

Possible Role of Regulatory B Cells in Different Behçet's Disease Phenotypes and Therapies: First Report from Egypt

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Background and Aim: The data about the role of regulatory B cells (Breg) in Behçet Disease (BD) are scarce. We aimed to evaluate the frequency of total B lymphocytes and Breg cells in different BD phenotypes and therapies attempting to unravel their function.

Methods: This cross-sectional study included 35 BD patients and 39 healthy controls (HCs). The demographic data of the study subjects were collected including age and gender. Current medications including disease-modifying anti-rheumatic drugs (DMARDs) were recorded. All patients underwent testing for baseline laboratory investigations including full blood count, liver and kidney function tests, erythrocyte sedimentation rate (ESR) by Westergren blot and C-reactive protein (CRP). Measurement of the total B lymphocytes and their subtypes B regulatory lymphocytes by flow cytometric assay. Assessment of BD activity was done using the revised Behçet's Disease Current Activity Form (BDCAF) 2006 and Behçet's Syndrome Activity Score (BSAS) ¹¹¹¹¹¹¹¹¹¹. All participants were assessed for the presence of erectile dysfunction using the International Index of Erectile Function (IIEF-5 score), and for depression using the Beck Depression Inventory.

Results: A dramatic drop in the number of B cells, total and regulatory, was observed in the patients compared to the HCs. Regulatory cells (Bregs) tend to be upregulated with genital ulcers or vascular disease. Bregs but not B lymphocytes were associated with BSAS and ESR. Neither the total B lymphocytes nor the Bregs correlated with CRP or the sexual function or depression scores. Of all the used medications, low-dose aspirin was seen with markedly high Bregs proportions.

Conclusion: This study supports the role of B cells in BD pathogenesis and strongly suggests a possible role for Bregs in the resolution of different BD manifestations.

Keywords: Behçet's disease, B lymphocytes, Breg

Introduction

Behçet's disease (BD) is a chronic multi-organ vasculitis characterized by recurrent oral and genital aphthae, skin lesions, widespread thrombosis and aneurysmal formation, and serious involvement of the eyes and central nervous system.² The evolution of the disease is incompletely understood, and different mechanisms were hypothesised to take place in the development of the disease, particularly, the environmental triggers.³ Bacterial infections and heat shock proteins have been thought to play an important role in the initiation of the disease via molecular mimicry.^{4,5} Furthermore, the genetic factor of the disease was strongly linked to the presence of HLA-B51.⁶ However, an array of single-nucleotide polymorphisms

encoding for cytokines, chemokines, receptors and signal transducers were also detected in BD patients, and were linked to increased susceptibility to the disease.⁷

The innate and adaptive immune cells integrate into the pathogenesis of BD. It appears that the inflammatory process is initiated by neutrophils and perpetuated by $\gamma\delta$ T cells which induce strong T helper (Th)-1, Th-2 and Th17 responses.⁸ Interestingly, the gene expression analysis of BD patients and healthy controls revealed the modulation of a range of genes involved in biological processes in BD, such as inflammation, apoptosis, angiogenesis, blood coagulation, vascular damage, signalling pathways, and immune responses, particularly, in innate immune cells, Th17 cells and B cells.⁹

Earlier, evidence of B cell involvement in the disease had grown through the observation of increased specific B cell subsets in BD compared to healthy controls and other autoimmune diseases,¹⁰ and the detection of abnormalities in B cell functions which were demonstrated to be associated with disease pathogenesis and disease activity.¹¹ Moreover, B cells were detected in active mucosal ulcers,¹² and the depletion of B cells was shown to be effective in improving the ocular disease.¹³ The regulatory function of Breg in autoimmune diseases was extensively studied in animal models. It was found to act as antigen-presenting cells and produce autoantibodies leading to targeted tissue damage. Thus, it was claimed to play a pathogenic role in different autoimmune diseases as rheumatoid arthritis, lupus, autoimmune diabetes mellitus and autoimmune encephalomyelitis.^{14–20}

Yet, the regulatory role of B cells in BD has not been thoroughly investigated,²¹ and their role in different disease presentations has not been studied. Therefore, the aim of the current study is to identify the proportions of total B lymphocytes and their regulatory subset in different BD phenotypes and therapies attempting to unravel their function in BD.

Methods

Subjects and Methods

This observational, cross-sectional study was conducted on patients attending the Rheumatology Clinics in Assiut University Hospitals, Egypt. It was approved by the ethical committee of Assiut Faculty of Medicine in accordance with the declaration of Helsinki. Clinical trial identification number NCT04376411. Written consents were collected from all the participants before recruitment. We

enrolled 35 patients aged ≥ 18 years who fulfilled the diagnosis of BD after scoring four or more points of the International Criteria for Behçet's Disease (ICBD).²² Thirty-nine age and sex-matched healthy controls (HCs) were also recruited after being consented. The patients and healthy controls were informed about the purpose of the trial.

Clinical and Laboratory Assessment

After full history taking and clinical examination, assessment of the patients' disease activity was done using the revised Behçet's Disease Current Activity Form (BDCAF) 2006 of the original BDCAF^{23,24} And Behçet's Syndrome Activity Score (BSAS).¹

Current medications including disease-modifying anti-rheumatic drugs (DMARDs) were recorded. All patients underwent testing for baseline laboratory investigations including full blood count, liver and kidney function tests, erythrocyte sedimentation rate (ESR) by Westergren blot and C-reactive protein (CRP). An expert cardiologist performed a full cardiac examination to exclude cardiac involvement including blood pressure and left ventricular function. Patients and HCs were assessed for the presence of erectile dysfunction using the International Index of Erectile Function (IIEF-5 score),²⁵ and for the presence of depression using the Beck Depression Inventory.²⁶

Flow-Cytometric Assay

Using flow cytometry, circulating Bregs were detected using PE-conjugated CD38, FITC-conjugated CD24 (Bioscience, USA) and PerCP-conjugated CD19 (BD Bioscience, USA).

One hundred microliters of the blood sample was incubated with 10 μ L of CD24, CD38 and CD19 for 20 minutes at 4 °C in the dark. Following incubation, red blood cells lysis, washing and analysis by FACS Calibur flow cytometry with CellQuest software (Becton Dickinson Biosciences, USA) were done. An isotype-matched negative control was used for each sample. Forward and side scatter histogram was used to define the lymphocytes population. Then, CD19⁺ B cells were gated. Then, the expression of CD38 and CD24 on the CD19⁺B cells was detected. Bregs were identified as CD19⁺CD24^{hi}CD38^{hi} cells (Figure 1).

Statistical Analysis

The data were subjected to normality tests and were presented as means and standard deviations or medians and

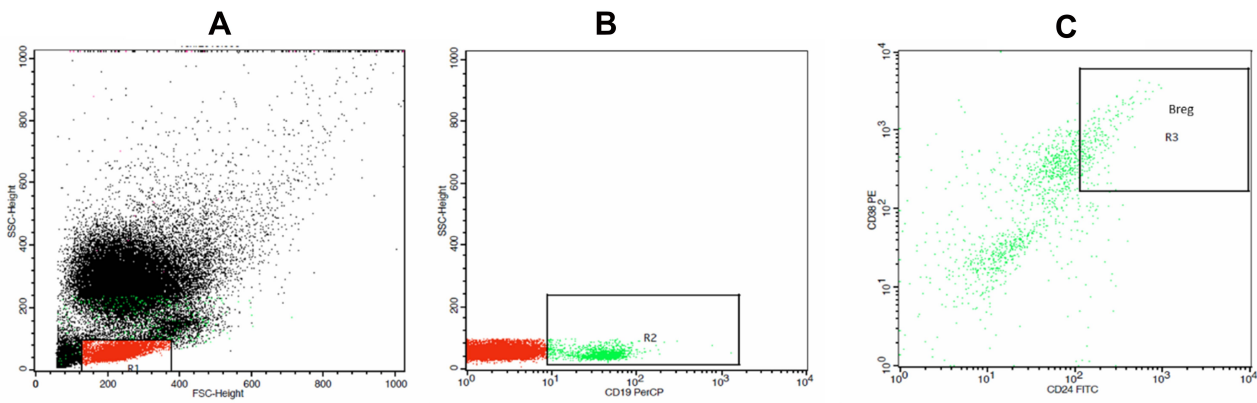


Figure 1 Flow cytometric detection of regulatory B cells.

Notes: (A) Forward and side scatter histogram was used to define the lymphocytes population (R1). (B) The CD19+ cells (R2) were assessed within the lymphocyte population. (C) The expression of CD24 and CD38 was assessed in CD19+ cells to define CD19+CD24^{high}CD38^{high} cells (regulatory B cells).

interquartile ranges as appropriated. For categorical variables, data were presented as numbers and percentages. To compare between two or more groups, Independent *T* test, Mann Whitney *U*-test and Kruskal–Wallis test were used as appropriate. Chi-square test and Fisher's exact test were employed to compare categorical data as appropriate. Spearman's rho correlation coefficient was used to determine the correlations between groups. P-values less than 0.05 were considered significant. The statistical program used for all statistical analyses is the Statistical Package for Social Science (SPSS) version 24 (SPSS Inc.; Chicago, IL, USA).

Results

A total number of 35 BD patients and 39 health controls (HCs) were gathered. The mean age of the participants' was 35.2 ± 7.6 for patients and 36.7 ± 5.7 for HCs. Twenty-six of the patients and 27 of the HCs were of the male gender. Comparable numbers of the patients and HCs were smokers (22.9% and 30.8%, respectively). The clinical and immunological profile of the study subjects, including the proportions of total and regulatory B cells, and therapy of the BD patients were shown in Tables 1 and 2. A dramatic drop in the number of B cells, total and regulatory, was observed in the patients compared to the HCs. A non-significant correlation was found between the frequency of both B lymphocytes and B regs with all of the following: heart rate, systolic blood pressure and diastolic blood pressure ($P > 0.05$).

Studying the trends of total and regulatory B cells in different disease phenotypes revealed a tendency of the B regulatory cells (Bregs) to be upregulated in different involvements, in particular, in the presence of genital

ulcers or vascular disease despite the markedly low number of B cells in the arterial diseases as demonstrated in Table 3. On the contrary, there were no significant differences in either total B lymphocytes or Bregs in the presence or absence of ocular involvement or in different ocular manifestations. Furthermore, they showed no significant differences with favourable or unfavourable responses, to first-line therapy, or with the number of relapses per year.

Though both BSAS and ESR did not correlate with each other (data not shown), there were significant relationships between the proportions of total B cells, on one side, and BSAS ($\rho = -0.36$, $p = 0.033$) and ESR ($\rho = 0.34$, $p = 0.047$), on the other side, as illustrated in Figure 2. On the other hand, BSAS and ESR displayed no associations with Bregs. Noticeably, neither the total B lymphocytes nor the regulatory subset correlated with CRP or the sexual function or depression scores.

Studying the changes in B cells linked to the therapeutic profile of the patients revealed no substantial changes in B lymphocytes or Bregs with the administration of different DMARDs. Nevertheless, the use of low-dose aspirin was seen with markedly high Bregs proportions, and there was a similar trend with the use of anticoagulants despite the lack of a significant difference as illustrated in Figure 3.

As all patients were on different doses of prednisolone, B cell numbers were tested against the dose and duration of corticosteroid administration. It was observed that the doses of prednisolone correlated positively with the proportions of Bregs ($\rho = 0.394$, $p = 0.019$) but not with those of total B lymphocytes ($\rho = 0.081$, $p = 0.65$). The durations

Table 1 Clinical and Immunological Characteristics of the Study Subjects

	BD Patients N=35 N (%)	Control N=39	p-value
Arterial disease	17 (48.6%)	NA	NA
DVT	16 (45.7%)	NA	NA
GIT	10 (28.6%)	NA	NA
Skin lesions/Pathergy test positive/Oral ulcers	35 (100%)	NA	NA
Genital ulcers	16 (45.7%)	NA	NA
Urethritis, n (%)	6 (17.1%)	NA	NA
Ocular disease		NA	NA
• Anterior uveitis	2 (5.7%)		
• Pan uveitis and retinal vasculitis	6 (17.1%)		
• Pan uveitis and retinal vasculitis with optic neuritis/atrophy	12 (34.3%)		
• No ocular signs	15 (42.9%)		
Arthralgia/arthritis	20 (57.1%)	NA	NA
Headache	5 (14.3%)	NA	NA
Seizures	0	NA	NA
Cognitive dysfunction	6 (17.1%)	NA	NA
Peripheral neuropathy	10 (28.6%)	NA	NA
Psychosis	0	NA	NA
Inflammatory back pain	14 (40%)	NA	NA
Activity within the past four weeks (BDCAF 2006)	21 (60%)	NA	NA
Erectile dysfunction (IIEF-5 score) #	15 (9–20)	20 (20–25)	<0.001*
Depression score (Beck questionnaire) #	13 (8–19)	10 (7–14)	0.042*
BSAS #	30 (30–60)	NA	NA
ESR#	25 (17–34)	20 (16–26)	0.02*
CRP§	14.4 ± 9.8	2 ± 3.4	<0.001*
B lymphocytes % §	11.1 ± 1.9	14.2 ± 3	<0.001*
B reg.% §	1.9 ± 0.5	3.3 ± 1.4	<0.001*

Notes: Independent *T* test, Mann–Whitney *U*-test and Chi-square test were used as appropriated. *Significance was considered when $P \leq 0.05$

Abbreviations: BD, Behcet's disease; SD, standard deviation; DVT, deep venous thrombosis; GIT, gastrointestinal manifestations; BDCAF, Behcet's Disease Current Activity Form; #, median (interquartile range); §, mean ± standard deviation; BSAS, Behcet's Syndrome Activity Score; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein.

Table 2 DMARDs Administration in BD Patients

Drug Used	BD Patients N=35, N (%)
Prednisolone doses	
10 mg	9 (25.7%)
20 mg	11 (31.4%)
30 mg	7 (20%)
40 mg	7 (20%)
60 mg	1 (2.9%)
Prednisolone duration (years) #	4 (3–5)
CYC	8 (22.9%)
CS	11 (31.4%)
MTX	9 (25.7%)
AZA	13 (37.1%)
LDA	12 (34.3%)
Anticoagulant	4 (11.4%)

Abbreviations: DMARDs, disease-modifying anti-rheumatic drugs; BD, Behcet's disease; #, median (interquartile range); CYC, cyclophosphamide; CS, cyclosporine; MTX, methotrexate; AZA, azathioprine; LDA, low dose aspirin.

of corticosteroid administration did not correlate with either total B lymphocytes or Bregs. Unfortunately, within the recruited patients, none of them had started biologic therapy prior to enrolment; therefore, the effect of its commencement was not studied.

Discussion

Multiple innate and adaptive immunological responses are proven to be implicated in the pathogenesis of BD.⁹ However, the participation of these different responses to initiate diverse manifestations is still unclear particularly the role of B cells. The current study aims to identify the aberrations in B lymphocytes and B regs in different BD manifestations and therapy.

In this study, patients diagnosed with BD had significantly lower proportions of B cells and a remarkably lower B regulatory fraction in the peripheral blood compared to

Table 3 The Differences in Total B Lymphocytes and B Regulatory Subset with Different Disease Characteristics

	% of B Cells in (+) Group Median (IQR)	% of B Cells in (-) Group Median (IQR)	p-value	% of B Reg. in (+) Group Median (IQR)	% of B Reg. in (-) Group Median (IQR)	p-value
Activity within 4 weeks	12 (11–12.4)	11.3 (10.4–12)	0.14	1.86 (1.5–2.3)	1.8 (1.5–2.2)	0.5
Arterial disease	11 (9–12)	12 (11.3–12.5)	0.013*	2.2 (1.7–2.4)	1.7 (1.5–2)	0.067
DVT	11.8 (10.7–12.1)	11.5 (10.4–12.4)	0.8	2.1 (1.8–2.4)	1.6 (1.4–2.1)	0.052
GIT	12 (11.3–12.4)	11.4 (10.4–12.2)	0.3	1.9 (1.7–2.6)	1.8 (1.5–2.2)	0.4
Genital ulcers	11.6 (10.2–12.1)	11.9 (10.8–12.4)	0.4	2.1 (1.7–2.5)	1.7 (1.4–1.9)	0.04*
Urethritis	11.8 (11.2–12.2)	11.6 (10.4–12.2)	0.7	2.5 (2.2–2.6)	1.8 (1.5–2)	0.002*
Arthralgia/arthritis	11.9 (10.5–12.6)	11.4 (10.4–12.1)	0.46	1.8 (1.4–2.4)	1.86 (1.6–2.1)	0.88
Headache	12.2 (11.2–12.4)	11.6 (10.4–12.2)	0.57	2 (1.6–2.16)	1.8 (1.5–2.2)	0.84
PN	11.3 (7.2–12)	12 (10.6–12.4)	0.12	1.9 (1.5–2.3)	1.86 (1.5–2.2)	0.76
Cognitive dysfunction	12.2 (11.2–12.4)	11.5 (10.4–12)	0.38	2 (1.6–2.16)	1.8 (1.5–2.2)	0.69
Inflammatory back pain	11.6 (10.6–12.2)	11.9 (10.4–12.2)	0.93	1.8 (1.5–2.2)	1.86 (1.6–2.2)	0.82
Smoking	10.6 (9.4–12.2)	11.9 (10.8–12.2)	0.32	2.3 (1.5–2.5)	1.8 (1.5–2.1)	0.24
Family history of AIDs	12 (10.4–12.3)	11.5 (10.4–12.2)	0.92	2 (1.7–2.4)	1.8 (1.4–2.2)	0.3

Note: Mann–Whitney *U*-test was used to identify the significance between the groups. *Significance was considered when $P \leq 0.05$.

Abbreviations: IQR, interquartile range; DVT, deep venous thrombosis; GIT, gastrointestinal manifestations; PN, peripheral neuropathy; AID, autoimmune diseases.

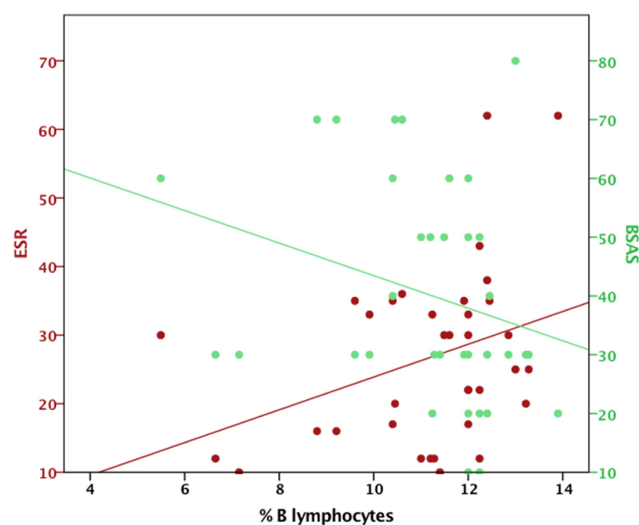
the healthy controls. Further studying of changes in their numbers in different disease manifestations revealed a marked drop in B lymphocytes in arterial diseases. However, there was a trend of B regs to be upregulated in the presence of major vascular diseases, genital ulcers and urethritis.

Consistently, it was noticed that B regs were higher in patients who were commenced on LDA and possibly in those who received anticoagulants, as those medications were used exclusively as adjuvant therapy in major vascular diseases. On the other hand, the use of

immunosuppressives, which were indicated for different disease manifestations, did not exhibit significant variations in B regs percentages. Actually, all the used immunosuppressing medications were working on the T cells and none of the patients in this study had received medications that target the B cells like Rituximab.

Noticeably, the total proportions of B cells correlated negatively with the scores of disease activity, whereas they correlated positively with ESR. Moreover, the increment in the dose of prednisolone was associated with an increment in B regs numbers.

In line with our findings, a reduction in numbers of B cells in BD patients compared to HCs has been reported before²¹ and attributed to a drop in certain subsets such as CD27⁺ memory B cells.¹² Additionally, a decline in the peripheral blood B regs of the patients compared to the controls was also observed by Yoon et al despite the lack of a significant difference in their study.²¹ B regs play an immunomodulatory and suppressive role against auto-inflammation and auto-reactivation, as was noticed in other diseases, via IL-10 dependent and independent mechanisms, through maintaining T regulatory cells and negatively regulating T cell differentiation.^{17,18,27} Thus, the observed drop in B regs numbers may be accounting for the increased risk of acquiring BD. An inverse relationship between the total number of B cells and the disease activity scores was present in the current study which was also reported by van der Houwen et al who observed shifting of B cells from the blood to the sites of

**Figure 2** The relationship of B lymphocytes with ESR and BSAS scores.

Abbreviations: ESR, erythrocyte sedimentation rate; BSAS, Behçet's Syndrome Activity Score.

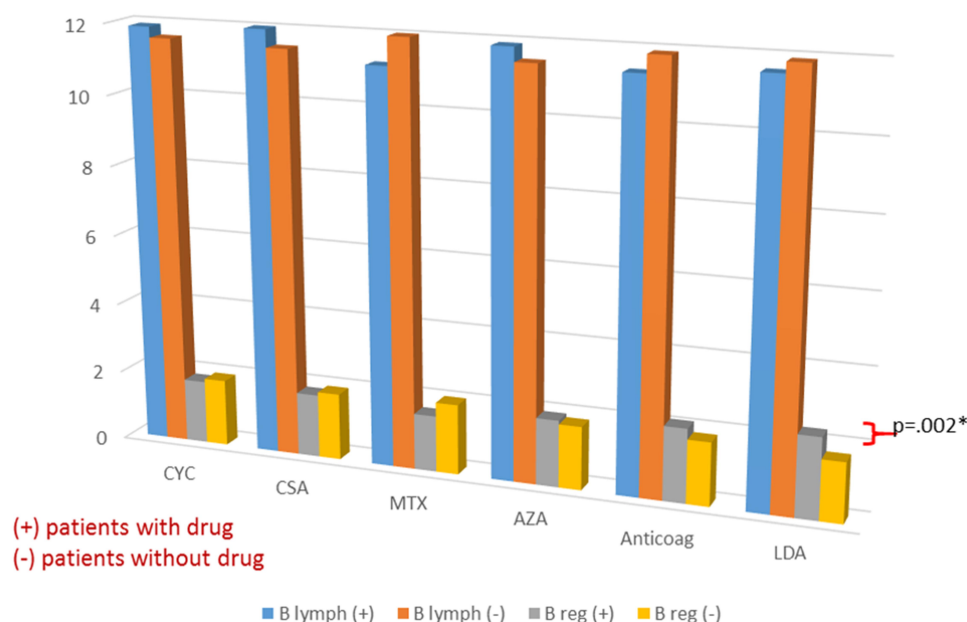


Figure 3 The differences in medians of total B lymphocytes and B regs. Percentages with versus without different drug administration. *Significant difference. **Abbreviations:** CYC, cyclophosphamide; CSA, cyclosporine-A; MTX, methotrexate; AZA, azathioprine Anticoag; anticoagulant; LDA, low dose aspirin.

inflammation, and normalization of the numbers following anti-TNF therapy.¹² In this context, the role of B cells in BD arterial disease was highlighted, previously.²⁸ CD20⁺ B cells were demonstrated in the vascular wall of a ruptured pulmonary artery aneurysm²⁸ suggesting its role in the pathogenesis of arterial disease and mostly explaining the marked drop of these cells in the peripheral blood with this phenotype.

Although there was a lack of association between the clinical activity and ESR, a laboratory marker of inflammation, an increased ESR was linked to a parallel rise in B cells. The value of ESR in determining the activity of different BD manifestations or differentiating the activity of other inflammatory diseases was previously questioned.^{29,30} Besides that, the disease activity scores used for Behcet disease in this article were measuring patients' reported outcomes (PROs). No laboratory work was considered in these scores and the PROs depend on the patients' feelings and perception of disease activity status that could be affected by several personal, psychological and environmental factors regardless of the laboratory landmarks of activity. This might explain the independent association of ESR with increased numbers of B cells which may be a predictor of a progressive disease rather than an active one.

In the present study, B regs tend to expand in certain clinical presentations, namely, vascular and urogenital

diseases. It was noticed that B cells play a role in the resolution of venous thrombosis which was defective after splenectomy or B cell-depletion.³¹ It is possible that they exert a role in the resolution of thrombosis similar to effector T cells.³² However, in vitro studies of the regulatory function of B reg in BD venous and arterial thromboses are warranted to support our findings.

Despite the lack of significant changes in B cell numbers with different therapies, longitudinal studies are required to detect their exact effects along the course of the disease. The observed rise in B regs in patients receiving LDA is expected to be secondary to the vascular nature of their involvement, which entailed the use of LDA to modify the prothrombotic conditions, rather than an effect of the drug. This was less evident with anticoagulants probably due to the much fewer number of patients on them at the time of recruitment.

The regulatory effect of corticosteroids on T cell functions has been gaining a lot of attention,^{33–35} nonetheless, their effects on the regulatory function of B cells have seldom been investigated. The current study strongly suggests a regulatory effect of corticosteroids via upregulating B regs.

One of the limitations of the current work is the observational nature of the study. Small study population is another limitation. Therefore, longitudinal tracking of the dynamic changes in B cells throughout the course of the

disease in larger patients' number is warranted. It was also unclear, in this study, whether B regs were upregulated with other disease presentations; this is partly due to the scarcity of some involvements, such as different neurological manifestations. In addition, all the patients exhibited oral ulcers and skin lesions, and showed positive pathergy result which hindered to demonstrate the probable upregulation of B regs in these contexts.

In conclusion, this study supports the role of B cells in BD pathogenesis and strongly suggests a possible role for B regs in the resolution of different BD manifestations.

Data Sharing Statement

Data will be available upon request from the corresponding author.

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Disclosure

The authors report no conflicts of interest for this work.

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