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Th17/Treg Ratio in COPD Patients with Normal and High Pulmonary Arterial Hypertension

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ABSTRACT

The airflow limitation is one of the characteristics of chronic obstructive pulmonary disease (COPD), which is not entirely reversible. It seems that factors such as inflammation, hypoxia, and remodeling of pulmonary vessels can increase pulmonary hypertension (PH). This increase in pulmonary arterial hypertension leads to aggravation of disease complications.

Considering the role of immune cells in causing pathological inflammation in the pathogenesis of COPD, it seems that Th17/Treg axis imbalance can be one of the main reasons for the difference in life expectancy in patients with COPD with and without PH.

By measuring and comparing some inflammatory biomarkers in patients with COPD with and without PH, this study tries to introduce these biomarkers to predict the occurrence or nonoccurrence of this complication. This study aims to compare the ratio and activity of Th17 to Treg in patients with COPD with high (20 patients) and normal (20 patients) pulmonary arterial pressure. Five milliliters of blood containing anticoagulant were obtained to isolate peripheral blood mononuclear cells (PBMCs). Then, the ratio of Th17 to Treg, their number, and their activity were evaluated by ELISA, real-time polymerase chain reaction (PCR), and flow cytometry.

Our results show that the amount of inflammatory factors and the population of Th17 cells in patients with COPD with PH is associated with a significant increase in PH compared to patients with COPD without PH, which leads to damage caused by pathological inflammation to the lung tissue and a decrease in the overall survival of the patients.

Keywords: Chronic obstructive pulmonary disease; Th17; Inflammation; Pulmonary hypertension; Treg

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INTRODUCTION

The primary characteristic of chronic obstructive pulmonary disease (COPD) is airflow limitation, which is not entirely reversible. COPD was the third cause of death in the world in 2020.[1] This disease can lead to injuries and complications that not only affect the quality of life of patients but also impose a substantial economic burden on the health system, and ultimately, there will be high mortality due to complications.[2] One of the complications caused by COPD is an increase in pulmonary artery pressure, which occurs in a high percentage of patients.[3] There are several pathophysiological causes for this condition, including airflow limitation, emphysema, alveolar hypoxia, hyperkalemic acidosis, polycythemia, systemic inflammation, and lung inflammation.[4]

Various inflammatory factors in the course of COPD are responsible for systemic inflammation, which causes extrapulmonary manifestations such as increased pulmonary arterial pressure, followed by right heart failure in patients, worsens the course of the disease, and is also associated with the prevalence of complications and increased mortality in these patients.[3,5] According to the 6th Symposium on Pulmonary Hypertension, PH is the mean pulmonary arterial pressure of more than 20 mmHg at rest position.[6,7] However, it has been recommended that an average pulmonary artery pressure higher than 25 mmHg is considered high pulmonary arterial pressure, and a pressure of 21–24 mmHg should be decided based on the condition of each patient.[8] PH has also been shown to be associated with COPD exacerbations and appears to play an essential role in this disease.[9,10]

Structural and functional changes in pulmonary arteries in normoxic patients with COPD are also observed during the early stages of the disease. Therefore, chronic inflammation can be considered one of the main factors in changing the shape of pulmonary vessels.[11,12] Suppose there is a relationship between the level of systemic inflammatory factors and indicators, including C-reactive protein (CRP) and interleukin 17 (IL-17), IL-22, IL-23, IL-35, transforming growth factor β (TGF-β), and Th17/Treg ratio.[13] In that case, a higher level of these proteins and inflammatory factors are expected to be present in patients with PH compared to patients without pulmonary arterial hypertension secondary to COPD.[14]

The severity of COPD is divided into 4 stages based on the Global Initiative for Chronic Obstructive Lung Disease (GOLD) criteria.[15] If it is possible to identify patients at risk of developing pulmonary arterial hypertension in the early stages and, as a result, provide them with the necessary treatment, the development of these complications can be reduced or postponed. Increased PH is a sign of poor prognosis in COPD, which affects the quality of life and mortality of patients.[16] The prevalence of elevated PH in COPD is unknown, but it ranges from 20% to 91% across studies. PH seems to be more common in patients with COPD living in high-altitude areas.[17] Recent studies indicate that pathways associated with hypoxia and inflammation significantly contribute to the development of COPD-PH. Key hub genes, such as *CXCL9* and *CXCL12*, along with inflammatory cytokines, may serve as potential diagnostic or therapeutic biomarkers for COPD-PH.[18] Patients with COPD-PH exhibit a more distinctive inflammatory profile compared to patients with COPD alone. Notable increases in certain inflammatory cytokines have been observed, which correlate with the severity of PH. The inflammatory patterns in COPD-PH may indicate specific molecular mechanisms involved in the development of PH.[19] These findings can lead to further research aimed at understanding the disease mechanism and developing targeted therapies. Consequently, investigating the inflammatory pathways involved is crucial.

Investigating the effect of PH on the number and function of Th17/Treg cells in patients with PH and patients without PH (WPH) can determine the role of these cells and PH in the progression of this disease. Based on data from in vitro studies, the proportions of immune system cells can be determined, enabling more appropriate treatments to reduce inflammation in these patients. This project was conducted to compare the ratios of Th17 to Treg cells in patients with COPD with high and normal PH to identify effective treatment strategies for this group of patients.

MATERIALS AND METHODS

Study Design

Forty patients diagnosed with COPD, according to the guidelines of the European Respiratory Society (ERS) and the American Thoracic Society (ATS), who visited the clinic at Imam Reza Hospital in Tabriz from April 1, 2021, to October 31, 2021, were included in the

study. Eight milliliters of blood containing an anticoagulant was collected from 20 patients with COPD with and 20 without PH to isolate peripheral blood mononuclear cells (PBMCs). Venous blood gas (VBG) measured partial pressure of carbon dioxide ($PaCO_2$) and pH. All patients with COPD participating in this study were stable, and COPD exacerbation was an exclusion criterion. PH was diagnosed in accordance with the updated 2022 ESC/ERS Guidelines, defined as a mean pulmonary artery pressure (mPAP) of ≥20 mmHg at rest, measured by right heart catheterization. Patients with mPAP <20 mmHg were classified as non-PH controls. The present study has been approved by the Ethics Committee of Tabriz University of Medical Sciences (IR.TBZMED.REC.1400.446). This study fully complied with all items of the Declaration of Helsinki and the ethical codes of the National Medical Ethics Committee.

Echocardiography

Echocardiography was performed in the patients after checking peripheral blood oxygen saturation with pulse oximetry and determining COPD severity, according to the GOLD criteria. Pulmonary arterial systolic and diastolic pressure was determined with the help of tricuspid regurgitant (TR) and pulmonary insufficiency (PI) gradients, plus right atrium (RA) pressure. Pulmonary artery systolic pressure was determined based on TR velocity and gradient, and pulmonary artery diastolic pressure was determined based on PI velocity and gradient. The proper atrial pressure was calculated from inferior vena cava diameter measurements and their respiratory changes, and the TR gradient was added to the RA pressure obtained above to obtain the pulmonary artery pressure. Mean arterial pressure (MAP) can be estimated using a formula that doubles the diastolic blood pressure and adds it to the systolic blood pressure. The composite sum is then divided by 3. Patients with non-sinus rhythm or poor view were excluded from the study.

Spirometry

We used spirometry to confirm the diagnosis and establish COPD staging based on the GOLD criteria.

Venous Blood Gas

VBG was performed to measure the acid-base balance (pH levels) and the PCO_2.

Cytokine Quantification in the Supernatants of PBMCs

The concentrations of Treg-related anti-inflammatory (IL-10, IL-35, and TGF-β) and Th17-related inflammatory (IL-17, IL-22, and IL-23) cytokines, and CRP, were measured in serum from patients with COPD-WPH and COPD-PH, and healthy individuals using an enzyme-linked immunosorbent assay (ELISA) kit (MyBioSource) according to the manufacturer's instructions.

RNA Isolation and Complementary DNA Synthesis

The samples were processed to determine the mRNA levels of Treg/Th17 cytokines and related genes, including *FOXP3*, *RORγt*, *IL-10*, *IL-17*, *IL-22*, *IL-23*, *IL-35*, and *TGF-β*. According to the manufacturer's method, total cellular RNA was extracted from cultured peripheral blood cells and PBMCs from 2 groups of patients (with high and normal pulmonary arterial pressure) using Qiagen's RNeasy Mini Kit for reverse transcription PCR (RT-PCR). For comparison, RNA extracted from untreated cells was used as a control. RNA was then reverse-transcribed into complementary DNA for amplification using complementary DNA synthesis kits (Thermo Fisher Scientific).

Real-time Quantitative Polymerase Chain Reaction

The expression of 8 genes involved in the inflammatory pathways was profiled. The purity of the extracted RNA was estimated using a UV spectrophotometer to determine the A260/280 and A260/230 ratios. Quantitative analysis of mRNA expression was performed using a SYBR Green real-time PCR assay, and the LightCycler real-time PCR instrument (Roche Molecular Biochemicals) was used to detect fluorescence. cDNA (5 µL) was added to each well of a 96-well plate, resulting in a total reaction volume of 10 µL containing 8 µL of SYBR Green and 0.5 µM of primers. A melt curve was performed at the end of the PCR. As a housekeeping gene, *β-actin* was used as an internal control, and the amount of all mRNA targets in test samples was normalized to the corresponding *β-actin* transcript. The PCR reaction was set up in duplicate for each sample.

Flow Cytometry Analysis

After isolation of $CD4^+$ T cells, the percentages of $CD4^+IL\text{-}17^+$ Th17 and $CD4^+CD25^+FOXP3^+$ Treg cells were assessed in PBMCs by flow cytometry. 5×10^6 of

PBMCs were washed with phosphate-buffered saline (PBS) followed by a 5-hour incubation with phorbol-12-myristate-13-acetate (PMA) (50 ng/mL) plus ionomycin (0.5 mM) at 37 °C in a 5% CO_2 humidified incubator. The cells were stimulated with monensin and stained with fluorescence-labeled antibodies against surface and intracellular markers. The mean fluorescence intensity was measured by fluorescence activated cell sorting (FACS) and analyzed by FlowJo software.

Statistical Analysis

The Shapiro-Wilk test was used to assess whether the data were normally distributed. To examine the statistical differences in immunologic parameters between the healthy control group and the COPD patient group, and between pretreatment and posttreatment patient groups, the unpaired Student *t* test and the paired Student *t* test were used. Also, a 1-way analysis of variance (ANOVA) test was used for multiple comparisons. The scale data were reported using mean ± standard deviation (SD), and a $p<0.05$ was considered statistically significant. GraphPad Prism (version 8.00) was used to create the graphs.

RESULTS

Clinical Manifestations

In Table 1, among the variables including smoking pack-years, forced expiratory volume in the first second (FEV1)/forced vital capacity (FVC) (mean ± SD), leukocytes ($\times10^9$/L), lymphocytes ($\times10^9$/L), and granulocytes ($\times10^9$/L) in healthy controls, COPD without PH, and COPD with PH had statistically significant differences ($p<0.05$). The numbers of leukocytes, lymphocytes, and granulocytes were significantly lower in healthy people than in the other 2 groups (COPD without PH and COPD with PH). Also, in the COPD group, PH was significantly lower than in the COPD group with PH. Also, the level of CRP in patients with COPD with and without PH was significantly higher than that of healthy people, indicating inflammation in patients with COPD compared to healthy people (Table 1).

Table 1. Summary of study results in healthy participants, COPD-PH patients, and COPD-WPH patients.

Item	Healthy Controls (n=20)	COPD Without PH (n=20)	COPD With PH (n=20)	*P**	*P***	*P****
Age, years	62.3±6.6	63.2±9.5	64.2±10.8	0.618	0.435	0.772
Gender (Male/Female)	12/8	11/9	12/8			
BMI (kg/m^2), mean±SEM	23.5±1.54	22.9±1.25	23.1±2.1	0.651	0.794	0.907
History of smoke, n (%)	9 (45)	13 (65)	7 (35)			
History of bakery (biomass fuels exposure)	0	5(25)	11(55)			
Smoking pack-years	6.12	35.80	47.45	<0.001	<0.001	<0.001
FEV1/FVC (mean±SEM)	84.1±1.75	59.5±3.45	44.7±4.26	<0.001	<0.001	0.008
FEV1% (% predicted), mean±SD	93.235±6.8	47.4±9.5	48.3±9.2	<0.001	<0.001	0.570
Leukocytes ($\times10^9$/L)	6.7 (4.5; 7.45)	7.5 (6.2;10.1)	9.4 (7.4;15.21)	<0.001	<0.001	<0.001
Lymphocytes ($\times10^9$/L)	2.35 (1.25; 3.45)	2.5(1.75; 3.9)	4.35 (1.7; 6.9)	0.004	<0.001	<0.001
Granulocytes ($\times10^9$/L)	3.22 (1.58; 4.45)	4.22 (1.98; 6.9)	6.5 (2.6; 9.5)	0.001	<0.001	<0.001
CRP	2.35±1.11	14.4±2.1	17.23±1.23	<0.001	<0.001	0.08
PaCO2 (mmHg)	41.4±5.3	47.4±7.3	49.4±6.5	0.04	0.02	0.5
HCO3 (Meq/L)	24.5±2.11	23.2±3.2	22.9±2.6	0.8	0.8	0.9
PaO2(mmHg)	92.5±3.7	78.3±7.8	76.5±8.3	0.009	0.005	0.3
pH	7.41±0.032	7.39±0.042	7.38±0.028	0.875	0.791	0.691

Ratio of Th17/Treg

As mentioned, given the role of T cells and the importance of the Th17/Treg branch, flow cytometry was used to count these cells in healthy individuals and 2 patient groups, using PBMCs isolated from patients' blood samples. As seen in Figure 1, the percentage of Treg cells in patients with COPD with PH is not significantly different from that of patients with COPD without PH. The number of Treg cells in patients with COPD with PH is significantly lower than in healthy people (2.883% vs 4.518%; p=0.002). However, as mentioned, there was no significant change in Treg proportion between the healthy group and the COPD group without PH, or between the COPD group with PH and the COPD group without PH.

In addition, the percentage of Th17 cells in patients with COPD with PH is significantly higher than in patients with COPD without PH (5.280% vs 3.625%; p=0.005) and in healthy controls (5.280% vs 2.338%; p<0.001) (Figure 2). The percentage of these cells in patients with COPD without pulmonary hypertension (WPH) compared to healthy individuals was significantly different (3.625% vs 2.338%; p<0.001). Therefore, considering the role of Th17 cells in the pathogenesis of airway-related diseases and the importance of Treg cells in regulating inflammatory responses, the increase in Th17 cells following a decrease in Treg cells can lead to harmful inflammatory reactions in the airways. However, the increase in cell numbers alone cannot reflect the functions of these cells; that's why we have continued to investigate the molecular functions of Treg and Th17 cells.

Treg and Th17-related Gene Expression

Genes associated with Treg and Th17 functions were selected to investigate the roles of these cell subsets. The expression levels of *FOXP3*, *IL-10*, *IL-35*, and *TGF-β* genes were evaluated by real-time PCR to investigate Treg functions, and those of *IL-17*, *IL-22*, *IL-23*, and *RORγt* to investigate Th17 functions. RORγt and FOXP3 are specific transcription factors for each T-cell subgroup and are responsible for some of these cells' functions.

After extracting total RNA and converting it to cDNA, the expression level of the target genes was quantified using previously designed primers. The expression level of *RORγt* in PBMCs isolated from patients with COPD with high PH and those with low PH was reported as 0.47 ± 1.57 (p=0.004) and 0.16 ± 1.23 (p=0.004), respectively, indicating increased expression of this transcription factor in patients. Considering the role of RORγt in the production of cytokines associated with Th17, the expression levels of *IL-17*, *IL-22*, and *IL-23* are significantly increased in patients with COPD. The expression level of *IL-17* in COPD-PH compared to the control group was 1.59 ± 0.48 (p<0.001), and this level was reported for patients with COPD without PH at 1.28 ± 0.15 (p<0.001). *IL-22*, as another inflammatory cytokine responsible for the functions of Th17 cells, showed increased expression in both COPD-PH (1.55 ± 0.30; p<0.001) and COPD-WPH groups (1.29 ± 0.14; p<0.001) compared to the control group. *IL-23* expression was also significantly increased for COPD-PH (1.46 ± 0.71; p<0.001) and COPD-WPH patients (1.29 ± 0.12; p<0.001) compared to healthy controls (Figure 3).

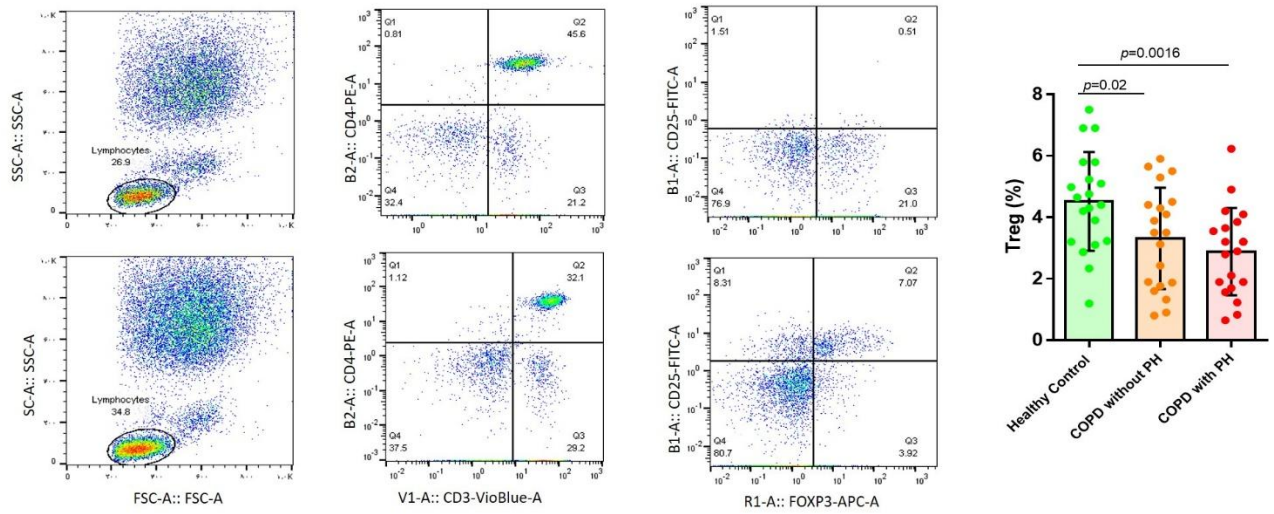

Figure 1. Treg cell populations in PBMCs of COPD patients with normal and high pulmonary arterial pressure. A gating strategy to investigate the frequency of Treg cells in PBMCs of different groups is shown. The number of Treg cells in the COPD-WPH group is associated with a significant decrease compared to the healthy control group. This reduction was also observed in the comparison between the healthy group and COPD-PH, and the percentage of Treg cells in the COPD-PH group is lower than in the COPD-WPH group.

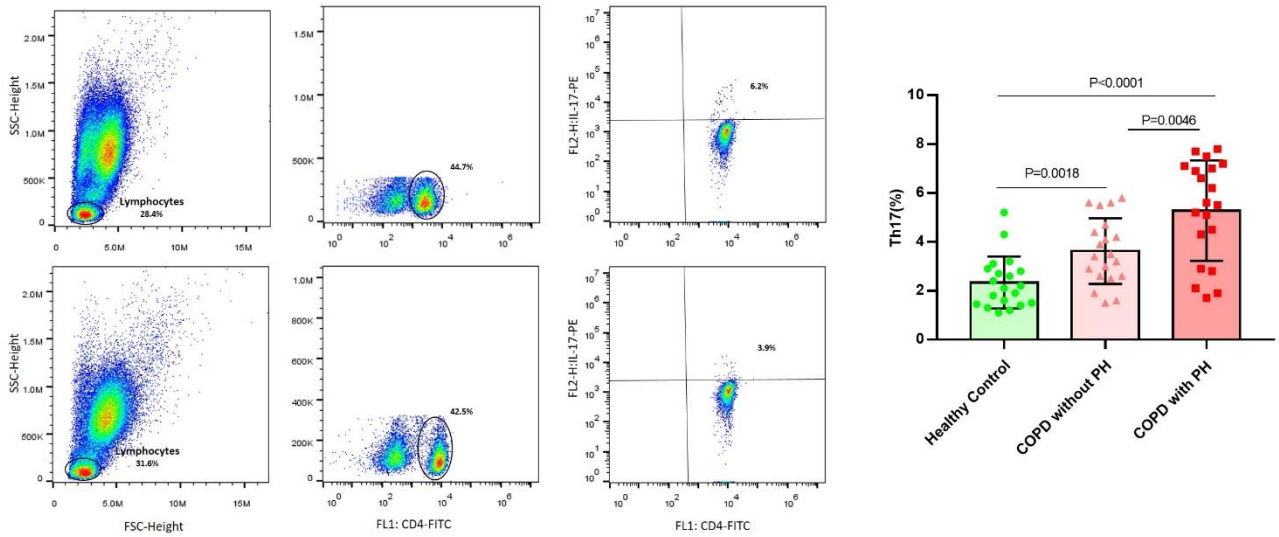

Figure 2. Th17 cell populations in PBMCs of COPD patients with normal and high pulmonary arterial pressure. A. A gating strategy to investigate the frequency of Th17 cells in PBMCs of different groups is shown. B. The number of Th17 cells in the COPD-WPH group is associated with a significant increase compared to the healthy control group. This increase was also observed in the comparison between the healthy group and COPD-PH, and the percentage of Th17 cells in the COPD-PH group is lower than in the COPD-WPH group. C. The number of Th17 cells in the COPD-PH group was associated with a significant increase compared to COPD-WPH and healthy controls. This shows an increase in the ratio of Th17/Treg in the COPD-PH group, which reveals the role of inflammation in the pathogenesis of this disease.

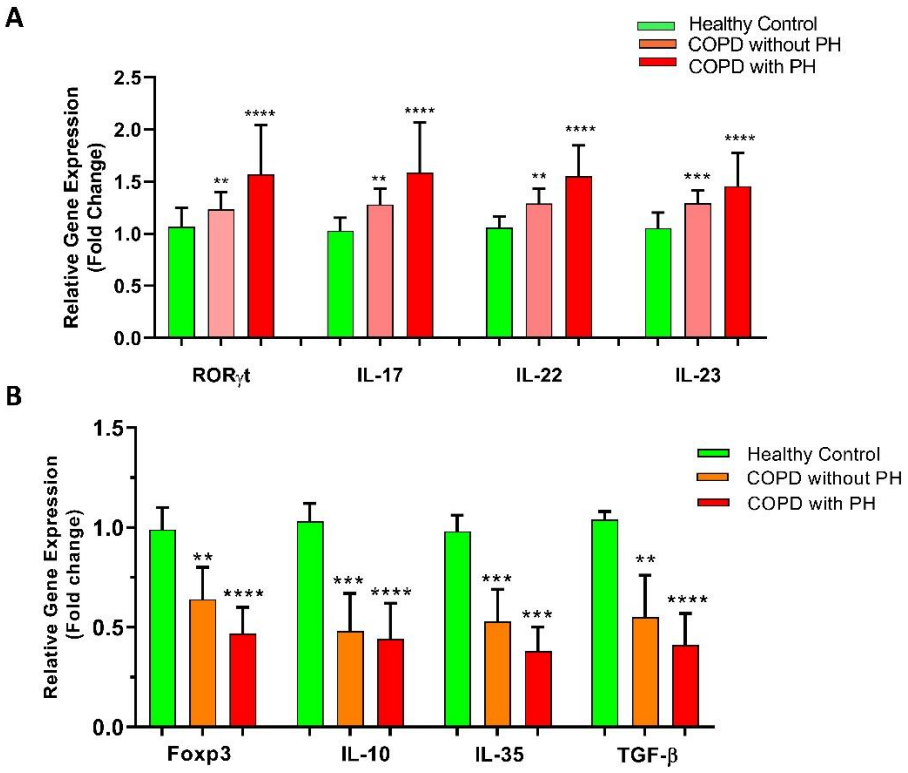

Figure 3. Expression level of important candidate genes in the differentiation and induction of Th17 and Treg cells. A. Important genes, including the gene related to the transcription factor of Th17 cells (*RORγt*), as well as genes related to the functions of this cell, including cytokines *IL-17*, *IL-22*, and *IL-23*, show the increased expression of these genes in PBMCs isolated from COPD-PH patients. B. Genes related to the differentiation (*FOXP3*) and function (*IL-10*, *IL-35*, and *TGF-β*) of Treg cells also show a significant decrease in the differentiation and function of these cells.

Cytokine Concentration in Serum

Considering the critical role of cytokines in immune cell function, the primary inflammatory factor, CRP, and cytokines produced by Th17 cells (IL-17, IL-22, and IL-23) and Treg cells (IL-10, TGF-β, and IL-35) were evaluated in serum across different groups. As shown in Figure 4A–C, the concentration of Treg cell cytokines, including IL-10 (p=0.02) and TGF-β (p=0.002) within the COPD-WPH group, is associated with a significant decrease compared to healthy controls. However, this reduction was not observed when comparing the control group and COPD-WPH for IL-35. Additionally, the healthy controls and COPD-PH comparison show a significant reduction in all cytokines. However, the decrease in anti-inflammatory cytokine concentration in the COPD-PH group appears greater than in the COPD-WPH group, but this difference was insignificant.

As shown in Figure 4D–F, for cytokines associated with Th17 cells, including IL-17, IL-22, and IL-23, their values are significantly higher (all p<0.001) in the COPD-WPH group compared to the healthy control group. Also, comparing serum cytokine concentrations between COPD-WPH and COPD-PH patients showed significant increases in IL-22 (p=0.01) and IL-23 (p=0.04) in the COPD-PH group compared to the COPD-WPH group. Meanwhile, serum IL-17 concentrations in the 2 COPD-WPH and COPD-PH groups did not differ significantly. As mentioned, the levels of 2 cytokines, IL-22 and IL-23, increased significantly in the COPD-PH group, indicating that these cytokines could serve as biomarkers distinguishing between COPD-WPH and COPD-PH patients.

In addition, the results of the CRP concentration examination across different test groups show that its level has increased significantly in patients with COPD (Figure 5), both in the groups with and without PH

(p<0.001). Also, in COPD-PH patients, serum CRP levels were significantly higher than in COPD-WPH patients. Since CRP is an important proinflammatory mediator produced by the liver, it seems this factor also plays an essential role in the inflammation and pathology of COPD.

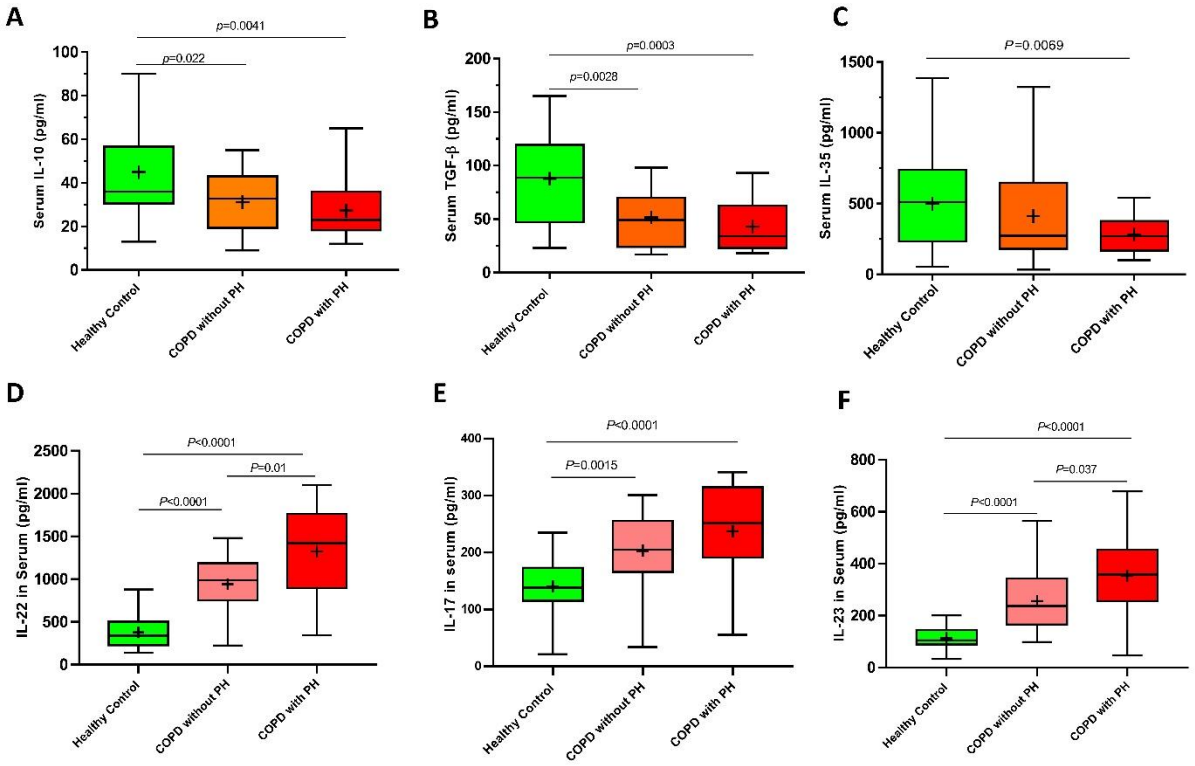

Figure 4. Concentration of cytokines related to the functions of Treg and Th17 cells in patients' serum pneumonia. As shown, the amount of cytokines related to the functions of Treg cells (A–C) in COPD-PH patients was significantly reduced compared to the control group. Also, the amount of cytokines associated with Th17 functions (D–F) shows an increase in the function of Th17 cells in COPD-PH patients compared to COPD-WPH patients and the healthy control group.

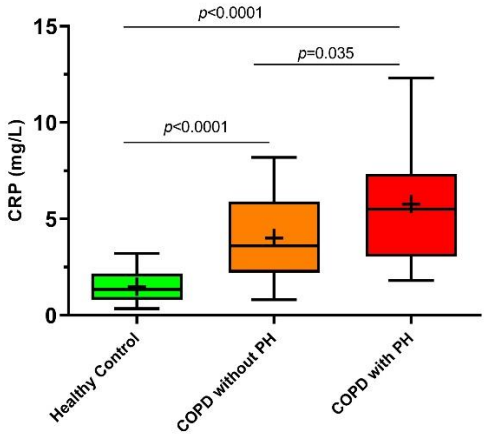

Figure 5. Concentration of C-reactive protein (CRP) in different groups. In COPD-PH patients compared to control and COPD-WPH patients the serum level of CRP was associated with a significant increase.

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DISCUSSION

Lymphocytes and cytokines produced by them play an important role in the pathophysiology of diseases, and the number and activity of immune system cells, along with environmental factors, are essential in the development of various diseases, especially inflammatory diseases like COPD.[20,21] The cells involved in this disease include Th1, Th2, Th17, and Treg types of B lymphocytes, as well as other inflammatory cells such as macrophages, neutrophils, and natural killer (NK) cells.[22] These cells play a significant role in the pathogenesis of this disease by producing mediators and cytokines.[23] Treg cells play an anti-inflammatory role and maintain tolerance to self-antigens.[24] Most Treg cells differentiate in the thymus and are called natural Treg (nTreg). Treg cells may also arise in peripheral tissues from the differentiation of naïve T cells called inducible Treg (iTreg).[25] The expression of markers, such as CD4, CD25, CTLA-4, and GITR characterizes Treg cells.[26] The differentiation of Treg cells from naïve TCD4$^+$ cells depends on the presence of TGF-β, which causes the expression of the FOXP3 transcription factor. FOXP3 plays a vital role in the development and function of regulatory T cells, regulating the immune system, especially in the tolerance to self-antigens and in the inhibition of autoimmune disease.[27] These cells help inhibit other immune cells by producing immunoregulatory cytokines and direct cell-to-cell interaction. IL-10 is a pleiotropic, immune-regulating cytokine that protects the host against immune damage caused by infections, autoimmunity, and allergy.[28] IL-10 was initially characterized as a Th2-specific cytokine; however, further research revealed that IL-10 production is also associated with Treg cell responses.[29]

Also, various studies have shown that these cells can play a role in lung diseases, including COPD. COPD is characterized by a persistent inflammatory process of the airways that leads to airflow obstruction or restriction and is mainly associated with exposure to air pollution and cigarette smoke.[30-32] In addition, it is currently considered a severe public health problem and is the fourth leading cause of death worldwide. Many immune cells participate in the pathophysiology of COPD, the most important of which are neutrophils, macrophages, and CD4$^+$ and CD8$^+$ T cells.[33,34] Migration of neutrophils to the inflammatory site can be mediated mainly by cytokines associated with CD4$^+$ Th17 lymphocytes, as IL-17A, IL-17F, and IL-22 have been shown to stimulate the secretion of CXCL8, CXCL1, CXCL5, G-CSF, and GM-CSF by airway epithelial cells.[35] The goals of these molecules are to differentiate, proliferate, and recruit neutrophils. These cells increase in the respiratory tract during COPD exacerbations. Therefore, increases in the number of neutrophils and macrophages in the airways, as well as in proinflammatory cytokines, are directly related to the severity of the exacerbation, underscoring the importance of the Th17 profile in this disease.[36] Th17 cells are important mediators of tissue damage in immune-mediated inflammatory diseases, such as inflammatory bowel disease, and in cardiovascular diseases, including systemic hypertension.[37] Th17 cells act by attracting neutrophils, stimulating the release of matrix metalloproteinases, and increasing the release of factors from quiescent cells. The differentiation of Th17 cells primarily depends on high levels of IL-6. An increase in the number of circulating Th17 cells has been reported in COPD and pulmonary arterial hypertension patients.[38,39]

Humbert et al[40] showed that the serum levels of IL-1β and IL-6 increase in severe primary PH, and proinflammatory cytokines play a role in this disorder. The result of this study was consistent with the result of our research. In our study, a statistically significant relationship was seen between these cytokines and the presence of PH. The results and figures show that the number of Th17 cells in PBMC isolated from patients with COPD is significantly higher than in healthy controls. Additionally, the number of these cells is significantly higher in patients with PH than in those without PH. Also, evaluating the function of these cells by measuring the cytokine levels in the serum confirmed the above results.

In the study of Joppa et al,[41] increased pulmonary artery pressure in patients with COPD is associated with increased serum levels of CRP and TNF-α, raising the possibility of the role of low-grade systemic inflammation in the pathogenesis of PH in these patients, which shows similar results and confirms the results of our study. In the study by Ahmed et al,[42] serum CRP and TNF-α levels were significantly higher in patients with elevated pulmonary artery pressure than in those without, a finding also observed in our study.

Considering that inflammation and cytokines can affect different populations of the immune system, investigating immune cell populations and their ratios can

be effective for understanding the pathogenesis of these diseases. In Yang et al's study,[43] an imbalance between Treg cell subpopulations was also shown in people with COPD after an exacerbation. Yang et al found an increased percentage of Treg cells secreting cytokines and a decreased percentage of resting Treg cells in patients with stable COPD. In our study, the number of Tregs in healthy people is significantly higher than in the other 2 groups, and in the COPD group without PH, it is significantly higher than in the group with PH.

Hou et al[44] evaluated the proportion of Treg cell phenotypes (rTregs, aTregs, and Treg-Frll) in peripheral blood and bronchoalveolar lavage of nonobstructive smokers, nonsmokers, and subjects with stage II and III COPD. Blood samples showed a decrease in the rTreg-to-aTreg ratio and an increase in Treg-Frll in COPD subjects compared with smokers without obstructive disease, consistent with our results. These findings show that the imbalance between anti-inflammatory subpopulations (aTreg+rTeg) and proinflammatory subpopulations (Treg-Frll) of Tregs plays an essential role in the progression of COPD. Therefore, considering the role each of these immune cells plays, it has been shown that the ratio of Th17 to Treg cells is altered from the normal state in many lung-related diseases.[45]

In the study by Li et al,[46] the ratio of Th17 to Treg cells in PBMCs from participants with and without COPD was analyzed to determine whether this ratio is a marker of COPD progression. The proportions of Th17 and Treg cells among $CD3^+/CD4^+$ T cells differed significantly between COPD and non-COPD groups, as confirmed in our study ($p<0.05$). Also, in this study, the levels of IL-17 and TGF-β1 were determined using ELISA, and their concentration significantly differed between groups. In our study, the level of TGF-β in healthy controls is significantly higher than in the other 2 groups (COPD-PH and COPD-WPH), and in the COPD-WPH group, it is significantly lower than in the COPD-PH group. Additionally, the level of IL-17 in the 3 investigated groups was significantly lower in healthy individuals compared to the other 2 groups, and in the COPD-WPH group, it was significantly higher than in the COPD-PH group.

In the study by Zhu et al[47] to evaluate the role of Th17 cells in COPD-PH patients, the frequency of Th17 cells was first investigated using IL-17 expression. Directly in vivo and in the absence of stimulation, a small number of IL-17-circulating $CD4^+$ T cells were found. Therefore, to improve detection, circulating $CD4^+$ T cells were activated using the anti-CD3 (OKT3) and the anti-CD28 antibodies.[47,48] The frequency of IL-17-secreting cells in each participant was evaluated using ELISpot, and it was significantly higher in COPD-PH patients than in healthy subjects, consistent with our results. In the study of Hautefort et al,[49] it has been shown that PH caused the activation and proliferation of $CD4^+$ T cells, which was accompanied by a decrease in the expression of IL-4 (Th2 response) and a higher expression of IL-17. Finally, there was significant hypomethylation of the *IL-17* promoter in COPD-PH-derived PBMCs compared to the control group. In our study, IL-17 secretion from COPD-PH $CD4^+$ T cells was significantly higher than that of $CD4^+$ T cells derived from healthy control. Also, the expression of TGF-β as a main cytokine of Treg[50] in the control group was significantly higher than in COPD-PH patients, which is consistent with our study.

By showing upregulation of Th17 response and downregulation of Treg cell response, the expression of transcription factors, including Th17 transcription factor RORγt[51] and Treg-lineage transcription factor FOXP3[52] in $CD4^+$ T cells, was investigated. In vivo, and following CD3/CD28 activation in ex vivo, *RORγt* expression in $CD4^+$ T cells was significantly higher in the COPD-PH group than in controls. In contrast, *FOXP3* expression in the COPD-PH group was significantly lower compared to COPD-PH controls. Upregulation of Th17 responses, including *IL-17, IL-22, IL-23*, and *RORγt* expression, was associated with downregulation of Treg responses, including *TGF-β, IL-10, IL-35*, and *FOXP3* expression. Overall, the findings here indicated that an imbalance in the ratio of Th17/Treg cells is a hallmark of COPD-PH.

The results of our study show that there is a significant increase in the frequency and function of Th17 cells as inflammatory cells in the COPD-PH group compared to the COPD-WPH group. Also, the frequency and function of Treg cells in the COPD-PH group had a significant decrease compared to the COPD-WPH group. Therefore, it seems that the imbalance in the Th17/Treg axis is one of the main factors that affect the quality of life and mortality of COPD-PH and COPD-WPH patients.

STATEMENT OF ETHICS

This study was conducted with the ethics code (IR.TBZMED.REC.1400.446) at Tabriz University of Medical Sciences.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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Not applicable.

DATA AVAILABILITY

Upon reasonable request (ahmadi.m@tbzmed.ac.ir).

AI ASSISTANCE DISCLOSURE

Not applicable

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