Behçet's disease heterogeneity: cytokine production and oxidative burst of phagocytes are altered in patients with severe manifestations

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ABSTRACT

Objective. To test the hypothesis that classical phagocytic functions are constitutively stimulated in patients with Behçet's disease (BD).

Methods. Four study groups were analysed: active BD (aBD; n=30), inactive BD (iBD; n=31); septic patients (SP; n=25); healthy controls (HC; n=30). Microbicide activity against Streptococcus pneumoniae, Streptococcus sanguinis and Candida albicans was determined by means of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction and absorbance read by ELISA. Flow cytometry analysis evaluated phagocytosis (zymosan particles and microrganisms) and oxidative burst by dihidrorhodamine oxidation before and after stimulation with phorbol myristate acetate (PMA). The supernatant of PBMC cultures under TLR or microbial stimuli and of neutrophil cultures under PMA, LPS or microbial stimuli were used for determination of cytokine production by ELISA.

Results. We found no significant differences between the BD patient groups and control groups with regard to oxidative burst, phagocytic activity, microbicide activity or cytokine production. However, the cells from patients with severe BD (based on clinical manifestation) exhibit significantly higher oxidative burst activity, both before and after PMA stimulation, compared to cells from patients with mild BD. Furthermore, we found significant correlations between the BD patients' scores on the simplified Behçet's Disease Current Activity Form adapted for Portuguese (BR-BDCAFs) and Streptococcus sanguinis-stimulated production of IL23 by PBMC and IL8 by neutrophils, and between BR-BDCAFs score and constitutive production of TNF-α, IFNγ, IL6 and IL23 by PBMC.

Conclusion. Patients with severe active BD do exhibit phagocytic dysfunction and some evidence of constitutive activation regarding oxidative burst and cytokine production.

Introduction

Behçet's disease (BD) is a heterogeneous idiopathic systemic vasculitis and prevalent along the ancient "Silk Route", suggesting that underlying genetic (1) and/or environmental factors may have spread along this avenue and in different regions of the world (2). Intestinal involvement is more frequent in the Far East (3), but is uncommon in Turkish patients (4). Moreover, a positive pathergy test is frequently observed in patients from the Mediterranean region to Japan, but is rarely observed in patients from Northern Europe and the United States (5), and only sometimes observed in patients from Brazil (6).

Increased neutrophil activation is a marked characteristic of BD (for example, the pathergy test) (7). Several studies suggest a role for exposure to microorganisms in BD pathogenesis. Neutrophils from BD patients overreact to *Streptococcus sanguinis* by increasing the oxidative burst, whereas lymphocytes produce excessive IL8, IL12 and IFNγ, indicating a Th1 response (8, 9). This cellular hyperactivity is known as "streptococcal hypersensitivity" (10). HSV-1 (11, 12), *Staphylococcus aureus*, *Mycobacterium tuberculosis* (13) and some species of *Prevotella* have also been implicated in the disease pathogenesis (14).

However, it is unclear whether neutrophil activation occurs constitutively or if it is secondary to as yet unknown stimuli. Additional phagocyte func-

tional abnormalities may be involved, including changes in cytokine production (15-17), oxidative burst (18-20) and phagocytic (21, 22)/microbicide activity (23), but this remains controversial (7, 24). Monocytes from BD patients seem to produce more IL1β and IL23 under LPS stimulus, while T naïve produced more IL17 than healthy controls (17). While several studies suggest that phagocytic activity of BD neutrophils is not significantly different from that of healthy controls (21, 22), at least one study found the opposite result (25).

There is considerable controversy regarding the oxidative burst in BD phagocytes. Carletto *et al.* (18) found no difference in superoxide production by neutrophils from active BD, inactive BD and healthy controls after stimuli with zymosan, PMA or fMLP. However, Yoshida *et al.* (19) found an increase in superoxide production after the same stimuli of BD neutrophils. Similar findings were obtained by Takeno *et al.*, who showed increased reactive oxygen species production not only by BD neutrophils, but also by those from healthy controls carrying the HLA-B51 gene, what may suggest a link between genetic background and the involvement of phagocyte dysfunction in the disease pathogenesis (20). In contrast, two studies reported diminished oxidative burst in neutrophils from BD patients (21, 22). The above findings are intriguing and appear to indicate some connection between infectious agents and phagocyte dysfunction in BD pathogenesis. Neutrophils appear to be involved in BD pathogenesis, but their exact role is unclear. We hypothesise that classical phagocyte functions, including oxidative burst, cytokine production, and phagocytic and microbicide activities, are constitutively stimulated in BD patients. In this study we present in depth investigation of phagocytic functions of neutrophils, monocytes and PBMC of BD patients compared to healthy and septic controls.

Materials and methods
Study subjects and diagnostic criteria
This study included four subject groups:
1) 30 healthy controls (HC);
2) 25 septic patients (SP);
3) 31 patients with inactive BD (iBD);
4) 30 patients with active BD (aBD).
All BD patients met the *International Study Group for Behçet's Disease* criteria (26). Active BD was defined as a score equal to or greater than two on the simplified *Behçet's Disease Current Activity Form* adapted for Portuguese (BR-BDCAFs) (27). Inactive BD was defined as BR-BDCAFs equal to zero. For subgroup analysis according to disease severity, patients were classified as described by Krause *et al.* (28) with minor modifications in severe (previous or current presence of at least one episode of posterior uveitis, retinal vasculitis, optic neuritis, neurological, gastrointestinal, or cardiovascular involvement) or mild/moderate (previous or current presence of oral or genital ulcers, cutaneous manifestations, arthritis/arthralgia and anterior uveitis) disease.

The healthy control group comprised healthy volunteers without any evidence of autoimmune diseases. The septic group, a positive control for neutrophil activation, comprised inpatients from emergency room and intensive care unit from UNIFESP University Hospital meeting the sepsis criteria of *American College of Chest Physicians/Society of Critical Care Medicine Consensus Conference* (29). The research was approved by UNIFESP Ethics Committee (CEP n. 0013/11).

Oxidative burst of phagocytes
Dihydrorhodamine (DHR) flow cytometry assays were performed as previously described (30). For every flow cytometry assay, samples were analysed in FACSAria III (Becton & Dickinson®, New Jersey, USA) and monocytes and neutrophils were gated by forward and side scatter. Acquired data were analysed using Flowjo software (Tree Star Inc®, Ashland, USA).

Microorganism culture and labelling
Samples of *Candida albicans* (ATCC 26279) and of bacterial strains *Streptococcus pneumoniae* (ATCC 6305) and *Streptococcus sanguinis* (ATCC 10556) were cultivated at 37ºC in separate bottles with Sabouraud medium for 18h (BD DIFCO®, Franklin Lakes, USA) and BHI medium for 24h (BD DIFCO®,

Franklin Lakes, USA), respectively, as previously described (31). Microorganism concentration was adjusted to 2.4×10^9/mL using a spectrophotometer (absorbance 2.5, wavelength 620 nm) calibrated by Mac Farland's scale, and then centrifuged at 1100g for 10 minutes at room temperature and washed with 10mL of sterile saline twice. At this point, microorganisms were ready for microbicide activity assay. For phagocytic activity assay, microorganisms were then killed by heat (60°C for 30 minutes) and the suspension was centrifuged at 1100g for 10 minutes at room temperature and washed with 10 mL of sterile saline. To assure the microorganisms death, this process was repeated twice. The killed microorganisms were conjugated with 25 μL 5% propidium iodide for 1 mL of cell suspension for 30 minutes at room temperature in the dark. Finally, the pellet was suspended in PBS with 5 mM glucose and 0.1% gelatin, aliquotted and stored at -80ºC. The labelling procedure was verified in one sample of each.

Phagocytic activity assay
Phagocytic activity assays were performed as previously described (32) in the presence of zymosan labelled with FITC (Sigma-Aldrich®, St. Louis, EUA), or *Streptococcus pneumoniae, Streptococcus sanguinis* or *Candida albicans* labelled with propidium iodide (as described above).

Microbicide activity assay
Neutrophils and PBMC were separated by density gradient, using dextran (Sigma-Aldrich®, St. Louis, USA) and Ficoll-Hipaque 1077 (Gibco®, Grand Island, USA), respectively. Duplicate 2×10^5-cell aliquots (neutrophils or PBMC) in RPMI 1640 (Gibco®, Grand Island, USA) were incubated in 96-well plates and stimulated with 2×10^6 *Streptococcus pneumoniae, Streptococcus sanguinis* or *Candida albicans*, opsonised or not with 10 μL with fresh pooled human serum. Two microorganism dilutions in PBS (100% and 10%) were used as reference for the calculation of killing units, as described below. Non-stimulated samples were also included as controls for contamination

with other microorganisms. Plates were incubated for 120 minutes with shaking at 37ºC and 5% CO_2 atmosphere. 100 µL 1.5% Triton X-100 (Sigma-Aldrich®, St. Louis, USA) was added to lyse leukocytes. Plates were centrifuged at 1100 g for 10 minutes at room temperature and washed with sterile PBS twice. Finally, 10 µL 1% MTT (Sigma-Aldrich®, St. Louis, USA) was added to each well and samples were incubated at room temperature in the dark for 2h. The reaction was stopped by adding 200 µL DMSO (Sigma-Aldrich®, St. Louis, USA) and the plate was read at 560 nm using a Victor X3 2030 Multilabel Reader (Perkin Elmer®, Singapore). Results were calculated using the formula below for each microorganism stimulus, and expressed as arbitrary "Killing Units", which can be defined as the percentage of variation of microorganism concentration between basal state and 120 minutes leukocyte activity. Positive values represent microbial killing while negative values represent microbial proliferation.

$$\text{Killing units} = 1 - \left\{ \left[\frac{(\text{OD sample}) - (\text{OD 10\% microorganisms})}{(\text{OD 100\% microorganisms}) - (\text{OD 10\% microorganisms})} \right] \times 0.9 \right\}$$

Quantification of cytokine production in culture
Triplicate 2×10^5 PBMC aliquots suspended in RPMI 1640 (Gibco®, Grand Island, USA) were incubated in 96-well plates for 48h and stimulated by:
1) 0.8 ng PAM3CSK4 (TLR2 ligand; Invivogen®, San Diego, USA);
2) 3 µg Poly(I:C) (TLR3 ligand; Invivogen®, San Diego, USA);
3) 0.4 ng *Salmonella Minnesota* Re595 LPS (TLR4 ligand; Sigma-Aldrich®, St. Louis, USA);
4) 4ng flagellin (TLR5 ligand; Invivogen®, San Diego, USA);
5) 150 ng imidazoquinoline (TLR7/8 ligand; Invivogen®, San Diego, USA);
6) 2×10^6 *Streptococcus pneumoniae* (as described in "microorganism culture and labelling");
7) 2×10^6 *Streptococcus sanguinis*;
8) 2×10^6 *Candida albicans*. PBMC culture supernatants were evaluated for the presence of IL10, IL12p70, IFN-γ, TNF-α, IL23 and IL6 (Becton & Dickinson®, San Diego, USA) by capture ELISA as described previously (33).

Similarly, neutrophil culture supernatants were evaluated for the presence of IL8 (Becton & Dickinson®, San Diego, USA) and IL1β (human IL1β ELISA Ready-SET-go, eBioscience®, San Diego, USA) after a 4h activation by *Salmonella Minnesota* Re595 LPS (TLR4 ligand), *Streptococcus pneumoniae, Streptococcus sanguinis, Candida albicans* or 30 ng PMA.

Statistical analysis
Continuous variables with normal distribution were analysed with the Student's *t*-test and those with non-parametric distribution were analysed with the Mann-Whitney test. Qualitative parameters were analysed by the Chi-square test and the Fisher's exact test when appropriate. Multiparametric analyses were performed with the one way ANOVA test with Bonferroni's test as a post-hoc test when appropriate. Correlation analysis was performed by the Pearson's correlation method. Statistical inference level was set at 0.05.

Results
Study subject characteristics
The female:male ratio was 19:11 in the aBD group and 15:16 in the iBD group, with age ranging from 19 to 61 years old (38.50±11.47) and 22 to 63 years old (39.38±10.50), respectively. The female:male ratio was 17:13 in the HC group and 14:11 in the in SP group, with age varying from 22 to 60 years old (36.86±11.94) and 20 to 82 years old (46.00±25.76), respectively. Patients and controls did not differ regarding age and gender distribution.
One aBD patient also had antiphospholipid syndrome and two iBD patients had psoriasis. Non-autoimmune diseases (miscellaneous) were present in 20 SP (80%), 21 iBD (67.74%) and 21 aBD (70%) patients, with no statistical difference. The clinical characteristics and therapeutic regimens of BD patients are shown in Tables I and II. There were no significant differences between aBD and iBD patients with regard to previous disease manifestations (Table I) or medications used (Table II). In fact, all but one patient had well-established diagnosis and were, therefore, under treatment.

Cytokine production from BD neutrophils and PBMC is similar to healthy controls
Cultures of PBMC isolated from aBD, iBD, HC and SP subjects were incubated for 48 hours with TLR 2, 3, 4, 5, or 7 ligands, or with *Streptococcus pneumoniae*, *Streptococcus sanguinis* or *Candida albicans*, and culture supernatant was assayed for the presence of IFNγ, TNF-α, IL23, IL10, IL6 and IL12. As shown in Figure 1, no difference was observed between cytokine production by PBMC from BD patients compared to HC for any cytokine tested. There was a significant decrease in IFNγ, TNF-α, IL23 and IL10 production by PBMC from SP compared to the other groups, however (Fig. 1A-D). Neutrophils from the four groups were also cultured and incubated with PMA or TLR4 ligand, or with *Streptococcus pneumoniae*, *Streptococcus sanguinis* or *Candida albicans*, and culture supernatant was assayed for the presence of IL1β and IL8. As shown in Figure 1E-H, production of these two cytokines by neutrophils was similar among the four groups. Thus, these experiments did not detect any difference in cytokine production by BD phagocytes compared to HC.

Oxidative burst does not differ significantly among phagocytes from BD patients and from controls
Neutrophils and monocytes from blood samples were assayed for oxidative burst, in the resting state or after stimulation with PMA, using DHR oxidation and flow cytometry. The four groups presented equivalent oxidative burst profile as assayed by DHR oxidation. There was no difference in oxidative burst of phagocytes (neutrophils or monocytes, Fig. 2), either at resting state or after stimulus with PMA stimulation, within among the four groups.

Phagocytic cells from patients with severe active BD present constitutively increased oxidative burst and cytokine production
We investigated possible associations between BD disease severity and phagocyte function, taking two approaches. In the first, we classified aBD

Table I. Distribution of inactive (iBD) and active (aBD) Behçet's disease patients according to clinical manifestations.

	iBD n=31 (%)	aBD n=30 (%)	*p*-value
Current manifestations			
Oral ulcers	0	17 (56.66)	<0.001
Genital ulcers	0	9 (30)	<0.001
Anterior uveitis	0	10 (33.33)	<0.001
Posterior uveitis	0	3 (10)	NS
Other ocular manifestations[a]	0	3 (10)	NS
Pseudofoliculitis	0	5 (16.66)	<0.05
Erithema nodosum	0	7 (23.33)	<0.01
CNS	0	5 (16.66)	<0.05
Gastrointestinal	0	1 (3.33)	NS
Cardiovascular	0	2 (6.66)	NS
Others[b]	0	2 (6.66)	NS
Previous manifestations			
Oral ulcers	31 (100)	29 (96.66)	NS
Genital ulcers	25 (80.64)	25 (83.33)	NS
Anterior uveitis	12 (38.70)	11 (36.66)	NS
Posterior uveitis	14 (45.16)	4 (13.33)	NS
Other ocular manifestations[a]	8 (25.80)	7 (23.33)	NS
Pseudofoliculitis	17 (54.83)	16 (53.33)	NS
Erithema nodosum	16 (51.61)	14 (46.66)	NS
Central nervous system	6 (19.35)	9 (30)	NS
Gastrointestinal	1 (3.22)	2 (6.66)	NS
Cardiovascular	7 (22.58)	2 (6.66)	NS
Pathergy	5 (16.12)	4 (13.33)	NS
Others[b]	13 (41.93)	15 (50)	NS
Disease duration (median/min-max)	9/1 – 29	4/1 – 16	<0.01
Age at BD onset (mean±SD)	29.22±9.75	33.16±10.43	NS
BR-BDCAFs (median/min-max)	0/0 – 0	2/2 – 6	<0.001

BR-BDCAFs: simplified Behçet's Disease Current Activity Form validated to Portuguese.
[a] Included cases with optic neuritis, vitreous hemorrhage, sudden blindness, etc.
[b] Other manifestations: BD induced dementia, arthralgia/arthritis, decalvans folliculitis, etc.

subjects as having mild disease (defined as oral or genital ulcers, cutaneous manifestations, arthritis/arthralgia and anterior uveitis; n=21) or severe disease (defined as posterior uveitis, retinal vasculitis, optic neuritis, neurological, gastrointestinal, or cardiovascular involvement; n=9) and evaluated oxidative burst of each group and inactive BD patients. For both neutrophils and monocytes there is a significant increase in oxidative burst in cells from aBD patients with severe disease compared to cells from aBD patients with mild disease (Fig. 3). We also considered the fold increase in oxidative burst after PMA stimulation and found, again, a significant increase in subjects with severe disease compared to those with mild or inactive disease (Fig. 3C). In the second approach, the Pearson correlation coefficient was calculated to identify any association between various BD patient characteristics and oxidative burst, phagocytic activity, microbicide activity or cytokine production of their cells, as reported in Figures 1–5. No associations were found between use of overall immunosuppressant, clinical activity, or peculiar clinical manifestations and any phagocyte function (data not shown).

We specifically studied possible correlations between the duration of current immunosuppressant therapy and phagocyte function. A negative correlation was observed between the duration of treatment with azathioprine and the percentage of neutrophils (Rho=-0.549, *p*<0.01) or monocytes (Rho=-0.618, *p*<0.001) expressing fluorescence after PMA-induced oxidative burst. Similarly, we found a negative correlation between the duration of treatment with cyclosporine and the percentage of neutrophils (Rho=-0.900, *p*<0.05) expressing fluorescence after PMA-induced oxidative burst.

Importantly, the exclusion of patients using azathioprine or cyclosporine did not affect the association of increased oxidative burst activity and severe forms of the disease (data not shown). No other correlation between immuno-suppressant therapy duration and other phagocyte functions was observed.

We specifically investigated potential associations between patients' BR-BD-CAFs score and phagocyte function. While no correlations were observed between BR-BDCAFs and oxidative burst, phagocytic activity or microbicide activity (data not shown) we did find significant associations between BR-BDAFs and constitutive production of TNFα (Rho=0.623, *p*<0.01), IFNγ (Rho=0.664, *p*<0.01), IL6 (Rho=0.604, *p*<0.05) and IL23 (Rho=0.516, *p*<0.05) by PBMC and *Streptococcus sanguinis*-stimulated production of IL23 (Rho=0.759, *p*<0.05) by PBMC and IL8 by neutrophils (Rho=0.836, *p*<0.01).

Phagocytic activity in BD patients is similar to healthy controls
Neutrophils and monocytes from aBD, iBD, HC and SP subjects were assayed for their ability to phagocytose fluorescently labelled zymosan particles or fluorescently labelled *Streptococcus pneumoniae*, *Streptococcus sanguinis* or *Candida albicans* As shown in Figure 4, there was no difference among the four groups regarding phagocytic activity of neutrophils or monocytes on zymosan or microorganisms.

Microbicide activity in phagocytes from BD patients is similar to controls
Neutrophils and PBMC from aBD, iBD, HC and SP subjects were isolated and assayed for microbicide activity against *Streptococcus pneumoniae*, *Streptococcus sanguinis* or *Candida albicans*, with and without opsonisation with fresh human serum, using the MTT assay to evaluate phagocyte effect on number of live organisms. Microbicide activity of both neutrophils and PBMC was similar among the four groups for all microorganisms tested, regardless of opsonisation (Fig. 5). Thus, as with phagocytosis, microbicide activity in BD patients is similar to controls.

Table II. Distribution of inactive (iBD) and active (aBD) Behçet's disease patients according to medication use.

		iBD n=31 (%)	aBD n=30 (%)	p-value
Current medication		28 (90.32)	27 (90)	NS
Colchicine		11 (35.48)	12 (40)	NS
Azathioprine		11 (35.48)	16 (53.33)	NS
Thalidomide		6 (19.35)	1 (3.33)	NS
Methotrexate		1 (3.22)	1 (3.33)	NS
Cyclophosphamide		0	2 (6.66)	NS
Penicillin G Benzatine		5 (16.12)	3 (10)	NS
Anti-TNF		3 (9.67)	4 (13.33)	NS
Corticosteroids	High doses	3 (9.67)	7 (23.33)	NS
	Low doses	10 (32.25)	5 (16.66)	NS
Cyclosporine		1 (3.22)	4 (13.33)	NS
Pentoxifylline		0	2 (6.66)	NS
Minocycline		1 (3.22)	1 (3.33)	NS
Dapsone		1 (3.22)	0	NS
Chlorambucil		0	0	NS
Sulfasalazine		0	0	NS
Previous medication		26 (83.87)	25 (83.33)	NS
Colchicine		8 (25.80)	7 (23.33)	NS
Azathioprine		9 (29.03)	6 (20)	NS
Thalidomide		9 (29.03)	5 (16.66)	NS
Methotrexate		5 (16.12)	4 (13.33)	NS
Cyclophosphamide		5 (16.12)	7 (23.33)	NS
Penicillin G Benzatine		4 (12.90)	5 (16.66)	NS
Anti-TNF		0	1 (3.33)	NS
Corticosteroids	High doses	15 (48.38)	10 (33.33)	NS
	Low doses	16 (51.61)	11 (36.66)	NS
Cyclosporine		9 (29.03)	5 (16.66)	NS
Pentoxifylline		1 (3.22)	3 (10)	NS
Minocycline		2 (6.45)	1 (3.33)	NS
Dapsone		0	1 (3.33)	NS
Chlorambucil		1 (3.22)	0	NS
Sulfasalazine		1 (3.22)	0	NS

Discussion

In the present study, we tested the hypothesis that classical phagocytic functions are constitutively activated in BD patients by investigating oxidative burst, phagocytic activity, microbicide activity and cytokine production of aBD patients and iBD patients compared to healthy controls and septic subjects. We found some classical phagocytic functions constitutively activated in patients with severe BD, however there are some caveats to this conclusion.

The impact of phagocyte functional abnormalities in BD pathogenesis is controversial, especially the role of oxidative burst. Previous studies investigated neutrophils in the peripheral blood and tissues using the nitroblue-tetrazolium reduction technique, which is based on the same principle as the DHR oxidation technique, and found no difference in oxidative burst in cells from BD patients (34, 35). Carletto *et al.* found similar results by measuring indirect superoxide production (18). In contrast, other studies showed decreased oxidative burst in the neutrophils from BD patients (21, 22), while others demonstrated increase (19, 20, 36-39). Another study evaluated oxidative burst by DHR oxidation in BD patients compared to healthy controls, and showed that constitutive and PMA-induced oxidative burst is higher in patients, although no comparison regarding disease activity was performed and no difference on fMLP-induced oxidative burst and overall reactive oxygen species production was identified (40).

The discrepancy observed in the literature may be due to several factors, including differences in study subjects' ethnicity, disease presentation, and disease activity, as well as differences in methodology and the source of samples used in each study (*e.g.* skin window neutrophil *versus* peripheral neutrophils). The remarkably pleiotropic clinical manifestation of BD probably also contributes to the heterogeneity in results. For instance, the immune activation in patients with muco-cutaneous symptoms may be distinct from those with more systemic process, such as neurological and vascular involvement. Our results suggest that increased oxidative burst and cytokine production is observed preferentially in patients with severe disease, which raises two possible explanations:

1) in a subset of patients with a more severe form of disease, phagocytes are constitutively activated and this abnormality plays a direct role in the pathogenesis of the disease;

2) the increased oxidative burst profile is a consequence of the severe immune system activation, typical of severe forms of BD. One possible bias was the absence of other stimuli such as f-MLF or zymosan to test whether there could be a modification in the signalisation pathway of the NADPH-oxidase that cannot be tested with PMA, which, in turn, directly stimulates the protein kinase C pathway, thus bypassing the entire kinase cascade. However, PMA is the prototypical stimulus used in routine DHR assay and could indicate the presence of usual differences between groups.

Concerning our finding that high BR-BDCAFs score correlates with increased production of some cytokines, it is important to point out the difficulty in defining disease activity in BD through BR-BDCAFs. Although BR-BDCAFs is extensively used in trials, the weight ascribed to the various disease manifestations may be not appropriate. For instance, uveitis and gastrointestinal involvement may have different pathogenesis, but both are scored equally. However, despite this possible caveat, we currently do not have a better-standardised method to evaluate disease activity.

Our finding that high BR-BDCAFs score correlates with cytokine production by resting PBMC is in accordance with several studies showing that BD patients with multiple systemic manifestations present a Th1 (TNF-α, IFNγ, IL6) and Th17 (IL23) skewed immune response (16, 41-44). Regarding this and similarly to our results, Mege *et al.* (39)
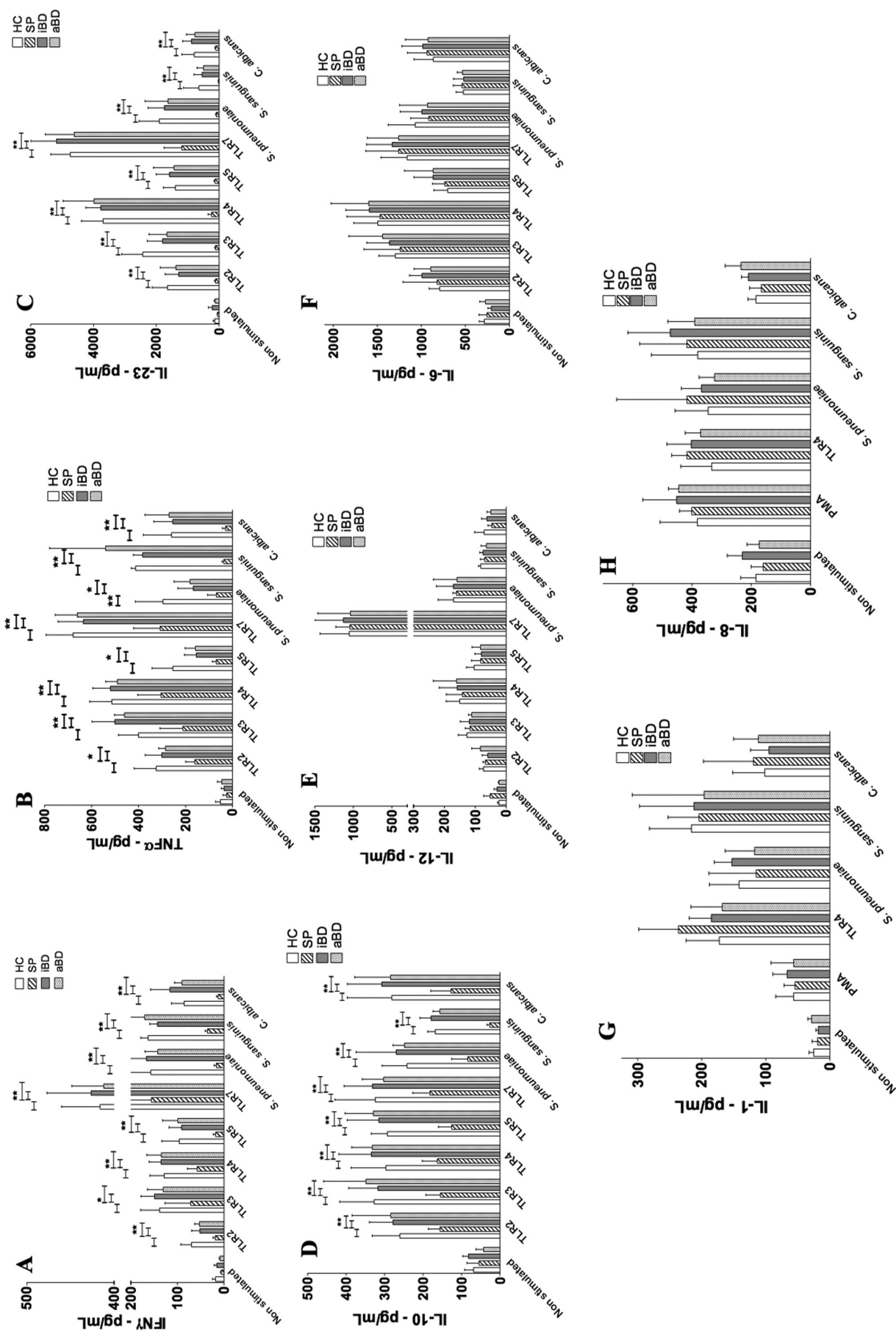
Fig. 1. Cytokine production by PBMC from BD patients and controls. PBMC were isolated, cultured and incubated with TLR ligands or microorganisms as shown, and as described in *Materials and Methods*. Cytokine concentrations in culture supernatant were determined using capture ELISA. Quantification of IFNγ (**A**), TNFα (**B**), IL-23 (**C**), IL-10 (**D**), IL-12 (**E**) and IL-6 (**F**) production by PBMC and of IL-1 (**G**) and IL-8 (**H**) by neutrophils of healthy controls (HC), septic patients (SP), inactive (iBD) and active Behçet's Disease (aBD) is shown. Bars represent mean±SE. Data is representative of three experiments. Student's *t*-test was performed to determine statistically significant difference. * *p<0.05*; ** *p<0.01*
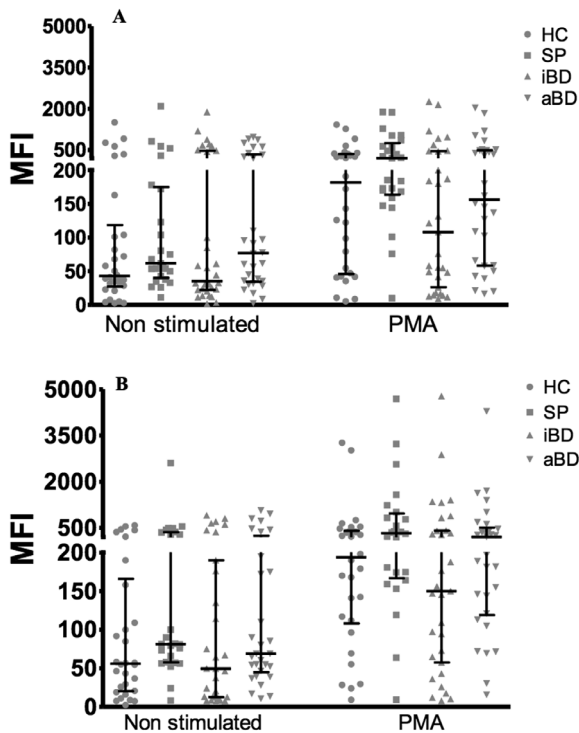
Fig. 2. Oxidative burst of neutrophils and monocytes from Behçet's disease patients and control subjects. Neutrophils (**A**) and monocytes (**B**) were isolated and assayed for oxidative burst by measuring fluorescence released after DHR oxidation. Cells were assayed at resting state (non-stimulated) or after stimulation with 30ng PMA for 60 minutes. Mean fluorescence intensity (MFI) of cells from healthy controls (HC), septic patients (SP), inactive (iBD) and active (aBD) Behçet's Disease patients is plotted. Bars represent median and interquartile range. Data is representative of two experiments. Mann-Whitney test was performed to determine difference. No significant differences were observed.
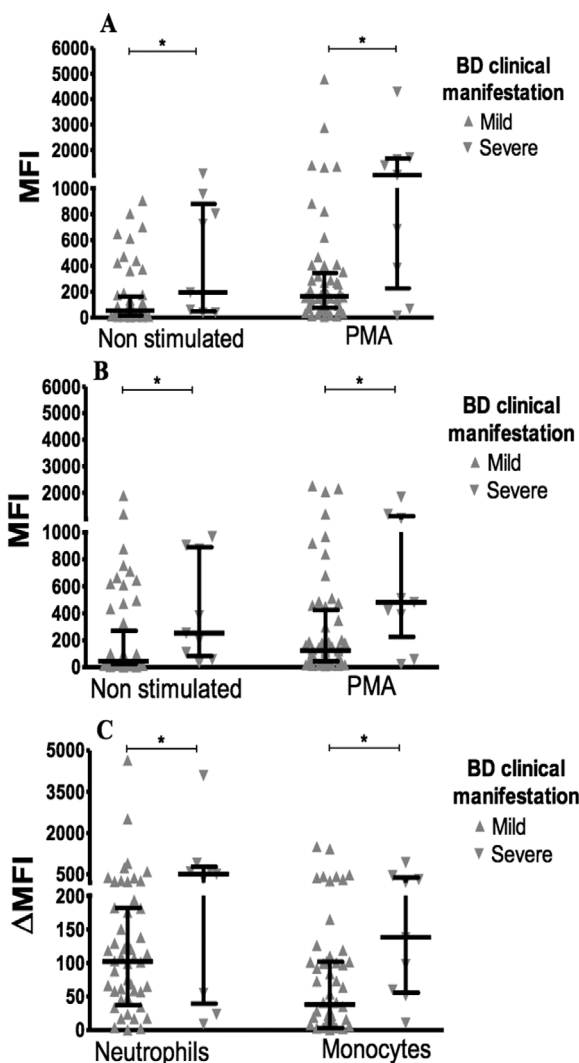

Fig. 3. Oxidative burst profile of neutrophils and monocytes from patients with mild or inactive versus severe active Behçet's disease (BD). Oxidative burst profiles, expressed as mean fluorescence intensity (MFI), of resting (non-stimulated) and PMA-stimulated neutrophils (**A**) or monocytes (**B**) from all BD subjects (taken from data shown in Figure 2) were graphed according to patient disease severity. Inactive BD (n=31) or mild active BD (oral or genital ulcers, cutaneous manifestations, arthritis/arthralgia and anterior uveitis; n=21) compared to severe aBD (posterior uveitis, retinal vasculitis, optic neuritis, neurological, gastrointestinal, or cardiovascular involvement; n=9). (**C**) Fold increase in MFI (ΔMFI) after PMA stimuli in neutrophils and monocytes from patients with mild or severe BD. *p<0.05.

found that the spontaneous secretion of TNF-α and IL-6 by PBMC was significantly increased in patients with active BD. Interestingly, the authors described an increased production of TNF-α, IL-6, IL-1 and IL-8 in LPS-stimulated cultures of PBMC from BD patients, without any correlation with disease activity. In this context, our finding of a strong positive correlation between BR-BDCAFs score and the production of IL6 and IL23 by BD PBMC stimulated with *Streptococcus sanguinis* is noteworthy. Hypersensitivity to several antigens from *Streptococcus sanguinis* has been associated with BD (10, 15, 45-47). These results suggest that PBMC from BD patients overreact to streptococcal components and exacerbate the standard immune response. Phagocytes from BD patients did not present higher microbicide activity, however. Therefore, it is possible that the *Streptococcus sanguinis*-induced PBMC response is triggered by the adaptive immune system, especially by T cells directly stimulating phagocytes in response to *Streptococcus sanguinis* stimulus. This hypothesis is supported by the previous experiments, which showed that neutrophils from patients with active BD were stimulated with supernatants of cultures of lymphocytes primed with streptococcal peptides (48), directly potentiating chemotaxis, phagocytosis and superoxide production compared to healthy controls, rheumatoid arthritis or septic patients. There is also evidence that BD patients' plasma increases chemotaxis (49) and oxidative burst (19) in normal and BD neutrophils. All these findings suggest that the cytokine mileu (44, 50) or unknown factors produced by different cell groups, possibly stimulated by mucosal streptococcal microbiota, are carried by the plasma and are able to activate phagocyte function in BD patients. Taken together these findings suggest that the striking neutrophil hyperactivity observed in BD may not be constitutive, but instead may be secondary to unknown stimuli.

Despite the hypersensitivity to *Streptococcus sanguinis* antigens, we did not observe an increase in microbicide activity of BD phagocytes against two different streptococcal species and *Can-*
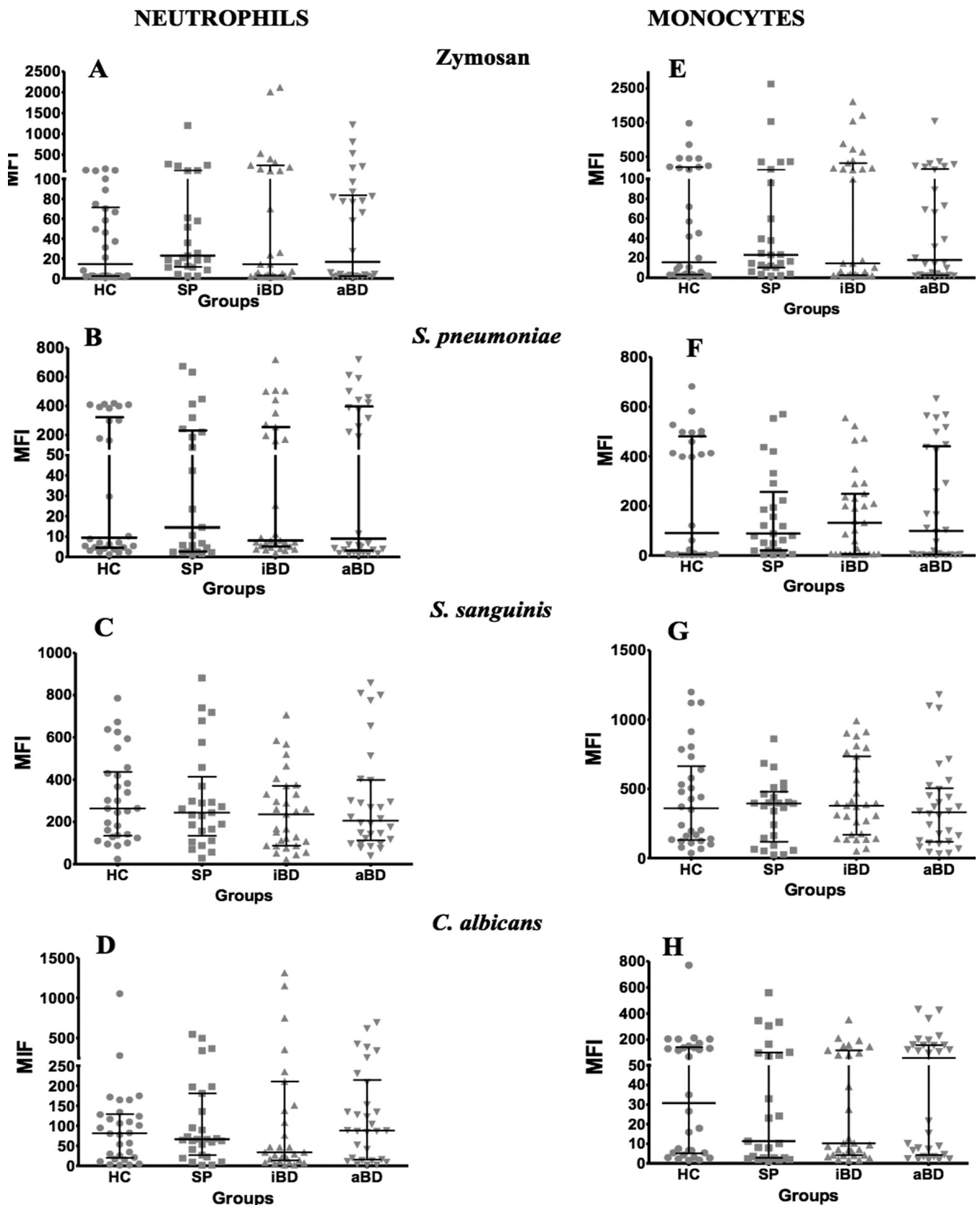
NEUTROPHILS **MONOCYTES**

Zymosan

S. pneumoniae

S. sanguinis

C. albicans

Fig. 4. Phagocytic activity of neutrophils and monocytes from BD patients and control subjects. Neutrophils (**A-D**) and monocytes (**E-H**) were assayed for phagocytic activity on FITC-labelled zymosan particles (**A**, **E**), or propidium iodide-labelled S. pneumoniae (**B**, **F**), S. sanguinis (**C**, **G**) or C. albicans (**D**, **H**). Mean fluorescence intensity (MFI) of cells from healthy controls (HC), septic patients (SP), inactive (iBD) and active Behçet's disease (aBD) is shown. Bars represent median and interquartile range. Data is representative of two experiments/person. Mann-Whitney test was performed to determine difference. No significant dyfferences were observed.
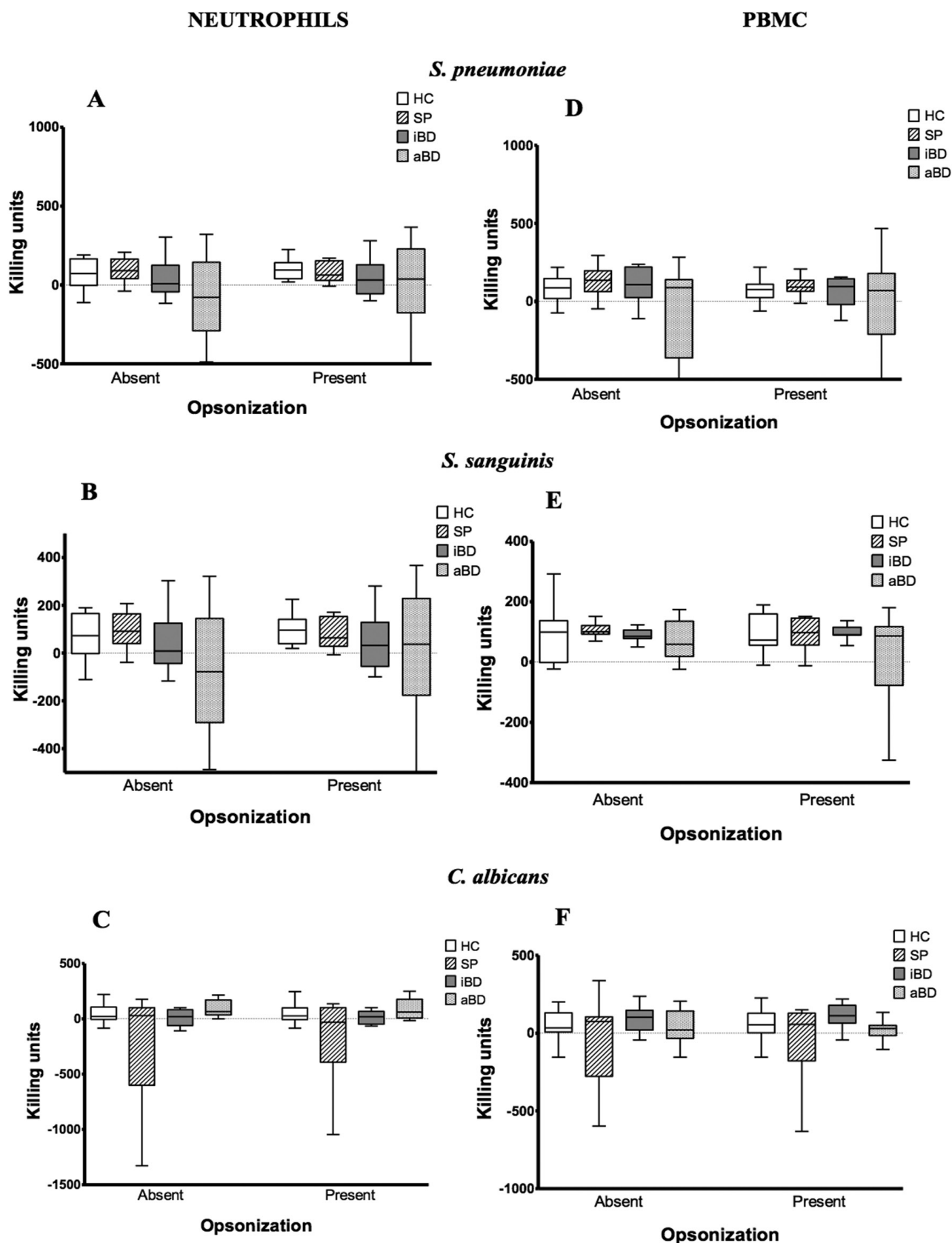
NEUTROPHILS

PBMC

S. pneumoniae

S. sanguinis

C. albicans

Fig. 5. Microbicide activity of neutrophils and PBMC from BD patients and control subjects. Neutrophils (**A-C**) and PBMC (**D-F**) were isolated and as-sayed for microbicide activity against S. pneumoniae (**A**, **D**), S. sanguinis (**B**, **E**) and C. albicans (**C**, **F**) Microbicide activity of cells from healthy controls (HC), septic patients (SP), inactive (iBD) and active Behçet's disease (aBD), opsonised or not with fresh human serum, was expressed of arbitrary "Killing Units" as defined in *Materials and Methods*. Data is representative of two experiments/person. Mann-Whitney test was performed to determine difference. No significant differences were observed.

dida albicans compared to HC or SP phagocytes. Similar results were previously obtained against *Candida albicans* and non-albicans (24, 35). Our results on the phagocytic activity of BD phagocytes confirm several previous studies, and extend them by adding analysis of *Streptococcus sanguinis* (21, 22, 35, 40, 51). There is at least one study that shows constitutively increased phagocytic activity of BD neutrophils, but that study used the non-specific Black India ink methodology (25). The consistent findings obtained by several groups with an array of microorganisms and by different methods indicate that phagocyte microbicide activity is not primarily disturbed in BD patients.

There is also a concern that the functional findings on BD phagocytes are biased by the long-term immunosuppressant therapy delivered to BD patients. In the present study, we observed no difference in the studied parameters registered in patients receiving and not receiving immunosuppressant therapy. However, we cannot formally exclude an effect of immunosuppressant therapy, since some of the patients with inactive disease had been under immunosuppressant for months and were still under maintenance immunosuppressant regimen. This was specially exemplified by the negative correlation observed between oxidative burst and the treatment duration of azathioprine and cyclosporine, even though the exclusion of these patients did not alter the observed association between increased oxidative burst and severe forms of BD.

Although it was not the main aim of the present study, we observed that PBMC from septic patients produced significantly lower levels of Th1 (IFNγ e TNF-α), Th17 (IL23) and Treg (IL10) cytokines. This may reflect these patients' exhaustion against different stimuli, since they are already immersed in a strong pro-inflammatory environment. Previous studies have reported similar results and associate this finding with a poor prognosis in sepsis (52, 53).

In conclusion, the current study shows that phagocytes from aBD patients with severe disease present constitutive increases in Th1 and Th17 cytokine production and oxidative burst activity.

However, phagocytic and microbicide functions are not altered in BD patients, even in those with severe disease. These findings suggest that a subset of BD patients may have pathophysiology related to the spectrum of auto-inflammatory syndromes. These preliminary results suggest new avenues for further studies exploring the role of phagocyte functions in BD pathophysiology.

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