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Th 9 cells in Behçet disease: Possible involvement of IL-9 in pulmonary manifestations

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ABSTRACT

Behçet disease (BD) is a multisystemic disease some of whose manifestations are characterized by pulmonary involvements. The purpose of the study was to evaluate the level of T-helper type 9 (Th9) cells and the cytokine interleukin (IL)-9 in peripheral blood and in bronchoalveolar lavage (BAL) of patients with Behçet's disease (BD) affected by pulmonary manifestations. Nevertheless, until recently there have been no studies on its role in BD.

The Th9 ($CD4^+IL\text{-}9^+T$) cell, transcription factor PU.1 and IL-9 mRNA levels, as well as serum and BAL IL-9 concentration, were measured in BD patients and healthy controls.

The Th9 cell percentage and absolute number, PU.1 and IL-9 expression levels of BD patients were all increased significantly compared with the control group. Absolute number of Th9 cells was particularly increased in patients with active BD compared to inactive BD patients. The levels of IL-9 associated to Th9 expression depended on BD severity. These parameters were markedly expressed in the BAL of BD patients with pulmonary manifestations. IL-17 and the epithelial inflammatory cytokine TSLP were significantly correlated to IL-9 levels. This cytokine trio decreased in inactive BD patients after corticosteroïd treatment. In addition, IL-9 levels were correlated to $CD4^+$ $IL\text{-}9^+$ cells in BAL and in PBMCs. LPS stimulated PBMCs and macrophages induced increased secretion of IL-9 and the encoding transcription factors PU.1 and IRF4.

In conclusion, the expansion of the Th9 cell subset, up-regulation of the PU.1 transcription factor and increased secretion of the IL-9 cytokine may contribute to the pathogenesis of BD, which may be supported by the increased release of IL-17 and TSLP. We provide evidence that Th9 T cells are increased in BD patients with pulmonary manifestations. This suggests an important role of IL-9 in the pathogenesis of BD particularly in patients suffering from lung involvement.

1. Introduction

Behçet's disease is a multisystemic inflammatory disorder characterized by immune dysregulation involving T cells with hyperactive neutrophils, supposedly triggered by infectious agents, in addition to genetic predisposition [1,2]. Recurrent oral and genital ulcers and uveitis characterized Behçet's disease (BD). Besides this classical clinical pattern, other organ engagements including gastrointestinal tract, musculoskeletal, cardiovascular, central nervous system and pulmonary manifestations were associated with BD manifestations [1,2]. Pulmonary artery aneurysms, arterial and venous thrombosis, pulmonary infarction, recurrent pneumonia, bronchiolitis obliterans organized pneumonia, and pleurisy are the main features of pulmonary involvement in BD [3–7]. These clinical manifestations are most often associated with immunological variations. The link between innate and adaptive immunity in patients with BD has been further clarified demonstrating that T cells are skewed toward Th1 and Th17 polarization with increased Th17/Treg ratio inducing rather disorganized homeostasis [8–11]. The epithelial cell-derived cytokines thymic stromal lymphopoietin (TSLP) and IL-33 characterized as "alarmins" are

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released by the barrier epithelium in response to external insults. These mediators have recently been implicated in inflammatory manifestations in patients suffering from BD. However, rare data have been reported on the inflammatory characteristics of bronchoalveolar lavage (BAL) in BD patients with pulmonary involvement [5,6].

Lung inflammation is the result of the activation of macrophages, lymphocytes and resident cells causing an imbalance in cytokines in situ and the arrival of new mediators [4–6,12]. Among diverse cytokines present in lung of BD, IL-17 and TSLP were believed to be critical mediators of inflammation [5]. The epithelial cell-derived cytokines TSLP was released by the barrier epithelium. Recent data support the role of TSLP cytokines in BD [13,14]. Damaged epithelial cells produce TSLP and stimulate myeloid dendritic cell maturation through the thymic stromal lymphopoietin receptor (TSLPR) heterocomplex. The TSLPR heterocomplex is composed of the IL-7Rα chain and TSLPR chain. The human TSLP gene is located on chromosome 5q22.1 [15]. It can promote the early differentiation of T and B cells. It also promotes B cell proliferation and prevents cell apoptosis [15]. Many cells can produce TSLP, including epithelial cells and keratinocytes in the skin, lung and eye tissue [16].

A new independent $CD4^+$ cell subset, characterized by expression of high amounts of IL-9, which do not express T-bet, GATA-3, retinoic acid-related orphan receptor gamma T (RORγt) or FoxP3, subset-determining transcription factors associated with Th1, Th2, Th17 and Treg cells, had been recognized as Th9 cells [12]. Th9 cells were defined in humans, which activate the transcription factors PU.1 and IRF4 [17,18]. Th9 cells have been implicated in disease states such as allergic asthma, atopic dermatitis, and autoimmune conditions including multiple sclerosis and ulcerative colitis [19–21], while the precise contribution of Th9 cells in BD remains unknown. IL-9 is a pleiotropic cytokine that has documented effects on lymphocytes, mast cells, and resident lung cells. IL-9 demonstrates proinflammatory activity in the development of allergic airway inflammation in several mouse models [22] as well as in human models [23]. An important function attributed to IL-9 in lung physiology is the induction of mucus production [24], goblet cell hyperplasia and other features of airway remodelling [25]. A possible interaction between IL-9 and TSLP could occur during inflammatory manifestations. Indeed, Yao et al [21] reported that the pleiotropic cytokine TSLP is able to increase IL-9 production in Th9 cells via direct activation of STAT5 with its binding to the *Il9* promoter.

There are no data concerning Th9 cell investigations in BD. The aim of our study is to determine the involvement of Th9 cells in peripheral blood and in bronchoalveolar and to establish correlations between IL-9, IL-17 and TSLP in order to define their possible role in the inflammatory process in BD.

2. Materials and methods

2.1. Ethical approval

The study was approved by the Institutional Review Board of our hospital (A. Mami hospital of respiratory diseases) in compliance with the ethic committee of the Medicine Faculty of Tunis. Informed consent was obtained from all participating subjects.

2.2. Patients

The patients' group consisted of 50 BD patients (active BD: 30, inactive BD: 20) who fulfilled the international study group criteria for Behçet's disease [26]. The mean age of BD patients was (age 42 ± 7 years; range 32–46 years). Inactive BD patients have lost all their clinical symptoms. The biological and clinical parameters of active BD were reported in Table I: oral ulcers and genital ulcers occurred in 100% of cases, eye lesion in 23 patients of cases (76.6%), erythema nodosum in 15 patients (50%) of cases, other skin lesions in the form of papulopustules and acneiform lesions in 15 patients (50%) of cases, positive pathergy test in 12 patients (24%), neurological manifestations in 7 patients (23.3%), gastrointestinal manifestations in 8 patients (26.6%), vascular involvement in 10 patients (33.3%) and pulmonary involvement, whether pulmonary vasculature or parenchyma, occurred in 18 patients (60%) of cases. The treatment consisted of immunosuppression with prednisone plus chlorambucil. Of the 30 patients with active BD, 18 patients have pulmonary involvement. Analysis of both vascular and parenchymal lung lesions were as follows: pulmonary artery aneurysm (PAA) in 10 patients; pulmonary embolism in 5 patients and alveolar haemorrhage in 3 patients.

The control subjects consisted of 20 non-smokers (men; mean age: 47.6 ± 6 years; range: 38–57 years) undergoing routine investigations for suspected bronchial carcinoma and whose chest X-ray (CXR), bronchial examination, and pulmonary function were normal. None of them had evidence of acute infection or chronic disease (e.g., other autoimmune or atopic disorders).

2.3. Peripheral blood mononuclear cells (PBMCs) isolation and expansion

Patients and healthy controls donated 30 ml of blood, which was collected in heparinised tubes. Lymphocytes were isolated from PBMC by Ficoll Hypaque gradient centrifugation (Histopaque; Sigma Aldrich, The Netherlands). For the in vivo studies several samples of the freshly isolated cells were fixed for flow cytometry analysis. For the in vitro studies, part of the PBMCs was cultured for 10 days in RPMI 1640 media with 20 units/ml IL-2 (R&D Systems), and 10% foetal bovine serum. After 7 days, cells were treated with cytokines and analysed as outlined in the text. Cultured PBMCs were stained with antibodies against CD3, CD19, and CD14, which were used for differentiating T-cells, B-cells, and monocytes respectively. The proportion of cell types making up the PBMCs of BD patients and healthy controls were (78–80%) T-cells, (4–7%) B-cells, (7–10%) monocytes, and (9–12%) of negative cells. A majority of negative cells probably represent natural killer (NK) cells based on their scatter properties. Eight ml of the PBMC was washed twice with HBSS. Adherent monocytes were cultured for one week in RPMI media, 15% fetal bovine serum, and 50 ng/ml GM-CSF [27]. Medium was replenished every 3 days and non-adherent cells were removed at the second feeding (at 6 days). For experiments, cells were cultured for an additional two days in the absence of GM-CSF, washed twice with PBS, and adherent cells were lysed in STAT-60 (Tel-Test, Friendswood, TX) for RNA analysis or in RIPA buffer [28] for protein analysis. Flow cytometry cells were detached by incubation in 5.0 mM EDTA for 10 min at 37 °C. The method consistently yielded more than 95% pure macrophages assessed by both morphological criteria as described [29,30] and expression of the myeloid lineage marker CD14 as determined by flow cytometric analysis. Ten millilitres of peripheral blood were collected from patients and controls, and stored at −80 °C for subsequent mRNA and Th9 ($CD4^+IL\text{-}9^+$) measurement. The cell samples were washed two times with phosphate-buffered saline (PBS); part of the collected cells was used for mRNA extraction, the other part was used for detecting $CD4^+IL\text{-}9^+$.

2.4. Bronchoalveolar lavage (BAL)

All patients and healthy subjects underwent bronchoscopy and BAL procedure with a flexible bronchoscope according to the guidelines of the ERS [31], as described before [5,6]. Flow cytometry, to determine lymphocyte subsets in BAL cellular fraction, was performed as described before [5,6]. Bronchoalveolar lavage fluid (BALF) were concentrated 10-fold using Amicon Ultra 4 (Millipore, Bedford, MA) for measurement, and the acquired raw data were multiplied by 10 to obtain the original concentrations in the BALFs.

2.5. Flow cytometry analysis

Cells were stimulated with 50 ng/ml phorbol myristate acetate

(Sigma–Aldrich, St. Louis, MO), 1 mg/mL ionomycin (Sigma–Aldrich), and 10 mg/mL Golgi Stop (BD Biosciences, San Jose, CA) at 37 °C under 5% (v/v) CO2 for 6 h then stained with fluorescein isothiocyanate-labeled anti-CD4 antibodies (BD Biosciences) and fixed and permeabilized using fix/perm solution (eBioscience, San Diego, CA) according to the manufacturer's instructions. The cells were then incubated with phycoerythrin (PE)-labeled anti-IL-9 antibodies (BD Biosciences). The percentages of cytokine-secreting $CD4^+IL\text{-}9^+$ T cells were determined by flow cytometry using a FACSCalibur instrument (Becton-Dickinson, Franklin Lakes, NJ, USA); these data were evaluated using FlowJo software 7.6 (TreeStar Inc., San Carlos, CA). Isotype controls were included for compensation and to confirm antibody specificity.

Lymphocytes percentages of Th9 cells were also expressed as absolute number, Th9 cells was calculated using a double platform method [32], i.e., the relative values obtained from the flow cytometry analysis were converted to the total number of cells/µl of blood using the following calculation: Absolute $CD4^+IL\text{-}9^+$ cells = % $CD4^+$ $IL\text{-}9^+$ cells × total WBC (white blood cell) count divided by 1000 (expressed as a decimal): this enables the absolute count per mcl of each cell type to be determined. The absolute WBC count was derived using a Coulter DxH 600 automated hematology analyser (Blood bank; Charles Nicolle Hospital, Tunis).

2.6. RNA extraction and real-time PCR analysis

RNA was isolated using TRIzol reagent, followed by reverse transcription using M-MLV reverse transcriptase and oligo(dT) primer (Invitrogen, Carlsbad, CA, USA). Approximately 1.5 µg RNA was converted to cDNA. For measuring the mRNA levels of IL-9 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 1 µl cDNA in triplicate was used for amplification by the TaqMan RT-PCR system (ABI Prism 7900 H T Sequence Detection System; Applied Biosystems, Foster City, CA, USA) with specific TaqMan primers. The expression of PU.1, IL-9 and IRF4 mRNA was analysed with the following primer sequences (all 5′ to 3′): IL-9: forward 5′ GGGC ATCAGAGACACCAAT-3′, reverse 5′-GGACGGAGAGACACAAGCA-3′;PU.1:forward 5′-CTTCCAGTTCTCGT CCAAGC-3′, reverse 5′-TTCTTCACCTCGCCTGTC TT -3′; IRF4: forward: 5′-ACA GCG CCT GGC CTA TTT TG-3′, reverse: 5′-TGC ATC TAT TAG GCT GGT GA-3′ and β-actin: forward 5′-GCAGAAGGAGATTA CTGC TCT-3′, reverse 5′-GCTGATCCACATCTGCTGGAA-3′ was used as an endogenous reference. Real-time RT–PCR was performed on a Rotor-Gene 3000 (Corbett Research, Sydney, Australia) and mRNA levels were quantified using SYBR Premix Ex Taq™ II. Each reaction was performed in duplicate. Data were analysed using the Rotor-Gene realtime analysis software version 6.0.

2.7. Measurement of serum IL-9, TSLP and IL-17 levels

Venous blood samples were collected into pyogen-free blood collection tubes, immediately immersed in melting ice, and allowed to clot 1 h prior to centrifugation. All the serum samples were stored at -80 °C before use. All the procedures used were standardized. Serum IL-9 levels were measured using specific ELISA kits (R&D Systems, Minneapolis, MN, USA). Each sample was tested in duplicate. The results were expressed as pg /ml and the detection limit of this assay was 0.5 pg / ml. We measured cytokine levels of TSLP and IL-17 using sandwich ELISA method as we reported recently [5; 13] with commercial human cytokine kits (R &D, Minneapolis, MN, USA). To detect TSLP, we read plate at 450 nm and subtracted the values of 570 nm from those of 450 nm performed with Spectra Max M5 multi-detection-microplate reader (Molecular Devices, Sunnyvale, CA, USA).

2.8. Statistical analysis

Results were expressed as the means ± standard deviation (SD). Statistical analysis of the data was performed using the GraphPad Prism (Version 4.0) statistical program. The nonparametric Mann Whitney *U* test was used to compare data between BD patients and healthy controls. Analysis of covariance was used to compare data among three groups. The association of the data with clinical and laboratory parameters of lung manifestations in BD patients was analyzed using the independent samples *t*-test. Pearson correlation coefficient was used and values were indicated in the figures. Two-sided *p*-values < 0.05 were considered to indicate a statistically significant difference.

Table 1
Clinical manifestations of active Behçet's disease (BD). Pulmonary manifestations and bronchoalveolar (BAL) characteristics were reported for the 18 patients with lung involvement compared to healthy controls.

Demographic	Active BD	Healthy controls	*p*
Males/Females	30/0	20 /0	
Age (years)	42 ± 7	47.6 ± 6	
Clinical features of patients	fulfilled the ISG for BD		
Oral ulcers	30 (100%)		
Genital ulcers	21 (70%)		
Skin lesions	20 (66.6%)		
Arthritis	17 (56.6%)		
Ocular lesions	23 (76.6%)		
Neurological involvements	7 (23.33%)		
Pulmonary manifestations	18 patients		
Asymptomatic functional abnormalities	22%		
Pulmonary artery aneurysm	50%		
Pulmonary artery embolism	11.1%		
Pulmonary venous abnormalities	16%		
Pulmonary manifestations (vascular)	18 (60%)		
Cell concentration (*106/L)	187 (140-310)	122 (109-270)	< 0.0001
Total cell number (*106/L)	26 (18-35)	15 (9-27)	< 0.0001
BAL differential cell counts			
% Macrophages	68.7 ± 8.4	55.7 ± 5.2	0.0006
% Lymphocytes	19.5 ± 5.4	11.7 ± 2.3	< 0.0001
% Neutrophils	0.78 ± 0.78	0.1 ± 0.2	
% Eosinophils	0.7 ± 0.2	0 ± 0.2	< 0.0001
CD4: CD8 ratio	2.13 ± 0.37	1.08 ± 0.24	< 0.0001
Blood count and CRP			
White blood cells	8.42 ± 1.76	8.07 + 5.72	0.007
Neutrophil to lymphocyte ratio	2.94 ± 0.62	1.86 + 0.85	< 0.0001
C-reactive protein (CRP)	1.97 ± 3.05	0.13 ± 0.85	< 0.0001

3. Results

3.1. BAL differential cell count and CD4/CD8 ratio

Table 1 represent clinical features, BAL and white blood characteristics of patients with active BD patients compared to healthy controls. BAL lymphocytes and macrophages were higher in active BD than in healthy controls. The CD4/CD8 ratio in BAL of active BD patients was increased (2.13 ± 0.37) than in inactive BD patients (1.2 ± 0.52; $p = 0.0003$) and healthy controls (1.08 ± 0.24; $p = 0.00027$).

In the peripheral circulation neutrophil to lymphocytes ratio was higher in active BD compared to controls and patients in inactive stage (2.01 ± 0.83; $p = 0.026$). High level of neutrophil to lymphocyte ratio in the sera of active BD patients compared to inactive BD and healthy controls support the view that neutrophils play a role in the inflammatory cascade of BD pathophysiology. CRP (C-reactive protein), a major component of inflammatory process was highly increased in active BD patients compared to values observed in inactive BD disease (0.34 ± 0.19; $p < 0.001$).

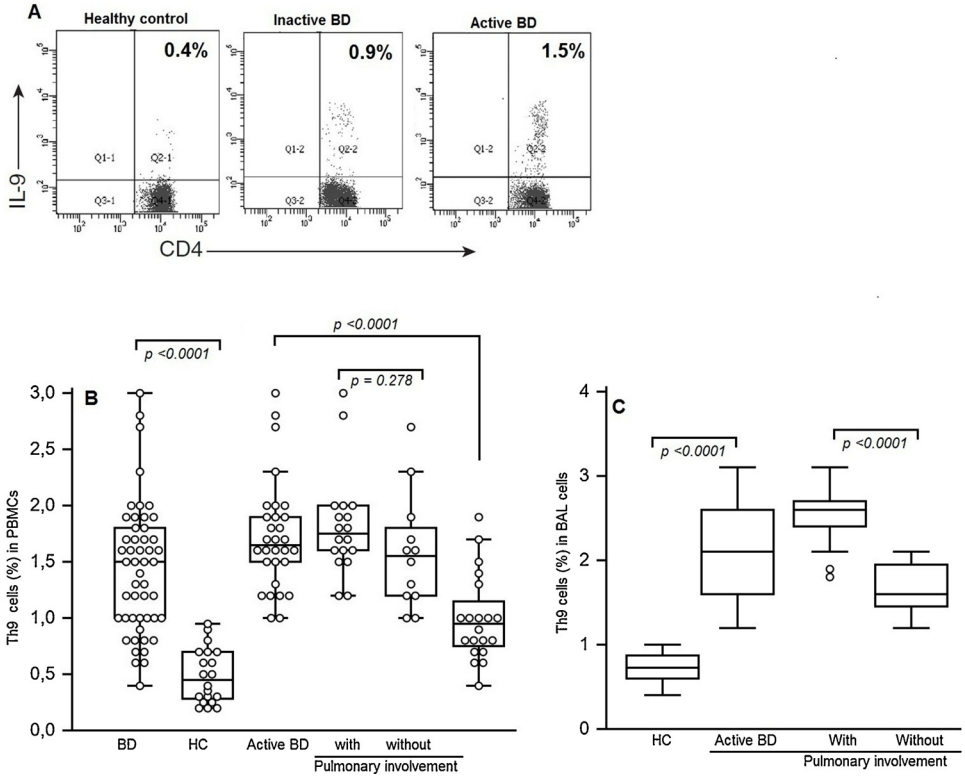

Fig. 1. Flow cytometric analysis of T helper type 9 (Th9) cells percentage in Behçet disease (BD) patients and healthy controls (HC). [A]: Peripheral blood mononuclear cells (PBMNCs) were isolated from BD patients in active and in inactive stages. Cells were stimulated with phorbol myristate acetate (PMA), ionomycin and Golgi Stop for 4 h. The numbers in the upper right quadrant represent the percentage of IL-9$^+$ cells with expression of CD4$^+$ cells. [B]: Expression of Th9% in the peripheral circulation (PBMCs). The percentage of Th9 cells in PBMCs was increased significantly in active and inactive BD patients compared with healthy controls. [C]: Expression of Th9 cell in bronchoalveolar lavage cells from BD patients in active and inactive stages. A p value < 0.05 were considered to be statistically significant. A line inside each box indicates the medians. The 25th and 75th percentiles are indicated by the box limits, the lower and upper error bars represent the 10th and 90th percentiles, respectively.

3.2. Th9 cells in PBMCs and in BAL

Fig. 1 and Fig. 2 represent Th9 cell percentage (CD4$^+$IL-9$^+$/CD4$^+$ T cells) and the absolute number of Th9 cells in the peripheral circulation (PBMCs) and in BAL-cells from patients with BD. Significant increase in the percentage and the absolute number of the Th9 cell in BD patients compared to healthy controls [Fig. 1A, 1B, 1C]. Active BD patients expressed more Th9 cell percentage than inactive BD patients [$p = 0.001$]. There is significant Th9 cell difference between inactive BD patients and healthy controls [$p = 0.0001$]. However, there is no significant difference in Th9 cell percentage and absolute number between active BD patients with and without lung involvements.

BAL-cells from active BD patients expressed more Th9 cells [2.15 ± 0.55%] than healthy controls [0.73 ± 0.16%; $p < 0.0001$] (Fig. 1). Active BD patients with lung involvement expressed more Th9 cell percentage [2.48 ± 0.42%] and absolute number (Fig. 2) comparatively to active BD free from this clinical manifestation [1.65 ± 0.29%; $p < 0.0001$].

3.3. IL-9 and PU.1 mRNA levels in PBMC and in BAL-cells

IL-9 and PU.1 mRNA were studied in the PBMCs and in BAL cells from BD patients compared to controls [Fig. 3A, 3B].

In the peripheral circulation, IL-9 and the PU.1 mRNA in BD were found increased in BD patients compared with healthy controls ($p < 0.0001; p = 0.0001$) [Fig. 3A]. Significant differences were found between active BD (n = 30) and inactive BD (n = 15) patients both in IL-9 percentage and PU.1 mRNA expression ($p < 0.0001$). No significant differences were observed in the IL-9 and PU.1 mRNA expression between active BD patients with lung involvements and active BD free from this manifestation ($p = 0.172; p = 0.146$)[Fig. 3A].

Bronchoalveolar lavage IL-9 and PU.1 mRNA were also tested in patients with BD compared to controls [Fig. 3B]. IL-9 mRNA and PU.1 mRNA exhibited an important increase in BAL from BD patients

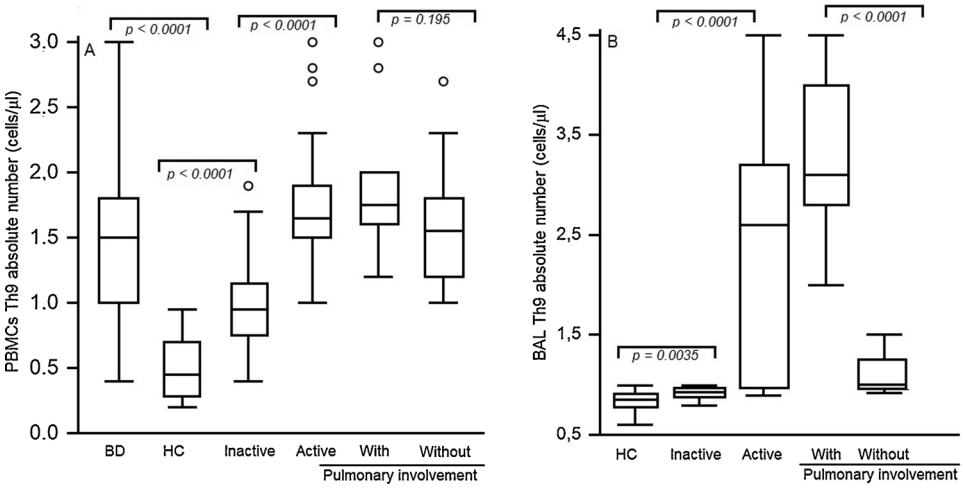

Fig. 2. Expression of absolute counts of Th9 cells in the peripheral blood mononuclear cells (PBMCs) and in BAL cells. A p value < 0.05 were considered to be statistically significant. A minimum of 5000 lymphocytes was collected to enumerate the absolute number of cell subpopulations. Absolute number of Th9 cells was calculated as reported in methods. A line inside each box indicates the median. The 25th and 75th percentiles are indicated by the box limits, the lower and upper error bars represent the 10th and 90th percentiles, respectively.

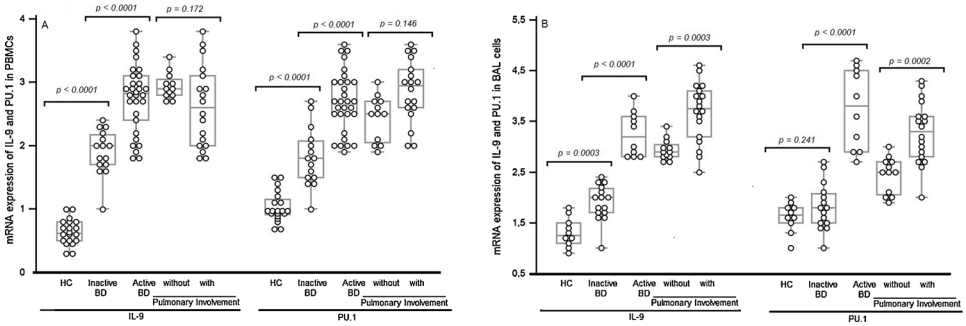

Fig. 3. Measurement of PU.1 and interleukin (IL)-9 mRNA levels in peripheral blood mononuclear cells (PBMCs) and Bronchoalveolar lavage cells (BAL- cells) from Behcet disease (BD) by real-time reverse transcription–polymerase chain reaction (RT–PCR). [A]: The expression of PU.1 and IL-9 mRNA levels in PBMNCs were all elevated significantly in BD patients compared to HC. Significant difference was observed between active (n = 30) and inactive (n = 15) BD stages. Significant differences were observed between active BD having pulmonary manifestations (n = 18) comparatively to patients free from this manifestation (n = 12). [B]: The expression of PU.1 and IL-9 mRNA levels in BAL cells were tested in HC (n = 20), inactive BD (n = 15) and active BD patients with lung manifestations (n = 18) and without lung involvement (n = 12). Significant differences were observed between HC and BD patients. Active BD patients with lung manifestations expressed more IL-9 and PU.1 mRNA than active BD free from this manifestation ($p < 0.0001$). A line inside each box indicates the medians. The 25th and 75th percentiles are indicated by the box limits, the lower and upper error bars represent the 10th and 90th percentiles, respectively.

compared to control (IL-9 mRNA: $p < 0.0001$ and PU.1 mRNA: $p = 0.0006$), this increase was more important between active BD comparatively to inactive BD stage (IL-9: $p < 0.0001$; PU.1: $p < 0.0001$). The most interesting result was the significant increase in IL-9 and PU.1 mRNA expression in active BD patients with pulmonary manifestations compared to those without such manifestations (IL-9: $p = 0.0003$; PU.1: $p = 0.0002$) [Fig. 3B].

Comparing the expression of IL-9 and PU.1 in the peripheral circulation (Fig. 3A) and BAL (Fig. 3B), we noted that IL-9 and PU.1 mRNA were more expressed in the BAL compartment of BD and active BD patients compared to the peripheral blood compartment.

3.4. Serum and BAL fluid IL-9 concentrations

In agreement with the previous results namely the percentages of Th9 cell and the real-time RT–PCR analysis of PU.1 and IL-9 mRNA, we studied IL-9 levels in serum and in BAL-fluid.

Serum IL-9 concentrations were increased in BD patients compared to healthy controls ($p < 0.0001$) (Fig. 4A) (Table 3). The increased serum levels of IL-9 are significantly different between active and inactive BD patients ($p < 0.0001$). There is significant difference in the IL-9 level between inactive BD patients and healthy controls ($p < 0.0001$)

In active BD patients we did not observed any significant difference in the serum IL-9 level according to the presence or not of pulmonary manifestations ($p = 0.911$). Values of serum IL-9 levels (pg/ml) were depicted in Table 3.

In BAL fluid, IL-9 concentration was highly expressed in active BD compared to healthy control ($p < 0.0001$) (Fig. 4B). Significant increase of IL-9 was observed in active BD patients compared to the inactive stage ($p < 0.0001$).

No differences in IL-9 level between active BD patients without lung involvement (14.91 ± 3.11 pg/ml) and inactive BD patients in their BAL-fluid (13.85 ± 2.88 pg/ml; $p = 0.339$). However, the increased IL-9 level is even more important in active BD patients with pulmonary manifestations compared to active BD patients free from lung manifestations ($p < 00001$).

The level of IL-9 in BAL clearly shows that active BD patients with pulmonary manifestations expressed high levels of IL-9 in their BAL fluid compared to those in the active phase do not exhibit such manifestations.

In active BD patients, serum and BAL fluid IL-9 were significantly

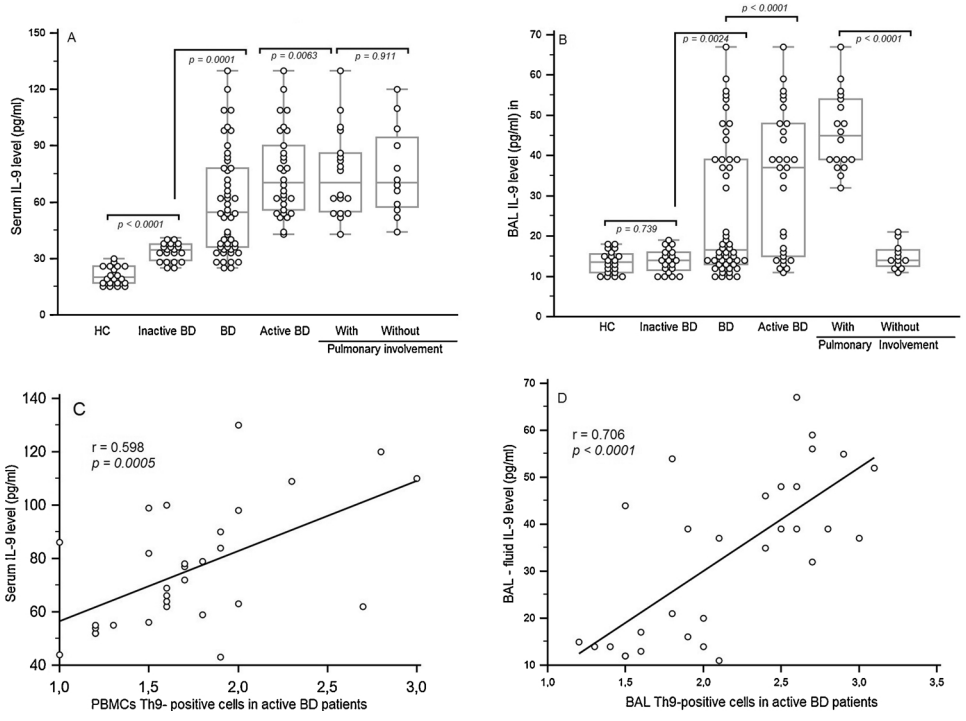

Fig. 4. Serum and Bronchoalveolar (BAL) fluid levels of IL-9 in Behçet's disease. [A]: Expression of serum IL-9. Increased IL-9 level in BD compared to HC. There is no difference in the IL-9 level between active BD patients with lung manifestations and active BD patients without pulmonary involvements ($p = 0.911$). [B]: IL-9 BAL levels were significantly elevated in BD patients compared to HC ($p < 0.0001$). High IL-9 levels were observed in active BD patients with pulmonary manifestations comparatively to patients free from these manifestations ($p < 0.0001$). Inactive BD patients expressed similar IL-9 than active BD patient do not having lung involvements ($p = 0.739$). [C, D]: Correlation between IL-9 levels in serum and BAL fluid with their respective Th9 cells in the PBMCs (r = 0.598; $p = 0.0005$) and BAL cells (r = 0.708; $p = 0.0001$). Spearman coefficient was represented in the figures.

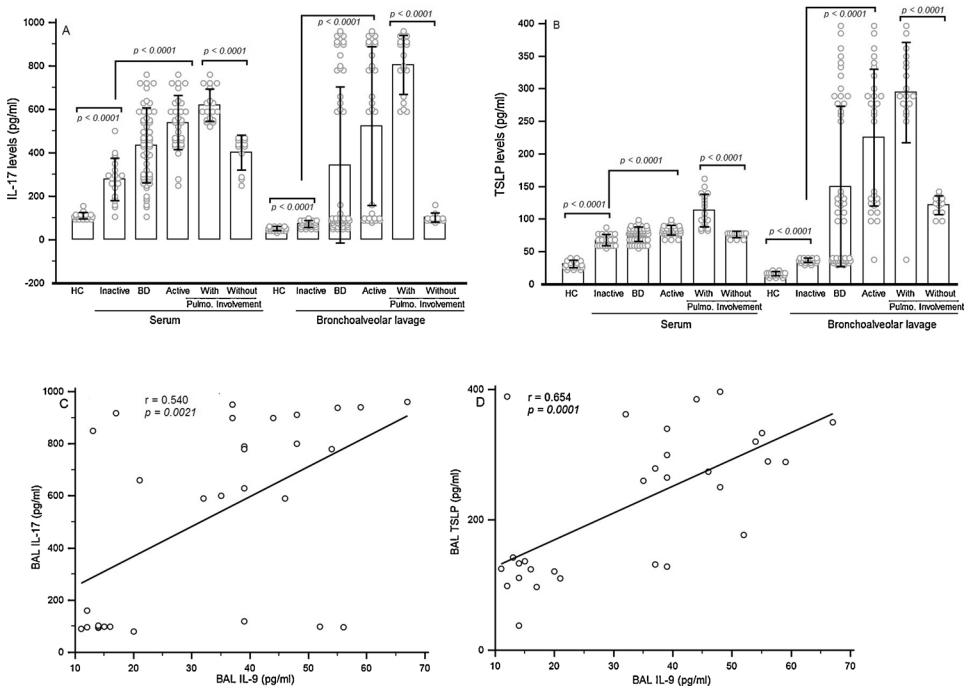

Fig. 5. Serum and Bronchoalveolar (BAL) fluid levels of IL-17 and TSLP in Behçet's disease. [A]: IL-17 levels of serum and BAL. Increased IL-17 level in BD compared to HC. There is significant difference in the IL-17 level between active BD patients with lung manifestations and active BD patients without pulmonary involvements ($p = 0.0001$), in serum and in BAL. [B]: TSLP-BAL levels were significantly elevated in BD patients compared to HC ($p < 0.0001$). High TSLP levels were observed in active BD patients with pulmonary manifestations comparatively to patients free from these manifestations ($p < 0.0001$). [C]: Correlation between IL-17 and IL-9 in BAL [D]: Correlation between IL-TSLP and IL-9 in BAL. Spearman coefficient was represented in the figures.

correlated with $Th9^+$ cell in PBMCs and in BAL (r = 0.59 $p = 0.0005$; r = 0.708; $p < 0.0001$ respectively) (Fig. 4C, 4D).

3.5. Serum and BAL fluid levels of IL-17 and TSLP

In this inflammatory context observed in BD, IL-17 and TSLP could be associated with IL-9 during disease exacerbation. Fig. 5 and Table 2 depicted elevated serum and BAL fluid of IL-17 (Serum: 434.78 + 172.78 pg/ml; BAL: 343.82 + 358.78 pg/ml) and TSLP (Serum: 76.98 + 10.86 pg/ml; BAL: 150.00 + 123.17 pg/ml) in BD patients compared to healthy controls. Table 3 indicated values of IL-17 and TSLP in active BD patients, which were more increased in patients suffering from pulmonary manifestations compared to active BD patients free from this lung manifestation.

Significant correlations were observed between serum IL-9 and TSLP levels of a par (r = 0.378; $p = 0.039$) and secondly between IL-9 and IL-17 (r = 0.383; $p = 0.036$). The significances of correlations found in the BAL-fluids between IL-9 and their respective IL-17 (r = 0.540; $p < 0.0021$) and TSLP (r = 0.654; $p = 0.0001$) levels were more important (Fig. 5C and Fig. 5D) than in the peripheral blood.

3.6. Comparative study of cytokines levels between active and inactive stage after treatment

Twelve patients were investigated during activity and inactivity of the disease. All active BD patients received corticosteroid treatment at the dose of 3–7 mg/ day. IL-9, IL-17 and TSLP decreased significantly in the remission stage after treatment (Fig. 6A, 6B, 6C). The decline in IL-9 and IL-17 expression is more dramatic than the variations observed for TSLP after treatment.

3.7. IL-9 expression in cultured lymphocytes and macrophages of active BD patients

The expression of IL-9, in cultured PBMCs was examined from 12 active BD patients and 10 healthy controls (Fig. 7A, 7B). PBMCs (10^5 cells/ml) were cultured in the presence of IL-2 for one week to expand the lymphocyte population. The proportion of cell making up the PBMCs of BD patients and healthy controls (as reported in materials and methods) were 82% T cells, 4% B cells and 12% monocytes. We further stimulated cultured PBMCs with phorbol myristate acetate (PMA) and ionomycin for 24 h. Following stimulation, the levels of IL-9 measured by ELISA were increased, and importantly PBMCs from active BD patients secreted significantly higher levels compared to healthy controls ($p < 0.0001$) (Fig. 7A).

Additionally, we examined the expression of IL-9 in macrophages (10^3 cells/ml) of normal subjects and active BD patients (Fig. 7B,7). Blood monocytes were expanded in the presence of GM-CSF for one week resulting in more than 95% adherent macrophages determined by morphology in microscope and CD14 staining.

Table 2
Serum and bronchoalveolar lavage (BAL) cytokine levels of IL-9, IL-17 and TSLP in Behçet's disease (BD).

Colonne1	IL-9	pg/ml	IL-17	pg/ml2	TSLP	pg/ml3
	Serum	BAL	Serum	BAL	Serum	BAL
HC	21.05 ± 4.87	13,55 ± 2.76	111.40 ± 14.17	52.50 ± 9.43	31.25 ± 5.40	16.55 ± 3.32
BD	58.90 ± 27.40†	25.64 ± 16.52†	434.78 ± 172.1†	343.82 ± 358.78†	76.98 ± 10.86†	150.0 ± 123.17†
Inactive BD	33.75 ± 4.77†	13.85 ± 2.88	227.95 ± 97.51†	73.45 ± 15.71†	67.85 ± 8.99†	37.15 ± 3.58†
Active BD	75.66 ± 22.95†	33.50 ± 17.21†	539.33 ± 74.97†	524.06 ± 365.12†	83.06 ± 7.11†	225.23 ± 104.8†
Active BD with lung involvement	75.27 ± 23.01	45.88 ± 9.63*	619.77 ± 74.97*	804.88 ± 135.66*	113.22 ± 25.26*	294.33 ± 77.5*
Active BD without lung involvement	74.25 ± 23.88†	14.91 ± 3.11	401.50 ± 80.32†	102.7 ± 20.14†	76.58 ± 5.01†	121.58 ± 14.50†

Values are expressed as (mean ± SD).
[*]: p ≤ 0.0001 compared to active BD patients without lung involvement.
[†]: p ≤ 0.001 compared to HC.

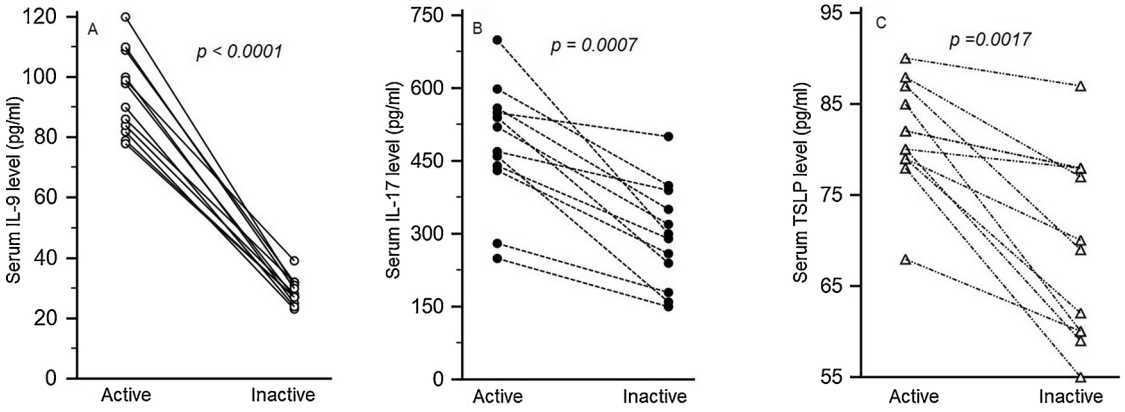

Fig. 6. Comparative study of cytokines IL-9, IL-17 and TSLP levels during BD activity and after a period of 6–8 months of treatment, when they go to the inactivity course of the disease confirmed by a specialist. Treatment was based on corticosteroids. All active BD patients received corticosteroid treatment at the dose of 3–7 mg/ day. IL-9, IL-17 and TSLP decreased significantly in the inactive stage after treatment (Fig. 6A, B, C). The decline in IL-9 and IL-17 expression is more dramatic than the variations observed for TSLP after treatment.

Macrophages (10^3 cells/ml) were then stimulated with LPS for 24 h and secreted IL-9 protein levels were determined with ELISA. The stimulated macrophages from active BD patient's secreted significantly higher levels of IL-9 (compared to macrophages from healthy controls ($p < 0.0001$).

Cultured PBMCs and macrophages were investigated for the expression of PU.1 and IRF4 mRNA expression. In the same way we find higher expression of PU.1 and IRF4 mRNA in BD compared to controls ($p < 0.0001$).

Importantly, the stimulated macrophages and PBMCs from active BD patients secreted significantly higher levels of IL-9 compared to macrophages and PBMCs from healthy controls. This increased IL-9 level (Fig. 7A, 7B) in active BD patients was supported by the significant increased expression of the transcription factors PU.1 (Fig. 7C, 7D) and IRF4 (Fig. 7E, 7 F).

4. Discussion

The importance of Th9 cells in BD has been demonstrated based on the increased expression of IL-9 protein, IL-9 mRNA and PU.1 mRNA in the peripheral circulation and in BAL. The present study showed that Th9 cells were markedly increased in BD patients as compared to healthy controls. IL-9 mRNA and PU.1 mRNA expression in the peripheral circulation and in BAL obtained from BD patients with lung manifestations were significantly higher than BD patients without pulmonary involvements and normal control. Our results show for the first time that the Th9 cell percentage was elevated significantly in BD patients compared with healthy controls, which indicate that Th9 cells may participate in the pathogenesis of BD.

It is well known that BD is characterized by an increased ratio of Th17/Treg cells and recent data suggested the implication of TSLP in its immunopathology [8,13,33,34]. Th17 (IL-17) cells are characterized by their transcription factor RORγt and display a great degree of context-dependent plasticity acquiring functional characteristics of Th1 cells [35]. In BD levels of the immuno-modulatory ratio was expressed as Th1/Th17 [8,36] and actually, Th17/Treg signifies strictly the inflammatory state during the exacerbations of the disease. Treg cells play a critical role in directing and regulating the dynamic plasticity required for balancing Th17/Treg ratio to suppress inflammation, and now we hypothesize the intimately related Th17/Th9 subpopulations as suggested by Muranski & Restifo [35].

Th17 may contribute to the protection against microbes, in return was associated with higher in vivo survival and self-renewal capacity and less senescence than Th1 polarized cells, which have less plasticity and more phenotypic stability inducing more inflammation [35]. Previous studies demonstrated that IL-9 is able to promote the expansion of Th17 cell populations in vitro. In contrast, IL-9 does not seem to facilitate the expansion or survival of FoxP3+ Treg cells [37]. It is however understandable the strong correlation between IL-17 and serum IL-9 in BD in BAL.

Lung microenvironment is greatly dependent on the intricate relationships between mediators providing from different subpopulations Th1, Th17, and the suggested Th9. Th17 subpopulation and their related cytokine profile lend to a state of sustained T cell-driven inflammation seen in autoimmune diseases and in BD. Th9 cells were proposed as a new Th subset because they were found to express IL-9 in the absence of coexpression of subset-defining cytokines and transcription factors [38,39]. IL-9 is expressed only transiently and is predominantly produced in vivo and may have a regulatory and pro-survival function for many lymphoid and myeloid cells as reported by Wilhelm et al. [38]. Th9 cells modulate Th17-mediated sustained inflammatory responses systemically [34,40–42]. The cooperation between IL-9 and IL-17 is also observed during their elevation during the active phase and the decrease of their expression during the inactive stage of BD after treatment. A tremendous situation of immunological cooperation. Therefore, we hypothesize that the development of Th9 cells in BD inflammatory sites requires a balance of signals from Th cytokines and from epithelial cytokines such as thymic stromal lymphopoietin (TSLP) to reprogram the composition of mediators favorable for the amplification of IL-9. Our results confirmed a significant correlation between the IL-17 and TSLP. In addition, the increased level of IL-9 is confirmed by its significant correlation with Th9 cells in the peripheral circulation and in the BAL in active BD patients.

PU.1 plays a fundamental role in the configuration of the Th9 phenotype and constitutes the Th9 cell transcription factor par excellence. PU.1 binds to the IL-9 gene and is required for IL-9 production in human T cells. Decreasing PU.1 expression either by a conditional deletion in murine T cells or siRNA in human T cells impaired IL-9 production, while ectopic PU.1 expression promoted IL-9 production [16].

In agreement with the high percentage of Th9 cells in BD, we first found that the expression of PU.1 and IL-9 mRNA in peripheral circulation and in BAL were significantly increased in active BD patients. More importantly, we found that the Th9 cell percentage, PU.1, and IL-9 expression levels all correlated with BD activity. The levels of IL-9, IL-17, and TSLP decreased significantly in inactive BD compared to patients in the active stage. This decrease was due to the treatment indicating that a probable cytokines homeostasis should be established in inactive BD.

The correlation observed between IL-9 and TSLP during disease

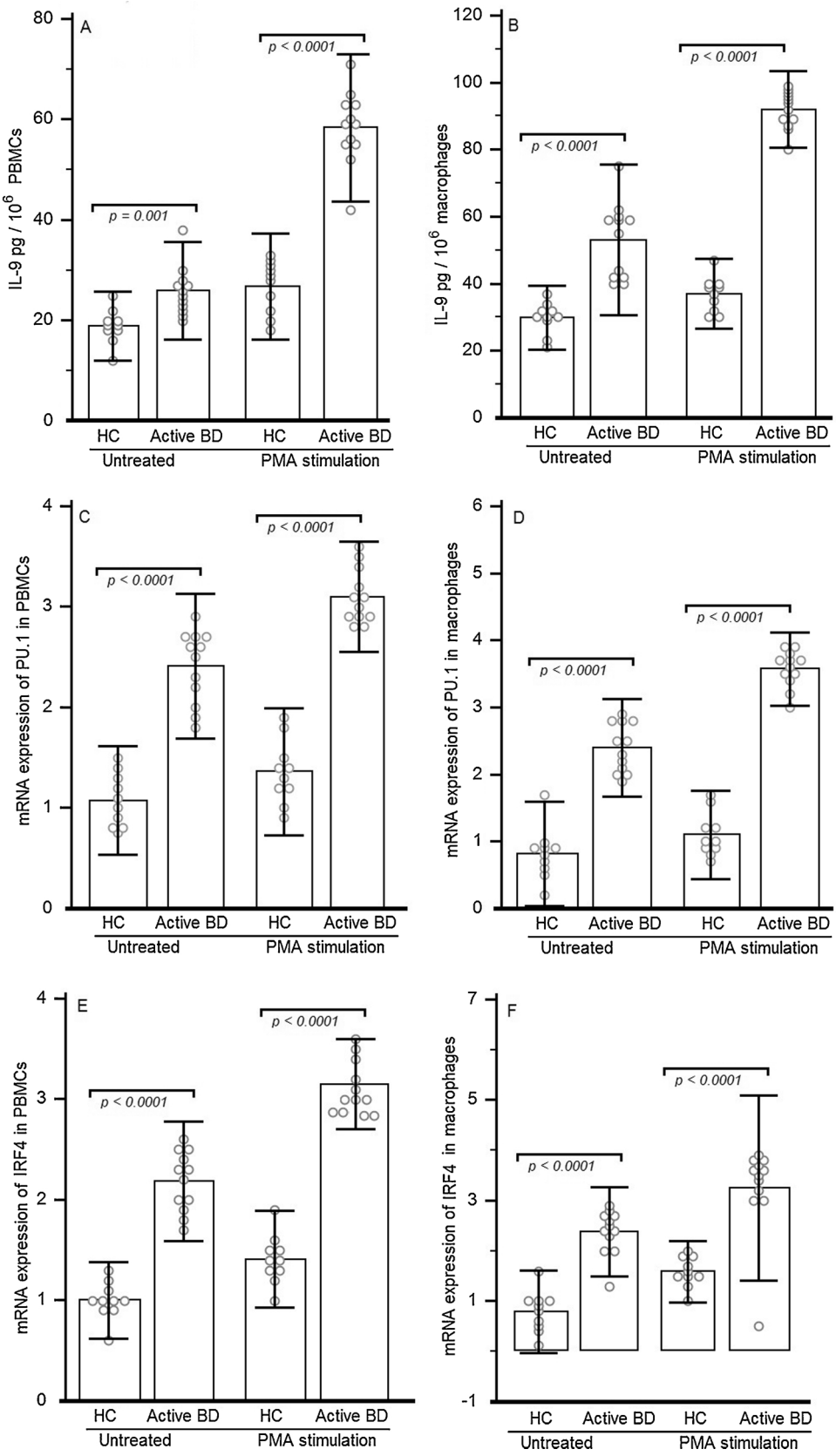

Fig. 7. Levels IL-9, PU.1 mRNA and IRF4 mRNA in cultured PBMCs and macrophages of active BD patients compared to healthy controls. [A; C; E]: PBMCs were cultured in the presence of IL-2 for one week and then stimulated with phorbol myristate acetate and ionomycin (PMA) for 24 h or media alone. Then IL-9 level was quantified in the supernatants by ELISA. Expression of PU.1 and IRF4 mRNA was measured by RT-PCR analysis. [B, D, F]: Adherent monocytes were expanded in the presence of GM-CSF for one week. Macrophages were then stimulated for 24 h with LPS and secreted IL-9 protein levels were determined with ELISA. PU.1 and IRF4 mRNA was investigated in cultured macrophages. IL-9 levels and PU.1 and IRF4 were highly expressed in the macrophage compared to PBMCs cultures.

activity in a first time and their simultaneous decrease in inactive BD patients suggests their intense involvement in the regulation of inflammation. Recently, it has been demonstrated that Th0 can differentiate into a specialized Th9 subset producing IL-9. The Th9 differentiation and IL-9 expression are enhanced by IL-4, TGF-β and particularly by TSLP [37,38] and induced by the activation of transcription factor PU.1 [43,44].

Recently Sugimoto et al. reported that IL-9 is retained at high levels

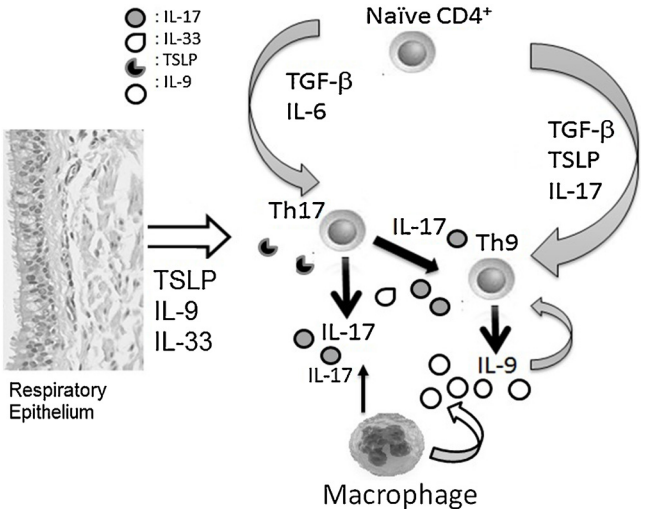

Fig. 8. Hypothetical figure describing the possible interactions between mediators secreted by epithelial cells (TSLP, IL-9), macrophages and cytokines produced by Th17, Th9 cell subpopulations (IL-17, IL-9). Th9 activation may cause the onset and progression of inflammation in the lung of Behçet's disease patients. In the pulmonary environment there is an inflammatory space in the presence of IL-9, IL-17, TSLP activating macrophages and lymphocytes.

in the BAL fluid of a silica-induced mouse lung fibrosis model, and the neutralization of IL-9 suppressed lung inflammation and fibrosis in this model, indicating that IL-9 plays a critical role in silica-induced mouse lung fibrosis [43]. Tan et al. reported that a local injection of recombinant murine IL-9 to the brain resulted in a marked up-regulation of VEGF-A in the striatum. The anti-IL-9-neutralizing antibody can reduce the severity of ischemic stroke partially by alleviating the destruction of the blood-brain barrier (BBB) via down-regulation of astrocyte-derived VEGF-A. This result suggests that targeting IL-9 could provide a new direction for the treatment of ischemic stroke [44]. This suggests that anti-IL-9 monoclonal antibody might be conceived as a possible anti-inflammatory mediator.

Pulmonary manifestations include different manifestations (parenchymal disease, thromboses, and pulmonary artery aneurysms) with hemoptysis, dyspnoea and chest pain. The prognosis is poor, with up to 80% mortality at 20 months [2,45]. The development of inflammation in the lung requires the presence of Th9 cells associated with other cytokines [46]. Given the pleiotropic functions of IL-9, Th9 cells might be involved in several types of inflammatory disease and characterized as circulating biomarkers of inflammation [47]. These bibliographic data and our findings suggest that Th9 cells may make an important contribution to adaptive immune responses occurring in BD and particularly in the BD with lung manifestations. However, Th9 expression in other inflammatory sites will be desired in BD.

In order to determine the possible cells producing IL-9, we found that in vitro PMA-stimulated macrophage from BD patients produced higher IL-9 levels compared to macrophages from healthy controls. The vast majority of IL-9 produced appears to be from macrophage activation in both controls and patients. This could be a favorable argument for the important secretion of IL-9 in BD-BAL from patients with pulmonary manifestations, as the number and activation of macrophages are demonstrated in BD. Diversity of cell types have been reported to express IL-9 mRNA including smooth muscle cells, epithelial cells, fibroblasts, keratinocytes, dendritic cells and particularly activated macrophages [46,48]. Previously published studies could demonstrate that in murine models of asthma, Th9 cells are found in the respiratory tract, especially during early phases of the disease [48,49].

In our study, we report a significant correlation between TSLP and IL-9 level in BD patients. IL-9 is required for TSLP-induced airway inflammation in vivo and acts directly on Th9 cells to enhance their function in vivo [46]. An interaction must necessarily exist between IL-9, IL-17, and TSLP at the inflammatory sites where the resident cells play a preponderant role. We present a hypothetical figure describing the possible interactions between mediators secreted by epithelial cells, macrophages, and cytokines produced by Th17, Th9 cell subpopulations (IL-17, IL-9) (Fig. 8). Therefore, we think that the serum level of IL-9, IL-17, and TSLP could be indicators of BD disease activity. Treatment of BD patients induces a significant decrease of the threesome of cytokines in inactive BD patients.

Recent studies have shown that IL-9 promotes inflammation principally by recruiting macrophages, mast cells, and eosinophils [49,50]. The separate stimulation of lymphocytes and macrophages by PMA show that the latter would be the main producers of IL-9 in BAL. Our data clearly demonstrate the increased expression of IL-9 levels in culture supernatants stimulated macrophages associated with the expression of PU.1 and IRF4 mRNA in Behcet's disease. Th9 cells represent a new member of the still-growing T helper cell family as reported by Staud et al. [17] identifying IRF4 as the essential transcription factor that drives IL-9 production and Th9 cell differentiation. IRF4, together with PU.1, was found to bind to the IRF/Ets response element in the IRF4 promoter, suggesting that IRF4 protein provides a positive feedback signal for its own gene expression [51]. Macrophages and myeloid DCs are important players in the interface between innate and adaptive immune responses. Blood monocytes give rise to both macrophages and DCs, two functionally distinct cell types [51]. Macrophages secrete a large number of inflammatory mediators (IL-17, IL-26, …) and probably IL-9, which remains to be confirmed [52,53]. IL-9 intervenes in the maintenance of barrier functions. It is important to identify the macrophage as a secretory cell of IL-9. Based on the observations that IL-9 can acutely stimulate inflammatory cells, the constant presence of Th9 cells might potentially activate innate immune cells. IL-9 is shown to promote the influx of neutrophils at the site of infections. It is important to establish the link between the important presence of IL-9 and the secretory cell in the BAL [52,53].

5. Conclusion

In conclusion, our results suggest that expansion of the Th9 cell subset, up-regulation of the PU.1 transcription factor and increased secretion of the IL-9 cytokine may contribute to the inflammatory process of pulmonary manifestations in BD, and the increased release of IL-9, PU.1, and IRF4 by stimulated cultured macrophages may further support this point. Targeting Th9 cells in BD could be a promising approach in the future. Therefore, further studies are needed to confirm and extend the current results.

Authors' contributions

Conceived and designed the experiments: AH; KH; KM; WK
Performed the experiments: WK; Ikh; BH
Analyzed the data: JA; KH, KM; WK
Wrote the paper: AH; KH; WK
Revised the manuscript: AH; KH

References

[1] H. Yazici, E. Seyahi, G. Hatemi, Y. Yazici, Behçet syndrome: a contemporary view, Nat. Rev. Rheumatol. 14 (2) (2018) 107.
[2] G. Hatemi, E. Seyahi, I. Fresko, R. Talarico, V. Hamuryudan, One year in review 2017: Behçet's syndrome, Clin. Exp. Rheumatol. 35 (Suppl 108) (2017) S3–15.
[3] E. Seyahi, H. Yazici, Behçet's syndrome: pulmonary vascular disease, Curr. Opin. Rheumatol. 27 (1) (2015) 18–23.
[4] K. Hamzaoui, A. Berraies, W. Kaabachi, J. Ammar, A. Hamzaoui, Pulmonary manifestations in Behçet disease: impaired natural killer cells activity, Multidiscip. Respir. Med. 8 (1) (2013) 29.
[5] W. Kaabachi, E. Bouali, A. Berraïes, I.B. Dhifallh, B. Hamdi, K. Hamzaoui, A. Hamzaoui, Interleukin-26 is overexpressed in Behçet's disease and enhances Th17 related − cytokines, Immunol. Lett. 190 (2017) 177–184.

[6] K. Hamzaoui, H. Abid, A. Berraies, J. Ammar, A. Hamzaoui, NOD2 is highly expressed in Behçet disease with pulmonary manifestations, J. Inflamm. 9 (1) (2012) 3.

[7] R. Deniz, A. Tulunay-Virlan, F. Ture Ozdemir, A.U. Unal, G. Ozen, F. Alibaz-Oner, I. Aydin-Tatli, G. Mumcu, T. Ergun, H. Direskeneli, Th17-inducing conditions lead to in vitro activation of both Th17 and Th1 responses in Behcet's disease, Immunol. Invest. 46 (5) (2017) 518–525.

[8] K. Hamzaoui, Th17 cells in Behçet's disease: a new immunoregulatory axis, Clin. Exp. Rheumatol. 29 (July-August (4 Suppl 67)) (2011) S71-6.

[9] N. Gholijani, M.R. Ataollahi, A. Samiei, E. Aflaki, S. Shenavandeh, E. Kamali-Sarvestani, An elevated pro-inflammatory cytokines profile in Behcet's disease: a multiplex analysis, Immunol. Lett. 186 (2017) 46–51.

[10] V. Dardalhon, A. Awasthi, H. Kwon, G. Galileos, W. Gao, R.A. Sobel, M. Mitsdoerffer, T.B. Strom, W. Elyaman, I.-C. Ho, IL-4 inhibits TGF-β-induced Foxp3+ T cells and, together with TGF-β, generates IL-9+ IL-10+ Foxp3− effector T cells, Nat. Immunol. 9 (12) (2008) 1347.

[11] N. Ambrose, E. Khan, R. Ravindran, L. Lightstone, S. Abraham, M. Botto, M. Johns, D. Haskard, The exaggerated inflammatory response in Behçet's syndrome: identification of dysfunctional post-transcriptional regulation of the IFN-γ/CXCL10 IP-10 pathway, Clin. Exp. Immunol. 181 (3) (2015) 427–433.

[12] G. Beriou, E.M. Bradshaw, E. Lozano, C.M. Costantino, W.D. Hastings, T. Orban, W. Elyaman, S.J. Khoury, V.K. Kuchroo, C. Baecher-Allan, D.A. Hafler, TGF-beta induces IL-9 production from human Th17 cells, J. Immunol. 185 (1) (2010) 46–54.

[13] O. Kacem, W. Kaabachi, I.B. Dhifallah, A. Hamzaoui, K. Hamzaoui, Elevated expression of TSLP and IL-33 in Behçet's disease skin lesions: IL-37 alleviate inflammatory effect of TSLP, Clin. Immunol. 192 (2018) 14–19.

[14] M.-Y. Son, Y.-D. Kim, B. Seol, M.-O. Lee, H.-J. Na, B. Yoo, J.-S. Chang, Y.S. Cho, Biomarker discovery by modeling behcet's disease with patient-specific human induced pluripotent stem cells, Stem Cells Dev. 26 (2) (2017) 133–145.

[15] P.A. Reche, V. Soumelis, D.M. Gorman, T. Clifford, M.-R. Liu, M. Travis, S.M. Zurawski, J. Johnston, Y.-J. Liu, H. Spits, Human thymic stromal lymphopoietin preferentially stimulates myeloid cells, J. Immunol. 167 (2001) 336–343.

[16] Y.J. Liu, TSLP in epithelial cell and dendritic cell cross talk, Adv. Immunol. 101 (2009) 1–25.

[17] V. Staudt, E. Bothur, M. Klein, K. Lingnau, S. Reuter, N. Grebe, B. Gerlitzki, M. Hoffmann, A. Ulges, C. Taube, Interferon-regulatory factor 4 is essential for the developmental program of T helper 9 cells, Immunity 33 (2) (2010) 192–202.

[18] L. Worley, S.G. Tangye, C.S. Ma, What can primary immunodeficiencies teach us about Th9 cell differentiation and function? Immunol. Cell Biol. (2018).

[19] S. Malik, A. Awasthi, Transcriptional control of Th9 cells: role of Foxo1 in interleukin-9 induction, Front. Immunol. 9 (2018).

[20] M. Baeck, A. Herman, L. de Montjoye, E. Hendrickx, P. Chéou, P.M. Cochez, L. Dumoutier, Increased expression of interleukin-9 in patients with allergic contact dermatitis caused by p-phenylenediamine, Contact Dermatitis 79 (6) (2018) 346–355.

[21] X. Yao, J. Zhao, Q. Kong, X. Xie, J. Wang, B. Sun, L. Xu, L. Mu, H. Li, Exogenous IL-9 ameliorates experimental autoimmune myasthenia gravis symptoms in rats, Immunol. Invest. 47 (7) (2018) 712–724.

[22] U.A. Temann, P. Ray, R.A. Flavell, Pulmonary overexpression of IL-9 induces Th2 cytokine expression, leading to immune pathology, J. Clin. Investig. 109 (1) (2002) 29–39.

[23] V.J. Erpenbeck, J.M. Hohlfeld, M. Discher, H. Krentel, A. Hagenberg, A. Braun, et al., Increased expression ofinterleukin-9 messenger RNA after segmental allergen challenge in allergic asthmatics, Chest 123 (Suppl. 3) (2003) 370S.

[24] P.D. Vermeer, R. Harson, L.A. Einwalter, T. Moninger, J. Zabner, Interleukin-9 induces goblet cell hyperplasia during repair of human airway epithelia, Am. J. Respir. Cell Mol. Biol. 28 (3) (2003) 286–295.

[25] J. Kearley, J.S. Erjefalt, C. Andersson, E. Benjamin, C.P. Jones, A. Robichaud, et al., IL-9 governs allergen-induced mast cell numbers in the lung and chronic remodeling of the airways, Am. J. Respir. Crit. Care Med. 183 (7) (2011) 865–875.

[26] F. Wechsler, F. Davatchi, Criteria for diagnosis of Behçet's disease, Lancet 335 (8697) (1990) 1078–1080.

[27] K.S. Akagawa, Functional heterogeneity of Colony-stimulating factor-induced human nonocyte-derived macrophages, Int. J. Hematol. 76 (1) (2002) 27–34.

[28] G.P. Christophi, C.A. Hudson, R. Gruber, C.P. Christophi, P.T. Massa, Promoter-specific induction of the phosphatase SHP-1 by viral infection and cytokines in CNS glia, J. Neurochem. 105 (6) (2008) 2511–2523.

[29] G. Krissansen, M. Elliott, C. Lucas, F. Stomski, M. Berndt, D. Cheresh, A. Lopez, G. Burns, Identification of a novel integrin beta subunit expressed on cultured monocytes (macrophages). Evidence that one alpha subunit can associate with multiple beta subunits, J. Biol. Chem. 265 (2) (1990) 823–830.

[30] K. Hamzaoui, W. Kaabachi, B. Fazaa, L. Zakraoui, I. Mili-Boussen, F. Haj-Sassi, Serum IL-33 levels and skin mRNA expression in Behçet's disease, Clin. Exp. Rheumatol. 31 (3 Suppl 77) (2013) 6–14.

[31] P. Haslam, R. Baughman, Report of ERS task force: guidelines for measurement of acellular components and standardization of BAL, Eur. Respir. J. (1999).

[32] M. Brereton, R. McCafferty, K. Marsden, Y. Kawai, J. Etzell, A. Ermens, International council for standardization in haematology. Recommendation for standardization of haematology reporting units used in the extended blood count, Int. J. Lab. Hematol. 38 (5) (2016) 472–482.

[33] C. Sonmez, A.A. Yucel, T.H. Yesil, H. Kucuk, B. Sezgin, R. Mercan, A.E. Yucel, G.Y. Demirel, Correlation between IL-17A/F, IL-23, IL-35 and IL-12/-23 (p40) levels in peripheral blood lymphocyte cultures and disease activity in Behcet's patients, Clin. Rheumatol. (2018) 1–8.

[34] E. Gündüz, H.Ü. Teke, N.Ş.Y. Bilge, D.Ü. Cansu, C. Bal, C. Korkmaz, Z. Gülbaş, Regulatory T cells in Behçet's disease: Is there a correlation with disease activity? Does regulatory T cell type matter? Rheumatol. Int. 33 (12) (2013) 3049–3054.

[35] P. Muranski, N.P. Restifo, Essentials of Th17 cell commitment and plasticity, Blood 121 (13) (2013) 2402–2414.

[36] Y. Nanke, T. Yago, S. Kotake, The role of Th17 cells in the pathogenesis of Behcet's disease, J. Clin. Med. 6 (7) (2017) 74.

[37] E.C. Nowak, C.T. Weaver, H. Turner, S. Begum-Haque, B. Becher, B. Schreiner, A.J. Coyle, L.H. Kasper, R.J. Noelle, IL-9 as a mediator of Th17-driven inflammatory disease, J. Exp. Med. 206 (8) (2009) 1653–1660.

[38] C. Wilhelm, J.-E. Turner, J. Van Snick, B. Stockinger, The many lives of IL-9: a question of survival? Nat. Immunol. 13 (7) (2012) 637.

[39] M.H. Kaplan, M.M. Hufford, M.R. Olson, The development and in vivo function of T helper 9 cells, Nat. Rev. Immunol. 15 (5) (2015) 295.

[40] P. Zhao, X. Xiao, R.M. Ghobrial, X.C. Li, IL-9 and Th9 cells: progress and challenges, Int. Immunol. 25 (10) (2013) 547–551.

[41] A. Ramming, D. Druzd, J. Leipe, H. Schulze-Koops, A. Skapenko, Maturation-related histone modifications in the PU. 1 promoter regulate Th9 cell development, Blood (2012) Blood-2011-11-392589.

[42] J.H. Bauer, K.D. Liu, Y. You, S.Y. Lai, M.A. Goldsmith, Heteromerization of the γc chain with the interleukin-9 receptor α subunit leads to STAT activation and prevention of apoptosis, J. Biol. Chem. 273 (15) (1998) 9255–9260.

[43] N. Sugimoto, M. Suzukawa, H. Nagase, Y. Koizumi, S. Ro, K. Kobayashi, H. Yoshihara, Y. Kojima, A. Kamiyama-Hara, A. Hebisawa, Interleukin-9 blockade suppresses silica-induced lung inflammation and fibrosis in mice, Am. J. Respir. Cell Mol. Biol. (2018).

[44] S. Tan, Y. Shan, Y. Lin, S. Liao, B. Zhang, Q. Zeng, Y. Wang, Z. Deng, C. Chen, X. Hu, Neutralization of interleukin-9 ameliorates experimental stroke by repairing the blood–brain barrier via down-regulation of astrocyte-derived vascular endothelial growth factor-A, FASEB J. (2019) fj. 201801595RR.

[45] M. Nasser, V. Cottin, The respiratory system in autoimmune vascular diseases, Respiration 96 (1) (2018) 12–28.

[46] W. Yao, Y. Zhang, R. Jabeen, E.T. Nguyen, D.S. Wilkes, R.S. Tepper, M.H. Kaplan, B. Zhou, Interleukin-9 is required for allergic airway inflammation mediated by the cytokine TSLP, Immunity 38 (2) (2013) 360–372.

[47] J.M. Hughes-Austin, K.D. Deane, L.A. Derber, J.R. Kolfenbach, G.O. Zerbe, J. Sokolove, L.J. Lahey, M.H. Weisman, J.H. Buckner, T.R. Mikuls, Multiple cytokines and chemokines are associated with rheumatoid arthritis-related autoimmunity in first-degree relatives without rheumatoid arthritis: studies of the aetiology of rheumatoid arthritis (SERA), Ann. Rheum. Dis. 72 (6) (2013) 901–907.

[48] S.T. Holgate, Innate and adaptive immune responses in asthma, Nat. Med. 18 (5) (2012) 673.

[49] C.P. Jones, L.G. Gregory, B. Causton, G.A. Campbell, C.M. Lloyd, Activin A and TGF-β promote TH9 cell–mediated pulmonary allergic pathology, J. All Clin. Immunol. 129 (4) (2012) 1000–1010 e3.

[50] B. Zhou, M.R. Comeau, T. De Smedt, H.D. Liggitt, M.E. Dahl, D.B. Lewis, D. Gyarmati, T. Aye, D.J. Campbell, S.F. Ziegler, Thymic stromal lymphopoietin as a key initiator of allergic airway inflammation in mice, Nat. Immunol. 6 (10) (2005) 1047.

[51] A. Lehtonen, V. Veckman, T. Nikula, R. Lahesmaa, L. Kinnunen, S. Matikainen, I. Julkunen, Differential expression of IFN regulatory factor 4 gene in human monocyte-derived dendritic cells and macrophages, J. Immunol. 175 (10) (2005) 6570–6579.

[52] N. Sugimoto, M. Suzukawa, H. Nagase, Y. Koizumi, S. Ro, K. Kobayashi, H. Yoshihara, Y. Kojima, A. Kamiyama-Hara, A. Hebisawa, K. Ohta, IL-9 blockade suppresses silica-induced lung inflammation and fibrosis in mice, Am. J. Respir. Cell Mol. Biol. 60 (2) (2019) 232–243.

[53] M. Corvaisier, Y. Delneste, H. Jeanvoine, L. Preisser, S. Blanchard, E. Garo, E. Hoppe, B. Barré, M. Audran, B. Bouvard, J.P. Saint-André, P. Jeannin, IL-26 is overexpressed in rheumatoid arthritis and induces proinflammatory cytokine production and Th17 cell generation, PLoS Biol. 10 (9) (2012) e1001395.