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Expression Of Programmed Cell Death-1 (PD-1) On CD4+T Cells In Pemphigus Vulgaris

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ABSTRACT

Background: Pemphigus is chronic autoimmune intra-epidermal bullous skin disease. Recent studies have found that programmed cell death-1 (PD-1) plays a role in autoimmune diseases. In this study PD-1 expression on CD4 cells were assessed by flowcytometry, soluble PD-1 were assessed by ELISA and corelated with activity and severity in pemphigus patients.

Methods: 20 patients with active pemphigus, 20 patients in remission on therapy and 40 age-matched healthy controls presented to the Dermatology department in Mansoura University Hospital. Blood samples from all precipitants were studied by flow cytometry to assess expression of PD-1 on CD4+ cells and assess the level of serum PD-1 by ELISA.

Results: A highly significant difference in PD-1 percentage on CD4+ was observed the active old group much higher expression than the remission group ($p < 0.001$), and in the remission group higher expression than the control group ($p < 0.001$). PD-1 percentage correlated positively with the PDAI scores ($p < 0.001$) indicating that higher PDAI scores are associated with higher PD-1% values. Serum levels of sPD-1 were significantly lower in PV patients than in controls ($p < 0.001$) and significantly lower in patients with active disease than those in remission ($p < 0.001$). Serum sPD-1 correlated negatively with the PDAI scores ($p < 0.001$, $r = -0.4$).

Conclusions: Elevated PD-1 expression on CD4+ cells in the highest PDAI score patients. may show the importance of PD-1 in the pathogenesis of PV. Further studies are required to confirm the role of PD-1 in pemphigus vulgaris.

key Words: pemphigus vulgaris, programmed cell death-1 (PD-1).

INTRODUCTION

Pemphigus is a life-threatening autoimmune blistering skin disorder, in which autoantibodies destruct desmogleins Dsg1 and/or Dsg3 (adhesion molecules of the epidermis) leading to destruction of junctions between keratinocytes and acantholysis of epidermal cells **[1]**. In the skin, isolated Dsg3 dysfunction is unable to produce blisters, as it is compensated by Dsg1. However, the low concentration of Dsg1 in the mucosa is not sufficient to compensate for the Dsg3 dysfunction as it is the main antigen in mucosal affection **[2]**. Oral lesions usually the first manifestation in 50%-70% of cases presented as painful erosions and blisters **[3]**. Cutaneous involvement shows flaccid blisters with clear content on normal or erythematous skin. characteristic blister breaks easily with positive Nikolsky's sign then covered by crusts. Healing is usually with post inflammatory hyperpigmentation **[4,5]**. Histopathological samples show suprabasal cleavage in the epidermis and acantholytic keratinocytes within the blister **[6]**.

Autoantibody formation originates from Dsg3-reactive CD4+ T cells .This Th2 driven process is critical for the induction and maintenance of autoreactive memory B cells as precursors of autoantibody-producing plasma cells **[6]**. Active PV had significantly elevated interleukin (IL)-6, IL-8 and interferon (IFN)-γ compared with the controls, indicating that NK cells responsible for CD4+ T cells induction to produce proinflammatory cytokines**[7]**. CD4+ T cells are important in pathogenesis of PV because of their association with MHC proteins and because the adoptive transfer of these cells is involved in antibody production**[8]**.

PD-1 is transmembrane glycoprotein composed of an IgV domain which shares identity with CTLA4, CD28, and inducible T-cell costimulatory molecule (ICOS) **[9]**. PD-1 was assigned to the CD279 cluster of differentiation **[10]**. PD-1 binds to its ligands PD-L1 (CD274) and PD-L2 (CD273) which are transmembrane glycoproteins**[11,12]**. Both PD-1 and PD-L have their soluble forms which are produced from cleavage of membrane bound forms by proteolytic effects **[13]**. Soluble PD-1 (sPD1) exhibits opposite functions may antagonist the membrane bound PD-1 or agonist to it , as the sPD-1 functions like the membrane form, exhibiting immune regulation by limiting TCR-induced events **[14,15]**. PD-1 is expressed on T cells (CD4+ and CD8+), B cells, natural killer (NK) cells, dendritic cells (DCs) and innate lymphoid cell precursors **[16]**. T cells express immune check point (PD-1), which normally operates as an off-switch function to protect the normal cell from T-cell attack **[9].** lacked PD-1 expression in mice lead to autoimmune disease development later in life **[17].** Additionally, when PD-1 on T cells interacts with PD-L1 on APC leads to inhibition of production of several cytokines, including IFN-γ, IL-2 and TNF-a, which promote T cell apoptosis through inhibition of the survival factors as Bcl-xL **[18].** The PD-1/PDL-1 pathway is a tissue protective pathway as it impairs T-cell survival and blocks its response such as cell proliferation, cytokine secretion, and cytotoxic ability. Moreover it enhances FoxP3 expression and regulates the differentiation of induced TReg cells **[19]**. Frequent induction of autoimmune diseases, including AIBD, as an adverse event of checkpoint inhibitor treatment in cancer further supports the central role of these molecules in maintaining self-tolerance **[20].**

PATIENTS AND METHODS

Our comparative study was approved by the Ethics Committee of Mansoura University Faculty of Medicine (16.04.2022; 22.03.620).

This study was conducted on 3 groups of subjects: the active group A includes 20 patients with active pemphigus who did not receive any topical or systemic therapy, or previously diagnosed and in excerpation as developing new lesions within the last month, the remission group B includes 20 patients in complete remission were patients without development of any oral or skin lesion for at least 3 months but post inflammatory hyperpigmentation was not considered as a sign of activity and the third group C includes 40 age-matched healthy controls were free of a history of autoimmune diseases and infection and did not receive any treatment within the previous 3 months, and had never received an immunosuppressive drug. presented to the Dermatology department in Mansoura University Hospital during the period between February 2023 and February 2024. Before participation in our study a written informed consent was taken from all subjects.

Using G power program version 3.1.9.4 to calculate sample size based on expected effect size = 1.20 (71.57±21.84 & 96.88± 11.93 for cases with active and complete remission, respectively), using 2-tailed test, α error =0.05 and power = 90.0%, the total calculated sample size will be 16 in each group and by adding 20% to compensate for possible drop out then sample size will be 20 in each group.

Patients with any other autoimmune disease or pregnancy were excluded. Detailed history was taken from patients including: age, occupation, marital status and smoking habits, history of any associated chronic diseases (diabetes and hypertension). Onset, course, duration of disease, and any precipitating factors, such as the intake of drugs as systemic steroid type and dose, ACE inhibitor, Cephalosporin, and Beta blockers. PV was diagnosed by clinical, histopathological criteria then assessment of the severity of PV was done by pemphigus disease area index (PDAI) [21]. The PDAI has a score ranging from 0 to 263 points, with 250 points representing disease activity, 120 for skin, 10 for the scalp, and 120 points for mucosal activity.

4 ml of peripheral blood venous sample were taken from patients and controls then separated as follows:

Flow cytometry

2ml were collected on tubes containing ethylene diamine tetra acetic acid (EDTA) anticoagulant and the specimen was submitted for flow cytometric assessment of Anti-CD4 (Conjugate: FITC, cat. Num. IM0448U, Beckman Coulter CO, FRANCE), Anti-PD-1 (Conjugate: PE, cat. Num. B30634, Beckman Coulter CO, FRANCE), Fluorescence signals were measured on a flow cytometer (Navios EX Flow Cytometer from Beckman coulter, Indianapolis, USA).

The percentage of CD4-positive cells is calculated from the total number of gated events. Expression of PD-1 was assessed on CD4-positive cells and was interpreted as a percentage of positivity on CD4-positive cells and also its mean fluorescence intensity (MFI) that reflects the density of PD-1 on the cell surface was reported.

ELISA

2ml were collected on plain tubes (without anticoagulant) from which serum was separated and stored at -20 C for eventual serum soluble PD-1 assay using ELISA kit that was supplied from DLDevelop Co, CHINA cat. Num.DL-PDCD1-Hu). The test was done according to the manufacturer's instructions. serum soluble PD-1 level was assessed by calculating the absorbance value at 450 nm wavelength in the spectrophotometer.

Statistical analysis

The collected data was revised, coded, and tabulated using the Statistical Package for Social Science (IBM Corp. Released 2017. IBM SPSS Statistics for Windows, Version 25.0. Armonk, NY: IBM Corp), the Mann-Whitney U test and One way ANOVA test was used between more than two study group parametric variables. The Kruskal-Wallis test was used between more than two study group non parametric variables. In addition, the relationship between continuous variables was analyzed using Pearson and Spearman correlation coefficients, a *p* value is considered significant if less than or equal to 0.05.

RESULTS

A total of 40 cases: group (A) 20 patients with active PV and group (B): 20 patients in remission PV and group (C): 40 healthy control subjects were included in the study.

Table-1: Demographic And Clinical Data

Characteristic	Active PV	PV in remission	Control group
Gender **Female** **Male**	 11 9	 10 10	 20 20
Mean age	35 (24-50)	37 (28-50)	33 (20-58)
Mean PDAI	84.50 (34.00- 107.00	2.00 (0.00-10.00	-
Duration of remission (Months) (mean ± SD)	-	21.93 ± 19.967	-
Mucosal affection, n (%)	18(90%)	12(60.0%)	-
Hypertension, n (%)	10(50%)	11(55.0%)	-
Diabetes mellitus, n (%)	10(50%)	8(40.0%)	-

Table-1 represents demographic and clinical data of the patient and control groups, there was no significant difference in gender and age between the studied groups.

Table-2. Comparison between study groups regarding different parameters.

		Active	Remission	Control	*P1*	
		n=20	n=20	n=40		*Statistical test*
Absolute CD4 (cells /mm^3)	Mean ± SD	445.05 ± 248.03	548.45 ± 235.59	957.4±231.25	<0.001*	F=38.875
	Median (Min-Max)	388.50 (60.00-837.00)	513.00 (208.00-928.00)	960(430-1350)		One way ANOVA test
CD4 %	Mean ± SD	9.91 ± 5.60	10.34 ± 6.10	11.18±3.44	0.374	H=1.969
	Median (Min-Max)	8.55 (1.50-19.70)	9.20 (2.30-23.20)	12.15(1.5-14.8)		Kruskal Wallis test
PD1 %	Mean ± SD	51.19 ± 9.07	37.09 ± 7.34	26.45±4.68		H=53.745

	Median (Min-Max)	52.00 (33.40-64.50)	35.05 (28.30-54.90)	25.8(20.2-34.7)	<0.001*	Kruskal Wallis test
MFI PD1	Mean ± SD	4.55 ± 0.83	3.74 ± 0.49	2.77±0.19	<0.001*	H=58.840
	Median (Min-Max)	4.33 (3.43-6.36)	3.88 (2.92-4.53)	2.74(2.43-3.15)		Kruskal Wallis test
MFI ratio	Mean ± SD	11.75 ± 2.75	10.15 ± 1.65	6.55±0.49	<0.001*	F=77.612
	Median (Min-Max)	11.52 (7.60-16.91)	9.78 (6.94-13.28)	6.51(5.2-7.3)		One way ANOVA test
serum sPD-1	Mean ± SD	47.20 ± 15.09	72.10 ± 18.81	106.55±18.23	<0.001*	H=53.172
	Median (Min-Max)	47.50 (27.00-84.00)	69.00 (51.00-114.00)	110.5(66-128)		Kruskal Wallis test

P, comparison between active and remission and control group

In our study absolute CD4 cell counts (multiplying the percentage of $CD4^+$ lymphocytes by the total WBC count (cells/µl) and dividing by 100) show significant differences when comparing the active group with the control group (p <0.001) with the control group exhibiting significantly higher values. but no significant difference between the active and remission groups. However, no significant difference of the percentage of CD4-positive cells across study groups (p = 0.536).

The percentage of positivity of PD-1 was studied on the CD4-positive cells and compared among the study groups. The PD-1 percentage (mean ± SD) was 51.19 ± 9.07 for the active group, 37.09 ± 7.34 for the remission group, and 26.45 ± 4.74 for the control group. A highly significant difference in PD-1 percentage was observed among the groups (p <0.001).

Significant differences are observed when comparing PD-1 MFI (mean fluorescence intensity) and PD-1 MFI ratio between active group and the remission group and the control group (p <0.001). ***but*** still remission group levels lower than normal control values indicating that PD-1 is still anormal despite immunosuppressive drugs, also these results indicate higher possibility of these patients to relapse.

Serum soluble PD-1 levels show significant differences across the groups (p<0.001). significant negative correlations were found between serum PD-1 levels and PD-1 percentage of expression on the surface of CD4-positive cells (p < 0.001), PD-1 MFI (p < 0.001), and MFI ratio (p < 0.001), demonstrating that higher serum PD-1 levels correspond to lower PD-1 percentage, MFI PD-1, and MFI ratio.

PDAI score showed a significant positive correlation with PD-1% (p < 0.001) and MFI PD-1 (p < 0.001), indicating that higher PDAI scores are associated with higher PD-1% and MFI PD-1 values which can predict the pemphigus disease activity. Conversely, a significant negative correlation was found between PDAI and serum PD-1 levels (p < 0.001), where higher PDAI scores are associated with lower serum PD-1 levels.

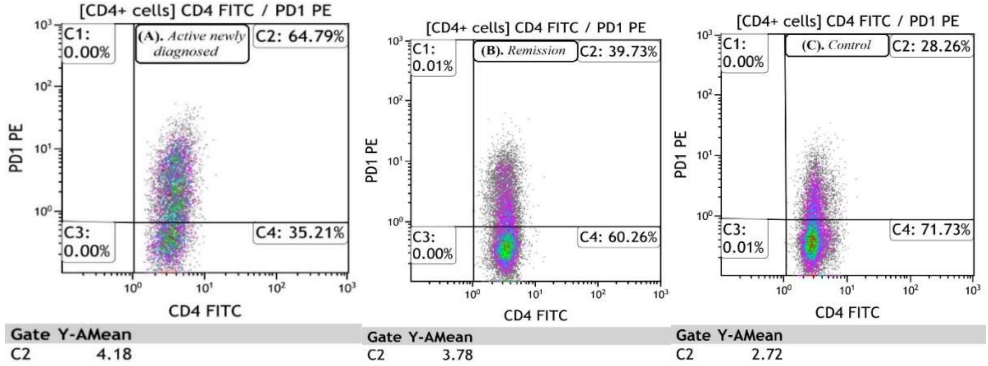

Figure 1. Flow cytometric plots showing PD-1 expression (percentage and MFI) on CD4+positive cells in different study groups

PD-1 percentages on CD4-positive cells are 64.79%, 39.73%, and 28.26%; while PD-1 MFI are 4.18, 3.78, and 2.72 in active newly diagnosed (A), remission (B) and control (C) groups respectively

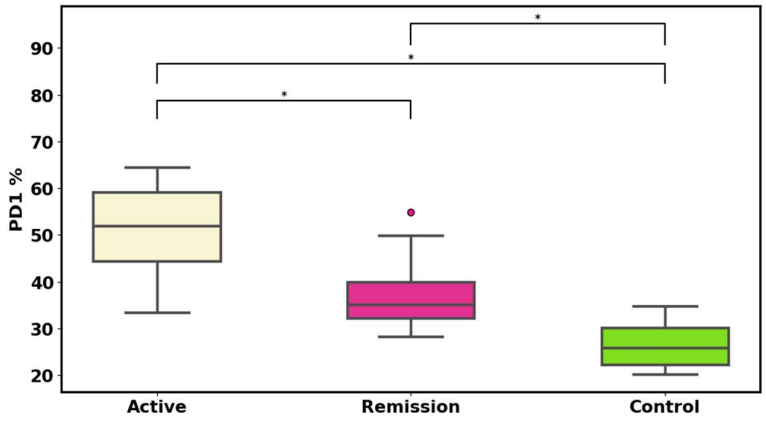

Figure 2. PD-1 percentage of positivity on CD4-positive cells (*P<0.001).

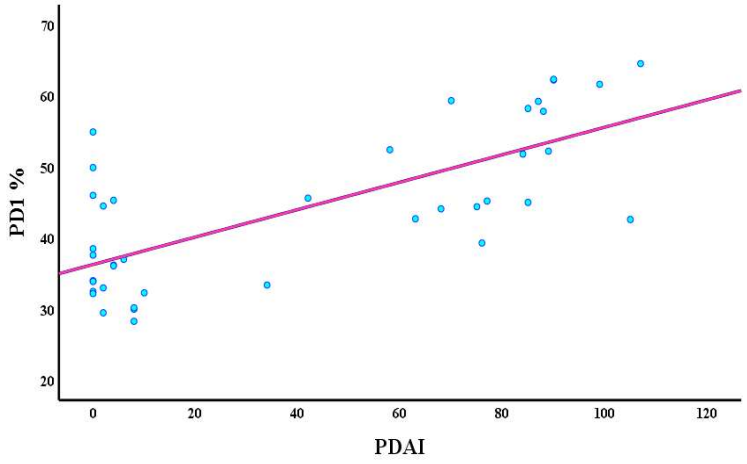

Figure 3. significant positive correlation between PD-1% on CD4 cells and PDAI .

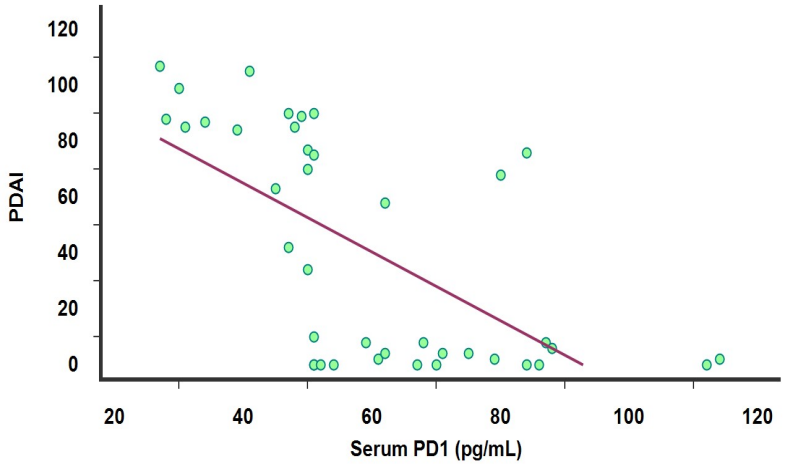

Figure 4. significant negative correlation between PDAI and serum PD-1.

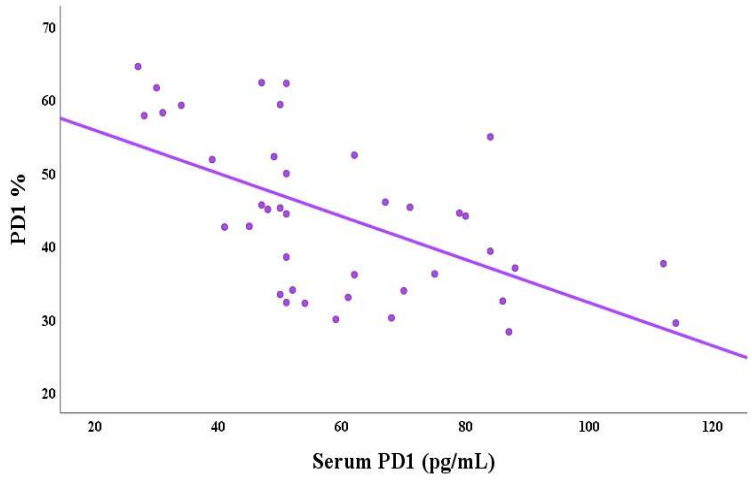

Figure 5. Correlation between PDI% and serum PD-I among PV patients.

DISCUSSION

A defective PD-1 pathway could be implicated in PV pathogenesis as the relapse of pre-existing PV under anti-PD-1 therapy as Nivolumab was described. Under nivolumab therapy the occurrence of PV seems to be a rare, but serious dermatological side effect [22]. A case of atypical PV without oral lesion was reported in a patient 2.5 weeks after discontinuation of nivolumab therapy in a patient with no prior history of autoimmune disease [23]. Ernst et al.,2021 reported increased expression of the checkpoint receptors PD-1 in lesional skin of PV. These studies directed us to study more about PD-1 role in PV pathogenesis [24].

In the present study, PV most frequently occurred in middle age: active group (36.25 ± 7.43), remission group (36.75 ± 5.95 years). Other previous studies also reported PV in the same age group [25,26]. While some reports detected PV in older age group with a mean age of 54.7±16 and 57.5 ± 17.3 years respectively [27,28]. Different geographic and ethnic natures of patients studied may be the cause of this discordance [29].

In our study, regarding gender distribution, the statistical test reveals that male to female ratio was 1:1.1 in total patients with nearly a slightly higher percentage of females in the PV cases group than control group, our results show lower percentage than other studies that reported higher female predominance (male to female 1:2) [30,28]. some authors reported male and female may be nearly equal in their distribution in PV [31].

Multiplying the percentage of $CD4^+$ lymphocytes by the total WBC count (cells/µl) and dividing by 100 to calculate absolute CD4 cell counts showed that PV cases exhibit significantly lower CD4 counts compared to the control group with a significant *p*-value (<0.001). The comparison between active and remission groups reveals no significant difference (p=0.184). Albers et al.,2017 found similar results and revealed low CD4 count (<400 cells/µL) in active cases and can predict the relapse as every CD4 value increase of 200 decreases the susceptibility ratio for relapse by 35% [32].

Naïve CD4+ T cells can differentiate into different types of T cells (Th1, Th2, Th17, Th follicular and inducible Treg cells) and every subset responds differently in pemphigus vulgaris with expected lower Th1 and Treg cells [33,34]. Higher Th2, Th17 and Th follicular [35,36]. This may explain insignificant differences we found when comparing the CD4 percentages between PV cases and control groups.

The percentage of positivity of PD-1 was studied on the CD4-positive cells was 51.19 ± 9.07 for the active group (with no significant difference between the active newly diagnosed and active old groups), 37.09 ± 7.34 for the remission group, and 26.45 ± 4.74 for the control group. A highly significant difference in PD-1 percentage was observed among the groups ($p1$<0.001). but still remission group levels lower than normal control values indicating that PD-1 is still anormal despite immunosuppressive drugs and the possibility of these patients to relapse.

Kim et al.,2020 showed that the circulating ICOS+ PD-1+CXCR5+CD4+ T cells in patients with pemphigus correlated with the anti-DSG3 autoantibody level. these data indicate that circulating TH2-like ICOS+CXCR5+PD-1+CD4+ T cells are important in autoantibody production in human patients with PV and in a mouse model of PV [37].

Hébert et al., 2024 observed the emergence of a Dsg-3-specific Th follicular subpopulation with a significant overexpression of the surface activation markers PD-1, ICOS, and CD25 that was not observed at the surface of non-autoreactive Th follicular cells of the same PV patients [38].

Karakaş et al.,2023 compared PV patients who received rituximab treatment and who did not receive rituximab therapy with the control group, the levels of CD3+CD4+CXCR5+PD1+ Th follicular cells were also significantly lower in patients receiving rituximab than the control group. their results ensure that CD3+CD4+CXCR5+PD1+Th follicular have important role in PV pathogenesis and activity [39].

To our knowledge it is the first study to show mean fluorescence intensity (MFI) that reflects the density of PD-1 on the cell surface in PV, and The MFI ratio (MFIR) is usually defined as meaningful way to measure expression intensity (mean of an PD1-positive population / mean of PD1-negative population). MFIR reduces subjective error which may present. the comparison of MFI PD-1 levels and MFI ratio between the PV cases and control groups shows a significant difference with (p <0.001). MFI PD-1 levels and MFI ratio between the active and remission groups, also showing a significant difference with (p<0.001), This significant finding highlights a considerable elevation in MFI PD-1 and MFI ratio concentrations in PV compared to control groups.

PDAI severity scores positively correlated with PD-1% (p < 0.001), MFI PD-1 (p < 0.001) and MFI ratio (p = 0.012), indicating that higher PDAI scores are associated with higher PD-1%, MFI PD-1 and MFI ratio values which can predict the pemphigus disease activity.

Serum PD-1 levels between the active (47.20 ± 15.09) and remission (72.10 ± 18.81) groups, showing a significant difference with (p <0.001), though patients in complete remission group showed significantly higher sPD-1 levels than patients in active group but didn't reach the highest level in control healthy group indicate high risk of recurrence. Also, patients with higher PDAI score demonstrated significantly lower serum levels of sPD-1 as a significant negative correlation was found between PDAI and serum PD-1 levels (p < 0.001), rendering sPD-1 a useful disease severity marker.

Our results go with Zeid et al., 2021 who found similar results as serum levels of sPD-1 in PV patients significantly lower than in healthy controls and show significant negative correlation with PDAI score [14]. however, Ernst et al.,2021 did not find significant difference between the soluble PD-1 in pemphigus patients and controls despite that they found the expression of PD-1 was upregulated in PV lesional immunohistochemistry samples than controls [24].

This is the first study to show the correlation between PD-1 expression percentage on CD4 T cells and soluble PD-1 level in pemphigus vulgaris. Significant negative correlations were found between and PD-1% (p < 0.001), and serum PD-1 levels, also significant negative correlations were found between MFI PD-1 (p < 0.001), MFI ratio (p < 0.001) and serum PD-1 levels demonstrating that higher PD-1%, MFI PD-1, and MFI ratio correspond to lower serum PD-1 levels.

Our results demonstrate that soluble PD-1 forms may be produced by detachment of the membrane form or alternate splice variants [40]. So, the membrane form density (PD-1 percentage and MFI PD-1 density) negatively correlates with the soluble form. This could be explained that immune system increases the inhibitory membrane bound PD-1 in trial to control the autoimmune reaction and suppress the autoreactive T cells. and this increase in PD-1 percentage and density is correlated with severity of the disease. As the most severe cases correlated with the highest PD-1 percentage and density.

Our results suggested that PD-1 pathway seems to be implicated in the pathogenesis of PV. Moreover, several ingoing studies have highlighted the advantages of immune checkpoint modulators in treating autoimmune diseases. Thus, targeting PD-1 pathways could be a future hope for new therapies for PV.

CONSLUSION

Our results cannot explain the exact role of PD-1 in PV disease; however, significantly higher detection of $PD\text{-}1^{+}$ on $CD4^{+}$ cell in PV patients than in the control group. and significantly lower detection of serum soluble PD-1 in PV patients than in the control group may play a role in the pathogenesis of the disease. It also seems to be influenced by disease severity and activity. more studies are needed to better understand the role of PD-1 in PV pathogenesis.

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