Serum immunoglobulin D levels in patients with Behçet's disease according to different clinical manifestations

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ABSTRACT

Background. *Behçet's disease (BD) is an autoinflammatory disorders mainly characterised by recurrent oral aphthosis, genital ulcers, and uveitis. The involvement of immunoglobulin D (IgD) in BD physiopathology is still unclear.*

Objective. *The aim of our study was to assess the role of IgD in BD by comparing circulating levels of IgD in a cohort of BD patients and healthy controls (HC), as well as by correlating IgD levels with BD activity and different clinical presentations.*

Methods. *Serum IgD and SAA levels were analysed by ELISA assay in ninety-nine serum samples collected from 72 BD patients and in 29 HC subjects.*

Results. *Serum concentration of IgD were higher in BD patients compared with HC (p=0.029), in patients with high serum amyloid A (SAA) levels compared with patients with normal SAA levels (p=0.035), and among subjects with active mucocutaneous involvement compared with other patients (p=0.036). No correlations were identified between IgD serum levels and disease activity assessed by the BD current activity form (BDCAF) (p=0.640). No differences were observed in the IgD serum levels between patients with and without specific disease manifestations. Increased SAA levels (Odds Ratio = 3.978, CI: 1.356 - 11.676) and active mucocutaneous BD manifestations (Odds Ratio = 4.286, CI: 1.192 - 15.407) were associated with a high risk for increased IgD serum levels.*

Conclusion. *Serum IgD levels are significantly increased in BD patients, especially among patients with active mucocutaneous manifestations, suggesting a possible role of IgD in BD pathogenesis and in the onset of mucosal and skin lesions.*

Introduction

Behçet's disease (BD) is a systemic autoinflammatory disease variably affecting vascular, gastrointestinal, neurological and musculoskeletal systems and mainly characterised by recurrent oral and genital ulcers, uveitis and skin lesions (1-3). Although several genetic and environmental risk factors have shown to contribute to disease development, BD pathogenesis is still widely unclear. However, impairment of both innate and adaptive immune responses has been reported in BD patients along with the involvement of several immune cells including monocyte/macrophage, neutrophils, B and T lymphocytes (4-6). The perturbation of T cells homeostasis, especially those involving Th1 and Th17 expansions, the decreased regulation by Tregs and hyperactivation of neutrophils or cytotoxic cells for endothelial dysfunction are known to be involved in BD pathogenesis (7-11). Moreover, the impairment of B cells regulators have also been found to play a key role in the pathogenesis of BD. In this regard, elevated levels of tumour necrosis factor (TNF)-α were observed in serum from BD patients and correlated with BD activity and severity (8, 12, 13). In addition, biological treatment with anti-TNF-α agents has largely demonstrated to be effective for BD treatment (13-15). Also other members of the TNF-α superfamily that regulates B-cell survival, proliferation and maturation were found up-regulated in BD patients. Recently, signs of chronic memory B cell activation indicating a persistent systemic B cell stimulation were related to disease activity in BD (16). In addition, regulators of B-cell survival and immunoglobulin class-switch recombination such as B-cell-activating factor of the TNF family (BAFF) were also identified up-regu-

lated in serum, skin and bronchoalveolar lavage from BD patients (17-19).

Although some studies demonstrated increased immunoglobulin production in BD, little is known about the role of immunoglobulin class D (IgD) in this disease. IgD is an antibody class, discovered almost 50 years ago, that is expressed as either membrane IgD in immature B-lymphocytes or in mature B-cells associated with IgM or secreted IgD (sIgD) (20). sIgD is involved in B-cell activation, generation and maintenance of B-cell memory and also seems to induce several cytokines including TNF-α, interleukin (IL)-1β and IL-6 (21), that are key players in BD pathogenesis. Accumulating evidences have suggested that serum IgD are elevated in several autoinflammatory and autoimmune diseases including hyper-IgD syndrome, due to *MVK* gene mutations, familial Mediterranean fever (FMF), owing to *MEFV* gene mutations, rheumatoid arthritis, systemic lupus erythematosus, and Sjögren's syndrome (20-24). Therefore, the aims of our study were to assess the circulating levels of IgD in a cohort of BD patients and to correlate their levels with the status of disease activity and the occurrence of specific clinical manifestations.

Materials and methods

Patients

Ninety-nine serum samples were routinely collected and analysed from 72 consecutive BD patients (37 males, 35 females), who met the International Study Group Criteria (ISGC) (25) and the International Criteria for BD (ICBD) (26). Twenty-nine healthy controls (HC) (11 males, 18 females) were also enrolled in the study. All patients and HC were Caucasians of Italian origin. Demographic and clinical data were retrospectively obtained from patients through review of medical records. Serum samples were collected every 3 to 4 months and in case of disease relapse. More than one serum sample was obtained from seventeen patients during an active phase of disease, resulting in a total of 99 BD samples. Disease activity was assessed by using the BD Current Activity Form (BDCAF), a worldwide used clinimetric tool for BD patients (27). Similarly to previous attempts (28, 29), patients showing a BDCAF score ≥5 (corresponding to ≥2 BD manifestations or organs involved during the 28 days preceding sample collection) were included in the active-BD group. Conversely, patients with a BDCAF score <5 were regarded as showing low disease activity.

The primary aim of the study was to compare serum IgD levels between BD patients and HC. The secondary aims were: i) to investigate whether serum IgD levels are associated with the occurrence of specific BD manifestations; ii) to examine whether serum IgD levels may reflect systemic disease activity; iii) to search for correlations between IgD levels and inflammatory markers including C-reactive protein (CRP), erythrocyte sedimentation rate (ESR) and serum amyloid A (SAA).

The primary endpoint of the study was represented by a statistically significant difference in the sIgD levels between BD patients and HC. The secondary endpoints of the study were as follow: i) to identify statistically significant differences in the sIgD levels between patients with and without BD-related clinical features including active mucocutaneous manifestations, ocular involvement, central nervous system (CNS) involvement, vascular disease, and gastrointestinal manifestations; ii) to identify any moderate-to-strong correlation between sIgD levels and BD activity as assessed by BDCAF; iii) to determine statistically significant differences in the sIgD levels between patients with and without a BDCAF score ≥ 5; iv) to identify any significant linear correlation between IgD serum levels and CRP, ESR or SAA; v) to explore the clinical variables associated with increased risk for higher sIgD (≥141 μg/mL, IgD cut-off value) at the time of sample collection.

Written informed consent was obtained both from patients and HC. The study protocol fulfilled the tenets of the Declaration of Helsinki and was approved by the local Ethics Committee of Azienda Ospedaliera Universitaria Senese, Siena, Italy.

Demographic and clinical information was obtained through structured interview, review of medical records, physical examination, and laboratory tests.

Determination of SAA and IgD serum levels

SAA and IgD serum concentrations were determined using a human solid phase sandwich enzyme-linked immunosorbent assay (Human SAA ELISA kit, Invitrogen, Carlsbad, CA) and Human IgD ELISA kit (Abcam) respectively, according to the manufacturer's protocol. According to several other studies, the IgD cut-off value was 141 μg/mL (22, 30, 31). Although literature data suggest a SAA cut-off level of 10mg/L (32), in our analysis we referred to a cut-off of 20 mg/L in order to minimise false positives and since it represents the threshold of our laboratory (33).

Statistical analysis

Statistical computations were performed using GraphPad Prism 5 software. Two-tailed Mann-Whitney test (for non-parametric data) and Student's t-test (for parametric data) were used for statistical comparisons between groups. Correlations were calculated using Spearman's correlation (two-tailed *p*-value) as well as Pearson's correlation test when required. Chi-square or Fisher exact tests were used for contingency tables as appropriate. Odds-ratio (OR) and its corresponding 95% confidence interval (95% CI) were used for the association between a putative risk factor and increased sIgD levels. Significance was defined as $p<0.05$.

Results

We included 72 BD patients (37 men and 35 women) with an average age of 42.27 years and 29 HC (11 men and 18 women), with an average age of 36.93 years. The main demographic and clinical characteristics of patients enrolled are summarised in Table I. At the time of serum collection, 15 (20.8%) patients were treated with colchicine and 6 (8.3%) patients with colchicine *plus* disease-modifying anti-rheumatic drugs (DMARDs). Anti-TNF-α and anti-IL1 agents were administered in

Table I. Demographic, laboratory and clinical characteristics patients affected by Behçet's disease (BD) recruited in our study.

	HC (n=29)	BD (n=72)
Females (%)	18 (62.06)	35 (49.30)
Age (mean ± SD), years	36.93 ± 11.70	42.27 ± 13.70
Disease onset (mean ± SD), years	-	29.14 ± 15.28
Disease duration (mean ± SD), years	-	13.01 ± 10.97
HLA-B51 positivity, n (%)	-	45 (69.23)
Disease activity, BDCAF	-	6.07 ± 3.56
Clinical manifestations (%)		
Oral/genital aphtosis	-	39 (54.17)
Skin manifestations	-	22 (30.56)
Uveitis	-	10 (13.89)
CNS involvement	-	11 (15.27)
Vascular involvement	-	5 (6.94)
Gastrointestinal manifestations	-	14 (19.44)
ESR (mean ± SD)	-	19.24 ± 20.9
CRP (mean ± SD)	-	0.39 ± 0.55
SAA range mg/L (mean ± SD)	-	60.80 ± 73.36

BD: Behçet's disease; BDCAF: Behçet's Disease Current Activity Form; CNS: central nervous system; CRP: C-reactive protein; ESR: erythrocyte sedimentation rate; HC: healthy controls; HLA: human leukocyte antigen; SAA: serum amyloid A; SD: standard deviation.

23 (31.9%) and 5 (6.9%) cases respectively, while 1 (1.4%) patient underwent IL-6 inhibition. Conversely, 16 (22.2%) patients were not treated at all. Serum levels of IgD were analysed in 99 serum samples from BD patients and in 29 samples from HC. Serum IgD concentrations were significantly higher among BD patients than in HC (126±185.6 *versus* 53±62.05 μg/mL, p=0.029), as reported in Figure 1. Subdividing BD patients into subgroups, no statistical difference was observed between HLA-positive and -negative patients (p=0.195). No differences were observed in sIgD levels between patients with active (BDCAF ≥5) and inactive (BDCAF <5) disease (p=0.758). As also illustrated in Figure 1, sIgD levels were significantly enhanced in BD patients with active mucocutaneous involvement compared with patients without skin and mucous manifestations at the time of sample collection (p=0.036). Conversely, as shown in Table II, no further statistical differences were observed in the sIgD levels between patients with and without specific disease manifestations at the time of sample collection.

Serum IgD levels did not differ between patients with and without treatment with colchicine (p=0.099), biological therapy (p=0.096) and cDMARDs (p=0.647) at the time of serum collection (Table II).

When serum IgD levels were evaluated between BD patients with increased SAA levels (>20 mg/L) and normal SAA levels (≤20 mg/L), serum sIgD were significantly higher in the former subgroup (p=0.035). Correlation study revealed a significant positive correlation between sIgD and SAA (r=0.2538, p=0.012) levels. Moreover, a stronger positive correlation was found between sIgD concentrations and SAA (r=0.525, p=0.047) in the active mucocutaneous BD subgroup (Fig. 2). In contrast, no significant correlation was observed between sIgD serum levels and CRP (p=0.098), ESR (p=0.3367), and BDCAF score (p=0.640).

Table III highlights statistical differences in the demographic, clinical and therapeutic features of patients enrolled between subjects with normal (<141 μg/mL) and increased (≥141 μg/mL) sIgD serum levels. In particular, BD with higher sIgD levels showed significantly enhanced levels of SAA (p=0.013).

The search for associations between higher sIgD levels and specific clinical features showed a significant higher risk for increased dosage of sIgD among patients with SAA levels >20 mg/L (OR=3.978, CI:1.356 - 11.676) or active mucocutaneous BD manifestations (OR=4.286, CI:1.192 - 15.407).

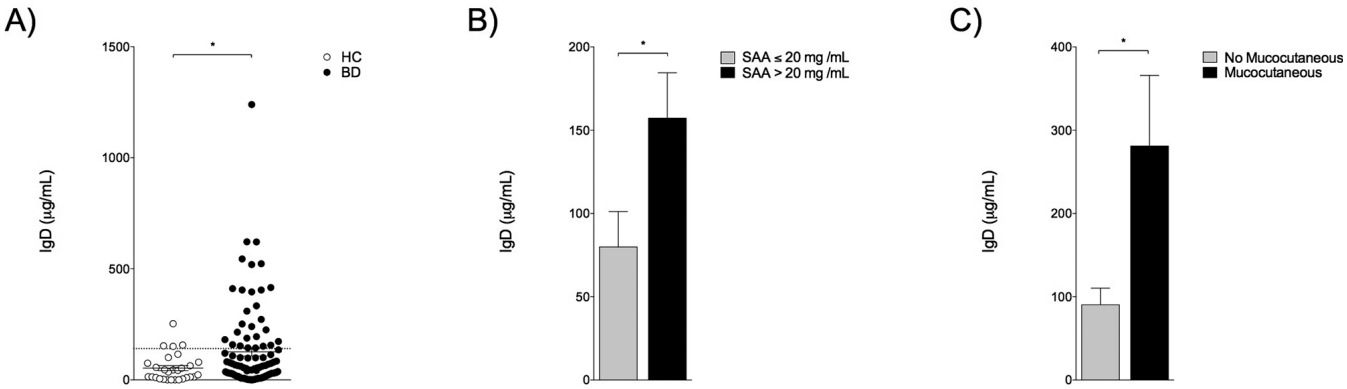

Fig. 1. IgD serum levels in patients with Behçet's disease (BD) and healthy controls (HC). **A**) Circulating levels of IgD were analysed in serum samples obtained from BD patients (n = 99) and HC (n = 29). The dashed horizontal line marks the cut-off value of 141 μg/mL. **B**) Serum IgD levels in patients with low-SAA levels (≤ 20 mg/L) compared with patients presenting high SAA levels (>20 mg/L). **C**) Serum IgD levels in patients with and without active mucocutaneous lesions. Data are expressed as mean ± SEM. Mann-Whitney U test and Student's *t*-test were carried out to check for statistical significance between groups (*p<0.05).

BD: Behçet's disease; HC: healthy controls; SAA: serum amyloid-A, SEM: standard error of the mean.

Table II. Comparison of circulating level of IgD between BD patients with (Yes) and without (No) specific disease manifestations, organ involvement and treatments (variables).

Variables		IgD μg/mL (mean ± SD)	*p* value
HLA-B51	Negative	113.1 ± 111.5	0.195
	Positive	107.5 ± 154.8	
Eye	No	124.8 ± 187.5	0.683
	Yes	134.4 ± 178.5	
CNS	No	138.2 ± 198.7	0.186
	Yes	60.78 ± 65.02	
Gut	No	136.4 ± 200.6	0.644
	Yes	75.78 ± 63.30	
Biological therapy	No	112.5 ± 151.6	0.096
	Yes	140.3 ± 216.7	
DMARDs	No	126.7 ± 200.9	0.647
	Yes	121.1 ± 159.8	
Colchicine	No	148.7 ± 212.4	0.099
	Yes	80.49 ± 103.2	
BDCAF	No	139.3 ± 229.2	0.758
	Yes	229.2 ± 146.9	

BDCAF: Behçet's Disease Current Activity Form; CNS: central nervous system; DMARDs: disease-modifying anti-rheumatic drugs; HLA: human leukocyte antigen; SD: standard deviation.

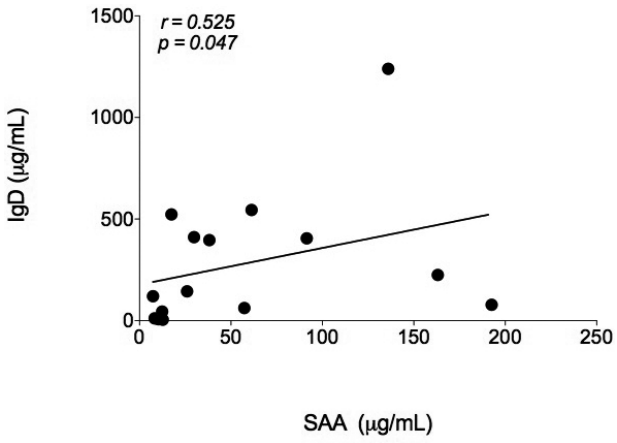

Fig. 2. Correlation analysis between serum IgD levels and serum amyloid-A (SAA) levels in the subgroup of patients with active mucocutaneous BD manifestations.

Discussion

Several studies have investigated the role of immune-related biomarkers in the pathogenesis of BD emphasising the complex nature of this disorder. In this context, sIgD levels have also been investigated as possible contributors to BD pathogenesis or biomarker of disease activity. Indeed, IgD have shown to promote inflammation and tissue damage by inducing IgD-interacting cells. Specifically, IgD may enhance proliferation of peripheral blood mononuclear cells along with production of several pro-inflammatory molecules including IL-1α, IL-1β, TNF-α and IL-6 (21).

Moriyama *et al*. firstly observed high levels of IgD in complete type of BD and in patients with disease remission (34). Accordingly, Kumano *et al*. displayed elevated levels of IgD in a cohort of 8/49 BD patients suffering from retinal vasculitis. Notably, serum IgD correlated with the percentage of sIgD-positive peripheral lymphocytes, suggesting IgD immune complexes as a possible cause of vasculitic lesions in BD (35). In contrast, Scully *et al*. identified raised IgD serum levels in BD patients compared with HC, but with no statistical significance (36).

On this basis, the purpose of our study was to shed more light on the role of sIgD in BD pathogenesis and evaluate the role of serum IgD levels as possible biomarker of disease activity or predictor of specific BD manifestations. In accordance with previous evidences (34, 35), we found significantly higher levels of sIgD in BD patients compared with HC. Nevertheless, no correlation was detected between serum IgD levels and disease activity assessed with BD-CAF; similarly, no statistically significant differences were identified in the frequency of increased serum IgD levels in patients with active and inactive BD. Basing on these results, although IgD might participate in the pathogenic process of BD, serum IgD levels do not appear to represent a marker of disease activity.

In our study, the risk for higher IgD levels was significantly associated with active mucocutenous BD subset (OR=4.286). In this regard, deregulation of mucosal IgD-armed basophils and IgD class-switched B cells has been previously observed in autoinflammatory disorders with periodic fever (37), suggesting a similar role in BD.

Of note, elevated sIgD promotes inflammation and tissue damage by inducing the activation and infiltration of IgD-interacting cells. Indeed, IgD enhance peripheral blood mononuclear cells (PBMCs) proliferation and stimulate PBMCs inflammatory responses by inducing several pro-inflammatory molecules including IL-1α, IL-1β, TNF-α and IL-6 (21). In this regard, we also found a moderate-strong positive correlation between sIgD concentrations and the acute-phase protein SAA in active mucocutaneous-BD subset.

Noteworthy, the microbiota has been shown to play an important role in driving class switch-mediated replacement of IgM with IgD (38). As microbiome changes have been considered to contribute to the pathogenesis of BD (39), our results on sIgD in patients with mucocutaneous manifestations may represent the epiphenomenon of a pathogenetic immune deregulation by non-invasive commensals and/or invasive pathogens. However, although interesting this hypothesis requires appropriate studies.

Apart from active mucocutaneous involvement, no statistically significant differences were observed in relation with specific BD characteristics includ-

Table III. Comparison of demographic, clinical and treatment features between BD patients with low IgD levels (Group A) and high IgD levels (Group B).

	Group A (with low IgD level) n=71	Group B (with high IgD level) n=28	*p*-value
Females, n (%)	32 (45.71)	9 (32.14)	0.240
Age (mean±SD), years	42.91 ± 13.81	40.86 ± 11.93	0.491
Disease onset (mean±SD), years	29.48 ± 15.26	26.74 ± 13.22	0.415
Disease duration (mean±SD), years	13.57 ± 11.16	13.22 ± 7.31	0.595
HLA-B51 positivity, n (%)	44 (68.75)	18 (69.23)	0.964
Disease activity, BDCAF	5.20 ± 3.55	5.54 ± 3.56	0.694
Clinical manifestations, n (%)			
Oral/genital aphtosis	33 (46.5)	16 (57.1)	0.339
Skin manifestations	17 (23.9)	9 (32.1)	0.404
Uveitis	8 (11.3)	4 (14.3)	0.679
SNC involvement	13 (18.3)	2 (7.14)	0.22
Vascular involvement	4 (5.6)	1 (3.6)	0.673
Gastrointestinal manifestations	14 (19.71)	3 (10.71)	0.382
ESR (mean±SD)	16.62 ± 14.41	24.33 ± 26.17	0.325
CRP (mean±SD)	0.38 ± 0.56	0.47 ± 0.59	0.155
SAA range mg/L (mean±SD)	52.91 ± 69.95	68.48 ± 62.96	**0.013**
Biological therapy, n (%)	36 (50.7)	12 (42.8)	0.482
DMARDs, n (%)	31 (43.7)	11 (39.3)	0.692
Colchicine, n (%)	27 (38)	6 (21.4)	0.115

HC: healthy controls; BD: Behçet's disease; HLA: human leukocyte antigen; SD: standard deviation; BDCAF: Behçet's Disease Current Activity Form; CNS: central nervous system; ESR: erythrocyte sedimentation rate; CRP: C-reactive protein; SAA: serum amyloid A; DMARDs: disease-modifying anti-rheumatic drugs. Significant *p*-values ($p<0.05$) are shown in bold.

ing neurologic, intestinal and ocular involvement. These data indicate that serum IgD levels are not able to discriminate patients with major BD manifestations and may not anticipate major organ involvement in patients with BD. Similarly, no statistical differences were identified in relation with different therapeutic approaches including anti-TNF-α agents, DMARDs and colchicine. These findings point up the lack of relationship between sIgD expression and current treatments for patients with BD.

Of note, a significant correlation was found between IgD serum levels and SAA values, as also supported by the high risk for increased IgD values among BD patients with high SAA levels (OR=3.978). In this regard, SAA is an apolipoprotein released by hepatocytes, adipocytes, activated macrophages and synoviocytes during the active phase of disease and has been investigated as a potential laboratory marker of disease activity and therapeutic response in many rheumatologic disorders, especially autoinflammatory diseases (40-44). In relation with BD, we have recently demonstrated a significant association between different SAA levels and the occurrence of oral aphthosis, neurological and ocular disease in BD patients (29). Furthermore, BD patients with increased levels of SAA (>20 mg/L) displayed significantly elevated levels of inflammatory mediators when compared with patients showing normal SAA levels, suggesting a potential relationship between SAA and pro-inflammatory cytokines in BD pathogenesis (33). In this context, the linear association between SAA and IgD serum levels further support a role for this class of immunoglobulins in BD pathogenesis.

The main limits of this study are represented by the relatively small number of patients enrolled and the lack of a disease control group. However, the preset study seeks to clarify the role of sIgD in the pathogenesis of BD shedding new light on clinical and therapeutic correlations. Nevertheless, in the next future further molecular studies are required to deeply understand how IgD are involved in the pathogenesis of BD.

In conclusion, IgD serum levels are significantly increased in BD patients compared with HC, suggesting a possible role of IgD in the pathogenesis of this disease, especially in relation with mucocutaneous manifestations. Nevertheless, IgD serum levels have not shown to represent a biomarker of disease activity and are not influenced by different treatment options.

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