

RESEARCH ARTICLE

Monocyte and Lymphocyte Activation in Bipolar Disorder: A New Piece in the Puzzle of Immune Dysfunction in Mood Disorders

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ABSTRACT

Background: This study tested the hypothesis that the low-grade inflammation presented in patients with bipolar disorder (BD) is associated with expansion of activated T cells, and this activated state may be due to a lack of peripheral regulatory cells.

Methods: Specifically, we investigated the distribution of monocytes and lymphocyte subsets, and investigated Th1/Th2/Th17 cytokines in plasma by flow cytometry. Twenty-one BD type I patients and 21 age- and sex-matched controls were recruited for this study.

Results: BD patients had increased proportions of monocytes (CD14+). Regarding lymphocyte populations, BD patients presented reduced proportions of T cells (CD3+) and cytotoxic T cells (CD3+CD8+). BD patients also exhibited a higher percentage of activated T CD4+CD25+ cells, and a lower percentage of IL-10 expressing Treg cells.

Conclusions: Our data shed some light into the underlying mechanisms involved with the chronic low-grade inflammatory profile described in BD patients.

Keywords: bipolar disorder, cytokines, lymphocytes, mania, monocytes

Introduction

There is a growing body of data showing that bipolar disorder (BD) is associated with a chronic low-grade inflammation. Increased circulating levels of pro-inflammatory cytokines have been consistently reported in BD patients, particularly tumor necrosis factor (TNF) and soluble TNF receptor type1 (sTNFR1;

Barbosa et al., 2011; Modabbernia et al., 2013; Munkholm et al., 2013, Barbosa et al., in press). Pro-inflammatory cytokines are soluble mediators produced by activated immune cells and are key messengers between the immune and non-immune cells. The underlying mechanisms of the immunologic imbalance

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observed in BD are largely unknown, and may include changes in circulating leukocytes.

The role of immune cells in the pathophysiology of mood disorders was hypothesized for the first time in the 1990's. Smith (1991) proposed the macrophage theory of depression, in which he associated depression with the excessive secretion of cytokines by macrophages. Data regarding immune cell subsets in BD patients are scarce and controversial. Few studies have evaluated monocytes (CD14+) count in BD patients, and they have had conflicting results. Torres et al. (2009) demonstrated decreased frequency of monocytes (CD14+), while Knijff et al. (2006) found an increased frequency of monocytes (CD14+) in BD patients. A third study demonstrated increased *in vitro* phagocytic activity of monocytes from BD patients (McAdams and Leonard, 1993). Data regarding total lymphocyte count and lymphocyte subsets in BD patients are also conflicting (Teixeira et al., 2013).

The present study aims to evaluate cellular immune subsets and circulating pro-inflammatory levels that may be associated with immunologic dysfunction in BD patients. Specifically, we evaluated monocytes (CD14+), B cells (CD19+), helper T (CD4+) and CD8+ T cells, regulatory T cells, and Th1/Th2/Th17 cytokines in the peripheral blood of BD patients and matched healthy controls. We hypothesized that the low-grade inflammatory profile presented by BD patients is associated with increased proportions of activated T cells and that this activated state may be due to a lack of peripheral regulatory cells.

Material and Methods

Subjects

This study included 21 BD type I patients in euthymia, and 21 age- and gender-matched controls. Patients were consecutively recruited from an outpatient psychiatric clinic specializing in BD. The local institutional review board approved the study, which is in accordance with the Helsinki Declaration of 1975. All participants were more than 18 years old. All volunteers provided their written consent after a complete explanation about the procedures involved in the research protocol.

Patients and controls were assessed with the Mini-International Neuropsychiatric Interview to confirm BD diagnosis (in patients) or to exclude a history of psychiatric disorders (in controls; Sheehan et al, 1998; Amorim, 2000). BD patients were also assessed with the Hamilton Depression Rating Scale, 17-item version (Hamilton, 1967), and the Young Mania Rating Scale (Young et al, 1978) to characterize the severity of depressive and manic symptoms, respectively. The healthy control group was recruited from the local population and participants did not have a personal psychiatric disorder (evaluated through Mini-International Neuropsychiatric Interview) or family history of major psychiatric disorder, suicide attempts, or completed suicide. Subjects with dementia, infectious or autoimmune diseases, or who had used steroids, anti-inflammatory drugs, or antibiotics within four weeks of evaluation were excluded from this research protocol.

Clinical assessment of subjects included the collection of demographic and clinical variables: gender, age, length of illness, and medications in use, and anthropometric measurement. Body mass index (BMI) was calculated by dividing the weight (in kilograms) by the squared height (in meters; $BMI = kg/m^2$).

Blood Collection

Twenty milliliters of blood was drawn between 8 and 10 AM from each subject by venipuncture into heparinized tubes. Ten milliliters of blood was immediately centrifuged at 3 000g for

10 min, 4°C, twice for the cytokines (IL-2, IL-4, IL-6, IL-10, IFN- γ , TNF, and IL-17A) analyses. The plasma was collected and stored at -80°C until assayed. Ten milliliters of blood were destined to the cell analysis as described below.

Isolation of Peripheral Blood Mononuclear Cells

Peripheral blood mononuclear cells (PBMCs) were isolated by density-gradient centrifugation for 40 min at 405g. Cells were counted by means of microscopy (100 x) and viability always exceeded 95%, as judged from their ability to exclude Trypan Blue (Sigma). Cells were re-suspended at the final concentration of 1×10^7 cells/mL in a medium composed of RPMI-1640 (Roswell Park Memorial Institute-1640) with L-glutamine (Cultilab), 40 IU/mL of penicillin (Ariston), 40 μ g/mL of gentamicin (Nova Farma), 25 mM of HEPES (4-[2-hydroxy-ethyl]-1-piperazine-ethane-sulfonic acid) buffer (Sigma), supplemented with 10% of heat-inactivated human serum (Sigma).

Immunophenotyping

A large panel of lymphocyte and monocyte subpopulations was identified by multi-color flow cytometry in freshly-isolated PBMC. Briefly, PBMC were washed in flow cytometry buffer (PBS containing 1% fetal bovine serum and 0.01% sodium azide) and treated with Fc Block solution for 20 min. In order to evaluate specific lymphocyte subsets, cells were stained for 30 min with combinations of the following monoclonal human antibodies: anti-CD3 FITC, anti-CD4 PE, anti-CD4 PEcy5, anti-CD25 FITC, anti-CD8 PEcy7, anti-CD19 PEcy5, anti-FOXP3 PE, and anti-IL10 PEcy7 (all from BD Biosciences). Immediately after staining, cells were washed, resuspended, and analyzed by flow cytometry. A minimum of 20 000 lymphocytes were identified by size (forward scatter (FSC)) and granularity (side scatter (SSC)) and acquired with a FACS Canto II flow cytometer (BD Biosciences). The instrument was checked for sensitivity and overall performance with Cytometer Setup and Tracking beads (BD Biosciences) prior to data acquisition. Data were analyzed using the Flowjo software (Tree Star) and Diva software (BD Biosciences).

Intracellular Staining for FoxP3 and IL-10

Briefly, 2.5×10^5 cells were placed in 96-well plates in 200 μ L cultures. The cells were then harvested, washed, and stained for surface markers, and fixed using 2% formaldehyde (Sigma-Aldrich). The fixed cells were permeabilized and stained using anti-FoxP3 and IL-10 monoclonal antibodies (BD Pharmingen) directly conjugated with phycoerythrin (PE). PE-labeled immunoglobulin control antibodies and a control of unstimulated PBMC were included in all experiments. Preparations were acquired on FACSCanto II (BD Biosciences). A minimum of 100 000 gated events on lymphocytes and monocytes population were acquired for analysis due to the low frequency of positive events being analyzed. The acquisition was processed using the Flowjo software (Tree Star) and Diva software (BD Biosciences).

Plasma Cytokine Determinations

Plasma cytokines (IL-2, IL-4, IL-6, IL-10, IFN- γ , TNF, and IL-17A) were measured using the cytometric bead arrays kit (BD Biosciences) according to the manufacturer's protocol. Bead flow cytometry allows the simultaneous quantification of various proteins in the same test. Fifty microliters of plasma per test were used. Samples were acquired in a FACSCanto flow cytometer (BD Biosciences) and analyzed using the FCAP Array v1.0.1 software (Soft Flow Inc.). Results are expressed as picograms per milliliter.

Flow Cytometry Data Analysis

Flow cytometry data files were analyzed using DIVA software (BD Biosciences) and Flowjo software (Tree Star). Lymphocytes were gated and analyzed for the expression of CD3⁺CD4⁺, for example. Limits for the quadrant markers were always set based on negative populations and isotype controls. A representative dot plot and a histogram analysis are shown on Figure 1.

Statistical Analysis

Statistical analyses were performed using SPSS software version 17.0 (SPSS Inc.). Descriptive statistics were used to report socio-demographic and clinical characteristics of the sample. Association between dichotomous variables was assessed with Pearson's chi-square test or Fisher's exact test when appropriate. All variables were tested for normality of distribution by means of the Shapiro-Wilk test, and all data were non-normally distributed. Therefore, differences between two groups were compared with Mann-Whitney U test. Spearman's correlation analyses were performed to examine the relationship between cytokines plasma levels and monocytes and lymphocytes subpopulation frequencies. All *p* values were two-tailed and a significance level of $\alpha = 0.05$ was chosen.

Differences between BD patients and controls were further examined with logistic regression modeling (stepwise

backwards logistic regression analysis). According to the backward elimination procedure, variables with the highest *p* value were progressively deleted from the model. The final model retained variables with a significance level ≤ 0.05 . The goodness of fit of the final model was tested by the Hosmer-Lemeshow method, and odds ratios with 95% confidence intervals are shown for each independent variable retained in the model.

Results

Demographic and Clinical Features

The mean age of BD patients was 55.05 years (standard deviation [SD] ± 10.64). The mean length of illness was 30.70 years (SD ± 14.38). Fifteen out of 21 BD patients (73.3%) were women. BD patients presented mean Young Mania Rating Scale and Hamilton Depression Rating Scale scores of 2.20 (SD ± 1.66) and 5.24 (SD ± 7.14), respectively. BD patients did not differ from controls in the frequency of arterial hypertension, diabetes mellitus, or dyslipidemia ($p > 0.05$). The mean BMI of BD patients was 28.13 Kg/m² (SD ± 4.36), and the mean BMI of controls was 29.49 Kg/m² (SD ± 5.89), not differing statistically. Demographic and clinical features of euthymic BD patients and controls are shown in Table 1.

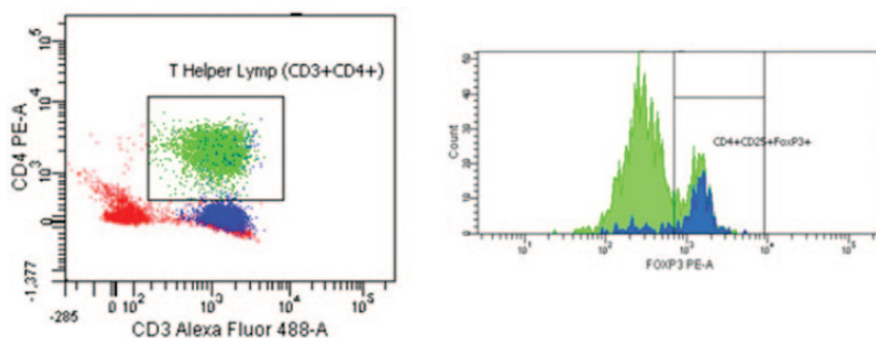


Figure 1. Representative dot-plots of analysis strategy. The immunophenotyping of lymphocytes was verified by flow cytometry assays. Peripheral blood mononuclear cells from bipolar disorder patients and controls were stained with surface markers and intracellular FoxP3. Total lymphocytes were gated and a fluorescent dot-plot for T helper lymphocytes (CD3+CD4+) is demonstrated (A). The histogram graphic demonstrates FoxP3 expression in CD4+CD25+ Activated T cell (B).

Table 1. Clinical, demographic features and cytokine plasma levels of euthymic BD patients and healthy controls.

	BD patients (N= 21)	Controls (N=21)	p Value
Female Gender (frequency, %)	73.3	75.0	0.92 [†]
Age in years (mean \pm SD)	55.05 \pm 10.64	51.95 \pm 5.12	0.43 ^{††}
YMRS (mean \pm SD)	2.20 \pm 1.66	---	---
HDRS (mean \pm SD)	5.24 \pm 7.14	---	---
Length of illness in years (mean \pm SD)	30.70 \pm 14.38	---	---
Arterial hypertension (frequency, %)	23.8	23.8	1.00 [†]
Diabetes Mellitus (frequency, %)	23.8	19.1	0.70 [†]
Dyslipidemia	42.8	23.8	0.33 [†]
Body mass index in Kg/m ² (mean \pm SD)	28.13 \pm 4.36	29.49 \pm 5.89	0.50 ^{††}
Medication in use (frequency, %)			
Lithium	66.7	---	---
Anticonvulsants	57.1	---	---
Antipsychotics	33.3	---	---

Abbreviations: BD = bipolar disorder; HDRS = Hamilton Depression Rating Scale; N= Number; SD= standard deviation; YMRS = Young Mania Rating Scale.

[†] Pearson's Chi-square test

^{††} Mann-Whitney test

Monocyte and Lymphocyte Subsets

PBMC from BD patients and controls were stained for surface markers in *ex vivo* condition. Monocytes and lymphocytes subpopulations were evaluated by the expression of the membrane-bound molecules CD14, CD19, CD3, CD4, CD8, and by the activation marker CD25 (Table 2 and Figure 2).

BD patients presented higher percentages of monocytes (CD14+) in comparison with controls ($p = 0.03$, Figure 2A). Regarding the lymphocyte subpopulations, BD patients presented lower percentages of T CD3+ cells ($p = 0.003$, Figure 2B), and particularly lower percentages of T CD3+CD8+ cytotoxic cells ($p = 0.004$, Figure 2C). BD patients presented higher percentages of activated T CD4+CD25+ cytotoxic cells ($p = 0.02$, Figure 2D), and lower percentages of IL-10 expressing Treg cells ($p = 0.047$, Figure 2E). With respect to possible effects of mood stabilizers (i.e., lithium, valproic acid, or antipsychotics), no significant association was found with the immunological measures.

Cytokine Production

Th1/Th2/Th17 cytokines (IL-2, IL-4, IL-6, IL-10, TNF, IFN- γ , and IL-17A) were assessed in plasma by cytometric bead arrays. There was no difference in plasma levels between BD patients and control group (see Table 3).

To further investigate the cytokine profile in BD we also compared cytokine ratios. BD patients showed higher IFN- γ /IL-4 ($p = 0.03$) and IFN- γ /IL-10 ($p = 0.04$) compared with controls, suggesting a bias towards a Th1 profile. There were no statistical differences regarding TNF/IL-4 or TNF/IL-10 (all $p > 0.05$).

With respect to possible effects of mood stabilizers (i.e., lithium, valproic acid, or antipsychotics), no significant association was found with the immunological measures ($p > 0.05$).

Correlation Analyses and Logistic Regression Model

In BD patients, IL-4 plasma levels were positively correlated with IL-10 expressing Treg cells ($p = 0.005$ and $\rho = 0.69$), and IL-10 plasma levels were negatively correlated with CD3+CD4+ T helper cells ($p = 0.04$ and $\rho = -0.53$). There were no correlations between length of illness, psychopathological scales (Hamilton Depression Rating Scale and Young Mania Rating Scale), and monocytes, lymphocytes, and cytokines.

A logistic regression model was performed to assess the likelihood of presenting BD. The model contained six independent variables: percentage of monocytes, B cells, T cells, T cytotoxic cells, activated T cells, and IL-10 expressing Treg cells. The model was statistically significant ($\chi^2 [6, N = 42] = 19.71, p = 0.004$), indicating that it was able to distinguish between BD patients and controls. The model as a whole explained between 55.6% (Cox & Snell R Square) and 74.1% (Nagelkerke R Square) of the variance

of subjects, and correctly classified 85.7% of cases. As shown in Table 4, four of the independent variables made a unique statistically-significant contribution to the model: B cells, T cells, activated T cells, and IL-10 expressing Treg cells.

Discussion

There is an extensive body of data showing that BD is associated with a chronic low-grade inflammation, but the pathways that explain this relationship remain elusive. Once changes in peripheral immune cells were examined to help to explain this pro-inflammatory imbalance in BD, we investigated a comprehensive panel of cell markers involved with cell activation and regulation. BD patients presented an increased proportion of monocytes (CD14+) and a lower proportion of T cells (CD3+), notably cytotoxic T cells (CD3+CD8+). Moreover, BD patients showed an increased proportion of activated T cells (CD4+CD25+) and a lower proportion of IL-10 expressing Treg cells (CD4+CD25+FoxP3+IL10+), suggesting increased monocyte and lymphocyte activation.

The increased proportion of monocytes (CD14+) on BD patients may indicate a systemic activation of the mononuclear phagocytic system. In accordance with this finding, Knijff, Breunis, Kupka, et al. (2007) showed that monocytes from BD patients presented an altered pro-inflammatory response—including higher production of IL-6—following lipopolysaccharide stimulation in comparison with monocytes from controls. The higher monocyte activation was also demonstrated in the offspring of BD patients (Padmos et al., 2008). There are some hypotheses to explain the association between BD and a state of monocyte overactivation: (1) BD or the stress associated with mood episodes is responsible for inducing a state of monocyte hyperactivity; (2) the state of monocyte hyperactivity is the trigger of the mood disorder (as suggested by Smith, 1991 in the macrophage theory of depression); (3) there is a common underlying factor to BD and monocyte overactivity; or (4) they are two independent underlying factors that share the same environment and lead to BD and the activation of monocytes (Padmos et al., 2009). Moreover, we may not exclude the possibility that the overactivation of peripheral monocytes represents the central nervous system activation of the mononuclear phagocytic cells in BD patients. Microglia is the main resident phagocyte cell of the brain, responding to stress and environment changes in the central nervous system and, hence, influencing neuronal plasticity and neurotransmitter synthesis. Postmortem studies demonstrated altered size and number of glia cells in the prefrontal cortex, amygdala, basal ganglia, and dorsal raphe nuclei (Soeiro-de-Souza et al., 2012). However, little is known about the activation of microglia in BD patients. Given that microglia helps to regulate cytokine production, there is an urgent need

Table 2. Immunophenotyping of monocytes and lymphocytes subsets.

Marker	Cell Type	BD patients (N=21)	Controls (N=21)	p Value ^{††}
CD14+	Monocyte	18.25 ± 10.42	12.33 ± 7.65	0.03
CD19+	B cell	11.59 ± 1.57	14.88 ± 7.25	0.12
CD3+	T cell	37.11 ± 15.37	52.11 ± 13.83	0.003
CD3+CD4+	Th	35.71 ± 11.52	31.91 ± 10.63	0.21
CD3+CD8+	Tc	12.08 ± 7.05	18.47 ± 7.01	0.004
CD4+CD25+	Activated T cell	2.52 ± 1.25	1.84 ± 1.98	0.02
CD4+CD25+FoxP3+	Regulatory T cell	0.26 ± 0.20	0.93 ± 1.57	0.42
CD4+CD25+FoxP3+IL10+	IL10 Treg cells	0.06 ± 0.06	0.16 ± 0.19	0.047

Abbreviations: BD bipolar disorder; Th = T helper cell; Tc = T cytotoxic cell; ^{††} Mann-Whitney test

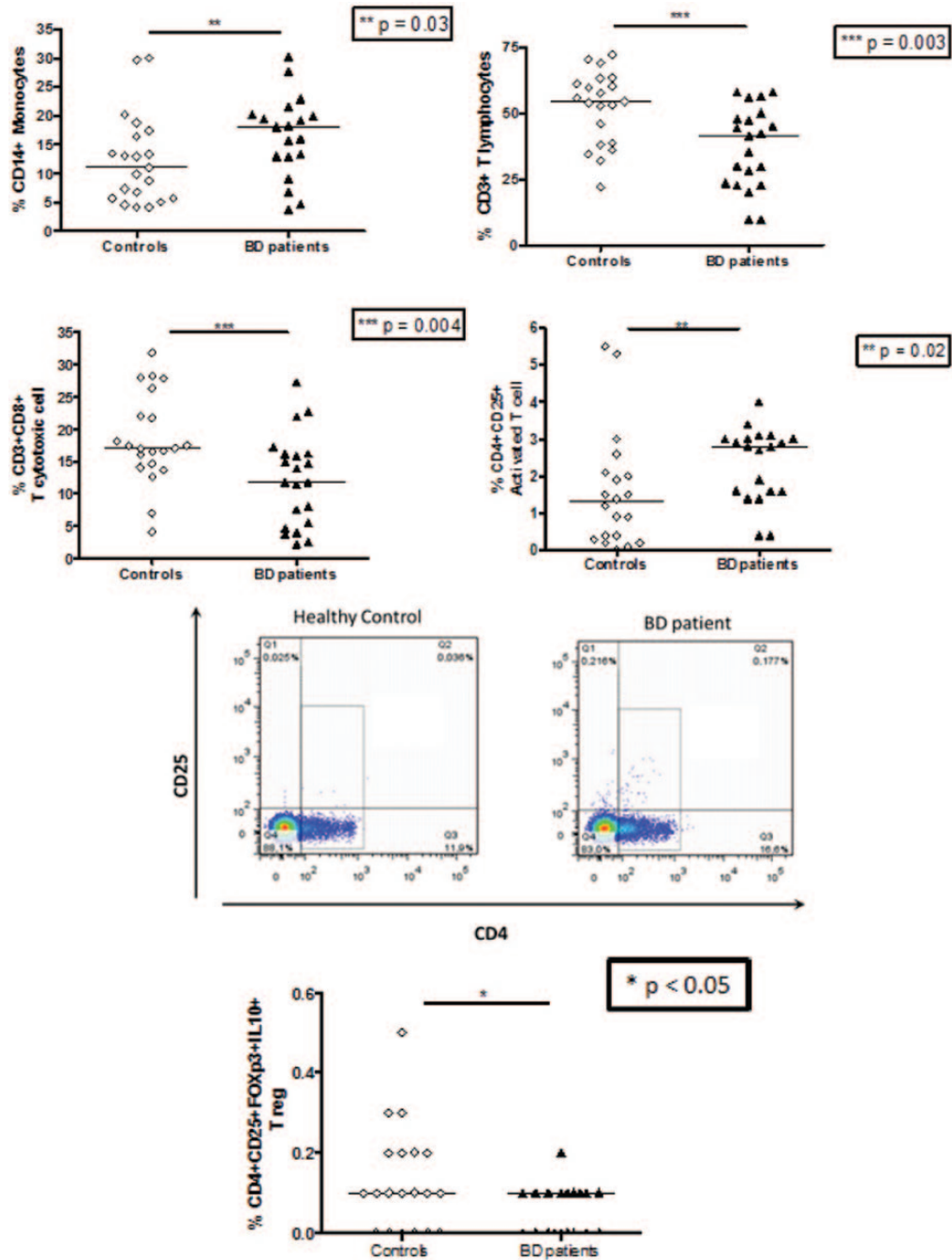


Figure 2. CD14+, total CD3+, CD3+CD8+, CD4+CD25+, and CD4+CD25+FoxP3+IL10+ ex vivo expression in monocytes and lymphocytes from bipolar disorder patients and controls. Figures show the percentages of: CD14+, monocytes (A); CD3+, T lymphocytes (B); CD3+CD8+, T cytotoxic cell (C); CD4+CD25+, Activated T cell (D); and CD4+CD25+FoxP3+IL10+, IL-10 Treg (F). E shows representative dot plots of CD4+CD25+ Activated T cell of gated peripheral lymphocytes. Statistical significant differences are indicated. Data were analyzed by Mann-Whitney Test.

Table 3. Plasma levels of Th1, Th2 and Th17 cytokines in BD patients and controls.

pg/mL	BD Patients (N=21)	Control (N=21)	p value ^{††}
IL-2 (median ± SD)	0.51 ± 0.69	1.58 ± 5.28	0.30
IL-4 (median ± SD)	1.08 ± 0.84	1.93 ± 2.77	0.48
IL-6 (median ± SD)	6.40 ± 7.92	5.84 ± 8.40	0.36
IL-10 (median ± SD)	1.06 ± 1.06	1.27 ± 1.64	0.78
TNF (median ± SD)	95.03 ± 203.94	51.23 ± 83.70	0.47
IFN-gamma (median ± SD)	0.49 ± 0.44	0.39 ± 0.42	0.62
IL-17A (median ± SD)	18.72 ± 30.58	16.89 ± 10.20	0.53

Abbreviations: BD = bipolar disorder; SD= standard deviation; †† Mann-Whitney test

Table 4. Logistic regression predicting likelihood of bipolar disorder.

	B	S.E.	Wald	Df	p Value	Odds Ratio	95% C.I. for Odds Ratio	
							Lower	Upper
CD19+	- 0.206	0.098	4.39	1	0.04	0.814	0.671	0.987
CD3+	- 0.113	0.050	5.25	1	0.02	0.893	0.810	0.984
CD4+CD25+	1.651	0.789	4.38	1	0.04	5.214	1.110	24.490
CD4+CD25+FoxP3+IL10+	- 41.506	17.854	5.40	1	0.02	0.000	0.000	0.001
Constant	7.986	3.241	6.07	1	0.01	2938.23		

for investigating activated microglia in BD patients (Watkins et al., 2014).

The decreased frequency of cytotoxic T cells (CD8+) in BD patients is discordant with previous studies that did not show differences in BD patients when compared with controls (Rapaport, 1994; do Prado et al., 2013). The classical role of cytotoxic T cells is to mediate the host defense against infectious agents (i.e., bacteria, virus, and parasite), and it is expected that a decreased number of these cells might be related with increased rates of infectious diseases. Since the 19th century a close relationship between BD and infectious agents has been described (Yolken and Torrey, 1995), and more recently studies confirmed increased rates of infectious diseases in BD patients compared with the general population, particularly hepatitis C and human immunodeficiency virus infection (McIntyre et al., 2007; Altamura et al., 2011).

We found an increased proportion of activated T cells along with a trend to Th1 activation in BD patients. In fact, the T-cell activation has been suggested as a possible trait in BD patients (Breunis et al., 2003; Knijff et al., 2006; Wieck et al., 2013). *In vitro* studies demonstrated an increased percentage of activated T cells in BD patients after stress exposure, showing an opposite direction from controls, suggesting aberrant control of immune activation in BD patients (Knijff, Breunis, van Geest, et al., 2007; Wieck et al., 2013). Moreover, activated T cells in BD patients are also less sensitive to the suppressive glucocorticoid effects (Knijff, Breunis, van Geest, et al., 2007; Wieck et al., 2013). These findings suggest that the immune activation on BD patients might be related to a relative steroid resistance.

The immune activation in BD patients is corroborated by the decreased proportion of IL-10 expressing Treg cells. There are limited studies evaluating Treg cells in BD patients despite these cells playing a pivotal role in immune regulation. For instance, Treg cells prevent immune responses against self-antigens (Bluestone and Abbas, 2003). In this line, decreased expression of Treg in BD might explain the increased rate of autoimmune diseases reported in BD patients (Kupka et al., 2002; Edwards and Constantinescu, 2004; Bachen et al., 2009; Eaton et al., 2010). Two previous studies demonstrated decreased expression of Treg cells in BD patients (do Prado et al., 2013; Wieck et al., 2013). A discordant study did not show differences in Treg cells between BD patients and controls (Drexhage et al., 2011). Differences in recruitment (inclusion of BD types I and II), gender, and age range could explain the discrepancy in these studies.

To the best of our knowledge this is the first study to demonstrate reduced number of IL-10 expressing Treg cells in BD patients. In fact, these cells are key players in immune response control, being able to inhibit both Th1 and Th2 type responses (O'Garra et al., 2004). Hence, the current findings contribute to explaining the chronic low-grade inflammatory profile in BD patients (Barbosa et al., in press).

BD patients exhibited unique immunological profiles in comparison with normal controls. In this sample, composed of euthymic BD patients with long-term disease, the profiles of cell activation and cytokines were not associated with clinical parameters, including drugs in use, length of illness, and severity of symptoms. It is possible that these profiles might change in BD patients during mania and/or depression episodes (Barbosa et al., in press). Moreover, it is uncertain whether the severity and/or frequency of mood cycles, variables difficult to address on long term diseases, impact the immune parameters. These issues must be controlled in future studies.

There are other limitations in this study to be discussed. One of the major limitations of our study is the relatively small sample size limiting the statistical power of the study. All BD patients were receiving mood-stabilizing agents (e.g., lithium, anticonvulsants, and antipsychotics) that may influence immune functions. However, no significant difference in immune parameters emerged when comparing patients using different mood stabilizing agents.

In conclusion, our data suggest that BD patients exhibit an immune imbalance associated with changes in monocytes and lymphocytes subsets.

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Statement of Interest

None.

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