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Serum and sputum levels of IL-17, IL-21, TNFα and mRNA expression of IL-17 in sulfur mustard lung tissue with long term pulmonary complications (28 years after sulfur mustard exposure)

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ABSTRACT

Background: Iranian veterans who had exposed to Sulfur Mustard (SM) suffer from long term complications such as Chronic Obstructive Pulmonary Disease (COPD) and bronchiolitis obliterate (BO). Th17 cells product IL-17A, IL-17F, IL-21, and IL-22. They have important roles in chronic inflammatory diseases. Also, TNFα has a major part in pathobiological processes of COPD. In this study, we evaluate the serum and sputum levels of IL-17, IL-21, TNF-α, and mRNA expression of IL-17 in the lung tissue of the patients 28 years after SM exposure.

Material and method: The cytokine levels of IL-17, IL-21 and TNFα were measured by ELISA method in serum and sputum of 455 SM-exposed and 123 unexposed people participated in Sardasht-Iran Cohort Study (SICS) of chemical victims. The mRNA expression of IL-17 was evaluated with qRT-PCR in lung biopsies (SM-exposed = 52, control = 33). Analyses of all data were accomplished with the SPSS software with P value ≤ 05.

Result: The results show the sputum level of IL-17 in the exposed group decreased significantly compared to control group ($P = 0.007$) and Veterans and Martyrs Affair Foundation (VMAF) assessment was significantly lower in abnormal/exposed than normal/exposed group ($P = 0.042$). There were no significant differences between control and exposed groups in serum level of IL-17; also serum and sputum levels of IL-21, TNF-α, and IL-17 mRNA expression.

Conclusion: Conclusively, The IL-17 level decreased in the exposed group. This decline could cause by mutation on transcription factors like Signal transducer and activator of transcription 3 gene (STAT3) or CCL20 as a chemokine.

1. Introduction

Sulfur mustard or bis(2-chloroethyl) sulfide (SM) is an alkylating and vesicant chemical agent. Although it was used during World War I for the first time, but according to reports of the United Nations, SM was extensively used by the Iraqi forces against Iranian veterans and civilians of border towns during Iraq-Iran war (1981–1989) [1,2].

SM exposure induces acute complications in different organs. In addition, it causes long-term adverse health effects. SM induces toxicity on skin, eyes, and respiratory tract of the victims many years after exposure [3,4]. Many studies have been conducted on the delayed SM complications, but its precise molecular mechanisms of action are still unknown.

The lung is the most common organ affected by SM. Cough, sneezing, sputum, hemoptysis, and chest pain are the respiratory symptoms of the exposure to this chemical agent [5] and in severe

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exposure dry cough and tracheobronchitis will appear, too [6]. Pulmonary complications are the most important long-term morbidity of SM exposure [7]. The most common injury caused by SM is chronic obstructive pulmonary disease (COPD) [7,8]. Also, obliterative bronchiolitis (OB) is one of the common pulmonary complications occurs after SM exposure [9,10]. Asthma is another pulmonary disorder in the SM-exposed victims that its outbreak may increase in the next generation [11,12]. It is also an important chronic disease after SM exposure [13]. Idiopathic pulmonary fibrosis (IPF) is a chronic disease that can be lethal [14], and SM inhalation can induce IPF, too [15].

Inflammation is an important base of pulmonary complications; the researchers documented alterations in immunological factors and inflammatory cells, including TCD4+, TCD8+, natural killer cells, immunoglobulins, and cytokines [16]. To identify the immunological parameters and inflammatory mediators in SM-exposed people, a cohort study named Sardasht-Iran Cohort Study (SICS) of chemical victims, was designed and conducted for ten years [17]. In this cohort study, long-term clinical complications induced by SM along with their underlying, especially immunological mechanisms are evaluated. Ghazanfari et al. investigated the serum level of inflammatory chemokines, including MCP-1/CCL2, RANTES/CCL5, IL-8/CXCL8, and fractalkine/CX3CL1 in the SM-exposure group and reported the increasing level of chemokines in the SM-exposed group compared to the control group [18]. Also, in another study, Ghazanfari et al. reported the positive association between high physical activity and the levels of anti-inflammatory cytokine, interleukin (IL)-10, in the SM-exposed group [19]. Yaraee et al. investigated the serum level of inflammatory cytokines (TNF [tumor necrosis factor]-α, IL-1α, IL-1β and IL-1Ra) and their results showed lower level of serum inflammatory cytokines in the SM-exposed group [20]. Pourfarzam et al. measured the serum levels of IL-8 and IL-6 in the long-term pulmonary complications made by SM and reported the decrease of IL-8 and IL-6 in the exposed group [21]. A member of TCD4+ superfamily is CD4+ T-helper 17 (Th17) that and has an important role in lung immunity. In chronic inflammatory states, the number of Th17 cells increases and they contribute to chronic inflammation associated with many inflammatory disorders [22,23]. Th17 cells produce mediators contributing in inflammatory responses, they release interleukin (IL) 17 family and the main IL-17 cytokines produced by human activated Th17 cells are IL-17A, IL-17F, IL-21, and IL-22 [24,25]. IL-17 as a proinflammatory cytokine has been recently described as an important element in chronic inflammatory diseases. Elevated IL-17 level has been associated with some inflammatory disorders. IL-17 is produced in lung and mucosa by specific innate and adaptive immune cells [24,26]. IL-17 affects the progression and development of COPD [27]. Also another chronic disease like asthma, has been associated with IL-17 response [28].

Some studies show an increasing level of IL-17 in COPD patients compared with the healthy controls. The elevated level of IL-17 has been demonstrated in both serum and sputum [23,29]. In addition to serum and sputum, IL-17 level increases in patient's lung tissue [30,31]. In asthmatic patients, a high level of IL-17 is seen in the airways [32]. High level of IL-17 is one of the characteristics of asthma [33]. Also, IPF is highly associated with IL-17 level [34].

IL-21, as a cytokine in Th17 profile, follows the IL-17 behavior and plays a role in lung immunity. It is one of the crucial cytokines produced by human activated Th17 [26]. IL-21 level in stable COPD is higher than that in acute exacerbations of OCPD [35].

TNF-α as a pro-inflammatory cytokine is high in the serum of the mouse model with high exposure to SM [36]. In serum and sputum of patients with COPD and asthma, TNF-α level has higher concentrations compared to the control group [37]. TNF-α has a critical part in many pathobiological processes of COPD [38].

The exact mechanisms of SM-induced pulmonary complications are not clear yet, but up to now, some similarities and differences in the immunopathology of SM-induced lung diseases to COPD, OB, and IPF have been reported. Although some studies investigated the IL-17 level in the serum and sputum of the patients, there is no study on Th17 type cytokines and especially IL-17 cytokine in the lung tissue of SM-exposed patients. In this study, the serum and sputum level of IL-17, IL-21, and mRNA expression of IL-17 were evaluated in the patients 28 years after their exposure to SM.

Table 1
Characteristics of all subjects.

		Control	Exposed	*p*-Value (chi square)
		Count (%)	Count (%)	
Gender	Male	85 (69.1%)	345 (75.8%)	0.13
	Female	38 (30.9%)	110 (24.2%)	
Smoker	No	98 (85.2%)	349 (82.1%)	0.345
	Yes	17 (14.8%)	76 (17.9%)	
Auscultation	Normal	116 (95.9%)	331 (79.6%)	< 0.001
	Abnormal	5 (4.1%)	85 (20.4%)	
		Mean (SD)	Mean (SD)	p-Value (*t*-test)
Height (kg)		164.545 (8.493)	166.556 (9.256)	0.038
BMI (kg/m^2)		28.151 (149.646)	28.193 (4.260)	0.932
FVC		93.449 (20.450)	87.379 (22.369)	0.008
FEV1		95.450 (20.812)	88.820 (24.926)	0.008

FEV1: forced expiratory volume in 1s, FVC: forced vital capacity, BMI: body mass index.

2. Participants and methods

2.1. Study design and participants

Serum and sputum of this study were collected from the participants of Sardasht-Iran Cohort Study (SICS). This study on chemical victims started in 2006 and continues until now. The particulars of the study design and methods of SICS have been explained formerly [17]. In 2014, after calling the SICS people, 455 SM-exposed people and 123 normal people volunteered for clinical visits and sampling. The SM-exposed group consisted of 345 men and 110 women from Sardasht City, Iran. The unexposed control group consisted of 85 healthy men and 38 healthy women. The age of the subjects ranged between 28 and 68 years. Table 1 presents other demographic data of the samples. Another part of this study was carried out on paraffin blocks of lung biopsies. All samples were gathered from archived paraffin blocks of the pathology departments of general hospitals of Tehran, Iran. The samples were SM exposed group with delayed pulmonary complications (n = 52) and the control group (n = 33). Table 2 presents the histopathological characteristics of the SM-exposed and control groups. The detailed characteristics of the samples have been presented in our previous study [39].

2.2. Ethical considerations

This study was executed following the World Medical Association Declaration of Helsinki, Ethical Principles for Medical Research Involving Human Subjects, amended in 2013. The study was approved by the Ethics Committee of the Board of Research Ethics of Shahed University (1395.21 and 41/198215). The individuals who took part in this study, signed an informed consent form.

2.3. Clinical evaluation

Three internists completed the study questionnaire containing pulmonary symptoms (chronic cough, sputum, hemoptysis, and dyspnea) and pulmonary findings (crackles, rales, and wheezing) and examined the patients at the same time. Spirometry was done in all contributors, according to American Thoracic Society Criteria with spirometry device

Table 2
Association of serum level of IL-17 with pulmonary classification in control, exposed and control-nonsmoker, exposed-nonsmoker individuals.

Pulmonary classification		Serum IL-17 (pg/mg)									
		Control					Exposed				
		N	Med	Q1	Q3	p^1	N	Med	Q1	Q3	p^2	p^3
All exposed												
Auscultation	Normal	106	4.06	2.92	6.13	**0.006**	313	4.06	2.92	5.28	0.778	0.519
	Abnormal	5	11.27	9.80	13.83		80	3.67	2.92	5.92		0.004
GOLD	Normal	110	4.06	2.92	6.57	–	397	4.06	2.92	5.28	0.908	0.226
	Abnormal	0					16	3.87	2.39	7.01		–
VMAF assessment	Normal	87	4.06	2.92	6.57	0.764	252	4.06	3.11	5.28	0.623	0.238
	Abnormal	22	3.68	2.92	8.38		150	3.67	2.92	5.70		0.939
Non smoker												
Auscultation	Normal	92	4.06	2.92	6.13	0.052	263	4.06	2.92	5.28	0.850	0.928
	Abnormal	3	11.27	8.38	13.83		65	3.67	2.92	6.13		0.035
GOLD	Normal	95	4.06	2.92	6.57		327	4.06	2.92	5.28	0.859	0.842
	Abnormal	0					13	4.06	2.92	7.01		
VMAF assessment	Normal	77	4.06	2.92	6.57	0.616	215	4.06	2.92	5.28	0.540	0.634
	Abnormal	17	3.29	2.92	6.13		116	4.06	3.11	6.35		0.629

The serum level of IL-17 was assessed by ELISA method in all participant including the control and exposed groups. A comparison was undertaken between the control and exposed groups, as well as, within each groups, with auscultation, GOLD classification and VMAF assessment. Also other comparison was undertaken between each of the exposed-nonsmoker group with the control-nonsmoker group, as well as, within each group, with auscultation, GOLD classification and VMAF assessment. Data was presented as Median (Q1–Q3). Auscultation and GOLD classification were presented as normal/abnormal. VMAF assessment is the classification of severity of pulmonary involvement in SM exposed patients according to the diagnostic protocol adopted by the Iranian Medical Committee of Veterans and Martyrs Affair Foundation and was presented as normal/abnormal. p^1: comparison of the control classification (Mann–Whitney). p^2: comparison of exposed classification (Mann–Whitney). p^3: comparison of control and exposed groups (Mann–Whitney). IL: interleukin, pg/mg: picogram per milligram.

(Chest 801 Spirometry) under the direction of a trained nurse in three subsequent measurements, and the greatest measure was selected. The classification of severity of pulmonary involvement was done for all samples agreeing to the Global Initiative for Chronic Obstructive Lung Disease (GOLD) and the diagnostic protocol adopted by the Iranian Medical Committee of Veterans and Martyrs Affair Foundation (VMAF) [21,40].

2.4. Serum collection

The participants' peripheral blood was drawn into Vacutainer tubes (BD Biosciences). The sera were divided by 20 min centrifugation at 2000*g* (4 °C), after that aliquated and labeled, finally kept frozen at −70 °C until laboratory measurements.

2.5. Sputum collection

Induced sputum was collected and prepared according to standard protocol; 1) separation of mucous, 2) measuring the mucous, 3) addition of *dithiothreitol* (0.1%) in the same volume as mucous, 4) vortex, 5) using of filter paper and collection of filtered liquid, 6) centrifuging at 300*g* for 30 min, 7) separation of supernatant and keeping at −20 °C.

2.6. Cytokine measurement

The concentration of serum and sputum of IL-17, IL-21, and TNF-α cytokines were measured by human IL-17, IL-21 and TNF-α DuoSet® ELISA Development Kits (R&D Systems, USA), according to kits protocols.

The mouse anti-human was the primary antibody and the biotinylated goat anti-human was the secondary antibody. Standards of the kits were diluted with 1% BSA (bovine serum albumin) in PBS (phosphate-buffered saline). Also, Wash buffer was made with 0.05% Tween 20 in PBS. 1% BSA in PBS was as Block buffer. PBS was contained 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, and 1.5 mM KH_2PO_4. The optical density (OD) of the samples was recorded by ELISA reader at 450 nm. According to the standard curves for each cytokine, their concentrations were detected by Gen5 software automatically.

2.7. RNA extraction and cDNA synthesis

RNA extraction from FFPE (formalin-fixed paraffin-embedded) tissue samples has been described in the previous study [41]. Temporarily, we used the RNeasy FFPE Kit (Qiagen, Germany) with a few modifications in the manufacturer's protocol for the total RNA extraction. The quantity and purity of the extracted RNA were measured with NanoDrop 2000 (Thermo Scientific, USA). The cDNA was synthesized with high capacity cDNA reverse transcription kit (Thermo Fisher Scientific, USA). Finally, cDNA was kept at −20 °C for future research.

2.8. Quantitative real-time PCR

We designed primers manually with amplicon length of < 125 bp to detect mRNA expression of IL-17 cytokine. Our previous study had shown that β-actin and *PGK1* were suitable housekeeping genes in SM-exposed lungs [39]. Each primer pair was designed separately by at least one intron on the corresponding genomic DNA to avoid genomic contamination. Table 3 presents the sequences designed for the primers. To evaluate the mRNA expression of IL-17 in FFPE lung tissue of the samples, we used quantitative RT-PCR using the StepOnePlus Real-Time PCR system (Thermo Fisher Scientific, USA). We performed the usual real-time PCR protocol. RealQ Plus Master Mix Green with high ROX (Ampliqon) was applied. The concentration of each forward and reverse primers were 10 pmol, and the samples were processed under the following steps: a holding stage at 95 °C for 15 min, amplifying stages consisting of 95 °C temperature for 30 s and 60 °C for 60 s (40 cycles), and a melting curve stage. Each measurement was performed in triplicate. Pfaffl equation was used to analyze the relative quantification of RT-qPCR.

2.9. Statistical analysis

The obtained data were presented as the mean ± SD for normally distributed variables and as the median and interquartile range (IQR) for cytokines. The correlation between inflammatory mediators and pulmonary function parameters was computed with the Spearman rank correlation coefficient. Differences were considered as statistically

Table 3
Correlation of the serum level of IL-17 and pulmonary function parameters in all-exposed, smoker-exposed and nonsmoker-exposed groups.

SerumIL-17 (pg/mg)		FVC	FEV1	(FEV1/FVC)*100	MMF	PEF	FEF25/75	FEF25	FEF50	FEF75	FEF25/75
All exposed	r	0.009	−0.023	−0.040	0.048	−0.020	−0.098	−0.006	−0.020	−0.060	0.081
	p	0.836	0.599	0.346	0.463	0.633	0.087	0.889	0.645	0.160	0.060
	N	548	548	549	236	548	307	548	547	546	546
Smoker-exposed	r	0.114	0.072	−0.002	−0.098	0.118	0.112	0.113	0.040	0.094	−0.017
	p	0.337	0.542	0.987	0.648	0.322	0.438	0.343	0.739	0.434	0.886
	N	73	73	73	24	73	50	73	73	72	73
Nonsmoker-exposed	r	0.001	−0.041	−0.066	0.039	−0.050	−0.090	−0.018	−0.013	−0.099	0.129*
	p	0.992	0.446	0.224	0.665	0.352	0.187	0.746	0.817	0.066	**0.018**
	N	340	340	340	123	340	215	340	339	339	339

The serum level of IL-17 and the pulmonary function parameters (FVC, FEV1, FEV1/FVC, MMEF, PEF, FEF2575, FEF25, FEF50, FEF75 and FEF25/75) were assessed. The correlation between the serum level of IL-17 and pulmonary function parameters was undertaken in the all-exposed, smoker-exposed and nonsmoker-exposed groups. r: Spearman's correlation coefficient, p: p-value, *: P-value < 0.05, IL: interleukin, pg/mg: picogram per milligram, FVC: forced vital capacity, FEV1: forced expiratory volume in 1s, MMF: maximum midexpiratory flow, PEF: peak expiratory flow.

significant when $P \leq 0.05$. Analyses of all data were performed in SPSS version 23 (IBM Co., Armonk, NY, USA).

3. Results

3.1. Comparison of serum and sputum levels of IL-17, IL-21, and TNF-α cytokines between the control and SM-exposed groups

Fig. 1 shows the SM-exposed and control groups regarding their serum and sputum levels of IL-17, IL-21, and TNF-α with and without smoking classification. There were no significant differences between the control and SM-exposed groups in serum levels of IL-17, IL-21, and TNF-α. However, the sputum level of IL-17 in the SM-exposed group was significantly lower compared to the control group ($P = 0.007$). Fig. 1.B shows the comparison between serum and sputum levels of these cytokines in the SM-exposed and control groups with smoking classification. There were no significant differences between the SM-exposed and control groups in non-smoker and smoker classes in serum levels of IL-17, IL-21, and TNF-α. The sputum level of IL-17 in the SM-exposed group of non-smoker class was significantly lower than that in the control group ($P = 0.011$); in smoker class, there were no significant differences between the SM-exposed and control groups regarding the sputum levels of IL-17 and IL-21.

3.2. IL-17 mRNA expression in lung biopsies

The mRNA expression of IL-17 was determined by qRT-PCR in SM-exposed and non-exposed FFPE lung samples. The results show no significant difference between the two groups in IL-17 mRNA expressions. The mean ± SD value of IL-17 mRNA in SM-exposed patients is 0.8403 ± 2.9519, and in the control people, it is 0.8016 ± 0.7870 ($P = 0.306$). Fig. 2 shows the boxplot curves of mRNA expression of IL-17 cytokine.

Further statistical analyses showed that age, gender, common drugs use for chronic respiratory diseases such as fluticasone/salmeterol, salbutamol, *N*-acetylcysteine, Atrovent, prednisolone, and smoking have no influence on the obtained results ($P = 0.838$).

3.3. Relationship between expression of IL-17 cytokine and pulmonary function parameters

In the SM-exposed group, IL-17 mRNA had non-significant inverse correlation with FEV1 (Forced expiratory value in one score), FVC (Forced vital capacity), and FEV1/FVC ($r = -0.393$, $P = 0.383$; $r = -0.643$, $P = 0.119$; $r = -0.200$, $P = 0.800$, respectively). There is no difference in IL-17 mRNA between mild (2.1046 ± 1.75), moderate (2.4794 ± 3.9909), and severe (5.027) cases of pulmonary involvements in the SM-exposed subjects ($P = 0.485$). Also, the association between mRNA of IL-17 in SM-exposed lung with pulmonary signs and symptoms of these patients such as cough, sputum, hemoptysis, dyspnea, and chest pain, was non-significant (supplementary table).

3.4. Association of serum and sputum levels of IL-17 with pulmonary classification

According to GOLD classification, auscultation of the patients was classified to normal and abnormal groups. The serum level of IL-17 in auscultation classification was significantly lower in the abnormal/SM-exposed group compared to the abnormal/control group ($P = 0.004$). Also, the level of IL-17 was significantly lower in the normal/control group compared to the abnormal/control group ($P = 0.006$). According to Table 2, there were no significant differences between the SM-exposed and control groups in serum level of IL-17, GOLD classification, and VMAF assessment. Considering the smoking, there were no significant differences between the SM-exposed-nonsmoker and control-nonsmoker groups in auscultation, GOLD classification, and VMAF assessment (Table 2). Based on Table 3 data, a significant positive correlation exists between serum level of IL-17 and FEF25/75 in the nonsmoker-SM-exposed group; there was no significant correlation between the smoker-SM-exposed and all-SM-exposed groups in the serum level of IL-17.

Our study results indicate that sputum level of IL-17 based on the auscultation classification was significantly lower in the normal/SM-exposed group compared to the normal/control group ($P = 0.039$) (Table 4). It shows abnormal/SM-exposed is significantly lower than normal/SM-exposed group ($p = 0.047$). According to Table 4, the sputum level of IL-17 in GOLD classification was significantly lower in the normal/SM-exposed group than that in the normal/control group ($P = 0.012$). There were no significant differences between the SM-exposed and control groups regarding the sputum level of IL-17 and VMAF assessment. Considering the smoking status, sputum level of IL-17 in auscultation classification was significantly lower in the normal/SM-exposed group compared to the normal/control group ($P = 0.041$), also the sputum level of IL-17 in GOLD classification was significantly lower in the normal/SM-exposed group compared to the normal/control group ($P = 0.017$). Finally, the sputum level of IL-17 in VMAF assessment was significantly higher in the normal/SM-exposed group compared to the abnormal/SM-exposed group ($P = 0.042$). The data presented in Table 5 indicate a significant positive correlation between sputum level of IL-17 and FEV1/FVC, MMF (mid-maximal flow), PEF (peak expiratory flow), FEF25 (forced expiratory flow at 25%), and FEF75 (forced expiratory flow at 75%)in all SM-exposed groups. Also, Table 5 shows a significant correlation between sputum level of IL-17 and variables of FEV1/FVC, MMF, FEF25, and FEF50 in the smoker-SM-exposed group. In the nonsmoker-exposed group, there were significant correlations between sputum level of IL-17 and FEV1/FVC and FEF25.

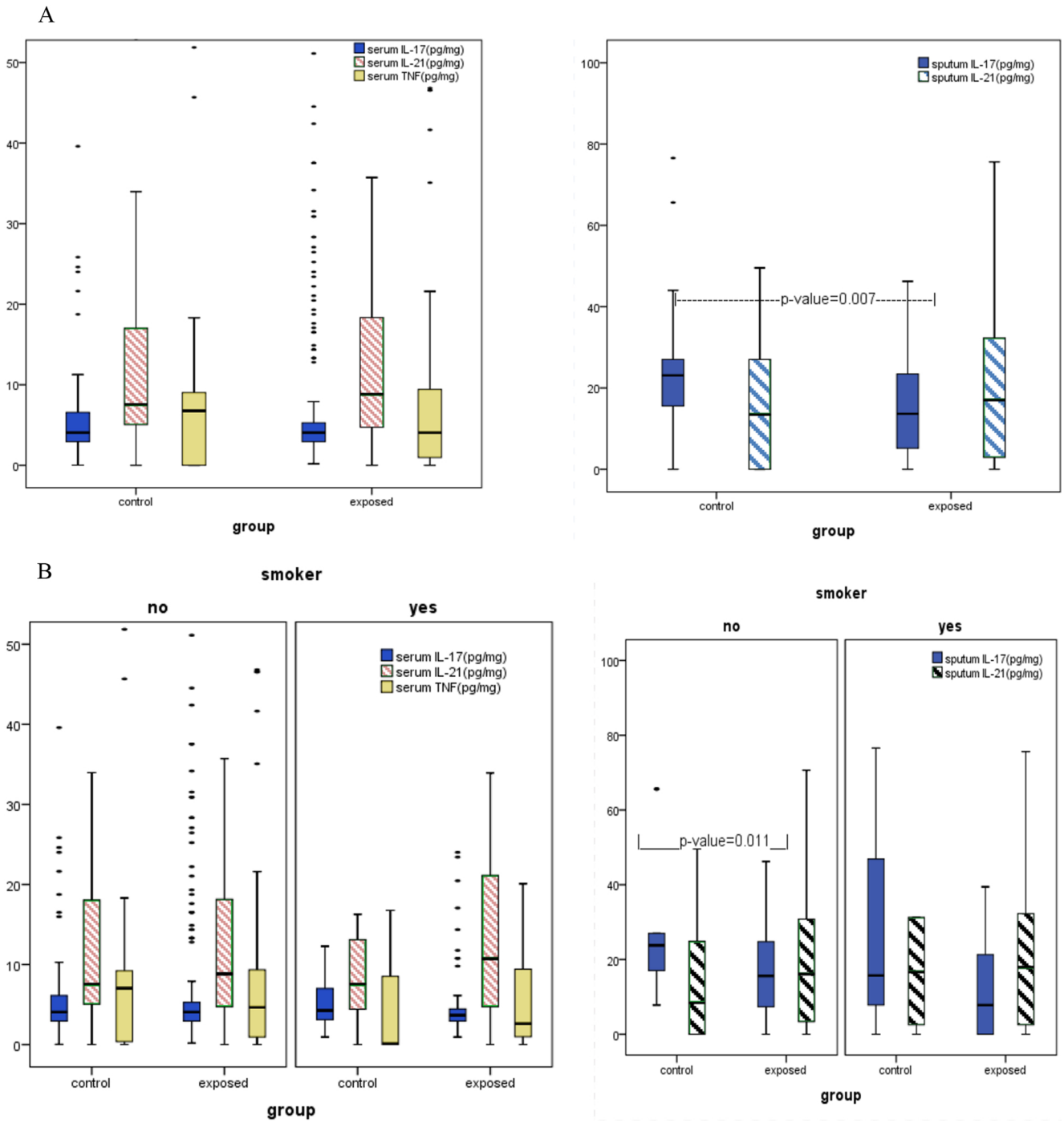

Fig. 1. Median of the serum and sputum levels of IL-17, IL-21 and TNFα in control and exposed groups (A), and median of the serum and sputum levels of IL-17, IL-21 and TNFα in control and exposed groups with smoking classification (B) was done with Mann–Whitney test and the starred data present significant differences ($P < 0.05$).

3.5. Association of serum and sputum levels of IL-21 with pulmonary classification

There were no significant differences between the SM-exposed and control groups regarding the serum level of IL-21 and auscultation, GOLD classification, and VMAF assessment. About smoking, there were no significant differences between the serum level of IL-21 and auscultation, GOLD classification, and VMAF assessment (Table 6). There was a significant negative correlation between serum level of IL-21 and pulmonary function parameters of FEV1/FVC, FEF2575, and FEF75. Also, a positive correlation existed between serum level of IL-21 and FEF25/75 in all SM-exposed groups (Table 7). There was no significant correlation between serum level of IL-21 and spirometry parameters in smoker-exposed group. The results show a significant negative correlation between serum level of IL-21 and FEV1/FVC in the nonsmoker-SM-exposed group.

On the other hand, there were no significant differences between SM-exposed and control groups regarding the sputum level of IL-21 and auscultation, GOLD classification, and VMAF assessment. Also, according to smoking classification, there were no significant differences between sputum level of IL-21 and auscultation, GOLD classification, and VMAF assessment (Table 8(. As shown in Table 9, there was no

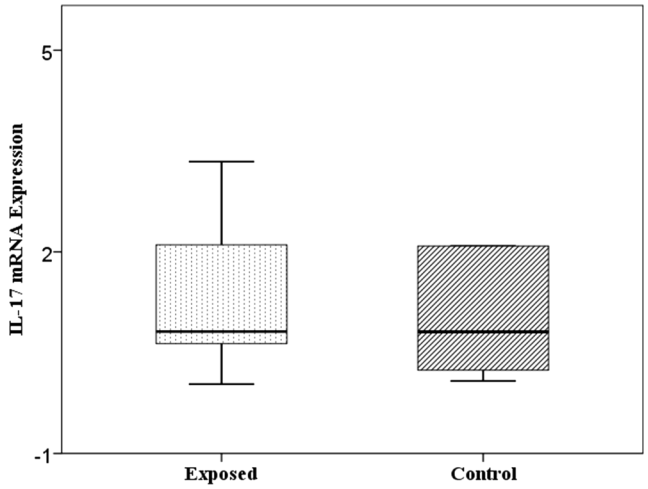

Fig. 2. The mRNA expression of IL-17 in lung tissue of SM-exposed patients and non-exposed control group. mRNA expression of 85 samples (52 SM-exposed and 33 control) was evaluated by RT-qPCR. Expression of this inflammatory cytokine had no significant different between SM-exposed and control groups.

significant correlation between sputum level of IL-21 and spirometry parameters in all SM-exposed and smoker-SM-exposed group, but there was a significant negative correlation between sputum levels of IL-21 and FEV1/FVC in the nonsmoker-SM-exposed group.

3.6. Association of serum level of TNF-α with pulmonary classification

Table 10 presents the serum level of TNF-α associations. According to this Table, There were no significant differences between the SM-exposed and control groups with respect to serum levels of TNF-α, auscultation, GOLD classification, and VMAF assessment. Also, according to smoking classification, there were no significant differences between serum levels of TNF-α and auscultation, GOLD classification, and VMAF assessment. There were significant correlations between the serum level of TNF-α and pulmonary function parameters such as a significant negative correlation with FEV1/FVC, FEF2575, and FEF75 also a significant positive correlation with FEF25/75 in the all-SM-exposed groups (Table 11). The data presented in Table 11 show a significant positive correlation between the serum level of TNF-α and FVC and FEF25/75 also a significant negative correlation with FEV1/FVC, FEF2575, FEF50, and FEF75 in the smoker-SM-exposed group. Thus, there was a significant negative correlation between serum level of TNF-α and FEF25/75 in the nonsmoker-SM-exposed group.

3.7. Correlation between serum and sputum levels of IL-17, IL-21, and TNF-α in the SM-exposed group

Regarding serum and sputum cytokines, there were positive significant correlation between serum levels of IL-17 and IL-21, positive significant relationship between serum levels of IL-17 and TNFα, a significant positive correlation between serum levels of TNF-α and IL-21, and finally a significant positive correlation between serum levels of TNF-α and IL-21 (Table 12).

4. Discussion

In this study, we presented an evaluation of serum and sputum Th17 cytokines level in SM-exposed victims and its correlation with long-term pulmonary complications (28 years after sulfur mustard exposure). Serum and sputum levels of Th17 cytokines were evaluated by ELISA method. Also, their association with pulmonary classification and pulmonary function parameters were investigated. Our results show no significant difference between the control and the SM-exposed groups in serum levels of IL-17, IL-21, and TNF-α. Only the sputum level of IL-17 in the SM-exposed group was significantly lower compared to that in the control group ($P = 0.007$).

Mutation of *STAT3* gene can decrease IL-17 level; this toxic gas could affect genes expression pathways in the long term. For instance, metallothionein-1A mRNA has been upregulated in patients exposed to sulfur mustard [26]. *STAT3* gene deficiency can lead to a decrease in Th17 cells number by non-differentiation in origin. Subsequently, the level of IL-17 as a main cytokine of Th17 cells will decline [26,42]. IL-17 is a cytokine that improves protection against extracellular bacterial

Table 4
Association of sputum level of IL-17 with pulmonary classification in control, exposed and control- nonsmoker, exposed-nonsmoker individuals.

Pulmonary classification		Sputum IL-17 (pg/mg)									
		Control					Exposed				
		N	Med	Q1	Q3	p^1	N	Med	Q1	Q3	p^2	p^3
All exposed												
Auscultation	Normal	23	23.45	15.60	27.02	0.347	98	17.03	6.93	24.57	**0.047**	**0.039**
	Abnormal	1	13.00	13.00	13.00		42	8.67	3.47	17.34		0.553
GOLD	Normal	24	23.09	14.30	27.02	–	145	13.65	5.20	22.33	0.355	**0.012**
	Abnormal	0					8	5.20	0.00	14.27		
VMAF assessment	Normal	20	23.45	16.31	27.02	0.561	72	17.03	6.93	24.80	0.065	0.085
	Abnormal	4	18.23	10.40	33.73		76	9.75	3.47	19.17		0.262
Nonsmoker												
Auscultation	Normal	17	24.16	17.03	27.02	–	73	17.84	8.67	25.59	0.058	**0.041**
	Abnormal	0					31	9.75	3.47	19.88		–
GOLD	Normal	17	24.16	17.03	27.02	–	101	17.03	7.80	25.02	0.057	**0.017**
	Abnormal	0					8	5.20	0.00	14.27		–
VMAF assessment	Normal	15	24.16	17.03	27.02	0.455	48	18.98	9.21	26.30	**0.042**	0.232
	Abnormal	2	33.73	23.45	44.01		57	10.40	3.90	19.88		0.105

The sputum level of IL-17 was assessed by ELISA method in all participant including the control and exposed groups. A comparison was undertaken between the control and exposed groups, as well as, within each groups, with auscultation, GOLD classification and VMAF assessment. Also other comparison was undertaken between each of the exposed-nonsmoker group with the control-nonsmoker group, as well as, within each group, with auscultation, GOLD classification and VMAF assessment. Data was presented as Median (Q1–Q3). Auscultation and GOLD classification were presented as normal/abnormal. VMAF assessment is the classification of severity of pulmonary involvement in SM exposed patients according to the diagnostic protocol adopted by the Iranian Medical Committee of Veterans and Martyrs Affair Foundation and was presented as normal/abnormal. p^1: comparison of the control classification (Mann–Whitney). p^2: comparison of exposed classification (Mann–Whitney). p^3: comparison of control and exposed groups (Mann–Whitney). IL: interleukin, pg/mg: picogram per milligram.

Table 5
Correlation of the sputum level of IL-17 and pulmonary function parameters in all-exposed, smoker-exposed and nonsmoker-exposed groups.

Sputum IL-17(pg/mg)		FVC	FEV1	(FEV1/FVC)*100	MMF	PEF	FEF2575	FEF25	FEF50	FEF75	FEF25/75
All exposed	r	−0.061	0.072	0.241**	0.349*	0.161*	0.083	0.238**	0.181*	0.150	0.102
	p	0.454	0.377	**0.003**	**0.014**	**0.047**	0.404	**0.003**	**0.026**	0.065	0.209
	N	153	153	153	49	153	104	153	152	152	153
Smoker-exposed	r	0.089	0.247	0.366*	0.660*	0.304	0.204	0.370*	0.350*	0.243	0.105
	p	0.605	0.147	**0.028**	**0.020**	0.072	0.328	**0.026**	**0.036**	0.153	0.541
	N	36	36	36	12	36	25	36	36	36	36
Nonsmoker-exposed	r	−0.132	0.006	0.265**	0.269	0.113	0.065	0.223*	0.165	0.174	0.061
	p	0.172	0.947	**0.005**	0.113	0.241	0.590	**0.020**	0.088	0.072	0.530
	N	109	109	109	36	109	72	109	108	108	109

The sputum level of IL-17 and the pulmonary function parameters (FVC, FEV1, FEV1/FVC, MMEF, PEF, FEF2575, FEF25, FEF50, FEF75 and FEF25/75) were assessed. The correlation between the sputum level of IL-17 and pulmonary function parameters was undertaken in the all-exposed, smoker-exposed and nonsmoker-exposed groups. r: Spearman's correlation coefficient, p: p-value, *: P-value < 0.05, **: P-value < 0.01, IL: interleukin, pg/mg: picogram per milligram, FVC: forced vital capacity, FEV1: forced expiratory volume in 1s, MMF: maximum midexpiratory flow, PEF: peak expiratory flow.

infections and plays particular roles in inflammation state [43,44]. Insufficient level of IL-17 can cause pneumonia by *S. aureus* [45]. According to reported pulmonary infection by *S. aureus* in some SM exposure cases [46,47], probably there is a relationship between low level of IL-17 and pulmonary infections.

Moreover, insufficiency of CCL20 as a Th17 attractive chemokine and mutation in the signaling pathway of CCL20 secretory cells can decrease IL-17 level [26]. Association of sputum level of IL-17 with pulmonary classification shows that the sputum level of IL-17 in the SM-exposed group with abnormal auscultation was significantly lower compared to that in the control group with normal auscultation. This finding could confirm our previous results of IL-17 level in sputum.

The measured mRNA of IL-17 in the lung tissue of SM-exposed individuals was the same as the unexposed ones. The expression of IL-17 in the lung tissue was not under the influence of pulmonary complications, drugs, or smoking, and there were no correlations with pulmonary function parameters. SM could not affect IL-17 gene expression in lung tissue. Although the sputum level of IL-17 in the SM-exposed group was significantly lower compared to that in the control group, this decrease has not been associated with gene expression; SM might be an agent that decreases IL-17 cytokine half-life in sputum, or it may affect the post-translational processes. It is not clear whether sputum could be an appropriate indicator for lung parenchyma changing, but it can be a common indicator. Bronchoalveolar lavage is undoubtedly a better indicator, but it is an invasive approach and not appropriate ethically for this group of patients.

Although there is no significant difference between smoker-SM-exposed and nonsmoker-SM-exposed groups regarding the serum levels of IL-17, the sputum level of IL-17 decreased significantly in smoker-SM-exposed group compared to the nonsmoker-SM-exposed group, the decrease could be because of the suppressing effect of smoking on the level of IL-17. In addition, there is a positive correlation between sputum level of IL-17 and MMF and FEV1/FVC as pulmonary function parameters in the smoker-SM-exposed group. Significant reduction of sputum level of IL-17 in the SM-exposed group with normal GOLD classification compared to the control group with normal GOLD classification could suggest that reduction of sputum level of IL-17 result in the reduction of normal pulmonary functions. It should be noted that this group of patients are mostly without lung complications and a few of them have lung problems; a positive correlation between the cytokine level and the disease indicates that there are more problems in the patients who have not controlled this cytokine.

Table 6
Association of serum level of IL-21 with pulmonary classification in control, exposed and control- nonsmoker, exposed-nonsmoker individuals.

Pulmonary classification		Serum IL-21 (pg/mg)									
		Control					Exposed				
		N	Med	Q1	Q3	p^1	N	Med	Q1	Q3	p^2	p^3
All exposed												
Auscultation	Normal	115	7.56	5.04	17.97	0.948	327	9.08	4.54	18.35	0.625	0.726
	Abnormal	5	7.56	6.05	9.08		83	8.07	4.54	17.43		0.914
GOLD	Normal	119	7.56	5.04	18.06	–	411	8.82	4.54	18.35	0.640	0.892
	Abnormal	0					16	10.71	5.04	17.89		
VMAF assessment	Normal	96	7.56	5.04	18.61	0.663	264	8.07	4.03	17.89	0.089	0.489
	Abnormal	22	7.56	5.04	13.61		155	10.40	5.04	19.73		0.304
Nonsmoker												
Auscultation	Normal	93	7.56	5.04	19.16	0.776	262	9.08	4.73	18.15	0.610	0.963
	Abnormal	3	7.56	6.05	9.08		64	8.45	3.78	18.58		0.705
GOLD	Normal	96	7.56	5.04	19.16	–	324	8.82	4.73	18.25	0.662	0.822
	Abnormal	0					13	13.87	5.67	18.35		–
VMAF assessment	Normal	78	7.56	5.04	19.87	0.767	213	8.51	4.54	17.89	0.333	0.641
	Abnormal	17	7.56	6.05	12.10		114	10.08	5.04	18.58		0.417

The serum level of IL-21 was assessed by ELISA method in all participant including the control and exposed groups. A comparison was undertaken between the control and exposed groups, as well as, within each groups, with auscultation, GOLD classification and VMAF assessment. Also other comparison was undertaken between each of the exposed-nonsmoker group with the control-nonsmoker group, as well as, within each group, with auscultation, GOLD classification and VMAF assessment. Data was presented as Median (Q1–Q3). Auscultation and GOLD classification were presented as normal/abnormal. VMAF assessment is the classification of severity of pulmonary involvement in SM exposed patients according to the diagnostic protocol adopted by the Iranian Medical Committee of Veterans and Martyrs Affair Foundation and was presented as normal/abnormal. p^1: comparison of the control classification (Mann–Whitney). p^2: comparison of exposed classification (Mann–Whitney). p^3: comparison of control and exposed groups (Mann–Whitney). IL: interleukin, pg/mg: picogram per milligram.

Table 7
Correlation of the serum level of IL-21 and pulmonary function parameters in all-exposed, smoker-exposed and nonsmoker-exposed groups.

Serum IL-21 (pg/mg)		FVC	FEV1	(FEV1/FVC)*100	MMF	PEF	FEF2575	FEF25	FEF50	FEF75	FEF25/75
All exposed	r	0.063	−0.031	−0.182**	0.068	−0.048	−0.242**	−0.058	−0.072	−0.162**	0.152**
	p	0.191	0.524	< **0.001**	0.405	0.321	< **0.001**	0.232	0.139	**0.001**	**0.002**
	N	427	427	427	150	427	276	427	426	426	426
Smoker-exposed	r	0.007	−0.062	−0.171	0.074	−0.137	−0.274	−0.125	−0.122	−0.173	0.080
	p	0.953	0.605	0.149	0.725	0.246	0.057	0.291	0.302	0.144	0.502
	N	73	73	73	25	73	49	73	73	73	73
Nonsmoker-exposed	r	0.072	−0.031	−0.174**	−0.095	−0.051	−0.078	−0.062	−0.075	−0.042	0.020
	p	0.187	0.572	**0.001**	0.296	0.348	0.259	0.259	0.173	0.440	0.716
	N	337	337	337	122	337	213	337	336	336	336

The serum level of IL-21 and the pulmonary function parameters (FVC, FEV1, FEV1/FVC, MMEF, PEF, FEF2575, FEF25, FEF50, FEF75 and FEF25/75) were assessed. The correlation between the serum level of IL-21 and pulmonary function parameters was undertaken in the all-exposed, smoker-exposed and nonsmoker-exposed groups. r: Spearman's correlation coefficient, p: p-value, **: P-value < 0.01, IL: interleukin, pg/mg: picogram per milligram, FVC: forced vital capacity, FEV1: forced expiratory volume in 1s, MMF: maximum midexpiratory flow, PEF: peak expiratory flow.

Table 8
Association of sputum level of IL-21 with pulmonary classification in control, exposed and control- nonsmoker, exposed-nonsmoker individuals.

Pulmonary classification	Sputum IL-21(pg/mg)											
	Control						Exposed					
	N	Med		Q1	Q3	p^1	N	Med	Q1	Q3	p^2	p^3
All exposed												
Auscultation	Normal	22	13.44	0.00	27.01	0.435	95	17.97	4.20	35.06	0.665	0.276
	Abnormal	1	31.25	31.25	31.25		41	19.87	3.36	37.50		0.535
GOLD	Normal	23	13.44	0.00	31.25		141	17.02	3.36	35.06	0.906	0.370
	Abnormal	0				–	8	18.45	17.02	21.76		
VMAF assessment	Normal	19	3.36	0.00	31.25	0.249	69	18.92	5.04	35.42	0.490	0.138
	Abnormal	4	24.46	17.68	29.13		75	16.07	2.52	32.29		0.351
Nonsmoker												
Auscultation	Normal	16	8.40	0.00	24.89	–	70	13.44	1.68	28.40	0.090	0.404
	Abnormal	0					30	21.76	5.88	38.54		–
GOLD	Normal	16	8.40	0.00	24.89	–	97	14.29	1.68	35.06	0.780	0.249
	Abnormal	0					8	18.45	17.02	21.76		
VMAF assessment	Normal	14	2.52	0.00	22.76	0.254	45	11.76	3.36	29.35	0.858	0.144
	Abnormal	2	24.46	21.91	27.01		56	17.97	2.52	33.16		0.391

The serum level of IL-21 was assessed by ELISA method in all participant including the control and exposed groups. A comparison was undertaken between the control and exposed groups, as well as, within each groups, with auscultation, GOLD classification and VMAF assessment. Also other comparison was undertaken between each of the exposed-nonsmoker group with the control-nonsmoker group, as well as, within each group, with auscultation, GOLD classification and VMAF assessment. Data was presented as Median (Q1–Q3). Auscultation and GOLD classification were presented as normal/abnormal. VMAF assessment is the classification of severity of pulmonary involvement in SM exposed patients according to the diagnostic protocol adopted by the Iranian Medical Committee of Veterans and Martyrs Affair Foundation and was presented as normal/abnormal. p^1: comparison of the control classification (Mann–Whitney). p^2: comparison of exposed classification (Mann–Whitney). p^3: comparison of control and exposed groups (Mann–Whitney). IL: interleukin, pg/mg: pictogram per milligram.

Table 9
Correlation of the sputum level of IL-21 and pulmonary function parameters in all-exposed, smoker-exposed and nonsmoker-exposed groups.

SputumIL-21(pg/mg)		FVC	FEV1	(FEV1/FVC)*100	MMF	PEF	FEF2575	FEF25	FEF50	FEF75	FEF25/75
All exposed	r	0.116	0.049	−0.092	−0.116	−0.016	−0.061	0.022	−0.027	−0.024	0.020
	p	0.161	0.549	0.266	0.433	0.843	0.543	0.788	0.743	0.773	0.811
	N	149	149	149	48	149	101	149	148	148	149
Smoker-exposed	r	0.128	0.120	0.158	0.543	0.148	−0.131	0.234	0.216	0.110	0.017
	p	0.456	0.486	0.356	0.068	0.388	0.534	0.169	0.206	0.523	0.921
	N	36	36	36	12	36	25	36	36	36	36
Nonsmoker-exposed	r	0.135	0.001	−0.211*	−0.293	−0.085	−0.082	−0.050	−0.156	−0.113	0.065
	p	0.170	0.989	**0.031**	0.088	0.390	0.503	0.609	0.115	0.252	0.508
	N	105	105	105	35	105	69	105	104	104	105

The sputum level of IL-21 and the pulmonary function parameters (FVC, FEV1, FEV1/FVC, MMEF, PEF, FEF2575, FEF25, FEF50, FEF75 and FEF25/75) were assessed. The correlation between the sputum level of IL-17 and pulmonary function parameters was undertaken in the all-exposed, smoker-exposed and nonsmoker-exposed groups. r: Spearman's correlation coefficient, p: p-value, *: P-value < 0.05, IL: interleukin, pg/mg: picogram per milligram, FVC: forced vital capacity, FEV1: forced expiratory volume in 1s, MMF: maximum midexpiratory flow, PEF: peak expiratory flow.

Serum level of IL-21 in the nonsmoker-SM-exposed group has a negative correlation with FEV1/FVC. According to our results on IL-21 and its inflammatory effects, reduction of this cytokine might be effective in restoring normal condition in pulmonary inflammatory disease.

Many studies on exposure to SM evaluated the inflammatory status of the victims. Yaraee et al. have reported the reduction of serum level of TNF-α, IL-1α, IL-1β, and IL-1RA in SM-exposed cases [20]. Emad et al. have shown an increasing level of IL-1β, IL-6, TNF-α, IL-12, and

Table 10
Association of serum level of TNFα with pulmonary classification in control, exposed and control- nonsmoker, exposed-nonsmoker individuals.

Pulmonary classification		Serum TNFα (pg/mg)									
		Control					Exposed				
		N	Med	Q1	Q3	p^1	N	Med	Q1	Q3	p^2	p^3
All exposed												
Auscultation	Normal	113	6.59	0.00	9.02	0.914	331	4.06	0.63	9.26	0.235	0.973
	Abnormal	5	7.68	0.00	10.41		85	2.56	0.52	7.05		0.730
GOLD	Normal	117	6.82	0.00	9.21	–	417	3.49	0.58	9.09	0.634	0.329
	Abnormal	0					17	5.50	1.45	10.80		
VMAF assessment	Normal	94	6.79	0.00	9.81	0.659	269	3.78	0.63	9.09	0.960	0.403
	Abnormal	22	6.82	0.00	8.05		157	3.36	0.92	9.66		0.951
Nonsmoker												
Auscultation	Normal	91	6.82	0.23	9.21	0.947	265	4.91	0.86	9.76	0.149	0.561
	Abnormal	3	7.68	0.00	10.41		66	3.20	0.00	7.49		0.667
GOLD	Normal	94	7.05	0.37	9.21		329	4.06	0.52	9.22	0.894	0.186
	Abnormal	0					14	2.00	1.38	11.33		
VMAF assessment	Normal	76	6.93	0.44	10.02	0.719	217	4.06	0.80	9.09	0.658	0.246
	Abnormal	17	7.05	0.23	8.00		116	4.14	0.39	9.69		0.684

The serum level of TNFα was assessed by ELISA method in all participant including the control and exposed groups. A comparison was undertaken between the control and exposed groups, as well as, within each groups, with auscultation, GOLD classification and VMAF assessment. Also other comparison was undertaken between each of the exposed-nonsmoker group with the control-nonsmoker group, as well as, within each group, with auscultation, GOLD classification and VMAF assessment. Data was presented as Median (Q1–Q3). Auscultation and GOLD classification were presented as normal/abnormal. VMAF assessment is the classification of severity of pulmonary involvement in SM exposed patients according to the diagnostic protocol adopted by the Iranian Medical Committee of Veterans and Martyrs Affair Foundation and was presented as normal/abnormal. p^1: comparison of the control classification (Mann–Whitney). p^2: comparison of exposed classification (Mann–Whitney). p^3: comparison of control and exposed groups (Mann–Whitney). IL: interleukin, pg/mg: picogram per milligram.

Table 11
Correlation of the serum TNFα level and pulmonary function parameters in all-exposed, smoker-exposed and nonsmoker-exposed groups.

SerumTNFα (pg/mg)		FVC	FEV1	(FEV1/FVC)*100	MMF	PEF	FEF2575	FEF25	FEF50	FEF75	FEF25/75
All exposed	r	0.128*	0.019	−0.216**	0.017	−0.045	−0.158*	−0.046	−0.066	−0.193**	0.218**
	p	0.018	0.730	< **0.001**	0.848	0.410	**0.021**	0.399	0.222	< **0.001**	< **0.001**
	N	343	343	343	127	343	214	343	342	342	342
Smoker-exposed	r	0.132**	0.003	−0.236**	0.023	−0.059	−0.210**	−0.058	−0.098*	−0.203**	0.211**
	p	**0.006**	0.947	< **0.001**	0.772	0.223	< **0.001**	0.227	**0.041**	< **0.001**	< **0.001**
	N	434	434	434	156	434	277	434	433	433	433
Nonsmoker-exposed	r	0.141	−0.026	−0.218	0.199	−0.122	−0.393**	−0.099	−0.175	−0.192	0.098
	p	0.234	0.829	0.063	0.340	0.304	**0.005**	0.405	0.138	0.103	0.412
	N	73	73	73	25	73	49	73	73	73	73

The serum level of TNFα and the pulmonary function parameters (FVC, FEV1, FEV1/FVC, MMEF, PEF, FEF2575, FEF25, FEF50, FEF75 and FEF25/75) were assessed. The correlation between the serum level of TNFα and pulmonary function parameters was undertaken in the all-exposed, smoker-exposed and nonsmoker-exposed groups. r: Spearman's correlation coefficient, p: p-value, *: P-value < 0.05, **: P-value < 0.01, IL: interleukin, TNFα: Tumor necrosis factor-α, pg/mg: picogram per milligram, FVC: forced vital capacity, FEV1: forced expiratory volume in 1s, MMF: maximum midexpiratory flow, PEF: peak expiratory flow.

Table 12
Correlation between serum and sputum levels of IL-17, IL-21 and TNFα in SM exposed group.

Exposed		Serum il-17	Serum tnfα	Sputum il-21	Sputum il-17
Serum IL-21(pg/mg)	r	0.139**	0.414**	−0.045	0.086
	p	**0.003**	< **0.001**	0.589	0.293
	N	444	447	147	152
Serum IL-17(pg/mg)	r		**0.370****	−0.039	0.102
	p		< **0.001**	0.641	0.206
	N		450	149	154
SerumTNF(pg/mg)	r			0.215**	−0.047
	p			**0.008**	0.562
	N			150	155
Sputum IL-21(pg/mg)	r				−0.082
	p				0.319
	N				151

The serum and sputum levels of IL-17, IL-21 and TNFα were assessed by ELISA method. Comparisons of the serum and sputum levels of named cytokines was undertaken in the SM exposed groups. IL: interleukin, TNFα: Tumor necrosis factor-α, r: Spearman's correlation coefficient, p: p-value, *: P-value < 0.05, **: P-value < 0.01, IL: interleukin, pg/mg: picogram per milligram.

IL-8 in bronchoscopy liquid of SM-exposed people [48] and in another study they reported that the levels of IL-8, IL-1β, IL-6, TNF-α, IL-12, TGF-β, EGF, and IGF-1 in bronchoscopy liquid in the SM-exposed group is higher than those in the control group [49]. Moreover, Eghtedardoost et al. studied TNF, TNFR, and IL-1 gene expression in the lung tissues of SM-exposed individuals and found no significant difference between SM-exposed group with lung disease and non-SM-exposed group with lung disease [41]. With regard to our results on TNF-α and studies on the ineffectiveness of suppressor drugs (like prednisolone in BO patients, reported by Ghanei et al. or corticosteroids on > 50% of SM-exposed cases, reported by Poursalehi et al.), immune-suppressive medicines could not be recommended as the first choice of therapy for these cases [50,51].

5. Conclusion

According to our study results, IL-17 is an effective cytokine on lung function that plays an important role in the determination of intensity and severity of lung complications. It means that in cases with a reduction of IL-17 level, the complications might be worse. This cytokine

might be a useful parameter to determine the lung status of SM-exposed individuals. IL-17 has two opposite effects; it can be beneficial through the control of infections but harmful through the intensification of inflammation. It is also useful to examine the level of the TGF-β and measuring of T17/TGF-β ratio. Because of the ability of sulfur mustard to mutate DNA, it is not possible to draw a clear pathway for these cytokines in SM-exposed individuals compared to common cases. Evaluation of *STAT3* gene expression and level of CCL20 are recommended in this regard.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.intimp.2019.105828.

Declaration of Competing Interest

The authors report no conflict of interest in this study.

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