Increased Genes Expression Levels of Cytokines Related to Th17/Treg Cells in Peripheral Blood Mononuclear Cell Correlate with Clinical Severity in COPD and Mustard Gas-exposed Patients

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ABSTRACT

The long lasting inflammation and immune dysregulation is one of the main mechanisms involved in lung complication of veterans exposed to sulfur mustard (SM) gas. Th17/Treg cells have an important role in immunopathogenesis of chronic obstructive pulmonary disease (COPD) and mustard lung disease.

In this study, expression of cytokines genes levels related to Th17/Treg cells was determined in peripheral blood mononuclear (PBMC) of mustard lung patients and was compared with COPD patients and healthy controls (HC). Real time-polymerase chain reaction was used to assay genes expression levels of Th17 related cytokines (IL-17, IL-6 and TGF-β) and Treg related cytokines (IL-10, TGF-β).

IL-17 gene expression level considerably was higher in SM patients (9.98±0.65, p<0.001), and COPD (4.75±0.71, p<0.001), compare to HC group. Also, gene expression level of IL-6 in the SM group (3.31±0.93, p<0.001) and COPD group (2.93±0.21, p<0.001) were significantly higher than the HC group. The IL-10 gene expression level showed a high increase in SM patients (4.12±0.91, p<0.01), and COPD (2.1±0.45, p<0.01). Finally, the TGF-β gene expression level was increased in SM patients (4.91±0.69, p<0.001) as well as in COPD group (5.41±0.78, p<0.001). In SM patients, IL-17 (R= -0.721, p<0.05), IL-6 (R= -0.621, p<0.05) and TGF-β (R= -0.658, p<0.05) had significant negative association with FEV1 (%). Inversely, IL-10 showed positive correlation (R=0.673) with FEV1 (%).

Th17/Treg cells related cytokines genes were highly expressed and imbalanced in peripheral blood mononuclear cells of SM and COPD patients which correlated with pulmonary dysfunction.

Keywords: Chronic obstructive pulmonary disease (COPD); Gene expression; Mustard lung patients; Peripheral blood mononuclear cell (PBMC); Th17/Treg related cytokines

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**INTRODUCTION**

Mustard Lung disease and chronic obstructive pulmonary disease (COPD) have many similarities in terms of pathophysiology and mechanisms involved in these diseases. Airways remodeling is one of the major problems and late complications in veterans exposed to mustard gas. Airways remodeling mainly can be seen in the form of epithelial cell damage, sub epithelial fibrosis, and airway angiogenesis which its clinical symptoms are manifested in the form of reduced lung function. The patients mostly suffer from severe cough, shortness of breath, chest pain and much sputum production. It seems that the airways remodeling occurs due to recurrent tissue damage and aberrant repairing of the airways tissues. Some effective mechanisms involved in the airways remodeling are oxidative stress, imbalance in the proteases and anti-proteases induction and dysregulation of immune system.

There are abundant reports on the increase or decrease of cells and molecules in the innate and adaptive immune system in these patients that the majority of them indicate that there are inflammatory cells, molecules and cytokines in lung tissue, as well as in the sera of these patients. However, immunopathogenesis of this disease is not well known.

In healthy individuals, Th17 cells play an important role in the defense against infectious agents and cancers, also, in some inflammatory diseases; Th17 cells play a central role in the development of tissue damage. On the other hand, Treg cells play a key role to control the inflammation and re-establish the immune system homeostasis.

In mustard lung patients, previous studies based on the high expression and the presence of TGF-β and IL-6 cytokines in lung tissue and serum of the patients, proposed involvement of the Th17 / Treg axis in immunopathogenesis. Our previous study showed that, despite the increase in Th17 and Treg cells, imbalance in the axis can be seen in favor of Th17 in the immune system of lungs and in the peripheral blood. Also, increase in the Th17 cells showed a negative correlation with pulmonary indexes such as FEV1 and FEV/FCV, and indicated the role of the axis in the airways remodeling. The next study indicated overexpression of Th17 axis cytokines genes such as IL-17, TGF-β, IL-6 in the lung tissue which was consistent with the results of immunohistochemistry based in an increase in the Th17 cells. Also, expression of the cytokine gene IL-10 increased which was consistent with the increase in Treg cells.

In the present study, we tried to show the genes expression levels of cytokines related to Th17/Treg cells (i.e. IL-6, IL-10, IL-17, TGF-β) in peripheral blood mononuclear cell (PBMC) and their correlation with clinical severity in COPD and mustard gas exposed patients.

**MATERIALS AND METHODS**

**Study Type and Patients**

This study was a cross-sectional-analytical research. Regarding medical documents and inclusion criteria, a total of 35 patients were selected for this study. They were divided into three groups: chemical veterans, patients with COPD and healthy subjects. Sulfur mustard group consisted of 15 patients who were exposed to a single dose of SM more than 30 years ago and had moderate symptoms. COPD group included 10 patients with GOLD stage II COPD, diagnosed >3 years prior to the study. Finally, 10 individuals with normal pulmonary function comprised the healthy group. A confidential questionnaire that included demographic data, specific disease records, spirometric indexes, as well as the evaluation of breathing questionnaire (SGRQ) was given to the people who referred to the desired department.

**Pulmonary Function**

After spirometry test, some parameters such as forced vital capacity (FVC), forced expiratory volume in the first second (FEV1), the ratio of FEV1/ FVC, forced expiratory flow between 25 and 75 percent MEF (MEF25-75) , residual volume (RV), total lung capacity (TLC) were collected for the three groups studied.

**Ethical Considerations**

This study was approved by a local ethical committee of Baqiyatallah Hospital (IR.BMSU.REC.1393.97). An overview of the project was described for each patient and then patients were enrolled voluntarily. An informed consent was received of each patient at the beginning of the project, and all of them were assured that the information is confidential and none of the trials and tests have any
risk for the patient. The patient could withdrawal from cooperation in the project at any time if he had no desire to continue the treatment due to complications or risks threatening. All patients were to be coded and recorded.

**Quantitative Analysis of the Genes Related to Th17 / Treg Axis in PBMC**

The Ficoll-Hypaque method was used to isolate mononuclear cells from peripheral blood.25 Briefly, 10 mL diluted blood was carefully layered on to a 3 mL Ficoll-Hypaque plus cushion (Pharmacia Biotech, Uppsala, Sweden) in a 15-mL centrifuge tube (Falcon 3033; Becton Dickinson, Franklin Lakes, NJ, USA) and centrifuged at 400 g for 15 min. Then, the mononuclear cells was carefully collected and washed twice with PBS and cells were used for RNA extraction. The total RNA from cells was extracted by Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s protocol. Finally, total purified RNA dissolved in 20 μL DEPC water. The quantity and quality of RNA were determined by spectrophotometry (ND-1000; Nanodrop, Wilmington, DE, USA) and electrophoresis.

The complimentary DNA (cDNA) with 1 μg of total RNA was performed by SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s protocol. Real-time PCR was carried out using SYBR Green Master mix reagents (Roche, Germany) in a Rotor-Gene RG 3000 (Corbett Research, Sydney, Australia) with the specific primers (Table 1).

PCR condition was initial denaturation at 94º C for 10 min followed by 45 amplification cycles consisting of denaturation at 94 C for 30 sec, annealing at suitable temperature for 30 sec and extension at 72º C for 30 sec. The changes in gene expression as fold change was examined in three groups of mustard lung patients, patients with COPD and healthy control groups. Threshold cycle values were normalized by GAPDH expression.26

**Statistical Method**

After the end of the reaction and calculating CT of the samples by device, the changes in gene expression was calculated using relative method and using the formula ∆∆CT and relative quantification (RQ)=2^(-∆∆CT). The statistical analyses were performed with SPSS software for Windows (Version 21, SPSS Inc., Chicago, Illinois, USA). Data were analyzed using analysis of variance (ANOVA). Statistical significance was accepted at \( p<0.05 \).

**RESULTS**

**Clinical Findings**

The patients belonging to the SM group had FEV1, FEV1 (%) and FVC (%) (1.98±1.01), (5024±10.28), (60.65±9.50) respectively. The FEV1 / FVC ratio and MEF25-75 were higher than the control group \( (p<0.001) \). The RV/TLC ratio and TLC (%) were higher than the control group \( (p<0.001) \). Also, mustard lung patients had a significant difference with the COPD group in terms of MEF25-75 and FEV1, and there was no significant difference between SM and COPD in terms of other factors evaluated. Patients with COPD according to two indices (FEV1/FVC<70%) and (50%≤FEV1 predicted<80%) were classified in the moderate COPD group.

**Table 1. Sequences of designed PCR primers used for assaying gene expression level of desired cytokines in mustard lung and COPD patients**

<table>
<thead>
<tr>
<th>Designation</th>
<th>Sequence</th>
<th>Ann. Temp</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6</td>
<td>Forward</td>
<td>5'-GTACATCCTCGACGGCATCT-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'-GTGCTCTTGTGCTTAGCAC-3'</td>
</tr>
<tr>
<td>IL-10</td>
<td>Forward</td>
<td>5'-GAGATGCGCTCAGACGAGTAAGA-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'-AGGCTTGCAACCCAGGTAAC-3'</td>
</tr>
<tr>
<td>IL-17</td>
<td>Forward</td>
<td>5'-GTCACCTGAACATCCATAACCG-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'-AAGCTCGCTTCACAGAC-3'</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Forward</td>
<td>5'-CATCCCCGCCACTTTCAC-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'-AACCCGTTGCTGGCAT-3'</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Forward</td>
<td>5'-TCGACAGTCGACGGCATCTT-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'-AATCCGTTGCTGGCATCTT-3'</td>
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</table>
Th17/Treg Related Cytokines in Mustard Lung Disease

Table 2. The demographic and baseline clinical characteristics of mustard lung and COPD patients

<table>
<thead>
<tr>
<th>Variables</th>
<th>HC (n=10)</th>
<th>COPD (n=10)</th>
<th>SM (n=15)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yrs.)</td>
<td>49.14 ± 1.32</td>
<td>50.26 ± 7.83</td>
<td>50.56 ± 4.92</td>
</tr>
<tr>
<td>BMI (Kg/m2)</td>
<td>23.60 ± 2.40</td>
<td>24.30 ± 2.40</td>
<td>23.80 ± 1.90</td>
</tr>
<tr>
<td>Tobacco, pack/ys.</td>
<td>–</td>
<td>36.00 ± 8.40</td>
<td>11.00 ± 3.00</td>
</tr>
<tr>
<td>FEV1 (%) pred</td>
<td>88.98 ± 10.68</td>
<td>49.18 ± 3.99**</td>
<td>50.24 ± 10.28**</td>
</tr>
<tr>
<td>FVC (%) pred</td>
<td>96.03 ±8.21</td>
<td>80.92 ± 4.63**</td>
<td>72.65 ± 9.50**</td>
</tr>
<tr>
<td>FEV1/FVC (%)</td>
<td>85.87± 5.80</td>
<td>59.94 ± 10.44**</td>
<td>62.34 ± 15.38**</td>
</tr>
<tr>
<td>RV/TLC</td>
<td>31.10 ± 6.58</td>
<td>44.94 ± 3.52**</td>
<td>46.64 ± 4.33**</td>
</tr>
<tr>
<td>SGRQ</td>
<td>28.43 ± 3.23</td>
<td>53.61 ± 4.50**</td>
<td>71.06 ± 7.21###</td>
</tr>
<tr>
<td>CAT</td>
<td>13.28 ± 1.21</td>
<td>28.90 ± 3.01**</td>
<td>33.18 ± 2.52**</td>
</tr>
</tbody>
</table>

All data expressed as mean ± SD (range) of the mean of individual groups. COPD: the GOLD stage II COPD patients; SM: sulfur mustard lung; BMI: body mass index; FEV1 (%): pred: % of predicted forced expiratory volume in 1 s; FVC (%): pred: % of predicted forced vital capacity; RV: residual volume; TLC: total lung capacity; SGRQ: St-George respiratory questionnaire; CAT: COPD assessment test.

*p<0.05
**: p<0.001 vs the HC group.
#: p<0.05
###: p<0.001 v.s. the COPD group.

Before entering of the individuals into the study, CAT questionnaire was evaluated to assess the patients with COPD and also the quality of life of individuals studied was examined using the SGRQ questionnaire. Results showed that patients with COPD and SM have a significant increase in both SGRQ and CAT indicators compared to the control group as specified in Table 2. The quality of life index in the SM group was increased 3 times compared to the control group; while the increase for CAT is almost less than 2 times. Also during this study, it was found that SM group had a significant difference with COPD group in both indicators of SGRQ and CAT at p<0.001.

mRNA Level of Th17/Treg Related Cytokines in PBMC of SM Lung, COPD and Normal Group

In this study, expression of cytokines related with Treg and Th17 cells were measured by real time PCR. It was found that IL-17 mRNA expression was extremely increased in the SM group (9.98±0.65), and COPD (4.75±0.71), compare to HC group (p<0.001) (Figure 1.A). Also, the cytokine expression of the IL-6 in the SM group (3.31±0.93) and COPD group (2.93±0.21) was significantly higher than the HC group (Figure 1.B.). In this study, cytokine mRNA expression of TGF-β was obtained in the SM group (4.91±0.69) and COPD group (5.41±0.78) was higher than HC group (Figure 1.C.). Regarding cytokines related with Treg, the IL-10 mRNA expression showed increase for the SM (4.12±0.91), and COPD (2.1±0.45) (Figure 1.D). In sum up, in SM patients, the Th17/Treg related cytokines expression level was higher than COPD patients and HC group (p<0.001) (Figure 1).

Cytokines mRNA Expression Level Correlation with Pulmonary Function Test

Statistical analysis showed that Th17 related cytokines genes expression level in PBMC was inversely correlated with mustard lung spirometry indexes. IL-17 (R=-0.721), IL-6 (R=-0.621) and TGF-β (R=-0.658) had significant negative association with FEV1 (%). On the other hand, IL-10 cytokine mRNA expression level showed positive correlation (R=0.673) with FEV1 (%) (Figure 2).
Figure 1. Comparison of mRNA levels of Treg/Th17 related cytokines in peripheral blood samples between study groups. Relative mRNA level of A) IL-17, B) TGF-β, C) IL-6 and D) IL-10 in SM patients (n=15), COPD patients (n=10) and healthy controls (n=10). SM: sulfur mustard lung, COPD: Chronic obstructive pulmonary disease, Cont: Healthy control.

Figure 2. Correlation between mRNA levels of Th17/Treg related cytokines and predicted FEV1 in mustard lung and COPD patients. A) IL-17 and FEV% Pred, B) TGF-β and FEV% Pred, C) IL-6 and FEV% Pred, D) IL-10 and FEV% Pred. Data indicate negative correlation in A, B, C (Th17 cytokines) and positive correlation in D (Treg cytokine). Treg related cytokines and lung function were shown to have a positive correlation and in contrast the Th17 related cytokines showed significant negative correlation with lung function in SM and COPD groups.

COPD: Chronic obstructive pulmonary disease; SM: Sulfur mustard; FEV1 (%) pred: % predicted of forced expiratory volume in 1 s; FVC (%) pred: % predicted of forced vital capacity; IL: Interleukin; TGF β: Transforming growth Factor β. Treg: Regulatory T cell; Th17: T helper 17
DISCUSSION

Imbalance in Th17/Treg cells is considered as a key player in long lasting inflammation and airways remodeling in mustard lung disease like as COPD. This study tries to show genes expression levels of cytokines related to Th17/Treg cells in peripheral blood mononuclear cell and their correlation with clinical severity in COPD and mustard gas exposed patients.

Several studies reported reduction of FEV1/ FVC in both SM and COPD groups which represents a similar obstruction in both groups. In this line, our spirometry results indicated a reduction in the FEV1/FVC ratio for SM (63.9±15.38) and COPD groups (59.4±10.44) compared to the healthy group (p<0.001) which confirm the airflow obstruction in both COPD and SM groups. Also, the same as other studies, the SGRQ questionnaire showed the quality of life in this category of veterans has reduced 3 times the control group (p<0.001). Moreover, evaluation based on CAT standard questionnaires to check the quality of respiratory has shown that SM patients in all three groups of symptoms of the disease (i.e. energy, sleep and related activities) were twice worse than the control group. In this regard, studies conducted by Razavi and Imani et al confirm that the social, mental problems have an impact on the quality of life of patients with SM. In this study, it was found that, patients with SM had quality of life and respiratory lower than the COPD patients.

More recently, in COPD patients, role of Th17/Treg axis in immunopathogenesis of disease and direct relationship with lung injury have been interested issues. There are some reports on the imbalance of Th17/Treg cells in the blood, lung tissue, bronchoalveolar lavage and sputum of COPD patients. Furthermore, these studies showed that reduction in FoxP3 (signature transcription factor of Treg) and increase in RORγt (main transcription factor ofTh17) expression in COPD patients with the excessive secretion of pro-inflammatory cytokines such as IL-17A, -21, -22, with their related cytokines have been increased in lung tissue and concluded that development and increase in regulatory T cells could not inhibit progression of Th17 inflammatory cells.

In the present study we sought to determine whether gene expression levels of cytokines related to Th17/Treg cells in peripheral blood mononuclear cell correlate with clinical severity in COPD and mustard gas exposed patients. The results showed an increase in expression of cytokines genes related in the Th17 (i.e. IL-17, IL-6, TGF-β) and Treg (i.e. IL-10) cells in PBMC of the SM and COPD patient. Albeit in SM patient, expression of these cytokines were higher than COPD patients. This increased cytokines expression showed an imbalance between Th17/ Treg related cytokines in favor of Th17 cell. These data were in compliance with cytokines pattern of lung tissue and are in agreement with previous flowcytometry and immunohistochemistry report of Th17/Treg in blood and tissue of SM and COPD patients. Moreover, Stefano studying on cytokines related to Th17 confirmed increase IL-17A, IL-2 and IL-23 in bronchial mucosa in patients with COPD.

Our results showed the increased genes expression levels of Th17 related cytokines has an inverse correlation with lung function. In this way, by increasing Th17 related cytokines, the pulmonary indexes such as FEV1 and FEV1/FCV should have been reduced.

However, the basses for isolation of Th17/Treg cells from PBMC of patients and assay their specific cytokines are to be defined in future studies in our laboratory.

The results of this study were consistent with the previous studies showing that an increase in Th17/ Treg related cytokines genes expression has a direct relationship with SM and COPD lung dysfunction.

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REFERENCES