

Original Article

Cite this article: Barbosa IG, Rocha NP, Vieira EL, Camkurt MA, Huguet RB, Guimarães FTL, de Brito-Melo GE, Mendonça VA, Bauer ME, and Teixeira AL. (2019) Decreased percentage of CD4⁺ lymphocytes expressing chemokine receptors in bipolar disorder. *Acta Neuropsychiatrica* **31**: 246–251. doi: [10.1017/neu.2019.5](https://doi.org/10.1017/neu.2019.5)

Received: 10 September 2018
Revised: 31 January 2019
Accepted: 31 January 2019
First published online: 14 March 2019


Key words:

bipolar disorder; chemokine receptors; psychoneuroimmunology; lymphocytes

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Decreased percentage of CD4⁺ lymphocytes expressing chemokine receptors in bipolar disorder

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Abstract

Objective: Although accumulating evidence supports the hypothesis that immune/inflammatory mechanisms are associated with the pathophysiology of bipolar disorder (BD), data about the profile of chemokines (chemotactic cytokines) and chemokine receptors are still scarce. The current study was designed to evaluate the expression of chemokine receptors on lymphocytes of patients with BD in comparison with controls. **Methods:** Thirty-three patients with type I BD ($N = 21$ in euthymia; $N = 6$ in mania/hypomania; $N = 6$ in depression) and 22 age- and sex-matched controls were subjected to clinical evaluation and peripheral blood draw. The expression of chemokine receptors CCR3, CCR5, CXCR4, and CXCR3 on CD4⁺ and CD8⁺ lymphocytes was assessed by flow cytometry. **Results:** Patients with BD had decreased percentage of CD4⁺CXCR3⁺ ($p = 0.024$), CD4⁺CCR3⁺ ($p = 0.042$), and CD4⁺CCR5⁺ (0.013) lymphocytes in comparison with controls. The percentage of both CD4⁺ and CD8⁺ lymphocytes expressing the chemokine receptor CXCR4 was similar in patients with BD and controls. Likewise, the percentages of CD8⁺CXCR3⁺, CD8⁺CCR3⁺, and CD8⁺CCR5⁺ lymphocytes were similar in patients with BD and controls. **Conclusion:** Our findings reinforce the hypothesis that immune pathways, especially involving CD4⁺ lymphocytes, are involved in the pathophysiology of BD.

Significant outcomes

- The percentage of chemokine receptor-expressing CD4 lymphocytes is altered in BD.
- BD is associated with decreased percentage of CXCR3⁺, CCR3⁺, and CCR5⁺ on CD4⁺ cells.
- No changes were observed in the percentage of CD8⁺ cells expressing chemokine receptors.

Limitations

- Sample size
- Cross-sectional design.

Introduction

Immunological aspects of psychiatric disorders, especially bipolar disorder (BD), have been a topic of great interest in recent years (da Rocha *et al.*, 2008; Barbosa *et al.*, 2014). In comparison with controls, patients with BD have increased frequency of autoimmune diseases, reduced proportions of circulating T regulatory cells, and increased blood levels of inflammatory cytokines such as tumour necrosis factor (TNF), interleukin (IL)-1 β , and IL-6. In addition, anti-inflammatory strategies have been regarded as a potential add-on treatment for BD (Kupka *et al.*, 2002; El-Sayed & Ramy, 2006; Nery *et al.*, 2008; do Prado *et al.*, 2013; Colpo *et al.*, 2018).



Peripheral blood samples are easily accessible and, therefore, a convenient alternative for investigating biological processes associated with the pathophysiology of psychiatric disorders. Indeed, peripheral lymphocytes have even been considered a neural probe in studies of psychiatric disorders (Gladkevich *et al.*, 2004). The basis of this suggestion relies on different levels of evidence, including: (i) alteration of lymphocyte function in neuropsychiatric disorders; (ii) lymphocytes and neural tissue may display similar responses to hormones; (iii) neural proteins can be expressed in lymphocytes (Gladkevich *et al.*, 2004). Lymphocytes express chemokine receptors CCR3, CCR5, CXCR3, and CXCR4, among others (Qin *et al.*, 1998).

Chemokines are small cytokines or signalling proteins primarily involved in promoting chemotaxis (Luster, 1998). Chemokines control the migratory pattern and positioning of immune cells. Chemokine function is critical for all immune cell migration, including physiological (e.g. immune organ development and tissue homeostasis) and pathological (e.g. inflammatory response to infection) processes. Such coordinated movement of cells requires the action of chemokines through their respective receptors on target cells (Griffith *et al.*, 2014). Structurally, chemokines are classified as CC chemokines, CXC chemokines, C chemokines, and CX3C chemokines (Zlotnik & Yoshie, 2000). Previous studies have found that the expression of chemokine receptors is changed in peripheral immune cells of patients with psychiatric disorders. For example, one study found increased expression of chemokine receptors (including CXCR3, CCR3, and CCR5) on peripheral lymphocytes of patients with autism spectrum disorder in comparison with control children with typical development (Ahmad *et al.*, 2018). In addition, patients with depressive disorders exhibit increased plasma levels of CXCR4 and CCR5 in comparison with controls (Ogłodek *et al.*, 2014). Patients with panic disorder and personality disorders also present increased plasma levels of CCR5 and CXCR4 in comparison with controls (Ogłodek *et al.*, 2016). Regarding BD, the evidence on chemokine receptor changes came from a polymorphism study describing that CCR5-Δ32 II and CXCR4-C138T C⁺ genotype frequencies contributed to an increased risk of BD (Tokac *et al.*, 2016).

Given the accumulating evidence that chemokines and their receptors may play a role in psychiatric disorders, the current study was designed to evaluate the expression of chemokine receptors CCR3, CCR5, CXCR4, and CXCR3 on lymphocytes of patients with BD in comparison with controls. To the best of our knowledge, this is the first study to investigate chemokine receptor expression on lymphocytes of BD patients.

Methods

Subjects and clinical evaluation

This study included 33 patients with type I BD and 22 age- and sex-matched controls. Patients were consecutively recruited from the BD outpatient psychiatric clinic, *Instituto de Previdência dos Servidores do Estado de Minas Gerais*, Belo Horizonte, Brazil. Controls were recruited from the local community and enrolled in the study protocol if they did not present a history of psychiatric disorder or a family history of major psychiatry disorder, suicide attempts, or completed suicide.

Patients and controls were subjected to 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). Patients with BD were also

assessed with the 17-item version of the Hamilton Depression Rating Scale (HDRS) and the Young Mania Rating Scale (YMRS) to determine the severity of depressive and manic symptoms, respectively (Hamilton 1967; Young *et al.*, 1978). Patients were considered euthymic when they had a score ≤ 7 in both YMRS and HDRS, for at least 8 weeks. Individuals with a diagnosis of dementia, infectious or autoimmune diseases, or who had used steroids, anti-inflammatory drugs, or antibiotics within 4 weeks of the clinical evaluation were excluded from this research protocol.

Local human research ethics committee approved this study protocol, which is in accordance with the Helsinki Declaration of 1975. All participants were >18 years and provided written consent prior to study enrolment.

Blood samples and immunophenotyping

Ten millilitres of blood was drawn by venipuncture in vacuum tubes containing ethylenediamine tetraacetic acid (Vacuplast, Huangyn, China) at the same day of the clinical assessment. Blood samples were kept at room temperature and used within 16 h of drawing for immunophenotyping analyses, which were performed according to the procedures recommended by Becton Dickinson (San Diego, CA, USA), briefly described as follows.

For staining, 100 μ L of whole blood samples was incubated for 30 min with the monoclonal antibodies for specific cell surface markers, followed by three times washing using phosphate buffered saline (PBS). Following incubation procedures, erythrocytes were lysed using 100 μ L lysing solution (Optlyse-B; Beckman Coulter, Inc., Miami, FL, USA) for 5 min, followed by addition of distilled water and 10 min incubation. Then, the cells were washed twice with 2 mL PBS containing 0.01% sodium azide. Cell preparations were fixed in 500 μ L of FACS fixing solution (10 g/l paraformaldehyde, 1% sodium cacodylate, 6.65 g/l sodium chloride, 0.01% sodium azide). The acquisition of cytofluorimetric data was performed using a Becton Dickinson FACScan instrument. A minimum of 10,000 lymphocytes, gated by size (forward scatter) and granularity (side scatter), were analysed using the CellQuest software (BD Pharmingen).

The specific antibodies used were: mouse anti-human CD4-PC5 (IOTest), mouse anti-human CD3-PE, mouse anti-human CD8-PE-Cy5 (BD Biosciences), mouse anti-human CXCR3-PE, mouse anti-human CCR5-PE (Pharmingen), rat anti-human CXCR4-PE (RD Systems), mouse anti-human CD8a-FITC, mouse anti-human CD3-FITC, and mouse anti-human CCR3-FITC.

Statistical analysis

Differences in dichotomous variables (sex and presence of clinical comorbidities) were tested with the chi-square test. All continuous variables were tested for Gaussian distribution with the Shapiro-Wilk normality test. Student's *t*- or Mann-Whitney *U*-tests were used for comparisons between two groups (patients vs. controls) when variables were determined to follow a normal distribution or not, respectively. All statistical tests were two-tailed and were performed using a significance level of $\alpha = 0.05$. Statistical analyses were performed using SPSS software version 22.0 (SPSS Inc., Chicago, IL, USA).

Results

Both groups were similar in terms of age and sex distribution. Table 1 summarises sociodemographic and clinical data of the

Table 1. Clinical and demographic features of patients with BD and controls

| Variable | Patients with BD (N = 33) | Controls (N = 22) | p-Values |
|--|---------------------------|-------------------|--------------------|
| Female sex, N (%) | 21 (63.64) | 14 (63.64) | 1.000* |
| Age, years (mean ± SD) | 50.87 – 14.31 | 46.95 – 8.58 | 0.254 [†] |
| Years of schooling (mean – SD) | 9.6 – 3.8 | 10.2 – 3.5 | 0.550 [†] |
| Duration of disease, years (mean – SD) | 26.50 – 14.33 | – | – |
| YMRS (mean – SD) | 5.06 – 7.03 | – | – |
| HAMD (mean – SD) | 4.52 – 5.69 | – | – |
| Psychiatric comorbidities (%) | | | |
| Generalised anxiety disorder | 27.27 | – | – |
| Nicotine dependence | 30.30 | – | – |
| History of past suicide attempt (%) | 30.30 | – | – |
| Clinical comorbidities (%) | | | |
| Arterial hypertension | 24.24 | – | – |
| Diabetes mellitus | 15.15 | – | – |
| Dyslipidaemia | 33.33 | – | – |
| Thyroid disease | 15.15 | – | – |
| Medication in use (%) | | | |
| Lithium | 48.48 | – | – |
| Antipsychotic | 39.39 | – | – |
| Antidepressant | 9.09 | – | – |
| Anticonvulsant | 54.54 | – | – |

HAMD, Hamilton Depression Rating Scale; SD, standard deviation; YMRS, Young Mania Rating Scale; BD, bipolar disorder.

*Fisher's exact test.

[†]Student's *t*-test.

participants enrolled in this study. Patients with BD had a mean educational level of 9.6 (± 3.8) years, and 11 out of the 33 patients (30%) were formally employed at study enrolment. Twenty-one out of the 33 patients were euthymic (64%), while the remaining 12 patients (36%) were experiencing mood episodes ($N = 6$ in mania/hypomania; $N = 6$ in depression). The most prevalent comorbid disorders in patients were generalised anxiety disorder ($N = 9$; 27%) and nicotine dependence ($N = 10$; 30%). In addition, psychotic symptoms were identified in four patients with BD (12%) at the clinical interview. Regarding patients' medical history, 28 (85%) had a history of psychosis, 10 (30%) had past suicide attempts, and 6 (18%) had a history of alcohol dependence.

Table 2 presents the results of the immunophenotyping analyses showing the expression of chemokine receptors (CXCR3, CXCR4, CCR3, and CCR5) on different T lymphocytes ($CD4^+$ and $CD8^+$) from patients with BD and controls. The percentage of both $CD4^+$ and $CD8^+$ lymphocytes expressing the chemokine receptor CXCR4 was similar in patients with BD and controls. Likewise, the percentages of $CD8^+CXCR3^+$, $CD8^+CCR3^+$, and $CD8^+CCR5^+$ lymphocytes were similar between patients with BD and controls. Conversely, patients with BD had decreased percentage of $CD4^+CXCR3^+$, $CD4^+CCR3^+$, and $CD4^+CCR5^+$ lymphocytes in comparison with controls. We did not find any correlation between chemokine receptor expression and clinical/demographic parameters.

Discussion

The main findings of our study were: (i) patients with BD and controls presented similar percentage of $CD8^+$ lymphocytes expressing chemokine receptors CXCR3, CXCR4, CCR3, and CCR5; and (ii) patients with BD presented decreased percentage of $CD4^+$ lymphocytes expressing CXCR3, CCR3, and CCR5.

Growing evidence suggests that a proinflammatory background may contribute to BD neurobiology (Barbosa *et al.*, 2014). Increased levels of inflammatory cytokines have consistently been reported in patients with BD (Kunz *et al.*, 2011; Modabbernia *et al.*, 2013). A meta-analysis showed that peripheral blood levels of IL-4, IL-6, IL-10, soluble IL-2 receptor (sIL-2R), sIL-6R, TNF, soluble TNF receptor-1 (sTNFR1), and IL-1 receptor antagonist (IL-1RA) were significantly elevated in patients with BD in comparison with healthy controls. This proinflammatory state seemed to be exacerbated during mood episodes, particularly during mania. Phasic difference was observed for TNF- α , sTNFR1, sIL-2R, IL-6, and IL-1RA (Modabbernia *et al.*, 2013). A more recent meta-analysis assessing peripheral biomarkers for different mood states in BD showed that a combination of high-sensitivity C-reactive protein/IL-6, brain-derived neurotrophic factor/TNF, and sTNFR1 can differentiate specific mood phases (Rowland *et al.*, 2018).

Patients with BD have increased frequency of autoimmune diseases in comparison with the general population, and the

Table 2. Percentage of T helper (CD4⁺) and T cytotoxic (CD8⁺) lymphocytes expressing chemokine receptors in peripheral blood samples from patients with BD and controls

| Marker | Controls (N = 22) | Patients with BD (N = 33) | p-Values* |
|--------------------------------------|---------------------------|---------------------------|--------------|
| %CD4 ⁺ CXCR3 ⁺ | 13.17 (5.99–21.73) | 4.05 (0.86–12.70) | 0.024 |
| %CD8 ⁺ CXCR3 ⁺ | 2.64 (0.93–4.58) | 1.51 (0.63–2.94) | 0.126 |
| %CD4 ⁺ CXCR4 ⁺ | 3.33 (1.26–7.54) | 2.86 (1.31–21.40) | 0.917 |
| %CD8 ⁺ CXCR4 ⁺ | 0.88 (0.61–1.81) | 1.01 (0.51–2.98) | 0.572 |
| %CD4 ⁺ CCR3 ⁺ | 3.56 (1.67–9.23) | 1.35 (0.79–3.20) | 0.042 |
| %CD8 ⁺ CCR3 ⁺ | 0.98 (0.67–1.40) | 0.83 (0.25–1.61) | 0.462 |
| %CD4 ⁺ CCR5 ⁺ | 12.08 (2.63–18.05) | 2.36 (0.98–7.63) | 0.013 |
| %CD8 ⁺ CCR5 ⁺ | 0.99 (0.63–1.61) | 1.19 (0.61–1.46) | 0.978 |

Percentages are related to total lymphocytes. Values are given as median (25th–75th percentiles). Significant differences are highlighted in bold. *Mann–Whitney U-test.

activation of cell mediated immunity is thought to be part of BD physiopathology (Barbosa *et al.*, 2014). We found that patients with BD exhibited changes in the percentage of circulating T helper (CD4⁺) lymphocytes – but not T cytotoxic (CD8⁺) lymphocytes – expressing CXCR3, CCR3, and CCR5. These results can be helpful to a better understanding of the immune basis of BD.

The lower number of circulating CD4⁺ cells expressing CCR3 might reflect a compensatory mechanism for the increased levels of its ligands. Panizzutti *et al.* (2015) reported elevated circulating levels of eotaxin/CCL11, a ligand of the CCR3 receptor, in patients with BD (Panizzutti *et al.*, 2015). It is worth mentioning that increased eotaxin/CCL11 levels were also found in patients with schizophrenia (Teixeira *et al.*, 2008). Increased eotaxin/CCL11 levels may be associated with enhanced reactive oxygen species (ROS) production (Tenscher *et al.*, 1996). In addition to activating chemotaxis, both eotaxin/CCL11 and eotaxin-2/CCL24 were capable of inducing the release of ROS by eosinophils (Elsner *et al.*, 1998). Another study showed that eotaxin/CCL11 induced microglial production of ROS by upregulating nicotinamide adenine dinucleotide phosphate-oxidase 1 (NOX1), thereby promoting neuronal death (Parajuli *et al.*, 2015). ROS can be used to define oxidative load (Apel & Hirt, 2004) and oxidative stress, which seem to play a critical role in the biology of BD. Two meta-analyses concluded that thiobarbituric acid reactive substances, nitric oxide, lipid peroxidation, and DNA damage are increased in BD (Andreazza *et al.*, 2008; Brown *et al.*, 2014). Accordingly, the finding of decreased CCR3 activity corroborates previous studies in BD.

We also found that the percentage of CD4⁺CXCR3⁺ lymphocytes was decreased in BD in comparison with controls. The interaction between CXCR3 and its ligands plays a role in T helper 1 cell differentiation. The expression of CXCR3 by newly activated CD4⁺ T cells is associated with their ability to produce IFN- γ and is required for optimal effector cytokine response (Groom *et al.*, 2012). Here again, we suggest that the decreased percentage of circulating CD4⁺CXCR3⁺ lymphocytes in BD might be compensatory to increased circulating levels of its ligands in BD. For instance, circulating levels of CXCL10, one of the most important ligands for CXCR3, were found to be increased in patients with BD in comparison with controls (Brietzke *et al.*, 2009; Barbosa *et al.*, 2013).

Finally, we found decreased percentage of CD4⁺CCR5⁺ lymphocytes in BD patients in comparison with controls. The CCR5 32-bp deletion allele has been described as a susceptibility factor for late-onset schizophrenia (Rasmussen *et al.*, 2006). Moreover, there are important ligands for CCR5, such as CCL3,

CCL4, and CCL5. While the CCL3 gene expression was found to be downregulated in dorsolateral prefrontal cortices of BD patients (Nakatani *et al.*, 2006), plasma levels of CCL3 were reported to be unchanged in BD (Barbosa *et al.*, 2013). As we evaluate our findings in the context of available evidence, CCR5 and its ligands should be investigated in more depth in BD.

Our study has several limitations, such as the small number of subjects, cross-sectional design, and lack of information about body mass index. Our relatively small sample size prevented additional analyses, such as assessing whether chemokine receptor expression varies according to mood state. It is worth mentioning that our group has previously shown that chemokine imbalance is related to BD trait and not to mood state (Barbosa *et al.*, 2013). The fact that we did not correct for multiple comparisons should also be taken into account as a limitation. Also, if more stringent statistical criteria were applied (e.g. Bonferroni correction), the observed differences would have lost significance. The observed differences were relatively small (except for %CD4⁺CCR5⁺), and further studies with larger samples are needed to confirm these. As all patients were medicated, the findings might be influenced by patients' ongoing treatment. By contrast, the strict exclusion criteria and the comprehensive clinical evaluation can be regarded as strengths of the study.

In conclusion, patients with BD have decreased percentage of CD4⁺CCR3⁺, CD4⁺CXCR3⁺, and CD4⁺CCR5⁺ lymphocytes in comparison with controls. Together with previous evidence, our findings reinforce the hypothesis that immune pathways, especially involving CD4⁺ lymphocytes, are involved in the physiopathology of BD. Future studies should include the evaluation of peripheral expression of chemokine receptors and their ligands to have a deeper understanding of this immune pathway in BD. Longitudinal studies can also provide valuable information about the association between chemokine receptors and illness course and/or mood stage.

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Acknowledgements. Authors would like to acknowledge the participation of volunteers for their magnificent support. The Neuropsychiatry Program is funded by the Department of Psychiatry and Behavioral Sciences, The University of Texas Health Science Center at Houston. NPR is a Huntington's Disease Society of America fellowship recipient.

Funding. This work was supported by the Brazilian funding agencies Conselho Nacional de Desenvolvimento Científico e Tecnológico; Coordenação de Aperfeiçoamento de Pessoal de Nível Superior; and Fundação de Amparo à Pesquisa do Estado de Minas Gerais.

Author's contributions. IGB, MEB, and ALT designed the study, wrote the protocol, and supervised all experiments and analyses. IGB, NPR, and RBH enrolled and evaluated the participants and applied the clinical scales. ELV, FTLG, GEBM, and VAM designed and performed flow cytometry experiments and analysed the data. MAC and NPR performed statistical analyses and wrote the first draft of the manuscript. All authors contributed to and have approved the final manuscript.

Declarations of interest. None.

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