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The Effect of Conventional Immunosuppressive Therapy on Cytokine Serum Levels in Pemphigus Vulgaris Patients

Hossein Mortazavi[1,2], Nafiseh Esmaili[1,2], Somayeh Khezri[1,2], Ali Khamesipour[3], Iman Vasheghani Farahani[4], Maryam Daneshpazhooh[1,2], and Nima Rezaei[5,6]

[1] Autoimmune Bullous Diseases Research Center, Tehran University of Medical Sciences, Tehran, Iran
[2] Department of Dermatology, Tehran University of Medical Sciences, Tehran, Iran
[3] Center for Research and Training in Skin Diseases and Leprosy, Tehran University of Medical Sciences, Tehran, Iran
[4] Sharif University of Technology, Tehran, Iran
[5] Research Center for Immunodeficiencies, Children's Medical Center, Tehran University of Medical Sciences, Tehran, Iran
[6] Molecular Immunology Research Center; and Department of Immunology, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran

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ABSTRACT

Pemphigus vulgaris is an autoimmune disease, in which the role of Th17 cytokines needs to be further explored. This study was performed to assess serum levels of three interleukins (IL) required for Th17 differentiation (IL-1β, IL-6 and IL-23) and two specific Th17 cytokines (IL-17 and IL-22) in a group of patients with pemphigus vulgaris, at baseline, 3 weeks and 6 months after of treatment. Correlations between anti-desmogleins and cytokines with disease severity as well as the influence of therapy on the above factors were assessed.

Forty-three first-admitted pemphigus vulgaris patients with the active disease entered the study, but only 31 completed the study. Forty-five healthy volunteers were recruited as a control group. The patients were treated with conventional immunosuppressive therapy (oral prednisolone and azathioprine). Cytokines and anti-desmogleins were measured, using enzyme-linked immunosorbent assay. General linear model was used to evaluate the changes over time.

In patients at baseline, mean serum level of IL-6 was higher, while mean levels of IL-1β and IL-22 were lower than the controls. After 3 weeks of therapy, IL-1β and IL-6 levels showed a decreasing trend, whereas IL-22 showed an increasing trend. Mean anti-desmogleins 1 and 3 values decreased significantly during the time. Anti-desmoglein values were significantly correlated with disease severity.

In conclusion, IL-1β and IL-6 could be involved in the pathogenesis of pemphigus vulgaris. The positive trend of IL-22 is a new finding and should be confirmed by further studies.

Keywords: Desmogleins; Interleukin-1beta; Interleukin-6; Interleukin-17; Interleukin-22; Interleukin-23; Pemphigus vulgaris

Corresponding Author: Hossein Mortazavi, MD;
Razi Hospital, Vahdat Islamic Square, 1199663911 Tehran, Iran.
Tel: (+98 21) 2206 6052, Fax: (+98 21) 2207 0142, E-mail: mortazma@sina.tums.ac.ir and mortazavikerman@gmail.com

INTRODUCTION

Pemphigus vulgaris (PV) is an autoimmune disease

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in which autoreactive T and B cells are involved in the pathogenesis of the disease with production of antibodies against desmogleins (dsg) 1 and 3.[1,2]

Most of the studies in the pathogenesis of PV are related to Th1 and Th2 cells, and corresponding cytokines.[1,3,4] A pathogenic role for Th17 in autoimmune inflammations such as psoriasis was reported,[5,6] but there are only a few reports in PV[7-9] and in only one study Th17 cells were shown in lesions of PV.[7]

Effector T cells, based on cytokine profile and types of inflammation promotion are divided into Th1, Th2, Th9, Th17 and Th22 subgroups.[10]

Specific cytokines for $CD4^+$ T cell subsets are IFN-γ for Th1; IL-4, IL-5, IL-13 for Th2; and IL-17 and IL-22 for Th17 cells.[11] IL-1β, IL-6, IL-23 and TGF-β are required for Th17 cell differentiation.[10]

The role of cytokines in the pathogenesis of PV and other autoimmune bullous diseases is controversial hence more studies are needed to elucidate this challenging issue.[12] Based on the high prevalence of PV in Iran, we decided to study the cytokines of the Th17 pathway (IL-1β, IL-6, IL-23, IL-17 and IL-22) in PV patients.[13]

In this study, we evaluated the three cytokines required for Th17 differentiation (IL-1β, IL-6 and IL-23) and two specific Th17 cytokines (IL-17 and IL-22). The levels of the cytokines were checked in PV patients before treatment when the patients were in the active phase, and again at 3 weeks and 6 months after the initiation of conventional immunosuppressive therapy (CIST). The baseline cytokine levels were compared with the control group. The changes of cytokine levels during the time and the correlations of cytokine levels and anti-desmoglein (anti-dsg) 1 and 3 serum antibodies with the severity of the disease with regard to the influence of CIST were evaluated.

MATERIALS AND METHODS

Patients

This study was carried out at the Autoimmune Bullous Diseases Research Center of Razi Hospital, Tehran, Iran, from March 2010 to September 2011. Forty three PV patients were recruited based on the inclusion/exclusion criteria, which were defined as newly diagnosed PV patients with a confirmed diagnosis by clinical, histological and direct immunofluorescence microscopic studies, with no history of previous steroid or immunosuppressive therapy.

Forty three PV patients (male=19, female=24) with the mean age of 41.12 ± 9.61 years (range, 21-64 years) were recruited. The mean duration of the disease was 4.88 ± 2.97 months.

The patients were treated with CIST (2 mg/kg/day oral Prednisolone and 2.5 mg/kg/day azathioprine (Imuran)). By considering the disease activity trend and response to therapy, prednisolone was gradually tapered by 30% (at doses greater than 80 mg/day); thereafter the doses was reduced by 10 mg/week until 30 mg/day was achieved, afterwards the reduction was slower.

The study was approved by the Ethics Committee of Vice-chancellor for Research of Tehran University of Medical Sciences. The patients who were willing to sign an informed consent form and participate in the study were included.

Controls

As the control group, 45 (male=16, female=29) healthy individuals without any skin disease or autoimmune disorders were included in this study. The mean age of the control group was 38.14 ± 10.51 years (range, 21-54 years).

The blood samples from the control group were collected once at the baseline.

Assessment

Baseline assessments of the enrolled patients were done before treatment (first visit). The PV patients were also assessed at 3 weeks (second visit) and 6 months after initiation of the treatment, when the patients were on maintenance therapy and in partial remission (third visit). In each visit, the severity and activity of the disease were checked according to the pemphigus vulgaris severity score[14,15] (Table 1) and 5 ml blood samples were collected. All the samples were taken between 9:00 am and 11:00 am. Sera were isolated and kept at -70 °C until use. The serum levels of IL-1β (Quantikine high sensitivity enzyme-linked immunosorbent assay, HS ELISA), IL-6 (Quantikine HS ELISA), IL-17 (Quantikine ELISA, human), IL-22 (Quantikine ELISA, human) and IL-23 (Quantikine ELISA, human) were measured using ELISA (USA & Canada, R&D systems Inc., Minneapolis, MN, USA) according to the manufacturer's instructions. The scales of measurement

Table 1. Cutaneous and mucosal severity

Grade of severity	Site of involvement	
	mucosal involvement (erosions)	cutaneous involvement (BSA*)
0	No erosion	No lesion
1	<3 erosion	<10%
2	4-10 erosions	10%-30%
3	>10 erosions	>30%

*Body surface area (BSA)

for interleukins were picogram/ml.

The anti-dsg 1 and 3 antibodies in serums were titrated using the ELISA method (Euroimmun AG, Lübeck, Germany) according to the manufacturer's instructions. The cut-off value for anti-dsg 1 and 3 was 20 relative units per milliliter (RU/ml). Values ≥20 Ru/ml were interpreted as positive.

Statistical Analysis

SPSS statistical software was used to analyze the data. Descriptive values are presented as mean (± SD) and interquartile range. At the baseline, Mann–Whitney U-test was used to compare the mean rank values between the patients and the control group. In order to find changes occurring during the study period, general linear model (repeated measurement) was used. Spearman's rank correlation was used to evaluate the relationship between autoantibodies (anti-dsg) and disease severity. *P*-value less than 0.05 was considered as statistically significant.

RESULTS

Among 43 patients, 31 patients (male=12, female=19) with the mean age of 40.32 ± 9.14 years (range, 21-64 years) completed 6 months of follow-up. The mean duration of the disease was 5.23 ± 3.28 months.

The values of anti-dsg 1 and 3 antibodies were positive (value ≥ 20RU/ml) for all 43 PV patients at the baseline assessment (active phase). The mean serum values of anti-dsg 1 antibodies decreased significantly from 218.106 ± 150.417 RU/ml at the baseline to 79.681 ± 149.216 RU/ml at 3 weeks after therapy and 88.965 ± 95.980 RU/ml at 6 months after therapy ($p<0.001$, Table 2). The mean serum values of anti-dsg 3 antibodies also decreased significantly from 219.545 ± 156.010 RU/ml at the baseline to 84.355 ± 143.823 RU/ml at 3 weeks after therapy and 67.316 ± 66.722 RU/ml at 6 months after therapy ($p<0.001$, Table 2).

Table 2. Cytokine and anti-dsg values at baseline, 3 weeks, and 6 months after therapy (31 patients)

Cytokine/anti-dsg*	Baseline Mean ± SD**	3 weeks Mean ± SD	6 months Mean ± SD	*P*-value
IL-1β	0.074 ± 0.093	0.229 ± 0.401	0.094 ± 0.112	0.020
IL-6	4.119 ± 5.901	5.090 ± 6.844	2.474 ± 2.169	0.181
IL-17	1.226 ± 4.792	0.006 ± 0.036	0	0.142
IL-22	4.855 ± 14.654	3.765 ± 9.212	11.768 ± 28.217	0.191
IL-23	0.019 ± 0.090	0.013 ± 0.071	1.487 ± 8.261	0.381
Anti-dsg 1	218.106 ± 150.417	79.681 ± 149.216	88.965 ± 95.980	<0.001
Anti-dsg 3	219.545 ± 156.010	84.355 ± 143.823	67.316 ± 66.722	<0.001

*Anti-dsg; Anti-desmoglein

**SD; standard deviation

Table 3. The comparison of cytokine levels between patients and controls at baseline (using Mann-Whitney U test)

Cytokine	Patients			Controls			*P* - value
	Mean ± SD	Median	Interquartile ranges [25th-75th]	Mean ± SD	Median	Interquartile ranges [25th-75th]	
IL-1β	0.074±0.093	0	0-0.1	0.148±0.284	0.1	0-0.1	0.209
IL-6	4.119±5.901	1.6	0.6-4.2	2.031±2.711	1.4	0.7-1.8	0.427
IL-17	1.226±4.792	0	0-0	0.064±0.432	0	0-0	0.339
IL-22	4.855±14.654	0	0-1.1	10.049±52.042	0	0-0.8	0.827
IL-23	0.019±0.090	0	0-0	0	0	0-0	0.136

SD; standard deviation

For each cytokine and anti-dsg, the mean and standard deviation at each phase of the study are presented in Table 2. In addition, the serum baseline levels of IL-1β, IL-6, IL-17, IL-22 and IL-23 in patients and the control group and a comparison of each cytokine between the groups are presented in Table 3.

The mean level of IL-1β was lower in PV patients than in the control group at the baseline and at 6 months of therapy (Tables 2, 3). Fluctuations in IL-1β levels were observed during 6 months of immunosuppressive therapy. The changes in IL-1β levels over the time were significant (p=0.02, Table 2). IL-1β levels and changes during the study period (trend) are shown in Figure 1.

The mean level of IL-6 was higher in PV patients compared to the control group at the baseline and during the therapy (Table 2, 3). Fluctuations also occurred in IL-6 levels during the period of study (increased after 3 weeks and decreased thereafter). Changes over time were not significant (p=0.181, Table 2). IL-6 levels and changes during the study period are shown in Figure 2.

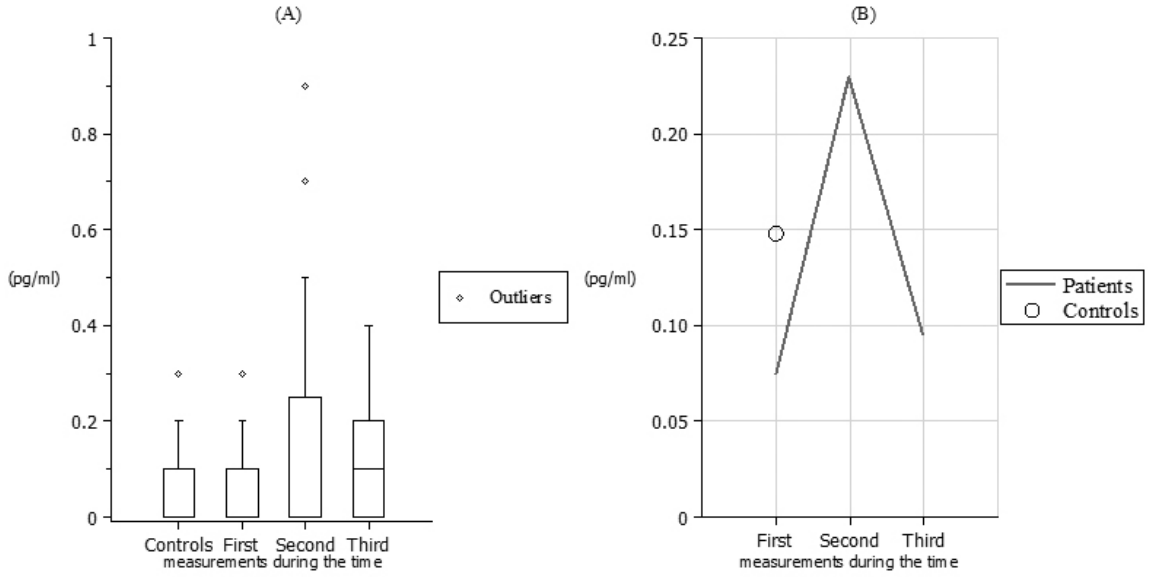

Figure 1. IL-1β levels and trend during the study period: A) Three measurements of IL-1β levels in PV Patients vs. Controls. Outlier data are shown as well. B) Changes in IL-1β levels during the time according to General Linear Model. The mean cytokine level of Control is also shown.

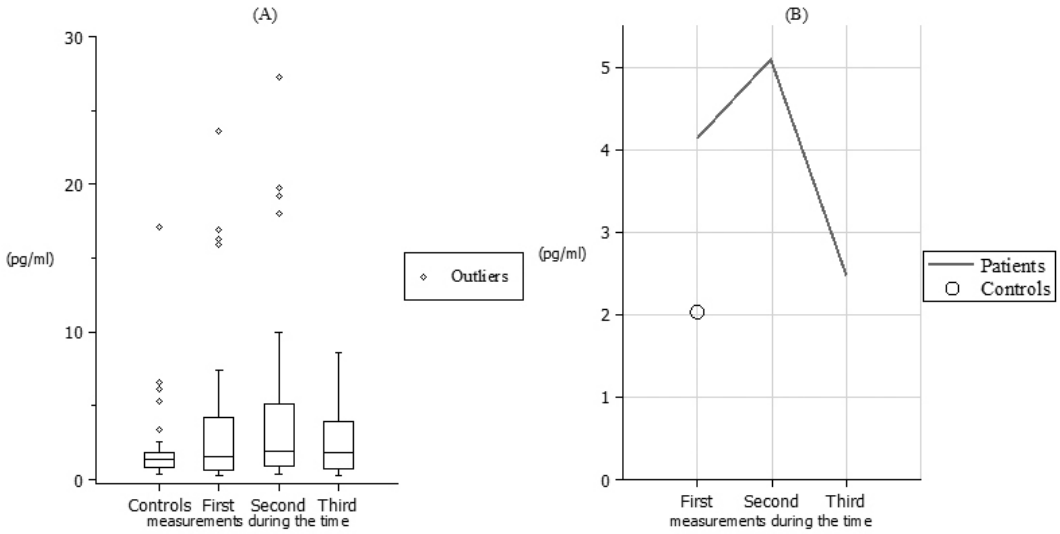

Figure 2. IL-6 levels and trend during the study period: A) Three measurements of IL-6 levels in PV Patients vs. Controls. Outlier data are shown as well. B) Changes in IL-6 levels during the time according to General Linear Model. The mean cytokine level of Control is also shown.

Due to the importance of IL-6 and IL-1β levels at each stage, these values in the PV patients and control group are shown in Table 4.

The mean level of IL-17 was higher in PV patients than in the control group at the baseline, 3 weeks, and 6 months of therapy (Table 2, 3). Changes over the time were not significant (p=0.142, Table 2). No specific trend was observed in IL-17 levels over the time.

Table 4. The cytokine levels of IL-1β and IL-6 in patients and controls during the study period

Cytokine	Baseline (Mean ± SD*) Median, Range**	3 weeks (Mean ± SD) Median, Range	6 months (Mean ± SD) Median, Range	Controls (Mean ± SD) Median, Range
IL-1β	(0.074 ± 0.093) 0, (0, 0.1)	(0.229 ± 0.401) 0, (0, 0.4)	(0.094 ± 0.112) 0.1, (0, 0.2)	(0.148 ± 0.284) 0.1, (0, 0.1)
IL-6	(4.119 ± 5.901) 1.6, (0.6, 4.2)	(5.090 ± 6.844) 1.9, (0.9, 5.4)	(2.474 ± 2.169) 1.8, (0.7, 4.2)	(2.031 ± 2.711) 1.4, (0.7, 1.8)

*SD; standard deviation

**Range; Interquartile ranges [25th-75th]

Table 5. Correlations between anti-dsg 1 with cutaneous severity and Anti-dsg 3 with mucosal severity

Anti-dsg1/3*	Cutaneous severity (correlation with anti-dsg 1)		Mucosal severity (correlation with anti-dsg 3)	
	r**	*p-value*	*r*	*p-value*
Baseline	0.673	<0.001	0.723	<0.001
3 weeks	0.539	0.002	0.482	0.006
6 months	0.906	<0.001	0.763	<0.001

*Anti-dsg; Anti-desmoglein

**Spearman's rho, Correlation Coefficient

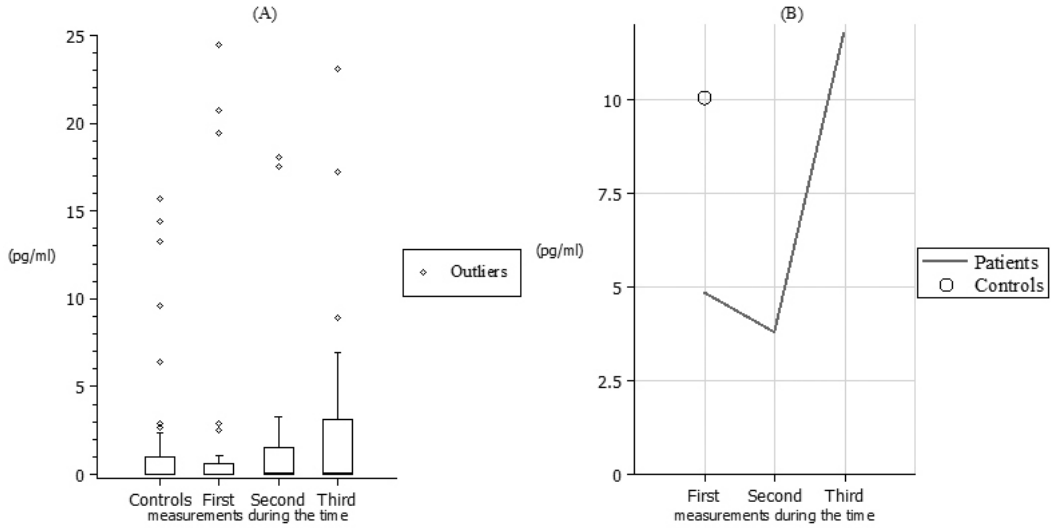

Figure 3. IL-22 levels and trend during the study period: A) Three measurements of IL-22 levels in PV patients vs. controls. outlier data are shown as well. B) Changes in IL-22 levels during the time according to general linear model. The mean cytokine level of control is also shown.

The mean level of IL-22 was lower in PV patients than in the control group at the baseline and 3 weeks of therapy (Table 2, 3). A decrease after 3 weeks and subsequent increase after 6 months of therapy were observed but changes over the time were not statistically significant (p=0.191, Table 2). IL-22 levels and changes during the study period (trend) are shown in Figure 3.

The mean level of IL-23 was higher in PV patients than in the control group at baseline, 3 weeks, and 6 months of therapy (Table 2, 3). A decrease after 3 weeks and increase after 6 months of therapy were observed. Changes over the time were not statistically significant (p=0.381, Table 2). No specific trend was observed in IL-23 levels over the time.

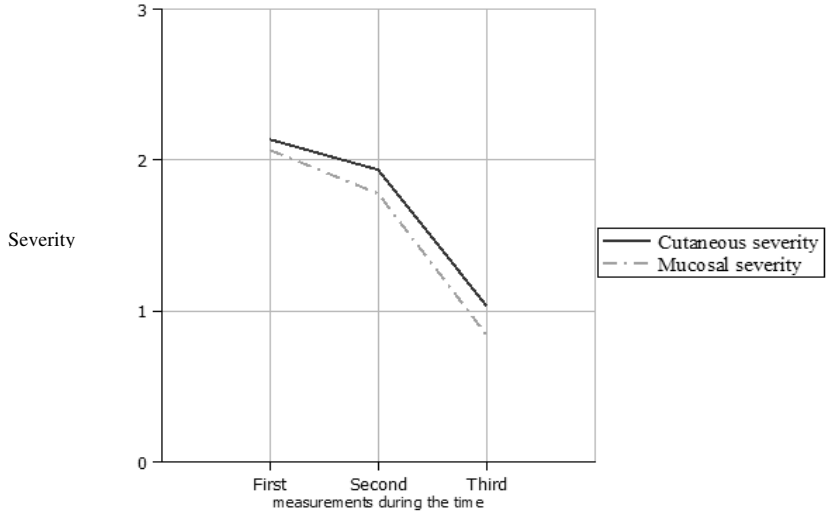

Figure 4. Cutaneous and mucosal severity changes during the study time

Cutaneous and mucosal severity coincidently decreased during the study time (both with $p<0.001$, Figure 4).

The correlation of anti-dsg 1 with cutaneous severity, and anti-dsg 3 with mucosal severity were statistically significant at baseline, 3 weeks and at 6 months after the therapy. The data are shown in Table 5.

The correlation of cytokines (IL-1β, IL-6, IL-17, IL-22, and IL-23) with cutaneous severity and mucosal severity were calculated at baseline, 3 weeks and after 6 months of therapy. Only the correlation of IL-6 with cutaneous severity at the baseline was nearly significant ($p=0.052$).

DISCUSSION

Understanding the cytokines profile pattern of PV leads to elucidate the pathogenesis of PV and possibly management of the disease. Changes in IL-1 (IL-1α and IL-1β) and IL-6, the two pro-inflammatory cytokines, have previously been reported in patients with PV.[16-19] Therefore, this study was performed to titrate the levels of IL-1β, IL-6, IL-23—the cytokines required for Th17 differentiation—and the two specific Th17 cytokines, namely IL-17 and IL-22 in PV patients.[9,20] The effect of CIST on the measured parameters was also assessed.

In the current study, at the baseline, the mean level of IL-17 of PV patients was higher than the controls, but the difference was not significant. Studies of Sinha's group showed a higher level of IL-17A in the active phase of PV.[8,9] In other autoimmune diseases such as systemic lupus erythematosus, increased serum level of IL-17 compared to the healthy control was reported.[21] In the present study, no significant change and no specific trend were observed in serum levels of IL-17 during the study period. Therefore, serum findings are not supportive of the role of IL-17 in the pathogenesis of PV.

In the current study, at the baseline, the mean level of IL-22 in PV patients was non-significantly lower than the controls. In addition, the general linear model curve showed a positive (increasing) trend in IL-22 levels in PV patients over the study period. Decreased serum IL-22 level in the active phase of the disease as well as increased IL-22 levels in the inactive phase of the disease—a positive trend—were observed in other autoimmune disease namely systemic lupus erythematosus.[22] IL-22 is a member of the IL-10 related family, mostly produced by Th17 cells and has a protective and inflammatory nature.[23,24] Since IL-22 has been shown to play a protective and regenerative role on epithelial cells, it seems logical that its positive trend coincides with the healing process of PV.[25] To the best of our knowledge, this is a new finding in PV; although further studies with a larger sample size are required to confirm this finding.

In this study, at the baseline, the mean level of IL-23 of PV patients was higher than the controls, but the difference was not significant. Similar to IL-17, no trend was found regarding serum IL-23 levels over time. As well as IL-17, the changes in IL-23 are negligible and with respect to the present results, IL-23 did not play any role in the pathogenesis of PV.

Increased levels of IL-1β and IL-6 on week 3 (Table 4) and a decreasing trend after this period may be explained as follows. First, the onset of action of azathioprine is delayed (6-8 weeks) and its effect does not begin until after week 3.[26,27] Second, on week 3 most of the patients on corticosteroid therapy are still in the active phase of the disease and it takes 4 weeks to control the active disease.[28] Thereafter, determination of IL-6 and IL-1β on week 3 did not show any decreased levels compared to the levels at the onset of therapy. After 3 weeks of therapy, due to the control of disease activity (decreased severity of the disease, Figure 4) and onset of the effects of the drugs, a decreasing trend in IL-6 and IL-1β levels was observed. As a result, it is concluded that the healing process is in accordance with decreasing trend of IL-6 and IL-1β levels.

In the current study, in the active phase of PV, the difference between serum levels of IL-1β in patients and controls was not significant (Table 3). IL-1β showed a decreasing trend and the changes in cytokine levels during the 6 months of immunosuppressive therapy and follow-up were statistically significant ($p=0.02$).

D'Auria et al. did not find any difference between IL-1β levels in PV patients during active or remission phases compared to controls.[29] However, Keskin et al. reported a reduction in IL-1β level after treatment.[30] Previously, noticeable reductions of IL-1α and IL-1β were reported in serum of PV patients in prolonged clinical remission.[17] Our findings on significant change and a decreasing trend in IL-1β levels are in accordance with these two latest studies.[17,30] In other words, the healing process was in accordance with the decreasing

trend in IL-1β levels.

In the current study, in the active phase (baseline) of PV, the mean serum level of IL-6 was higher in patients compared to the control group. Stern et al. co-cultured $CD4^+$ T cells from peripheral blood leukocytes with $CD56^+$, $CD3^-$ NK cells of the same PV patients. They showed that the supernatants from the co-cultured cells and serum of the PV patients from the active phase of PV induced a significantly higher level of IL-6, IL-8 and IFN-γ in comparison with the control group.[31] Production of higher levels of IL-6 in the active phase of PV was in accordance with the results of the current study. Sinha showed a higher level of IL-6, IFN-α, TNF-α, IL-5 and IL-2 either in the active or remission phase of PV in comparison with the control group.[8] Our findings on IL-6 were also in accordance with Sinha's results.

A decreasing trend of IL-6 levels (after 3 weeks) was probably due to CIST. This decrease was comparable with the non-significant reduction of IL-6 from active to remission phases of pemphigus by combination of prednisone and cyclophosphamide therapy in the study by Narbutt et al.[16] In Narbutt et al.'s study, the level of IL-6, even after at least six months of therapy, was more than the controls' which was similar to the finding in the current study (Table 4).[16] This means that in our study, the inhibition of IL-6 by CIST was incomplete.

Keskin et al. studied the serum levels of a panel of cytokines, including IL-6 and IL-1β in PV patients for 18 months. Cytokines were measured every 3 months during therapy in two groups of patients; group one was treated with intravenous immunoglobulin (IVIg) and group two with CIST.[30] In these two groups, the serum level of IL-6 and IL-1β gradually decreased due to therapy. In the IVIg group, the decrease in levels of IL-6 and IL-1β was more pronounced than that of the CIST group.[30] In the present study, the levels of IL-6 at the baseline and changes during the study period were also similar to Keskin et al.'s report.

According to Keskin et al., the decrease of pro-inflammatory cytokines (IL-6 and IL-1β) in the CIST group was due to general immunosuppression, while a more pronounced decrease in the IVIg group was due to changes in the cytokine network regulation.[30]

Chriguer et al. studied glucocorticoid sensitivity and pro-inflammatory cytokines in pemphigus patients (including PV and pemphigus foliaceus) and showed that the peripheral blood mononuclear cells (PBMC) of pemphigus patients produced higher levels of IL-6 and TNF-α in comparison with the control group. In the control group, the production of IL-6 and TNF-α were inhibited by low doses of dexamethasone, while in pemphigus patients, the levels of IL-6 and TNF-α remained elevated even when PBMC were treated with a high dose of dexamethasone. The study of Chriguer et al. showed that in vivo production of pro-inflammatory cytokines (IL-6 and TNF-α) were partially inhibited in pemphigus patients treated with dexamethasone (corticosteroid). The results of the current study along with Chriguer's study are supportive of partial inhibition of IL-6 level by CIST.[32]

Non-significant reduction in serum IL-6 after 6 months of CIST therapy in the current study could be explained with regard to the following studies:

- Keskin et al. showed more reduction in IL-6 levels during the time of IVIg treatment in PV patients in comparison with the CIST group[30] and
- Chriguer et al. showed partial and non-significant inhibition of IL-6 secretion even after high doses of dexamethasone in PV patients.[32]

Accordingly, we concluded that the reduction in IL-6 cytokine levels is not complete in CIST therapy and to induce more response to therapy, additional anti-cytokine (anti IL-6) or IVIg therapy might be needed for PV patients with severe and recalcitrant disease.

At the baseline, there was a correlation between serum IL-6 levels and cutaneous severity (p=0.052, nearly significant). Previously, our group showed the correlation of IL-6 levels with severity of PV.[18] These findings are supportive of a correlation between IL-6 levels and the disease severity.

In the current study, the values of anti-dsg 1 and 3 (in accordance with disease severity, Figure 4) were significantly reduced after 6 months of immunosuppressive therapy which implies that the values of anti-dsg 1 and 3 might be used to monitor PV status.[28]

In summary, this study showed that IL-6 levels in PV patients were higher than controls at the baseline. According to general linear model, IL-6 and IL-1β levels showed a decreasing trend during the treatment. In regard to IL-22, there was a positive trend during the study period. Our study showed that due to partial inhibition of pro-inflammatory cytokine IL-6, several cases of severe PV may be refractory to CIST. Thus, additional treatment using anti-cytokine therapy (anti IL-6) might be helpful in management of such

problematic cases.[9] Anti-cytokine therapy is in its infancy of emergence; however, several studies are supportive of anti-cytokine therapy (tumor necrosis factor-α inhibitor: infliximab) in recalcitrant cases of PV.[33,34] Our study, in agreement with some other studies, is supportive of the use of anti-cytokine, especially anti IL-6 in the treatment of recalcitrant PV.

Relatively, a short period of follow-up (6 months) and loss to follow-up (12 patients) are two major limitations of the present study. In this regard, further studies with more prolonged follow-up and larger sample sizes are required to confirm the present findings.

In conclusion, our findings on IL-1β and IL-6 agree with previous studies and are further evidence for participation of these two cytokines in the pathogenesis of PV.[16,17] The positive trend of IL-22, a cytokine of Th17 and Th22 cells, is a new finding and needs further studies to be proven.[10]

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