Acta Dermatovenerol Croat 2023;31(2):72-79 ORIGINAL SCIENTIFIC ARTICLE

The Role of T Follicular Helper Cells in Clinical Remission and Relapse in Patients with Pemphigus Treated with Rituximab

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ABSTRACT

Background: Pemphigus is a rare chronic autoimmune disease. Recent studies have found that T follicular helper (Tfh) cells may play a role in autoimmune diseases. In this study, Tfh cells frequency, BCL6 gene expression, IL-21, and IL-6 cytokines levels were examined, with the aim of understanding the effect of RTX on these cells in the onset of clinical remission or relapse in patients with pemphigus.

Methods: 20 patients with pemphigus vulgaris and 20 healthy controls without any autoimmune diseases that were admitted to the Dermatology and Venereology Clinic of the Akdeniz University Hospital were included. Peripheral blood sample was taken from all individuals and studied to analyze Tfh cell distribution, IL-21 and IL-6 distribution in CD3+CD4+CXCR5+ lymphocytes with flow cytometry, plasma IL-21 levels with ELISA, and mRNA levels that refer to BCL6 expression with PCR.

Results: Circulating Tfh cell distribution and IL-21 and IL-6 distribution in CD3+CD4+CXCR5+ lymphocytes and mRNA levels that refer to BCL6 expression showed no difference between patient and control groups. However, in patients who had received rituximab treatment there was a significant reduction in Tfh cells compared with other groups. Plasma IL-21 levels were significantly higher in the patient group.

Conclusions: We found that plasma concentrations of the cytokine IL-21 were greatly increased in the pemphigus compared with the control group. There were no significant differences in Tfh cell percentages between the patient and control groups. Tfh cells were decreased in patients who received rituximab treatment. Our findings show that the response to RTX in pemphigus causes a reduction in circulating T follicular helper cells, but not in the plasma IL-21 level. Further studies are required to clarify the role of Tfh cells in pemphigus vulgaris.

KEY WORDS: T follicular helper, pemphigus, BCL6, IL21

INTRODUCTION

Pemphigus is a rare, chronic, autoimmune bullous disease. The most common form is pemphigus vulgaris (PV) (1). The main pathology is characterized by the production of antibodies (Abs) to two desmogleins composing the desmosome, which are adhesion molecules of the epidermis: desmoglein (DSG)-1 and DSG-3 (3,4). Autoreactive T-cells assist B-cells in the production of pathogenic autoantibodies (5). T follicular helper (Tfh) cells are a recently defined subtype of CD4 + T helper cells and play a critical role in

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humoral immunity by assisting germinal center formation, affinity maturation, high-affinity antibody development, and B memory cell development (6). Tfh cells were first identified as active T-cells expressing C-X-C chemokine receptor type 5 (CXCR5) located in germinal centers. Gene expression assays showed that transcription profiles were different between Tfh cells and other effector CD4+ T-cells. Tfh cells express molecules such as BCL-6, inducible T-cell costimulator (ICOS), SLAM-related protein (SAP), CD84, as well as CXCR5 and others (7). The ICOS signal can stimulate the production of IL-21, which enhances Tfh cell growth and supports B-cell response. Tfh cells, primarily those on germinal center stage, express high doses of PD1. PD1 is a co-inhibitor from the family of CD28. PD1 signals provide peripheral tolerance by reducing interactions between T-cells and antigen-presenting cells (7). Although the role of this inhibitor pathway on Tfh cells is not completely understood, analysis on PD1 or PD1 ligand deficient mice supports the hypothesis that PD1 plays an important role in the B-cell cycle in the germinal center and for in memory B-cell formation (8). BCL-6 is the most important transcription factor for Tfh cells. High expression of BCL-6 promotes CXCR5 transcription. IL-6, one of the proinflammatory cytokines, is an important cytokine for Tfh cell development and has been reported to stimulate BCL-6 expression in active CD4+ T-cells and to be a potent inducer of IL-21 (9). IL-21 cytokine also plays a role in plasma cell differentiation and in production of different types of antibodies (10). A recent study reported that the long-term response to RTX in patients with pemphigus resulted in a reduction of circulating DSG-specific T follicular helper cells (11). Li *et al.* reported that, after effective treatment, the frequencies of Tfh cells as well as serum IL-21 levels decreased with clinical improvement in patients with BP (15). These findings demonstrated the role of Tfh cells after stimulation. Further studies are required to better understand the regulation of Tfh cells and the roles of these molecules involved in regulation. So far, little is known about the role of Tfh cells in autoimmune bullous diseases. Therefore, we investigated Tfh cell distribution and intracellular IL-21 and IL-6 distribution in CD3+CD4+CXCR5+ lymphocytes, plasma IL-21 levels, and, unlike other studies, mRNA levels expressing the BCL6 gene in the peripheral blood samples of patients with PV and healthy controls. In addition, we aimed to determine the frequency and phenotype of TFH cells and the effect of RTX on these cells at the onset of clinical remission or relapse in patients with pemphigus.

PATIENTS AND METHODS

Subjects

Identification of the patient and control groups

This comparative clinical study was approved by the Ethics Committee of Akdeniz University Faculty of Medicine (27.02.2014; 2014.04.0103.014) and was conducted according to the principles of the Declaration of Helsinki. The control group consisted of 20 people who were free of a history of autoimmune diseases and infection, had not received any treatment within the previous 3 months, and had never received an immunosuppressant drug. We recruited 20 patients who were diagnosed with PV by clinical, histopathological, immunofluorescence, and ELISA methods; diagnosed either recently or by the Dermatology and Venereology Clinic of the Akdeniz University Faculty. Informed consent was obtained from both the patient and control group participants, all of whom participated voluntarily in the study. Written informed consent was obtained from all participants. Demographic data, clinical characteristics, and Pemphigus Disease Area Index (PDAI) values at the time of inclusion were recorded on patient registration forms (Supplementary data).

ELISA

The IL-21 level in plasma samples of patients with pemphigus vulgaris and controls without autoimmune disease was determined using the Enzyme-Linked Immunosorbent Assay (ELISA) method. A sandwich ELISA IL-21 kit coated with IL-21 monoclonal antibody was used (YH biosearch, catalog # YH-B1723Hu, Shanghai, China). The test was performed according to the manufacturer's instructions. Measurements in the control group were performed at the same time as in patients with pemphigus vulgaris. Il-21 level was calculated by determining the absorbance value at 450 nm wavelength in the spectrophotometer (Bio-Rad, iMark Microplate Reader, California, USA).

Flow cytometry

Tfh lymphocyte profiles were determined as follows: anti CD45RO (FITC), anti-CD4 (APC-Cy7), anti-CXCR5 (PerCP-Cy5.5), anti-CD3 PE-Cy7 (1C6/CXCR3), anti-ICOS (PE), anti-PD1 (APC) (BD Bioscience, San Jose, CA, USA), and isotypic control antibodies (BD Bioscience, San Jose, CA, USA). Fluorescence signals of 10,000 cells per sample were measured on a flow cytometer (BD FACS CANTO II). In order to detect IL-21 and IL-6 cytokine levels in Tfh cells, these cytokines

were examined using intracellular direct fluorescent labeled antibodies. The following antibodies were used: anti IL-21 (PE), anti-IL-6 (APC-), and isotypic control antibodies of the same colors were used to determine the expression of these monoclonal antibodies (BD Bioscience, San Jose, CA, USA).

PCR (polymerase chain reaction)

Gene-specific exon-skipping primers were designed to determine the level of target BCL6 gene mRNA expression. In the presence of a cDNA pattern and primers, quantitative PCR (Rotorgene©) was performed using the SYBR Green containing polymerase enzyme. The beta actin gene was used as a reference. The cycle values for target (BCL6) and reference (beta actin) genes were normalized using the REST© program.

Statistical analysis

Data were analyzed using PASW 20 (SPSS / IBM, Chicago, IL, USA). Descriptive statistics such as frequency distribution, mean, and standard deviation were used to describe the sample. Suitability of normal distribution was examined using the Kolmogorov-Smirnov test. Since the parametric test assumptions were not performed, the Mann-Whitney U test was used for both groups. In addition, the relationship between continuous variables was analyzed using Pearson and Spearman correlation coefficients. A 95% significance level (or α=0.05 error margin) was used to determine the differences.

RESULTS

A total of 40 (20 patients with PV and 20 control cases without autoimmune disease) patients were included in the study. The PV group consisted of 7 men and 13 women, with a mean age of 52±13, while the control group consisted of 13 women and 7 men, with a mean age of 57±13. Demographic and clinical data of the patient and control groups are presented in Table 1. The mean duration of disease in patients with PV was 4.63±4.41 years. 18 of these patients were followed-up in our clinic, while 2 were recently-diagnosed patients with PV. Of the 18 patients undergoing follow-up, 9 had relapsed and the other 9 were in remission. This included the patients in relapse before initiation. Duration of disease, treatment, and PDAI values of patients with PV are presented in Table 2. Patients and controls were compared in terms of demographic characteristics, and there was no significant difference in gender and age (P=0.999, P= 0.172, respectively) between the two groups. When plasma IL-21 levels of the patient and control groups were examined using the ELISA method, median levels were 378 and 116 pg/mL, respectively. IL-21 level was significantly higher in the patient group compared with the control group (P<0.001), as shown in Figure 1. When compared by means of Tfh cell profiles, there was no significant difference between the two groups in terms of CD3+CD4+CXCR5+ (P=0.236), CD3+CD4+CXCR5+ICOS+, and CD3+CD4+CXCR5+PD1+ (P= 0.447) cells (Figure 2 A-E). Similarly, there was no significant difference between the two groups with regard to intracellular IL-21 and IL-6 levels in CD3+CD4+CXCR5+ lymphocytes (P=0.274) and mRNA levels expressing the BCL6 gene (P=0.890). CD3+CD4+CXCR5+ and CD3+CD4+CXCR5+PD1+ cell ratios, plasma IL-21 levels, intracellular IL-21 ratios of CD3+CD4+CXCR5+ lymphocytes, and distribution of BCL6 gene mRNA expression levels of groups. The control group was compared with patients who received rituximab treatment, patients who did not receive rituximab therapy (patients who only received systemic CS ther-

Table 1. Demographic and clinical data of the patient and control groups

Characteristic	Pemphigus vulgaris	Control group
Gender		
Female	13	13
Male	7	7
Mean age	52±13	57±13
Clinical course		
Recent diagnosis	2	-
Relapse	9	
Remission	9	
Mean disease duration (years)	4.63±4.41	-
Mean PDAI	6.25±10.07	-

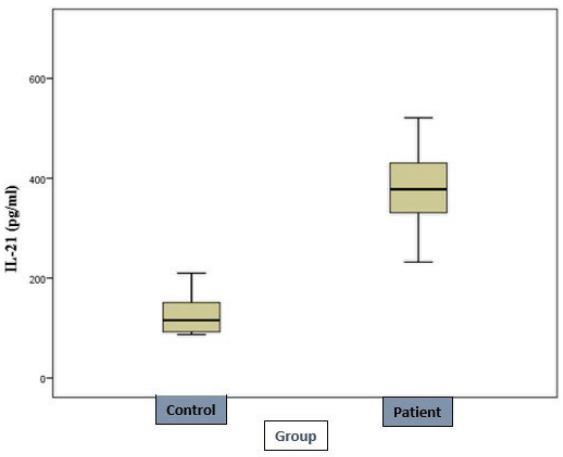


Figure 1. Plasma IL-21 levels in the control and patient groups (P<0.001).

Table 2. Characteristics of patients with PV

Gender	Age	Disease duration (years)	Treatment	PDAI
F	35	1.5	-	0
F	47	8	8 mg/g pred	0
F	75	0,5	40 mg/g pred	27
F	53	1	8 mg/g pred	4
F	50	8	- *	1
F	40	19	- *	0
F	51	4	-	12
M	58	1	4 mg/2g pred	0
M	39	0.83	20 mg/g pred	0
F	56	0.33	-	28
F	47	5	-	0
F	37	1	24 mg/g pred	0
M	66	6	-	0
M	58	7	24 mg/g pred	2
F	79	1	16 mg/g pred	3
M	51	7	8 mg/g pred*	21
F	35	3	- *	1
M	72	8	-	24
M	46	5	8 mg/g pred*	0
F	40	5.5	8 mg/g pred	2

*Patients who had previously received rituximab therapy

apy or were followed up without treatment at that time), patients receiving only systemic CS therapy, and patients in follow-up without treatment. Tfh cells in patients receiving rituximab treatment were found to be significantly lower compared with other groups; CD3+CD4+CXCR5+ (*P*=0.014, *P*=0.014, *P*=0.033, *P*=0.028, respectively) and CD3+CD4+CXCR5+PD1+ (*P*=0.044, *P*=0.018, *P*=0.039, *P*=0.035, respectively) (Figure 3, Figure 4).

DISCUSSION

In the present study, we examined the levels of Tfh cells, BCL6 gene expression involved in the induction of Tfh cells, and quantities of IL-21 and IL-6 cytokines in CD3+CD4+CXCR5+ lymphocytes, which increased BCL6 gene expression and regulated Tfh cells and IL-21 plasma levels in the peripheral blood of healthy controls and patients with PV.

Tfh cells are characterized by the expression of CXCR5 chemokine receptor and the production of IL-21, a cytokine that is essential for the generation of memory B-cells and plasma cells (10). They have been identified as a distinct T helper cell subset, based on their unique combination of surface markers rich expression of CXCR5, decrease of C-C CCR7, expression of ICOS and PD-1, cytokine production expression of high levels of IL-21, and specific transcription factor expression of Bcl-6 (10). In several ab-mediated autoimmune diseases such as SLE and RA, it has

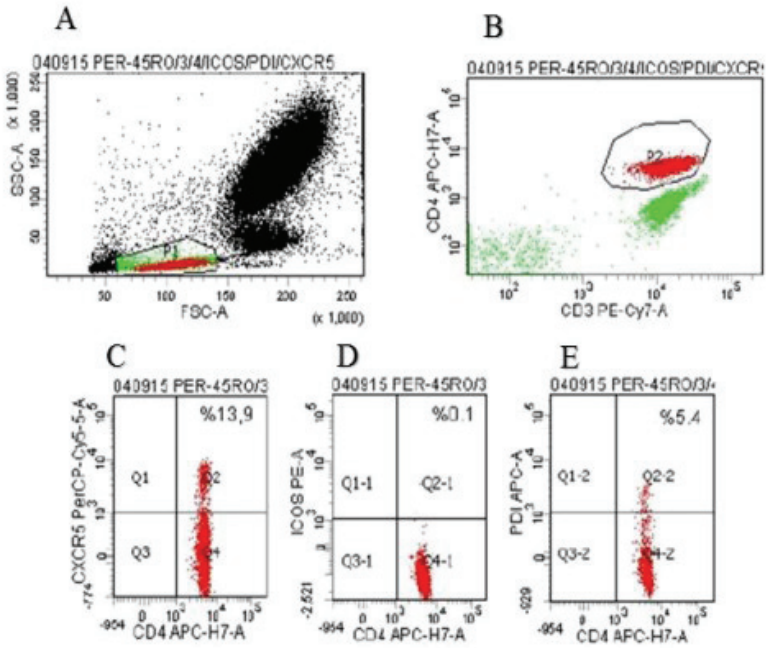


Figure 2. Determination of Tfh cells by flow cytometry. (A) The distribution of leukocytes in peripheral blood samples according to cell size and granularity was examined first, and the lymphocyte cell group was differentiated. (B) Cells positive for CD3 and CD4 were detected in selected lymphocyte group cells. Cell percentages detected in the CD4 + T lymphocytes: (C) CXCR5+, (D) CXCR5+/ICOS+, (E) CXCR5+/PD1+

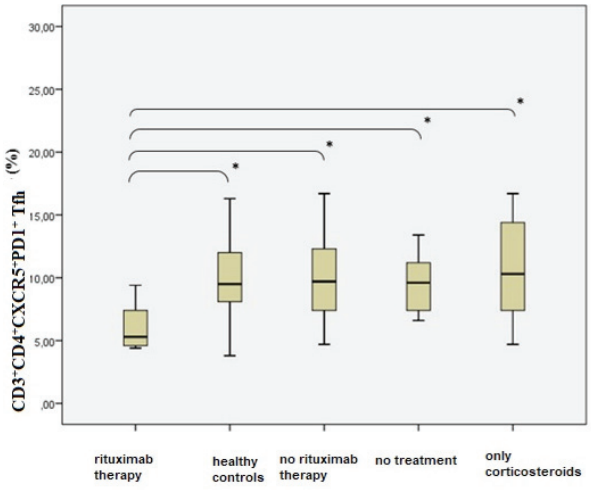


Figure 3. Distribution of CD3+CD4+CXCR5+PD1+ Tfh cells in the patient group according to treatment intake and in the control group.

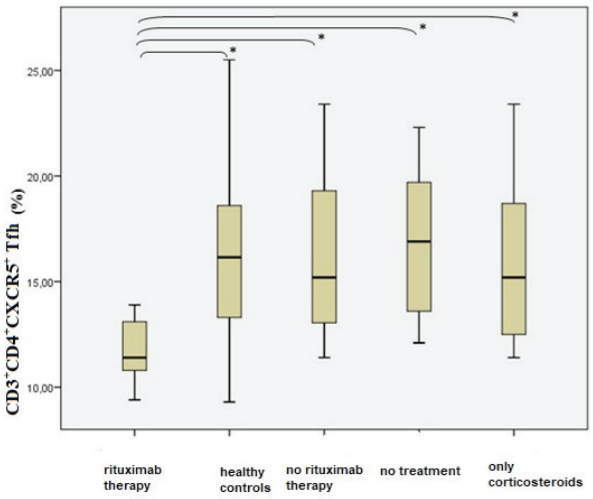
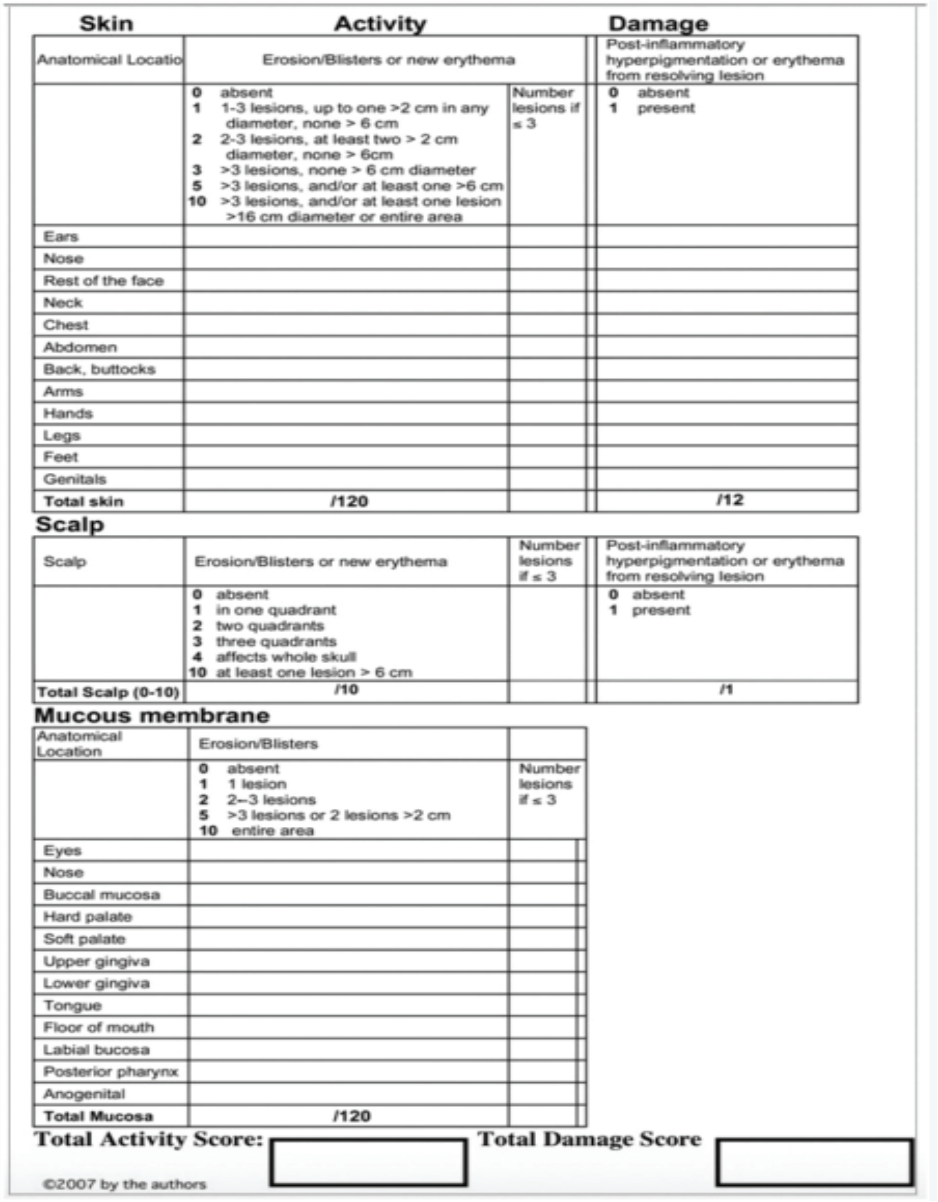


Figure 4. Distribution of CD3+CD4+CXCR5+ Tfh cells in the patient group according to treatment intake and in the control group.

been found that expansion of Tfh cells (defined as CD4+CXCR5+PD-1+ICOS+ T-cells) may be associated with disease activity (11-13,15-17).

A higher frequency of circulating TFH cells and increased serum IL-21 levels have been recently reported in patients with pemphigus relative to healthy controls (16). Hennerici *et al.* have included patients with pemphigus who received similar treatment to our study, and no significant difference was noted between the two groups in terms of CD3+CD4+CXCR5+ICOS+ and CD3+CD4+CXCR5+PD1+ cell groups, although there was a significant difference between patient and control groups in terms of CD3+CD4+CXCR5+ cells (16). When the circulating Tfh cell groups were evaluated in the present study, there was no significant difference between the patient and control groups in terms of CD3+CD4+CXCR5+PD1+ lymphocyte distribution, similar to this study (P=0.239). However, we did not find high expression levels of CD3+CD4+CXCR5+ cells in patients (P=0.460). In our study, we examined plasma IL-21 levels in patients and controls and found that IL-21 levels were significantly higher in the patient group (P<0.001). However, the absence of significant elevations in CD3+CD4+CXCR5+ cells and the presence of only elevated IL-21 plasma levels were inconsistent with our predictions and other studies (13,16). This suggests that the increase in IL-21 synthesis in patients with PV may also be caused by other cells beside Tfh cells. There was no significant difference between the two groups when we examined intracellular IL-21 prevalence in CD3+CD4+CXCR5+ T-lymphocytes in patients and controls (P=0.257). These findings also support the hypothesis that relatively higher plasma IL-21 levels in the patient group may be caused by cells other than Tfh cells. As a matter of fact, Th17 is a distinct CD4 T-lymphocyte subtype that is thought to play an important role in autoimmune disorders and pemphigus, and the major cytokines it synthesizes include IL-21 as well as IL-17 (21,22). IL-6 is a cytokine involved in the differentiation of Tfh cells, and this task induces expression of BCL6 gene required for Tfh differentiation in recently activated CD4+ T-lymphocytes through the IL-6 receptor (6). We also investigated intracellular IL-6 cytokine along with IL-21 in CD3+CD4+CXCR5+ T-lymphocytes. However, we could not detect an intracellular fluorescence signal with the IL-6 antibody on lymphocytes in patient and control groups. Since we did not perform any induction, intracellular staining may not have occurred.

BCL6 is a transcription factor that is necessary for the transition of Tfh cells from CD4 T-lymphocytes. It is required for early CXCR5 expression on Tfh cells (6). The mRNA levels of the BCL6 gene were measured in the PCR study performed on patients and controls, and no significant difference was found between the two groups (P=0.905). There were no study available in the literature investigating BCL6 in PV disease. In our study, there was no significant increase in Tfh cells of patients with PV compared with the control group; the fact that there was no significant elevation in mRNA levels reflecting the expression of the BCL6 gene involved in the differentiation of Tfh cells is a predictable outcome. In conclusion, we did not



Skin **Activity** **Damage**

Anatomical Locatio	Erosion/Blisters or new erythema		Post-inflammatory hyperpigmentation or erythema from resolving lesion
	0 absent 1 1-3 lesions, up to one >2 cm in any diameter, none > 6 cm 2 2-3 lesions, at least two > 2 cm diameter, none > 6cm 3 >3 lesions, none > 6 cm diameter 5 >3 lesions, and/or at least one >6 cm 10 >3 lesions, and/or at least one lesion >16 cm diameter or entire area	Number lesions if ≤ 3	0 absent 1 present
Ears			
Nose			
Rest of the face			
Neck			
Chest			
Abdomen			
Back, buttocks			
Arms			
Hands			
Legs			
Feet			
Genitals			
Total skin	**/120**		**/12**

Scalp

Scalp	Erosion/Blisters or new erythema	Number lesions if ≤ 3	Post-inflammatory hyperpigmentation or erythema from resolving lesion
	0 absent 1 in one quadrant 2 two quadrants 3 three quadrants 4 affects whole skull 10 at least one lesion > 6 cm		0 absent 1 present
Total Scalp (0-10)	**/10**		**/1**

Mucous membrane

Anatomical Location	Erosion/Blisters	
	0 absent 1 1 lesion 2 2–3 lesions 5 >3 lesions or 2 lesions >2 cm 10 entire area	Number lesions if ≤ 3
Eyes		
Nose		
Buccal mucosa		
Hard palate		
Soft palate		
Upper gingiva		
Lower gingiva		
Tongue		
Floor of mouth		
Labial bucosa		
Posterior pharynx		
Anogenital		
Total Mucosa	**/120**	

Total Activity Score: **Total Damage Score**

Supplementary data. PDAI Index

find any significant difference in Tfh cell distribution, mRNA level expressing the BCL6 gene, and intracellular IL-21 levels in Tfh cells of patients with PV compared with healthy volunteers. Plasma IL-21 levels were significantly higher in patients with PV than controls.

A significant number of patients in our study were receiving immunosuppressive therapy, and we cannot ignore the therapeutic effect this may have had. There was a significant decrease in CD3+CD4+CXCR5+ and CD3+CD4+CXCR5+PD1+ Tfh cell levels, especially in rituximab-treated patients. The low number of patients in our study, some of the existing patients being in remission, the majority of active patients being under immunosuppressive treatment, and the majority of patients therefore having mild to moderate disease severity may have been reasons why Tfh cell levels were not significantly elevated. An example that supports this view is the study of Li Q *et al.* on a bullous pemphigoid patient group. In this study, circulatory CD3+CD4+CXCR5+PD1+ cell ratios were determined in the pre-treatment stage, and the patients were given oral or intravenous methylprednisolone therapy. After treatment, CD3+CD4+CXCR5+PD1+ levels were re-evaluated; when compared with pre-treatment rates, a significant reduction was reported

in this cell group (15). When we evaluated the effect of treatment on Tfh cells in our study, which was similar to this study, we found that CD3+CD4+CXCR5+ Tfh cell levels were significantly lower in patients who received rituximab treatment compared with the control group and those who did not receive this treatment (*P*=0.014, *P*= 0.014, respectively). In particular, when we compared patients who received rituximab treatment with the control group and other patients who did not receive rituximab therapy, the levels of CD3+CD4+CXCR5+PD1+ Tfh cells were also significantly lower in patients receiving rituximab than the other two groups (*P*=0.044, *P*=0.018, respectively). In a study conducted by Xu *et al.*, circulatory Tfh cell ratios in patients with type 1 diabetes before and after rituximab treatment were analyzed; similarly to our study, there was a significant reduction in Tfh cell levels after treatment (18). These results also show that there was a significant decrease in Tfh cell levels, especially in rituximab-treated patients. The reasons for these differences may be factors such as the likelihood of discrepancy of Tfh cell subtypes that play a role in BP and PV, the difference in the kits used, or the fact that one study was performed on patients receiving treatment while the other study was conducted on patients who did not receive any treatment.

CONSLUSION

Studies in the literature are not sufficient to explain the exact role of Tfh cells and their subtypes in PV disease; however, significantly lower detection of Tfh cell ratios in rituximab-treated patients than in the control group may play a role in the pathogenesis of the disease. In order to better understand the role of Tfh cells in PV pathogenesis, subtype interpreting studies are required on wider patient groups, such as active patients who do not receive immunosuppressive treatment, and it is necessary to evaluate the cases before and after treatment.

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