Original Article

Iranian Journal of Immunology
https://iji.sums.ac.ir

Follicular Helper T Cells in Pulmonary Tuberculosis: A Retrospective Study

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ABSTRACT

Background: Follicular helper T lymphocyte (Tfh) promotes antibody production by B lymphocytes in various diseases, including Pulmonary Tuberculosis (PTB).

Objective: To explore the potential role of Tfh cells and assess the expression level of PD-1, and IL-21 in PTB.

Methods: 54 newly diagnosed smear-positive PTB, 27 people with latent tuberculosis (LTB) and 27 healthy controls (HC) were enrolled. The PTB group was further divided based on the range of lung field involved (focus number>=3, PTB-X3; <3, PTB-X2). After 6-month therapy, sputum smear (positive, PTB-SP; negative, PTB-SN) or imaging examinations (lesion reduction significant, PTB-os; insignificant, PTB-s) were used to evaluate the conditions of PTB patients. Blood samples were collected from PTB group at month six. CD4+CXCR5+Tfh, and its subsets, CD4+CXCR5+PD-1+Tfh and CD4+CXCR5+ICOS+Tfh in peripheral blood mononuclear cells (PBMCs) were detected. Serum IL-21 concentrations were measured.

Results: The frequencies of CD4+CXCR5+Tfh, CD4+CXCR5+ICOS+Tfh and CD4+CXCR5+PD-1+Tfh were higher in PTB group than in HC. IL-21, IL-4 and IFNγ concentrations were significantly higher in PTB group than in HC. The proportion of CD4+CXCR5+Tfh in PTB-X2 was lower than in PTB-X3 group. CD4+CXCR5+PD-1+Tfh proportion in PTB-X2 was lower than that in the PTB-X3. After treatment, CD4+CXCR5+Tfh proportion was significantly lower in the PTB-SN group. CD4+CXCR5+Tfh was lower in the PTB-os group than in the PTB-s group. However, the CD4+CXCR5+PD-1+Tfh and cytokine concentrations of IL-21 were not different.

Conclusions: CD4+CXCR5+Tfh level might predict the sputum results, and lesion decrease rate while CD4+CXCR5+PD-1+Tfh subset and IL-21 were not associated with sputum results or lesion decrease after treatment.

Keywords: Tfh, PD-1, IL-21, PTB, Immunological mechanisms

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Cite this article as:
Sun H, Liu J, Feng R, Wang C, Li Y, Wang X. Follicular Helper T Cells in Pulmonary Tuberculosis: A Retrospective Study. *Iran J Immunol.* 2022; 19(1):18-26, doi: 10.22034/IJI.2022.90588.2017.

Received: 2021-03-27
Revised: 2021-07-19
Accepted: 2021-07-20

INTRODUCTION

Tuberculosis is a chronic disease caused by microbacterium tuberculosis (Mtb) infection in humans. A third of the world's population was infected with the Mtb and a majority of them curbed underlying infection through autoimmunity (1). Only 5-10% of the Mtb-infected people developed active pulmonary Tuberculosis (PTB) (1).

$CD4^+$T cells play a considerable role in immune protection from exogenous pathogens due to their multi-facet functions (2). $CD4^+$T cells are discovered to be indispensable in Mtb containment in humans (3). However, the mechanisms of $CD4^+$ T cells in PTB pathogenesis are more than complicated and remain to be unveiled. $CD4^+$T cells were commonly classified into T helper 1 (Th1), T helper 2 (Th2), and T helper 17 (Th17) cells (2, 4). Th1 cells secrete IL-12, IL-2, TNF, and IFN-γ cytokines, and possess the ability to kill cells, and activate monocytes, improving the human immune response (5, 6), while Th2 cells secrete IL-13, IL-4, IL-6, IL10, are involved in B cell proliferation and antibody formation, thus promoting the humoral immune response[7]. Another subtype of $CD4^+$T cells was discovered to express the chemokine receptor CXCR5 and later $CXCR5^+CD4^+$T cells are named T follicular helper cells (Tfh), mainly located in the secondary lymphoid tissues (8). Tfh cells offer help to B cells in germinal center (GC) reaction (8). Furthermore, ICOS and PD-1 were identified in Tfh cells and normally Tfh cells were subtyped as $CXCR5^+CD4^+ICOS^+$Tfh cells and $CXCR5^+CD4^+PD\text{-}1^+$Tfh cells (9). As previously reported, Tfh cells could enhance protective autoimmunity against Mtb (10). In detail, Tfh-deficient mice tended to be susceptible to Mtb while in humans, it was discovered that PTB patients presented differential Tfh cells and IL-21 circulating levels compared with latent Tuberculosis patients (11). A previous study also revealed that IL-4 level reduced as the anti-PTB treatment functioned (12). In addition, IL-4 was discovered to participate in the GC formation through interaction with IL-21 (13). Similarly, IFNγ also engaged in autoimmunity and GC formation (14).

However, there is modest research evaluating the potential associations of Tfh subset level, IL4, IL-21, and IFNγ serum concentrations and the alleviation of PTB.

This study has examined the peripheral blood samples of PTB patients, LTB, HC groups, analyzed the potential roles of IL4, IL-21, and IFNγ serum concentrations in immunity against Mtb. Specifically, we have divided the PTB patients into sub-groups based on the range of lung fields, and further based on sputum smear results or imaging examinations, after six months of treatment, we divided the PTB group into PTB-SN/SP or PTB-os/PTB-s.

MATERIAL AND METHODS

Ethical Approval

All the patients were diagnosed and treated at Tianjin Haihe Hospital between January 1st, 2019 and December 31st, 2019 and agreed to take part in this study. So did the health control group. Informed consent was signed voluntarily by all participants. Ethical approval was authorized by the Ethical Committee of Tianjin Haihe Hospital.

Study Population and Enrollment Conditions

The health control group (HC) included 27 healthy people who did chest radiology examinations, and were diagnosed negative with TB at Haihe Hospital. There were twelve females, neither pregnant nor breeding. And there were fifteen males. The latent TB patients (LTB, n=27, male 10, female 17) underwent chest radiology examinations and showed no PTB symptoms but were diagnosed positive with TB. All the participants in HC and LTB groups were healthy and had no history of pneumoconiosis, HIV, TBS, diabetics, hepatitis, severe heart, liver, kidney diseases, immune system diseases,

or malignant tumors. Meanwhile, PTB group included 54 patients, 36 females, and 18 males. PTB group was further divided based on the range of lung fields involved (focus number>=3, PTB-X3;<3, PTB-X2). There were 24 patients in PTB-X2 and 30 in PTB-X3 group. All the 54 patients were diagnosed with PTB but had not started any treatment yet before the treatment at Haihe Hospital. The diagnosis criteria: two sputum samples were found to be TBS positive or to be positive after separate cultures and chest X-ray showed the tuberculosis symptom.

All the patients were consistently treated with 2HREZ/4HR or 2HREZ/4 (HR) 3 for six months. If the sputum test was still positive after two months of therapy, the session would be extended to 9 months.

Sputum Smear Sampling and Analysis Details

The sputum sample (5-10ml) was collected in a sputum bottle at the end of the 2nd, 5th and 6th month from each participant at the same time after washing their teeth. If the participant did not have enough expectoration, 5-10% normal saline was used to induce sputum. The sputum sample is only qualified when it meets the criteria that less than 10 squamous epithelial cells, more than 25 white cells, or the rate of squamous epithelial cells versus white cells was lower than 1/2.5 on average in each field. The sputum result at the 6th month divided the samples into two subgroups, negative, namely, PTB-SN, and positive, namely, PTB-SP. The sputum smear was analyzed using the fluorescent staining method. Based on the microscopy observations, the smear results were divided into 5 different sub-sets. Negative when no acid-fast bacillus (afb) was found in at least 50 different fields; 1^+ when 10-99 afb per 50 fields; 2^+ when 1-9 afb per field; 3^+ when 10-99 afb per field and 4^+ when above 100 afb per field.

Blood and Serum Sampling and Analysis

All participants were diet-banned for 8 hrs. at night and in the early morning, 3ml EDTA anticoagulant blood and a tube of 3ml ordinary serum were collected and kept in a fridge at 4◦ C. The samples were collected before therapy and at the end of the 2nd, 5th, and 6th month. And all collected samples were treated in 2 hrs. EDTA anticoagulant blood was used to separate peripheral blood mononuclear cells (PBMC), which further underwent Flow cytometry analysis for the ratio of $CD4^+CXCR5^+ICOS^+$Tfh cells / Tfh cells and the ratio of $CD4^+CXCR5^+PD\text{-}1^+$Tfh cells / Tfh cells. The ordinary serum was used for IL-21, IL-4, and IFNγ concentrations via ELISA method. PBMC separation was conducted as previously described (15).

Flow Cytometry Analysis

BB515-CD4 antibody (10ul), AF647-CXCR5 (2.5ul), PE-ICOS (2.5ul), and PerCP-cy5.5-PD-1 antibodies (2.5ul), or isotype controls with the same volume were added into tubes with 100 ul PMBCs (BD Biosciences, CA, USA). After 30-minute incubation in a dark room, the samples were examined on the FACS flow cytometer (BD Biosciences, CA, USA) and analyzed by Flow Jo software (Flow Jo CA USA). After gating for $CD4^+$T cells, the proportions of $CD4^+CXCR5^+ICOS^+$Tfh and the $CD4^+CXCR5^+PD\text{-}1^+$Tfh cells were determined.

ELISA Methods

The human ELISA kits for IL-4, IL-21, and IFN-γ, were used in ELISA experiments (KS12232, KS14488, KS12152, Keshun, Shanghai, China). The product instructions were strictly followed. TECAN microplate reader (TECAN, Austria) was used to read OD values at 450nm.

X-ray Examination

X-ray imaging was used to measure the lesion before treatment and at the end of the 6th month. Lesion absorption evaluation after six months of therapy: If the lesion reduction ratio was no less than 50%, the sample was

counted into the significantly absorbed group, namely, PTB-os; otherwise, the participant was counted into the non-significantly absorbed group, i.e., PTB-ns.

Statistical Methods

All data were processed using Graphpad Prism 8.2 (Graphpad, CA, USA). Mean and SD values were presented in each figure. An unpaired t-test with Welch's correction was performed for difference comparison between the two groups, where $P<0.05$ was considered significant. One-way ANOVA with Tukey's multiple comparison test was performed among HC, PTB, LTB in Figure 1, where $P<0.02$ was considered significant.

RESULTS

Tfh cells, PD-1/ICOS subtypes and cytokine concentrations of IL-4, IL-21, IFNγ in PTB, LTB, HC groups before treatment, IL-21 levels in PTB and HC groups before treatment

The ratio of $CD4^+CXCR5^+$Tfh/Lymphocytes (Lym) was (3.367±0.452)% in PTB group and (2.219±0.472)% in HC group ($P<0.0001$, Figure 1A) and there was no significant difference between LTB and HC groups ($P=0.226>0.02$, Figure 1A). Furthermore, the ratio of $CD4^+CXCR5^+ICOS^+$Tfh/$CD4^+CXCR5^+$Tfh was (1.924±0.157)% in PTB group, which was significantly higher than that in the HC group, (1.103±0.138)%, ($P<0.0001$, Figure 1B), furthermore, there was no statistically significant difference between LTB and HC groups ($P=0.574>0.02$, Figure 1B).

Based on the flow cytometry analysis, the ratio of $CD4^+CXCR5^+PD\text{-}1^+$Tfh/$CD4^+CXCR5^+$Tfh cells was (25.33±2.08)% in the PTB group and (8.42±2.31)% in the HC group ($P<0.0001$, Figure 1C). There was no significant difference between the LTB and

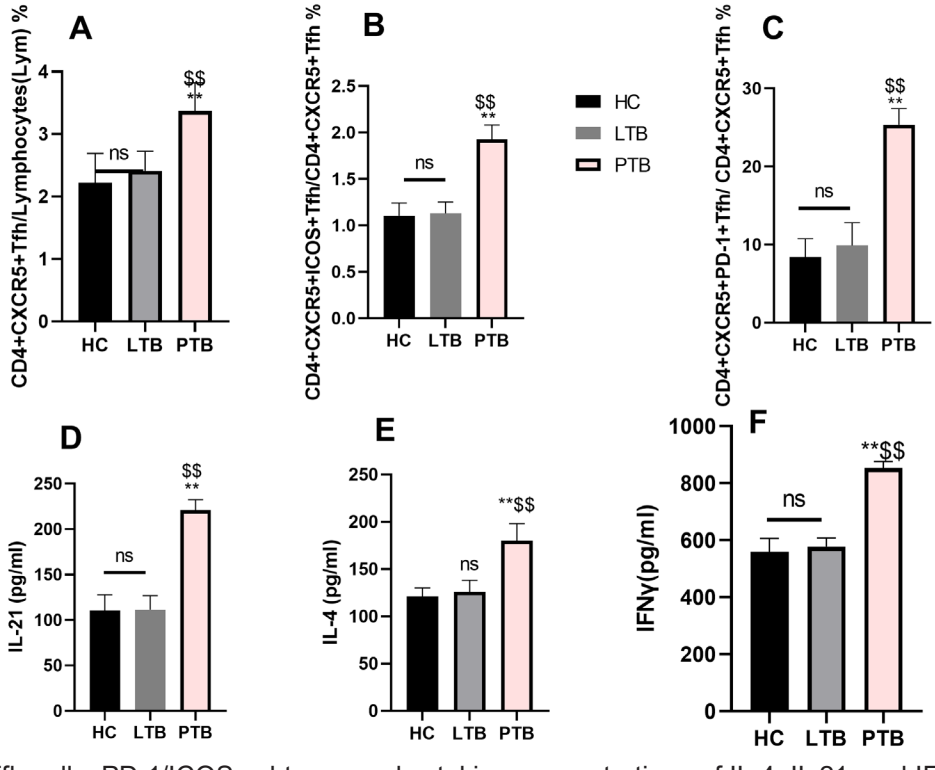

Figure 1. Tfh cells, PD-1/ICOS subtypes and cytokine concentrations of IL-4, IL-21, and IFNγ in PTB, LTB and HC groups before treatment. Before therapy, Flow cytometry methods were used to detect $CD4^+CXCR5^+$Tfh/Lymphocytes (A), (B-C) $CD4^+CXCR5^+ICOS^+/PD\text{-}1^+$Tfh subsets and ELISA method measured the serum IL-21, IL-4 and IFNγ concentrations in participants of HC group, LTB and PTB patients (D-F). One-way ANOVA analyzed the differences and Tukey's method corrected the P-value. ns, not significant. **P<0.02 vs HC. $$P<0.02 vs LTB. PTB: pulmonary tuberculosis patients. LTB: latent tuberculosis. HC: healthy control.

HC groups (P=0.062>0.02, Figure 1C).

In addition, ELISA analysis of IL-21 in serum showed that it was significantly higher in the PTB group (221±11.4) pg/ml than in the HC group (110.5±17.3) pg/ml (P<0.0001 , Figure 1D). There was no significant difference between the LTB and HC groups (P=0.96>0.02, Figure 1D). Similarly, IL-4 was (180±18) pg/ml in PTB group while it was (121±9) pg/ml in the HC (P<0.0001, Figure 1E) and there was no significant difference between the LTB and HCX groups (P=0.136>0.02). IFNγ concentration was elevated in PTB group (853±23) compared to HC group (559±47) (P<0.0001, Figure 1F) while there was no significant difference between LTB group and HC (P=0.108>0.02).

Evaluation of Tfh cell proportion and cytokine concentrations in the PTB-X2 and PTB-X3 groups before treatment.

Furthermore, before treatment, we also investigated the Tfh cells, PD-1$^+$Tfh proportion, and IL-21 differences in the PTB-X2 and PTB-X3 groups. Results showed that CD4$^+$CXCR5$^+$Tfh/Lym rate in PTB-X2 group (2.818±0.332)% was significantly lower than (3.916±0.521)% in PTB-X3 group (P<0.0001, Figure 2A). However, the rates of CD4$^+$CXCR5$^+$ICOS$^+$Tfh/CD4$^+$CXCR5$^+$Tfh presented no significant differences between PTB-X2 and PTB-X3 groups (P=0.2657>0.05, Figure2B). CD4$^+$CXCR5$^+$PD-1$^+$Tfh / CD4$^+$CXCR5$^+$Tfh rate in PTB-X2 (23.51±0.67)% was lower than that in the PTB-X3 (27.15±1.21)% (P<0.0001, Figure 2C). However, the results did not support the fact that IL-21 levels differed in the PTB-X2 and PTB-X3 groups (P=0.380>0.05, Figure 2D). Neither IL-4 nor IFNγ was significantly different between PTB-X2 and PTB-X3 groups (P=0.2950, Figure 2E; P=0.1478, Figure 2F).

Tfh Cell Proportion and Cytokine Concentrations in PTB Patients After Therapy

The PTB patients were grouped into the PTB-SP and PTB-SN subsets according to the sputum smear tests. Flow cytometry methods

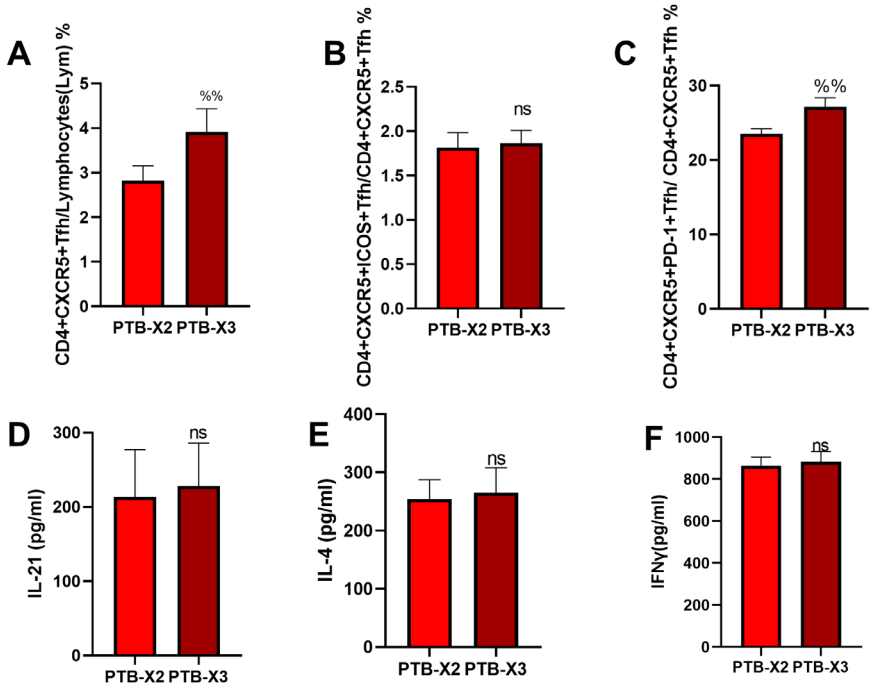

Figure 2. Evaluation of Tfh cell level and cytokine concentrations in PTB-X2 and PTB-X3 groups before treatment. (A-C) Before therapy, Flow cytometry methods were used to detect CD4$^+$CXCR5$^+$Tfh/ Lymphocytes, CD4$^+$CXCR5$^+$ICOS$^+$Tfh/CD4$^+$CXCR5$^+$Tfh, the relative percentage of CD4$^+$CXCR5$^+$PD-1$^+$Tfh cells. (D-F) ELISA method measured IL-21, IL-4, and IFNγ concentrations in PTB patients, PTB-X2 and PTB-X3. Student's t-test analyzed the difference between the two groups. %P<0.05. ns, not significant. PTB-X2: focus number<3. PTB-X3, focus number>=3.

showed that $CD4^+CXCR5^+$Tfh /Lym rate was significantly lower in the PTB-SN group (1.918±0.109)% in comparison with PTB-SP group (2.812±0.201)% at the 6th month (P<0.0001, Figure 3A). $CD4^+CXCR5^+PD\text{-}1^+$Tfh /$CD4^+CXCR5^+$Tfh was also investigated through Flow cytometry methods. As it turned out, there was no significant difference between the two groups, (P>0.05, Figure 3B). ELISA results showed that IL-4, IL-21, and IFNγ concentrations did not differ between the two groups (P>0.05, Figure 3C-E).

On the other hand, imaging examinations were performed, which divided PTB patients into PTB-os and PTB-s groups based on the lesion reduction rate. Moreover, $CD4^+CXCR5^+$Tfh / Lym rate was lower in PTB-os group (2.016±0.209)% than in PTB-s group, (2.611±0.133)% (P<0.0001, Figure 4A). However, Flow cytometry tests showed that $CD4^+CXCR5^+PD\text{-}1^+$Tfh/ $CD4^+CXCR5^+$Tfh rate did not present a significant difference in PTB-os and PTB-s groups (P>0.05, Figure 4B) and ELISA tests showed similar results in IL-21, IL-4, and IFNγ concentrations (P>0.05, Figure 4C-E).

DISCUSSION

Previously, Tfh cells were identified in blood and also GCs were reported to elevate antibody response (16). Tfh cells offer critical help to B cells in GCs of the secondary lymph tissues and thereafter promote the interaction with memory B cells, which facilitate efficient autoimmunity (17, 18). Recently, Tfh cells were reported to function in fibrosis-related diseases (19), encephalomyelitis (20), Systemic Lupus Erythematosus (21), etc. However, the role of Tfh cell level in tuberculosis has seldom been revealed before. One paper reported that a decrease in Tfh cell level was correlated with the reduced blood IL-21 level in PTB-active patients (11). Earlier, it was discovered that PD-1 was highly expressed in Tfh cells and controlled Tfh cell positioning and function through ICOS, and Tfh cells

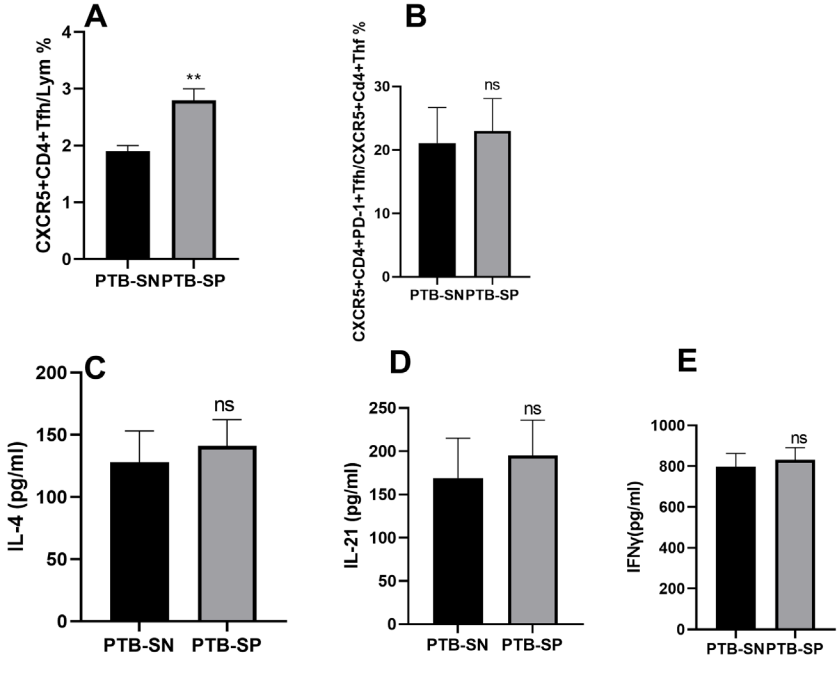

Figure 3. Tfh cell levels and cytokine concentrations in PTB patients (sputum positive group versus negative group) after the therapy. After six months of therapy, sputum tests were done to evaluate the therapeutic effect and divided PTB patients into two subgroups (sputum positive group, PTB-SP versus negative group, PTB-SN). Flow cytometry methods were used to analyze $CD4^+CXCR5^+$Tfh/ Lymphocytes, $CD4^+CXCR5^+PD\text{-}1^+$Tfh/$CD4^+CXCR5^+$Tfh, and ELISA method measured IL-4, IL-21 and IFNγ concentrations in the subsets, PTB-SN and PTB-SP. Vs PTB-SN, **P<0.05. ns: not significant. SN: sputum negative. SP: sputum positive group.

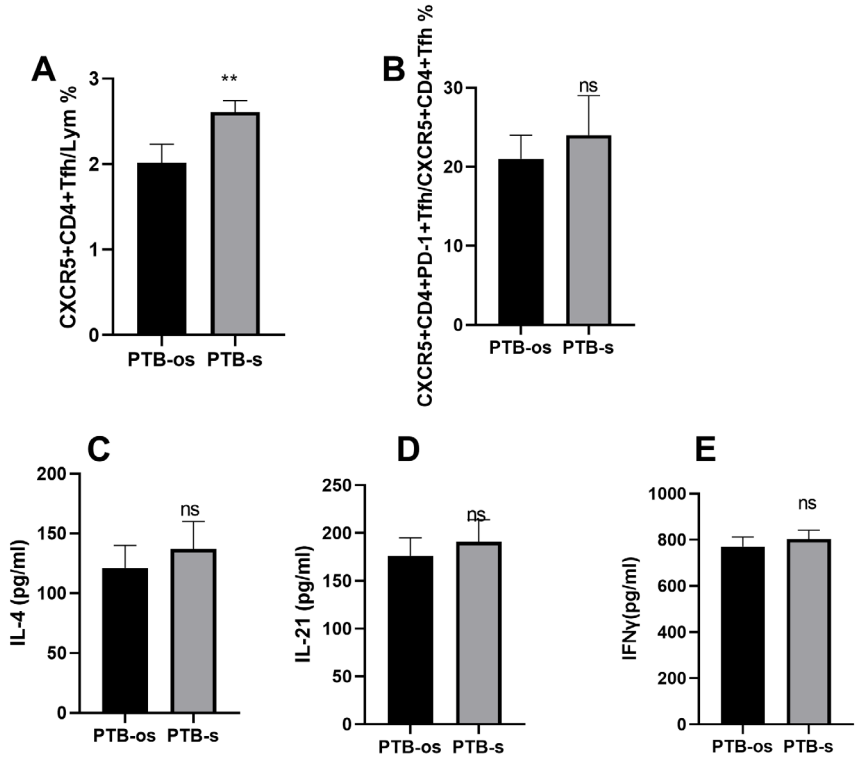

Figure 4. Tfh cell levels and cytokine concentrations in PTB patients with different lesion reduction rates after therapy. After six months of therapy, imaging examinations were performed to observe the lesion reduction rate, based on which, PTB group was divided into PTB-os and PTB-s. Flow cytometry methods were used to detect $CD4^+CXCR5^+$Tfh/Lymphocytes, $CD4^+CXCR5^+PD\text{-}1^+$Tfh/$CD4^+CXCR5^+$Tfh, and ELISA method measured IL-4, IL-21 and IFNγ concentrations in the subsets of PTB patients, PTB-os and PTB-s. **P<0.01. ns, not significant. PTB-os: lesion reduction significant. PTB-s: lesion reduction not significant.

were also divided into two subsets, which were $CXCR5^+CD4^+ICOS^+$Tfh cells and $CXCR5^+CD4^+PD\text{-}1^+$Tfh cells (22).

Different from the previous study on PTB and Tfh cells, this study has involved not only LTB group but also the health control as a negative control and further evaluated Tfh cell proportion and cytokine concentrations of IL-4, IL-21, and IFN γ before and after treatment. In this study, we have discovered that $CD4^+CXCR5^+$Tfh cells, $CD4^+CXCR5^+ICOS^+$Tfh cells, $CXCR5^+CD4^+PD\text{-}1^+$Tfh cells, and IL-4, IL-21, and IFN γ levels were higher in PTB patients compared with the healthy control and LTB group, which suggested that these factors might be involved in the occurrence of PTB in humans. And based on imaging evaluation, the more severe group presented the higher level of $CD4^+CXCR5^+$Tfh cells, $CD4^+CXCR5^+PD\text{-}1^+$Tfh subset but no significant difference in $CD4^+CXCR5^+ICOS^+$Tfh proportion nor IL-4, IL-21, and IFNγ. This fact suggested that $CD4^+CXCR5^+$Tfh cells and $CD4^+CXCR5^+PD\text{-}1^+$Tfh proportion might be used as biomarkers for the severity of PTB patients rather than $CD4^+CXCR5^+ICOS^+$Tfh subset and IL-4, IL-21, and IFNγ. Plasma IFNγ was reported to be stable during the treatment among extrapulmonary TB (EPTB) patients (23). Previously, it was also reported that IFN-γ, IL-4 plasma levels were not different between EPTB and PTB groups (24).

We have further explored the changes of $CD4^+CXCR5^+$Tfh cells, $CD4^+CXCR5^+PD\text{-}1^+$Tfh, and serum IL-21, IL-4, and IFNγ levels in PTB patients after the therapy. Based on the differential results from sputum tests and imaging evaluation, we have found that $CD4^+CXCR5^+$Tfh cell proportion was lower in PTB-SN (vs PTB-SP) and PTB-os (vs PTB-s) after six month of therapy. This

showed that $CXCR5^+CD4^+$Tfh cell levels were correlated with the lesion decrease rate and sputum results after therapy. Furthermore, neither $CD4^+CXCR5^+PD\text{-}1^+$Tfh subset nor IL-4, IL-21, and IFNγ levels were found to be associated with the sputum results, nor with the lesion decrease rate. However, this is not in agreement with previous findings in animal models, which reported that IL-21 receptor-knockout mice had a higher death rate, a higher burden of bacteria, lower antigen-specific T cells in lung tissues as well as lower IFNγ production compared with normal mice after the infection of Mtb (25). Tfh cells secret IL-21 cytokine (26) and other T cells including Th1, Th2, Th17, etc., also secrete IL-21 under certain conditions (27-29), which might help to explain why IL-21 level was not associated with PTB severity while Tfh cells were. Likewise, IL-4 and IFNγ could be secreted by other T helper cells (30, 31). Another clinical retrospective study has reported that there was no significant change of serum IL-4 concentrations before and after treatment (32), which is also validated in our study.

Nevertheless, due to a scarcity of massive data, the conclusions in this study need to be validated via a larger sample size.

CONCLUSION

$CD4^+CXCR5^+$Tfh cells, subsets $CD4^+CXCR5^+ICOS^+$Tfh cells and $CXCR5^+CD4^+PD\text{-}1^+$Tfh cells. Serum IL-4, IL-21, and IFNγ levels might be related to the occurrence of PTB in humans. Furthermore, $CD4^+CXCR5^+$Tfh cells and $CD4^+CXCR5^+PD\text{-}1^+$Tfh subset might be able to predict the severity of PTB lesions in patients. After six months of therapy, it was found that $CD4^+CXCR5^+$Tfh level might be used to predict the sputum results, and lesion decrease rate in patients while $CD4^+CXCR5^+PD\text{-}1^+$Tfh subset and serum cytokine concentrations of IL-4, IL-21, and IFNγ were irrelevant with sputum results or lesion decrease after treatment.

Statement: All authors allowed to publish to this journal.

Conflict of Interest: None declared.

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