD, patients with COPD; SM, sulfur mustard lung.
[a]$p<0.05$.
[b]$p<0.001$ versus the HC group.
[c]$p<0.05$ versus the COPD group.

fibroblasts and smooth muscle cells to secrete neutrophil recruiting chemokines and also increase IL-6 secretion by bronchial epithelial cells and fibroblasts (43–45). This evidentially results in neutrophil responses and localized tissue inflammation (21), which leads to increased production of proteins involved in tissue damage, including matrix metalloproteinase 9 (MMP-9) (46), mucin 5A (MUC5A) and mucin 5B (MUC5B) (47). Most importantly, Th17 cells were positively correlated with lung function parameters. These correlations indicate that Th17 cells may contribute to pathological injury and respiratory restrictions of SM and

COPD patients' lung during the progress of chronic inflammation. Moreover, in line with an increased Treg, we found a positive correlation between the circular Treg/Th17 ratio and FEV1%, which further linked the immune imbalance with airflow limitation in patients with SM and COPD in PB sample. The stronger correlation between the Th17 frequency and FEV1% provided more evidence to support this link. From these findings, we hypothesize that a increase in the immunosuppressive Treg populations, together with enhanced pro-inflammatory responses, induced by long-term exposure to inhalation of particles of tobacco smoking for COPD (37,48) and induced remodeling mediators in SM disease, for example TGF-β (9–11), lead to persistent airway inflammation dominated by CD4$^+$T cells.

Our results also show that Treg level was increased in SM and COPD lung tissue. Also, Treg cells increased in lung tissue to diminish Th17 cells. Here, we did not observe any reduction in Th17 level in SM and COPD lung, and we concluded that Th17 rate was not completely enough to balance between the ratios of Th17/Treg cells (49–51). Unexpectedly, we found a positive correlation between the increases in Treg rates and lung dysfunction (according to spirometry indices). Our literature review indicated that frequencies of Tregs cells in the immunopathogenesis of inflammatory lung diseases are controversial (36,52–54). The previous observations had demonstrated that IL-17 production is unaffected or even increased by Treg cells (55–57). There are some evidence for this finding: human Treg cells plasticity (property to able to express both ROR-γt and

Figure 4. (A). the representative double immunohistochemistry staining of Th17 and Treg. Original magnification ×400, The IL-17^{+} cells and Foxp3^{+} cells were observed brown dots (black arrow pointed) and the light blue dots (red arrow pointed). (B). Horizontal bars show mean ± SD of Th17/Treg cells ratios for SM, COPD and HC groups. HC, health controls; COPD, chronic obstructive pulmonary disease; SM, sulfur mustard.
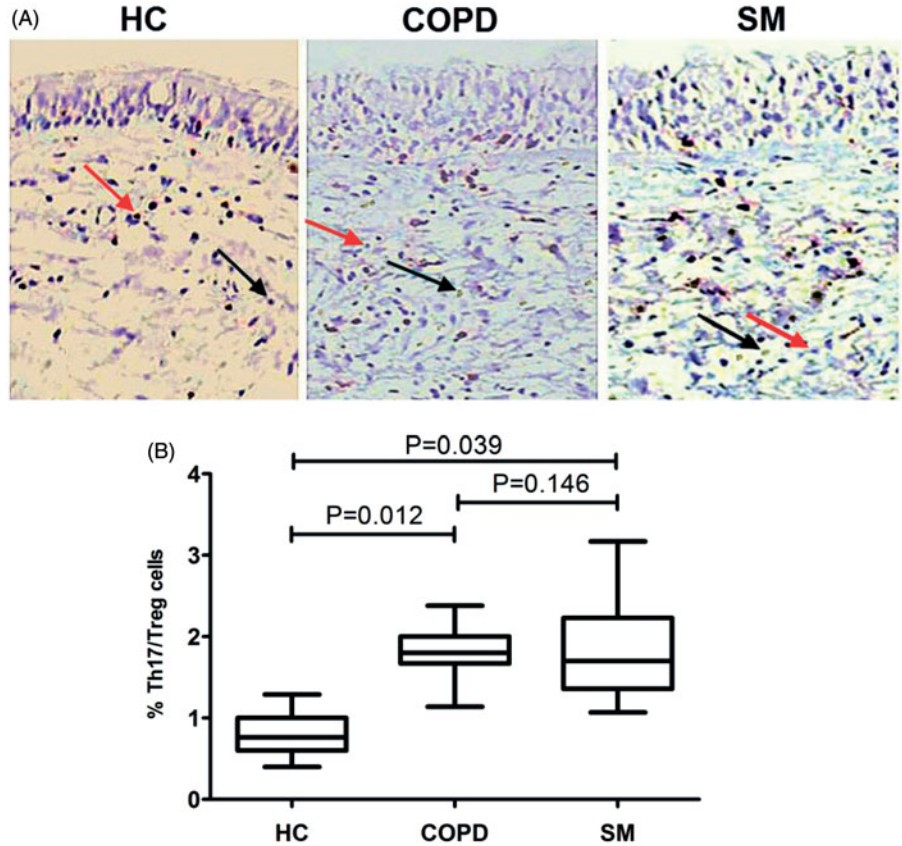
Foxp3) may cause loss of the suppressive function of Treg cells in the presence of high levels of IL-1β and IL-6 (11,58). Therefore, because of the high expression rate of IL-1β and IL-6 cytokines in SM lung (59–61), prolonged exposure of Treg to these cytokines may lead to conversion to Th17 cell and paralyze its suppressive function (62,63). Additionally, high concentrations of IL-6, IL-23 and IL-1β cytokines in inflammation area may inhibit Foxp3 expression. Then, Treg converts to producing IL-17 cells and no longer could retain its suppressive function. This mechanism has also been well characterized in COPD (64) and IBD (63). Also, another study highlighted that one subtype of human Tregs with CD25^{++}CD45RA^{−} cytokine-secreting (Fr III) phenotype has a pro-inflammatory function (37). This finding may also be true for our results.

The Th17/Treg ratio was also analyzed here to display Th17/Treg population balancing. As expected, this ratio was approximately 1:1 in the HC group indicating a stable situation and homeostasis in lung immune system, in line with previous reports (10,22,58,65). Interestingly, we observed that this ratio was disturbed in SM and COPD groups. It both groups, although two subsets of T cells were increased in the bronchial biopsies, Treg cells did not reach to Th17 cells level. Imbalances in Th17/Treg have been found in most other pulmonary inflammatory diseases, such as COPD and asthma (10,58). This imbalance status is present in the chronic phase of mustard lung disease and, more importantly, and has been associated with increased exacerbation phases (22,25,65–67). Accordingly, the lung injury and pulmonary alveolar destruction outcomes have been positively correlated with the frequency of Th17 cells and negatively with the frequency of Treg cells (10,65,66).

The circulating Treg and Th17 cells number in patients with COPD and SM were increased. But most importantly, in patients exposed to SM, the frequency of Treg and Th17 was increased ∼2-fold (8.71/4.95) and ∼3.5-fold (1.028/0.371), respectively, compared to HC. Despite spirometry indices being almost similar in both groups, the Th17 and Treg populations of patients exposed to SM was higher than the COPD group. During migration of the dendritic cells into the T cells area of lymph nodes and maturation, they are able to induce the naive T cells to differentiate to effector cells such as Th17 or Treg cells. Then, these effectors cells immigrate to inflamed or injured tissues via blood vessels (10,58,63). So, an increased level of Th17 and Treg in PB may be due to the recirculation of Treg and Th17 from lung lymph nodes toward injured lung tissues. At first glance, it seems that immune cells were recruited and employed in mustard lung to heal injuries and repair. But as we found, these immune cells act as the foe in the microenvironment and induce more injury and inflammation. This repeated injury and repair can subsequently create a ''vicious cycle'' in mustard lung tissue and lead to tissue remodeling. So it seems that Th17/Treg imbalance plays a role in this vicious cycle.

In summary, this study results demonstrate that Th17/Treg functional imbalance exists in SM-exposed patients and the significantly elevated CD4^{+}IL-17^{+} Th17 cells are accompanied by CD4^{+}Foxp3^{+} Treg cells increase and correlated with pulmonary function, suggesting a potential role in the immunopathogenesis of chronic phase of mustard lung disease. Of note, the correlations between the Th17/Treg ratio and FEV1% existed in lung tissue, and to a lesser extent, in PB, indicating a closer correlation between loss of lung
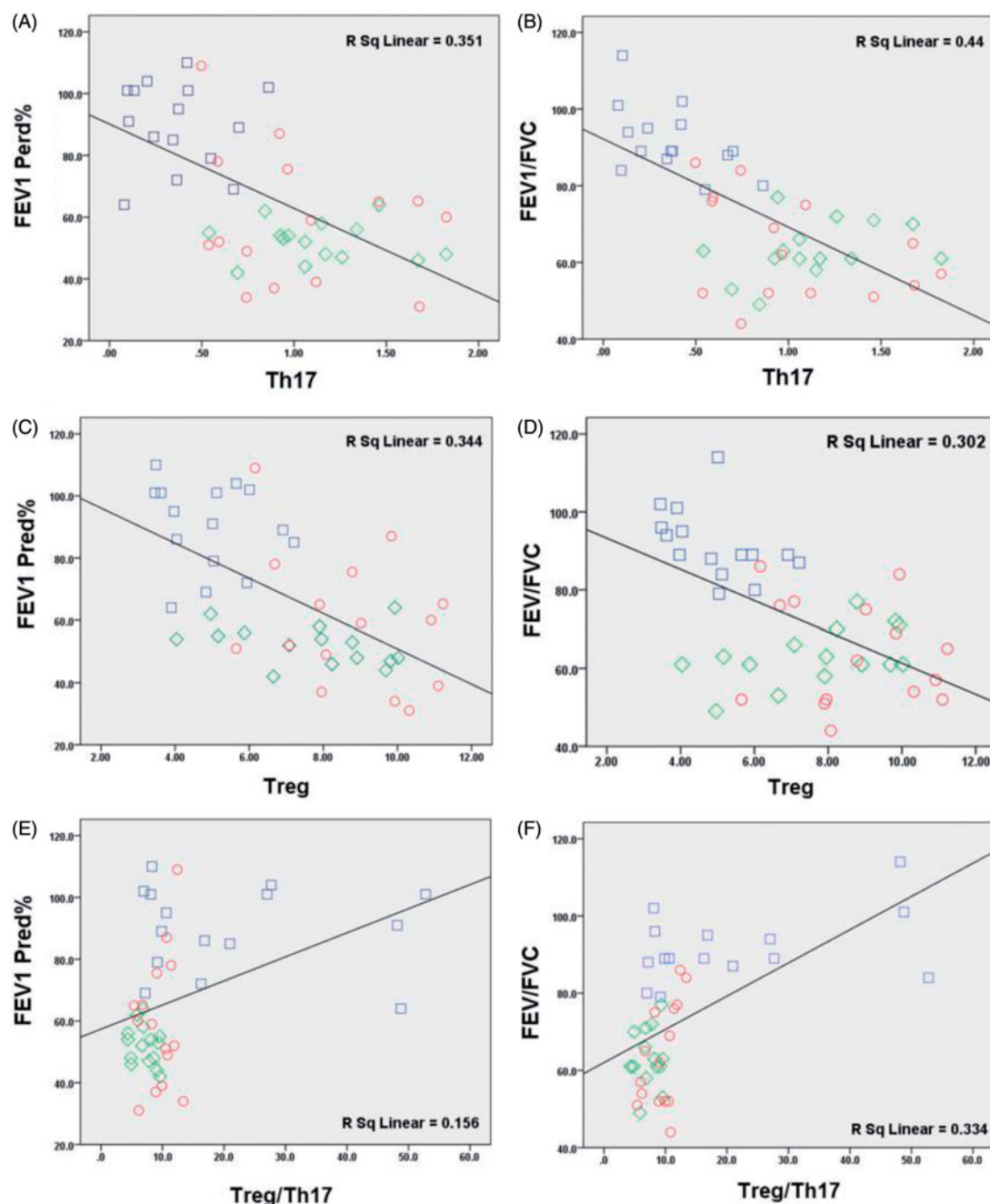
Figure 5. Correlations. Correlations between Th17 and FEV1%pred (A), Th17 and FEV/FVC (B), Treg and FEV1%pred (C), Treg and FEV/FVC (D), the ratio of Treg/Th17 and FEV1%pred (E), and the ratio of Treg/Th17 and FEV/FVC (F). Data indicate positive associations in (E) & (F) and an inverse association in (A), (B), (C) and (D). Squares represent the HC, and diamond indicates COPD, and circles represent SM patients. Data were analyzed by Spearman's rank correlation coefficient. In general, it was observed significant correlation between the imbalances of Th17/Treg and pulmonary function in the SM and COPD patients.

function and local immune activation. The results of the present study contribute to a better understanding of the $CD4^+ Foxp3^+$ Treg and $CD4^+$ IL-17$^+$ Th17 cells-related pathogenesis and progression of mustard lung disease in chronic phase. Despite of the current findings, further work is needed to explore the underlying mechanisms.

Declaration of interest

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