UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
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TRABALHO DE CONCLUSÃO DE CURSO

Gene expression of inhibitory receptors and immune regulatory molecules in polarized macrophages derived from early- and late-stage BD patients

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Trabalho de conclusão de curso apresentado como requisito parcial à obtenção do título de Farmacêutico pelo curso de Farmácia da Faculdade de Farmácia da Universidade Federal do Rio Grande do Sul.
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Title: Gene expression of inhibitory receptors and immune regulatory molecules in polarized macrophages derived from early- and late-stage BD patients

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Highlights

- Immune inhibitory receptors might play an important role in the progression of BD;
- Macrophages seem to regulate immunological response at different stages of BD;
- Inhibitory receptors expression remained unaltered in M1 macrophages at both stages of BD.
- M2 macrophages regulates immune response as a compensatory mechanism at early stage of BD;
- Chronic inflammation may lead to less responsive macrophages at late stages of BD.

Abstract

Background: Bipolar disorder (BD) has been associated with increased levels of peripheral inflammatory mediators and neuroinflammation. Previously, we observed different immune responses in macrophages of BD patients at different stages of the disorder. Thus, we aimed to further evaluate the regulation of immune response in BD by quantifying the expression of immune checkpoint receptors and respective ligands, as well as molecules involved in regulation and transcription of inflammatory mediators in polarized macrophages of early and late stages individuals with BD.

Methods: qRT-PCR was performed to analyze the expression of genes involved in immune regulation, such as *TLR1*, *TLR6*, *PD-1*, *NFKB1*, *PD-L1*, *PD-L2* and *TIM-3* in samples of proinflammatory M1 or M(IFNγ+LPS) and anti-inflammatory M2 or M(IL-4) macrophages derived from peripheral blood mononuclear cells (PBMCs) of euthymic BD patients (n=16), classified as early-stage BD (BD-E, n=9) and late-stage BD (BD-L, n=7) - according to Functional Assessment Short Test (FAST) - and healthy controls (HC, n=10).

Results: M(IL-4) from BD-E showed higher expression levels of *NFKB1* and *PD-L1* in comparison to HC ($p<0.05$), while BD-L only had higher expression levels of *PD-L1* compared to HC ($p<0.05$). No statistical differences were found between groups for the expression levels of *TLR1*, *PD-L2* and *TIM-3* in M(IL-4) phenotype, while expression levels remained unchanged in M(IFNγ+LPS) for all markers. *TLR6* and *PD-1* did not show PCR amplification in both macrophages phenotypes.

Conclusion: Our findings suggest an immunological regulation acting as a compensatory mechanism by M2 anti-inflammatory macrophages at early stage of BD, although decreased regulation in these cells seems to be observed at late stages of the disorder. On the other hand, the impairment function of proinflammatory macrophages at late stages of BD might underlie a different immunologic mechanism, such as senescence. We hypothesize that such alterations are possibly due to persistent chronic low-grade inflammation during the course of BD that results in the 'exhaustion' of macrophage response. However, further investigation is required to better comprehend the role of immune regulatory mechanisms in the disorder.

Keywords: Bipolar disorder, inflammation, macrophage polarization, immune regulation, inhibitory receptors, qPCR

Abbreviations

AKT: Protein Kinase B; APCs: Antigen-Presenting Cells; BD: Bipolar Disorder; BD-E: Early-Stage BD; BD-L: Late-Stage BD; CNS: Central Nervous System; DAMPs: Damage-Associated Molecular Patterns; DC: Dendritic Cells; FAST: Functional Assessment Short Test; Gal-9: Galectin-9; HAM-D: Hamilton Depression Rating Scale; HC: Healthy Controls; HCV: Hepatitis C; INF: Interferon; LPS: Lipopolysaccharide; NFATc1: Nuclear Factor of Activated T Cells 1; NF-κB: Nuclear Factor Kappa B; NK: Natural Killer; PBMCs: Peripheral Blood Mononuclear Cells; PD-1: Programmed cell Death protein 1; PD-L1: Programmed Death-Ligand 1; PD-L2: Programmed Death-Ligand 2; PI3K: Phosphoinositide 3-Kinase; Ps: Phosphatidylserine; PTEN: Phosphatase and Tensin Homolog; SASP: Senescence-Associated Secretory Phenotype; TIM-3: T cell Immunoglobulin and Mucin domain-containing-3; TGF: Transforming Growth Factor; TLRs: Toll-Like Receptors; T_{reg}: Regulatory T cells; YMRS: Young Mania Rating Scale.

Manuscript

1. Introduction

To date, immunological and inflammatory changes in the periphery and central nervous system (CNS) have been consistently reported as major players in the pathophysiology of bipolar disorder (BD) (1–6). Several studies have reported increased peripheral levels of proinflammatory cytokines in individuals with BD during euthymia and mood episodes (1,2,7–11). Peripheral inflammation has already been associated with systemic toxicity in BD patients (12), especially during mood episodes, and seems to contribute to a chronic low-grade inflammatory state (13,14). Hence, an exacerbation of the peripheral inflammatory response can upregulate the activation of immune cells, such as macrophages, and lead to detrimental consequences in the CNS (13).

Previously, we have shown an augmented secretion of proinflammatory cytokines - such as interleukin (IL)-1β and tumor necrosis factor (TNF) α - by cultured macrophages treated in vitro with serum of BD patients during mood episodes compared to the cells incubated with serum of euthymic patients (15). More recently, we observed that polarized macrophages derived from late-stage euthymic BD patients secreted lower levels of cytokines when stimulated by IFNγ and LPS (M1 phenotype, or proinflammatory) or IL-4 (M2 phenotype mainly anti-inflammatory) than macrophages from healthy individuals and patients at early-stage of the disorder (16). These interesting findings suggest a progressive dysfunction in the innate immune response of patients at late stages of BD; suggesting a chronic activation of the immune system and inflammatory pathways. However, the exact mechanisms underlying are still unclear.

The inflammation response observed in BD seems to be induced by damage-associated molecular patterns (DAMPs), which consist of intracellular molecules released during apoptosis (17). DAMPs are recognized by toll-like receptors (TLRs) expressed in peripheral immune cells (e.g., lymphocytes and monocytes/macrophages) but also in the CNS cells, including neuron and glia (18–21). The DAMPs/TLRs interaction regulates cytokines expression by ultimately activating the nuclear factor kappa B (NF-κB) (22). Interestingly, increased cell death and early apoptosis of peripheral blood mononuclear cells (PMBCs) have already been reported in euthymic BD (23), as well as upregulation of both TLR and NF-κB (1,5,24,25). Moreover, TLRs and its agonists seem to also regulate inhibitory receptors that are present on the surface of immune cells, such as the T cell immunoglobulin and mucin domain-containing-3 (TIM-3) and programmed cell

death protein 1 (PD-1) (26–30). These transmembrane receptors are expressed on lymphocytes, monocytes/macrophage and dendritic cells (31–37), and are part of an important immune regulatory mechanism that can modify intracellular signaling and inhibit cell activation or function in response to inflammatory stimuli (34,35,37–39). The mechanism underlying this regulation in the macrophage is shown in Figure 1. PD-1 and its ligands - programmed death-ligand 1 (PD-L1) and 2 (PD-L2) —, as well as TIM-3 have already been investigated in BD (40), which suggests their involvement in the pathophysiology of the disorder. However, evidence is still limited and lacks on accounting for illness progression in BD. Thus, to further elucidate the current knowledge on inflammatory mechanisms underlying BD (15,16), we aimed to investigate the expression of immune inhibitory receptors and related ligands, as well as other molecules involved in the regulation and transcription of inflammatory mediators in polarized macrophages. Specifically, we utilized derived from euthymic patients at early and late stages of BD and compare to healthy controls.

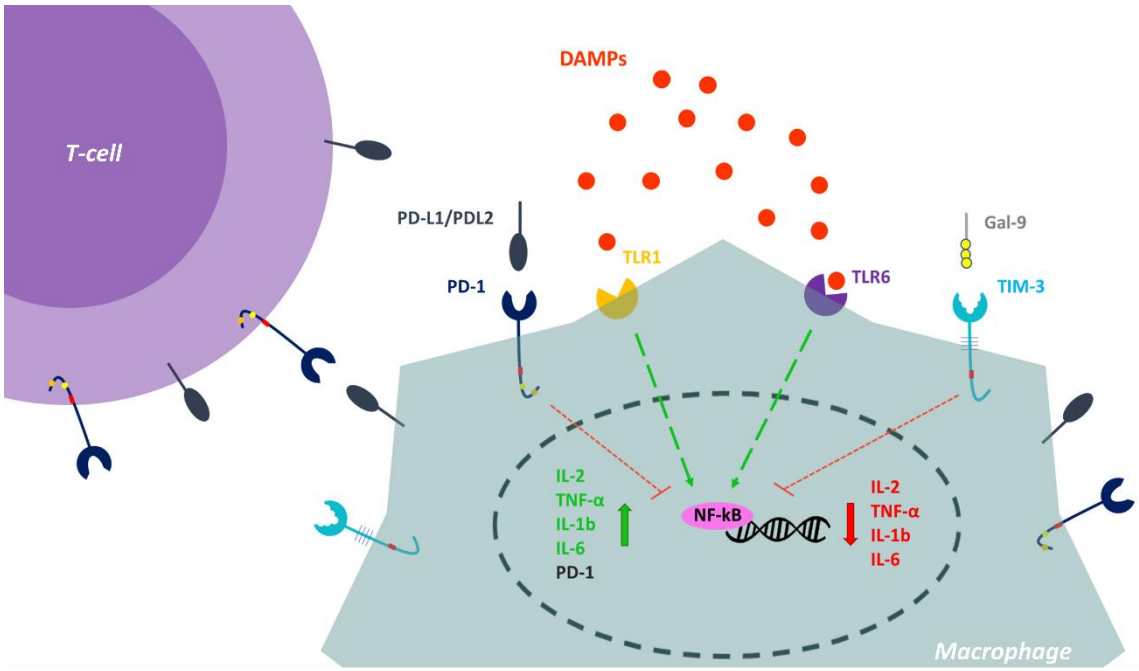

Figure 1. The role of inhibitory receptors and associated ligands on the inflammatory response in the macrophage. Toll-like receptors (TLR) recognize inflammatory mediators such as damage-associated molecular patterns (DAMPS) on the cell surface. Hence, nuclear factor kappa B (NF-κB) is activated resulting in the increase of the transcription of inflammatory cytokines. Following TLRs stimulation, these receptors can positively regulate the expression of programmed cell death protein 1 (PD-1) and T cell

immunoglobulin and mucin domain-containing-3 (TIM-3), which consist of inhibitory receptors responsible to regulate cell function during exacerbated inflammatory responses. The interaction of overexpressed PD-1 and TIM-3 receptors with their ligands — programmed death-ligand 1 (PD-L1) or 2 (PD-L2) and galectin-9 (Gal-9), respectively — on the macrophage inhibits inflammatory responses and, consequently, the secretion of cytokines. Also, it has been suggested that PD-Ls can induce a reverse inhibitory signaling on the macrophage without interaction or interacting with PD-1 on the surface of subjacent cell, such as lymphocytes, and that NF-κB can regulate the expression of PD-1 on macrophages (22,26,34-35).

2. Methods

2.1. Participants

The study included individuals with BD recruited from the *Hospital de Clínicas de Porto Alegre* (HCPA, Brazil). All patients met DSM-IV criteria for BD type I (Structured Clinical Interview for DSM-IV, SCID-I) and met the criteria for BD remission on both the Hamilton Depression Rating Scale (HAM-D) and the Young Mania Rating Scale (YMRS). Moreover, all patients included were receiving pharmacological treatment at the time of the study. Patients (n=16) were classified as early-stage BD (BD-E, n=9) and late-stage BD (BD-L, n=7) which was assessed by the Functional Assessment Short Test (FAST) (41). Total FAST score ≤11 and ≥40 were defined as BD-E and BD-L, respectively (Rosa et al., 2014). The control group (HC, n=10) comprised healthy volunteers selected according to the non-patient version of the SCID. All participants provided written informed consent before their inclusion, and this study was approved by the Institutional Review Board of Hospital de Clínicas de Porto Alegre (HCPA, Project Number 150396). Further information about the participants can be found elsewhere (16).

2.2. Sample collection and cell culture

PBMCs were obtained from peripheral blood for monocyte isolation and subsequent macrophage differentiation. Briefly, macrophages were induced into classical (M1 or M(interferon (IFN)γ + lipopolysaccharide (LPS)) – mainly proinflammatory – and alternative (M2 or M(IL-4)) – mainly anti-inflammatory – phenotypes, which was carried out as previously described (16).

2.3. RNA extraction

The RNA isolation of macrophages phenotypes M(IFNγ+LPS) and M(IL-4) was performed according to a standardized protocol based on Invitrogen™ RNA isolation protocol (42). First, cells were incubated in cold TRIzol reagent (Invitrogen, USA) followed by chloroform 100%. After incubation, samples were centrifuged (1200 xg, 15 min, at 4°C) producing an aqueous and organic layers. Thus, the aqueous interphase containing the RNA was collected. The RNA was precipitated with isopropanol 100% and washed with ethanol 75% to remove impurities. Then, RNA was resuspended in DEPC water and stored at -80°C until further analysis. The concentration of RNA in each sample was quantified using NanoDrop 1000 spectrophotometer (Thermofisher, USA).

2.4. Quantitative reverse transcription real-time polymerase chain reaction (qRT-PCR)

Reverse transcription was performed using High Capacity cDNA Reverse Transcription Kit according to manufacturer's instructions (Applied BioSystems, USA), operating with Veriti Thermal Cycler (Applied BioSystems, USA). The amount of RNA used for cDNA synthesis was 200 ng for all samples. RNase inhibitor (Applied BioSystems, USA) was used to prevent RNA degradation during the cDNA synthesis. The reverse transcription was performed in duplicate for each sample and mixtured for gene expression analysis, except when RNA concentration was insufficient for two cDNA synthesis. All cDNAs were stored at -20°C until qRT-PCR analysis.

For qRT-PCR, the synthesized cDNA was used to evaluate quantitative gene expression of *TLR1, TLR6, PD-1, NFKB1, PD-L1, PD-L2* and *TIM-3* genes in both M(IFNγ+LPS) and M(IL-4) macrophages phenotypes. Each reaction was perfomed in a final volume of 14 uL, containing 7 uL of TaqMan RT-PCR MasterMix (quatroG, Brazil), 0.7 uL of probe TaqMan Gene Expression Assay (Applied BioSystems, USA), 5.3 uL of UltraPure DNase/RNase-Free Distilled Water (Invitrogen, USA) and 1 uL of cDNA (200 ng/uL). The samples were analyzed using QuantStudio™ 3 Real-Time PCR System (Applied BioSystems, USA) using standard cycling conditions (50°C for 2 min, 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min), as recommended by the manufacturer. Beta-2-microglobulin (B2M) was used as a housekeeping gene to normalize the samples using the ΔCT method. All reactions were performed in triplicate, and probes information is presented in Table 1.

Table 1. Probes details.

Gene symbols	Gene name	Reference ID	Amplicon length
TLR1	Toll-like receptor 1	Hs00413978_m1	72
TLR6	Toll-like receptor 1	Hs04975840_m1	131
PDCD1, *PD-1*	Programmed cell death 1	Hs01550088_m1	127
CD274, *PD-L1*	Programmed cell death 1 ligand 1	Hs00204257_m1	77
PDCD1LG2, *PD-L2*	Programmed cell death 1 ligand 2	Hs00228839_m1	61
NFkB1	Nuclear factor kappa B subunit 1	Hs00765730_m1	66
HAVCR2, *TIM-3*	Hepatitis A virus cellular receptor 2, T-cell immunoglobulin mucin family member 3	Hs00958618_m1	60
B2M	Beta-2-microglobulin	Hs99999907_m1	75

2.5. Statistical Analysis

Normality of data distribution and variance were verified using Shapiro-Wilk's and Levene's test. BD stage was used as an independent variable (HC, BD-E and BD-L). Sample characteristics were compared using chi-square test and Student's t test or one-way ANOVA, as appropriate. Comparisons of gene expression levels among groups were performed using one-way ANOVA followed by Tukey post hoc, when applicable. Statistical significance was set at $p<0.05$. All statistical analyses were performed using SPSS software v. 19 (IBM, Chicago, IL, USA).

Samples that had (1) no amplification in gene expression data or (2) insufficient RNA/cDNA for PCR reaction were excluded from statistical analysis (M(IFNγ+LPS) - BD-L=2, and M(IL-4) - HC=1, BD-E=1, and BD-L=3).

3. Results

3.1. Sample characteristics and clinical data

Demographic and clinical data are summarized in Table 2. BD-L had higher number of suicide attempts (χ^2=3.87, p<0.05) and hospitalizations (t(14)=-2.89, p<0.05) when compared to BD-E patients. ANOVA indicated a difference in FAST scores among groups (F(2,23)=134.74, p<0.001). BD-L patients had higher FAST scores in comparison to BD-E and HC (BD-L vs. HC, p<0.001, 95% CI 34.28-48.09; BD-L vs. BD-E, p<0.001, 95% CI 32.89-47.02).

Table 2. Sample characteristics.

	HC(n=10)	**BD-E (n=9)**	**BD-L (n=7)**	**X^2, F, t**	**p value**
Sex[a]					
Females	7 (70.0)	8 (88.9)	5 (71.4)	1.12	0.573
Age (years)[b]	48.00 ± 14.34	56.89 ± 12.23	46.57 ±13.75	1.48	0.249
Marital status					
Married[a]	5 (50.0)	3 (33.3)	2 (28.6)	5.37	0.497
Years of education[b]	14.00 ± 4.69	10.00 ± 3.08	13.42 ± 5.56	2.12	0.143
Work status[a]					
Employed	4 (40.0)	3 (33.3)	1 (14.3)	20.14	0.064
Age of BD diagnosis (years)[b]		43.11 ± 13.27	34.14 ± 17.45	1.17	0.261
Length of illness (years)[b]		13.78 ± 9.61	12.43 ± 8.92	0.29	0.778
Suicide attempts		2 (22.2)	5 (71.4)	3.87	*0.049*
Number of hospitalizations[b]		1.44 ± 1.13	3.57 ± 1.81	-2.89	*0.012*
BD type I[a]		7 (77.8)	6 (85.7)	0.16	0.687
HAM-D[b]		3.00 ± 1.73	4.70 ± 1.98	-1,85	0.086
YMRS[b]		1.11 ± 1.96	2.00 ± 2.58	-0.78	0.446

FAST[b]	5.10 ± 6.24	6.33 ± 4.36	46.29 ± 6.02	134.74	*0.000* *
Medications[a]					
Lithium		3 (33.3)	4(57.1)	0.91	0.341
Anticonvulsants		6 (66.7)	4 (57.1)	0.15	0696
Antipsychotics		7 (77.8)	6 (85.7)	0.16	0.687
Antidepressants		3 (33.3)	2 (28.6)	0.04	0.838
Benzodiazepines		3 (33.3)	1 (14.3)	0.76	0.383

HAM-D: Hamilton Depression Scale; YMRS: Young Mania Rating Scale; FAST: Functioning Assessment Short Test; HC: Healthy Controls; BD-E: Early-Stage BD; BD-L: Late-Stage BD; [a] Data expressed as n (%); [b] Data expressed as mean ± standard deviation; *Tukey post hoc BD-L vs. HC, $p<0.001$; BD-L vs. BD-E, $p<0.001$.

3.2. Expression of inhibitory receptors and their ligands in macrophages of patients at early and late-stages of BD

One-way ANOVA indicated a different expression of *NFKB1* ($F(2,19)=5.14$, $p=0.016$) and *PD-L1* ($F(2,19)=9.75$, $p=0.001$) in M(IL-4) among groups (Figure 2). Following Tukey post hoc, M(IL-4) from BD-E had higher expression levels of *NFKB1* and *PD-L1* in comparison to HC (*NFKB1*, ddCt=0.54±0.18, $p=0.019$, 95% CI 0.08-1.00; *PD-L1*, ddCt=2.53±0.65, $p=0.003$, 95% CI 0.89-4.17), whereas BD-L only had higher expression levels of *PD-L1* compared to HC (ddCt=2.36±0.68, $p=0.007$; 95% CI 0.64-4.08). No statistical differences were found in the expression levels of *TLR1* ($F(2,16)=0.57$, $p=0.576$), *PD-L2* ($F(2,15)=1.75$, $p=0.208$) and *TIM-3* ($F(2,20)=3.04$, $p=0.070$) in M(IL-4) phenotype.

In M(IFNγ+LPS), one-way ANOVA did not show statistical significant differences for the expression levels of *TLR1* ($F(2,19)=0.02$, $p=0.976$), *NFKB1* ($F(2,23)=0.28$, $p=0.761$), *PD-L2* ($F(2,19)=1.94$, $p=0.170$), *TIM-3* ($F(2,23)=0.19$, $p=0.827$) and PD-L1 ($F(2,23)=3.22$, $p=0.580$). *TLR6* and *PD-1* were not detected in both macrophages' phenotypes.

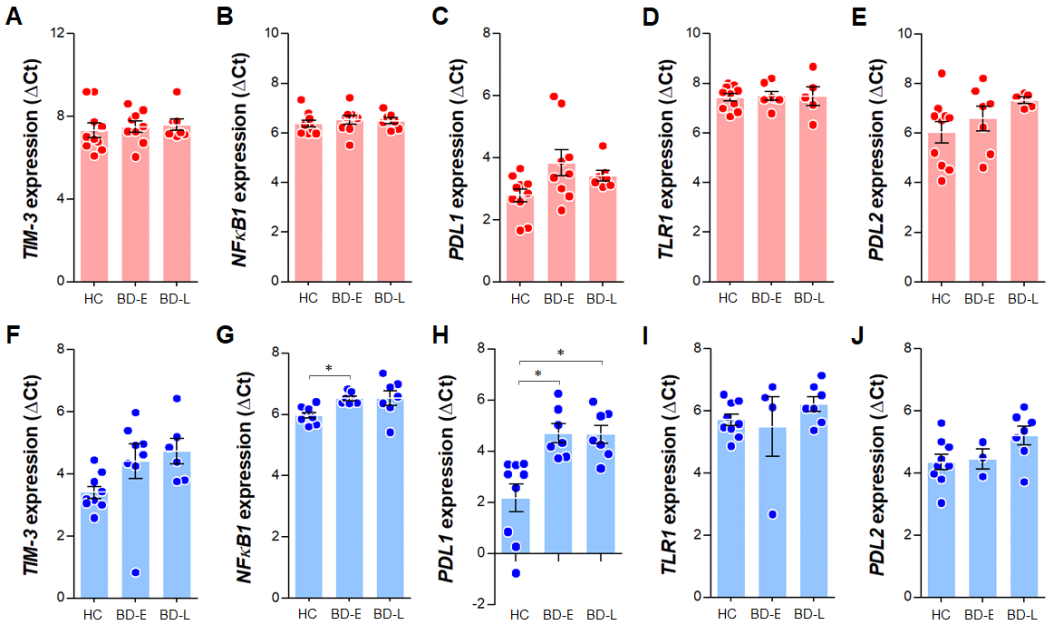

Figure 2. dCt values of *TIM3*, *NFKB1*, *PD-L1*, *TLR1* and *PD-L2* expression in M(IFNγ+LPS) and M(IL-4) macrophages (red and blue, respectively) derived from individuals with BD-E, BD-L and HC. One-way ANOVA followed by Tukey post hoc, data expressed by mean ± SEM, *p<0.05.

4. Discussion

As previously discussed, inflammation is considered an important component of BD pathophysiology. Previously, we demonstrated that cultured primary macrophages of euthymic BD-L patients produced lower levels of cytokines in comparison to macrophages of BD-E and healthy individuals, especially when stimulated with IFNγ+LPS (16). Thus, in the present work, we further investigated whether macrophage inhibitory receptors are involved in modulating immune activation at different stages of BD. Here, we found an increased expression of *NFKB* subunit 1 (p105/p50) and *PD-L1* in M(IL-4) macrophages of BD-E patients, whereas only *PD-L1* was upregulated in M(IL-4) macrophages of BD-L patients. To our knowledge, this is the first study to evaluate the role of inhibitory checkpoint receptors in polarized macrophages considering stage of illness in BD.

NF-kB is a major transcription factor of proinflammatory genes, such as those encoding cytokines and chemokines, and its pathway is usually triggered by TLRs and sustained by proinflammatory cytokines (e.g. TNFα and IL-1) (43,44). Increased levels of inflammatory markers, as reported in BD and other psychiatric disorders (e.g.,

major depressive disorder), are associated with depressed mood (45,46), which might account as a result of higher NF-kB activity (47). Besides its inflammatory role, NF-kB is a major transcription factor involved in the induction and perpetuation of the senescence-associated secretory phenotype (SASP) (48,49). Given the various NF-kB subunits, encoded by different genes, we evaluated only the expression of *NFKB1* that codifies the p105/p50 subunits for the canonical heterodimer P65/P50. It has been suggested that the loss of *NFKB1* increases cellular senescence and decreases apoptosis favoring early onset aging (50) and augmenting the secretion of inflammatory mediators through SASP (51,52). Both in vitro (i.e., macrophages) (51) and in vivo (i.e., mice and humans) (53,54) studies have found augmented expression of proinflammatory cytokines in the absence of *NFKB1* gene. However, the downregulation of proinflammatory genes and upregulation of anti-inflammatory genes have been found in *NFKB1*-deficient microglia, the CNS-resident macrophage (52). In our study, increased expression of *NFKB1* was observed only in M(IL-4) macrophages of BD-E patients. It is possible that, at earlier stages of BD, the anti-inflammatory macrophage phenotype is still responsive to immune activation. On the other hand, unaltered *NFKB1* expression in the M(IFNγ+LPS) macrophage were not expected. Our previous experiments suggest that at late stages of BD, M1 phenotype exhibit a reduced cytokine release. Although we have hypothesized that M1 polarized macrophages derived from BD-L might undergo a senescent state, which could be mediated by SASP, it is possible that other modulatory mechanisms are involved and require further elucidation.

Inflammatory pathways are strictly regulated, and immune inhibitory receptors play an essential role in this modulation (35,55). As these receptors are known to regulate the immune response and tolerance maintaining the homeostasis (35,55), dysfunctional activation inhibitory receptors may underly immune dysregulation in BD. One important inhibitory mechanism is exerted by PD-1/PD-Ls interaction, which has been well-described in T cells, but it has also a relevant role in regulating macrophage activation and production of inflammatory mediators (26,35,56). In T cells, PD-1 activation upregulates phosphatase and tensin homolog (PTEN) expression and suppresses phosphoinositide 3-kinase (PI3K)- protein kinase B (Akt) signaling pathway - an important route for cell metabolism, proliferation and survival (37,39,57–60). Hence, PD-1/PD-L binding results in T cell functional inhibition with reduction of cytokine secretion (35,60,61). In monocytes, PD-1 upregulation has been suggested

to promote cytokine release (e.g., IL-10) that mediates the downregulation of T cells response and proliferation (27). Conversely, TLR activation by cytokines and other agonists can also induce PD-1 expression on macrophages via NF-κB pathway (26,27), whereas this regulation is mediated by nuclear factor of activated T cells 1 (NFATc1) pathway in T cells (26). Unexpectedly, no amplification of *PD-1* was observed in both macrophage phenotypes even though M(IL-4) from BD-E showed increased *NFKB* expression. However, other factors may regulate constitutive and IFN-mediated PD-1 expression in macrophages (e.g., STAT1/2) (62).

Regarding PD-L1, our results show upregulation in M(IL-4) macrophages at both stages of BD, but unaltered in M(IFNγ+LPS). This result is in agreement with our previous work (15) that we observed augmented transforming growth factor (*TGF)-β* gene expression in cultured macrophages conditioned with serum from BD patients (manic and depressed). PD-L1 expression on antigen-presenting cells (APCs) such as macrophages, T cells and host tissue seems to prevent immune responses in naïve and effector T cells (63). While in vivo, PD1/PD-L1 interaction induces regulatory T cells (T_{reg}) development, important for immune downregulation, in the periphery, PD-L1 *per se* can also convert naïve T cells into T_{reg} cells and maintain Foxp3 expression as well as the suppressive function of T_{reg} cells (57). In fact, blunted immune response, induced by the suppressive effect of PD-1/PD-L1 signaling can induce apoptosis in T cells, another feature that we have previously reported ex vivo in PBMCs from BD patients (23). Similar to PD-1, inflammatory cytokines secreted by activated T and natural killer (NK) cells also induce PD-L1 expression during the immune response (64–66). For instance, IFNγ and IL-10 can upregulate PD-L1, while IL-4 upregulates both PD-1 ligands, but especially PD-L2, on APCs (64–66). Previously, we showed that lower levels of inflammatory cytokines are secreted by M(IFNγ+LPS) and M(IL-4) macrophages of BD-L patients compared to BD-E (16). Thus, our current work shows an increased PD-L1 expression in M(IL-4) in BD regardless of stage of illness. Even though cytokine levels were lower, IL-4 or IL-10 secreted by this macrophage phenotype could have contributed to PD-L1 upregulation. However, the expression of PD-L1 in M(IFNγ+LPS) and PD-L2 in M(IL-4) remained unchanged. Related to PD-Ls activity, it has been suggested that a reverse signaling in APCs might lead to an endogenous negative regulation in the cell function (67). Although overexpression of PD-L1 and PD-L2 in dendritic cells (DC) may not suppress cell proliferation, it reduces cell maturation following binding to soluble PD-

1 (67). However, it is proposed that PDLs without binding to PD-1 can mediate positive regulation in T cells (68).

It is worth mentioning that extant literature on inhibitory receptors is still limited in BD and, so far, it has only been studied by Wu et al. (40). The authors investigated PD-1 and its ligands in CD14+ monocytes and T cells of BD patients in depressive episodes. Despite no altered PD-1 and PD-L1 expression being found, PD-L2 was downregulated in the monocytes of patients compared to controls (40), which somewhat contrasts to our findings. Similar studies have been performed in other clinical samples, but results are still contradictory. For instance, the upregulation of PD-1 has already been identified in CD4 and CD8 T cells from patients with depression (69). In addition, a lower percentage of T cells expressing PD-L1 was found in individuals with posttraumatic stress disorder with no significant changes in PD-1 expression (70). Upregulation of PD-1 expression has also been found in macrophages of patients with cancer and hepatitis (56,71). Interestingly, such alteration was associated with attenuated macrophage inflammatory response in patients with hepatitis C (HCV) infection (71), as well as with increased time and stage of illness and decreased phagocytic capacity in macrophages of patients with colorectal cancer (56). Considering that neuroprogression and staging in BD have relevant clinical implications (72), the evaluation of PD-1 and its ligands may contribute to identifying new molecular targets involved with the disorder and its progression.

Another main inhibitory receptor is TIM-3, a transmembrane protein that binds to galectin-9 (Gal-9) and phosphatidylserine (Ps) ligands. The interaction with the first ligand regulates macrophage activation and cytokine production, while the later mediates the process of efferocytosis (i.e., phagocytosis and elimination of apoptotic bodies or cells) in macrophages and DCs (29,30,73,74). Resting macrophages constitutively express *TIM-3* and releases a limited amount of inflammatory cytokines, while, upon stimulation by TLR ligands, *TIM-3* is downregulated and cytokines are upregulated (29,30). This modulation supports the role of TIM-3 in immune tolerance. Similar to *PD-1*, enhanced *TIM-3* expression in macrophages has been shown to negatively regulate TLR activation by inhibiting NF-kB and upregulating PI3K-AKT pathways (28). Consequently, cytokine secretion is reduced preventing an excessive inflammatory response (28). However, evidence also suggests otherwise (29). In BD, Wei-wu et al. (40) reported enhanced expression of *TIM-3* in cytotoxic T cells during depressive episodes which was further associated with lower severity of depressive

symptoms (40). Despite the lack of evidence in the current literature, it is possible that macrophage inhibitory receptors, such as TIM-3, exert a protective and regulatory role in BD even in the presence of persistent stimulation of TLRs and immune response and, consequently, chronic low-grade inflammation. In addition, TIM-3 expression can induce a positive regulation and cell activation (32,73,75), where trans (different cells) and cis (same cell) TIM-3/Gal-9 interaction regulates TLR signaling negatively and positively, respectively (73). Interestingly, Zhang et al. have shown that acute inflammatory stimuli of macrophages increases TIM-3/Gal-9 interaction inhibiting M1 polarization and upregulating the M2, while the opposite effect occurs following chronic exposure (76). In our study, no significant difference of *TIM-3* expression was found in polarized macrophages in BD, but previous studies suggest that TIM-3 upregulates *PD-1* expression in macrophages (29,30). Hence, a positive regulation between TIM-3 and PD-1 might explain the unchanged expression levels of both receptors. In another aspect, when TIM-3/Ps interaction is blocked, apoptotic bodies are not removed leading to immune dysfunction and production of autoantibodies, which favors the development of autoimmune diseases (77,78). However, the unaltered *TIM-3* expression does not corroborate with a previous study that identified increased early apoptosis in cells from euthymic BD patients (23).

Also, we did not found alterations in *TLR1* and *TLR6* expression, but changes in TLRs expression have already been identified in psychiatric disorders (24,25,79–83). In BD, TLRs mediate an immune response induced by DAMPs contributing to the chronic low-grade inflammation state described in the patients (17,25). According to Mckernan et al., increased plasma cytokine levels in BD are induced by TLR2, TLR4 and TLR8 (24). Interestingly, Wieck and collaborators demonstrated that a higher percentage of monocytes expressing TLR1, TLR2 and TLR6 was associated with reduced secretion of cytokines in BD during euthymia (25). TLRs-mediated cytokines expression occurs via NF-kB activation (19,43). Although we observed an upregulation of *NFKB1* in M(IL-4) macrophages in BD-E, TLRs expression did not differ between groups. Previous studies suggest that TLR1, TLR2 and TLR6 cannot solely activate NF-kB. Specifically, TLR2 would require to be associated with either TLR1 or TLR6 to send precisely signals via NF-κB and, consequently, induce proinflammatory cytokines secretion (84–86).

Overall, considering the main role of chronic inflammation in BD, our previous and current findings support two main hypotheses. First, macrophages from BD

patients may undergo a senescent state, especially at late stages of the disorder, which is characterized by a lessened response to stimuli; hence, reduced cytokine secretion (16). Second, each macrophage phenotype might be responsible for regulating immunological processes at different stages of BD, and a balance of polarization could be required for better outcomes throughout the course of the disorder. Particularly, despite the persistent immune activation, the anti-inflammatory macrophage phenotype, M2 or M(IL-4), might still be able to exert an appropriate response to stimuli (i.e., upregulation of *NFKB1*) regulating immune response (i.e., upregulation of *PD-L1*) at early stages of BD. Thus, at late stages BD, macrophages (i.e., M(IL-4)) are less responsive, due to immune overactivation throughout the course of the disorder, and gene expression might be shifted to a latent and inhibitory profile. It is worth mentioning that increased *PD-L1* expression occurs in both physiological and exhaustion immune states. Similarly, *PD-1* is transiently expressed on T cells to prevent excessive immune responses at normal conditions. However, following persistent antigen-exposure and chronic T cell activation, *PD-1* is overexpressed resulting in a progressive loss of function. This phenomenon is characterized by a dysfunctional cell phenotype known as 'exhaustion', which is commonly identified in patients with chronic viral infections and cancer (61,87–89), and might also happen in BD. Additionally, gene expression regarding immune regulation remained unaltered for M(IFNγ+LPS) or M1 (proinflammatory) macrophages at both stages of BD. We predict that further mechanisms such as senescence (16) might be involved in the impaired function of these cells at late stage of BD, but additional investigations are required.

Although this is the first study of its kind, some limitations should be addressed. First, we had a small sample size. Second, we did not evaluate the expression of inhibitory receptors in the T cells, in which these receptors have been extensively studied. Third, it would be interesting to quantify the expression of TIM-3 ligands (i.e., Gal-9 and Ps) and understand their function in macrophages of BD patients. Fourth, protein levels of these markers were not analyzed to complement the gene expression data. Five, medications used by patients may have a potential influence in gene expression levels. Thus, future investigations should consider the experimental conditions investigated here and compare to symptomatic episodes in BD. This is especially relevant because increased levels of systemic toxicity (12) and

inflammatory markers are associated with mood episodes, and cognitive function seems to progressively decline throughout the course of BD.

5. Conclusion

Considering our previous findings (16), the current work was designed to further our knowledge on specific immunological regulatory mechanisms in BD. Thus, we expected to understand whether inhibitory receptors in different macrophage phenotypes mediate immune dysfunction at different stages of BD.

Here, we propose a possible immune regulation acting as a compensatory mechanism in anti-inflammatory macrophage phenotype (i.e., M2 or M(IL-4)) at an early stage of BD (Figure 3). Conversely, M2 macrophages showed less immune regulation at late stages of the disorder. Interestingly, no differences were found in the expression inhibitory receptors and associated molecules in M1 phenotype between groups. Along with our previous work, we speculate that the proinflammatory macrophage (i.e., M1 phenotype or M(IFNγ+LPS)) present with an impaired function at late stages of BD, possibly due to senescence or other immunologic pathways involved. However, such alterations might be induced by other mechanisms rather than those investigated in the current study. Overall, evidence points out to an impaired immune response in BD, especially at late stages, and this could be explained by a persistent chronic inflammation throughout the course of the disorder. Even though promising, the role of inhibitory receptors and associated regulatory mechanisms in BD warrant further investigation.

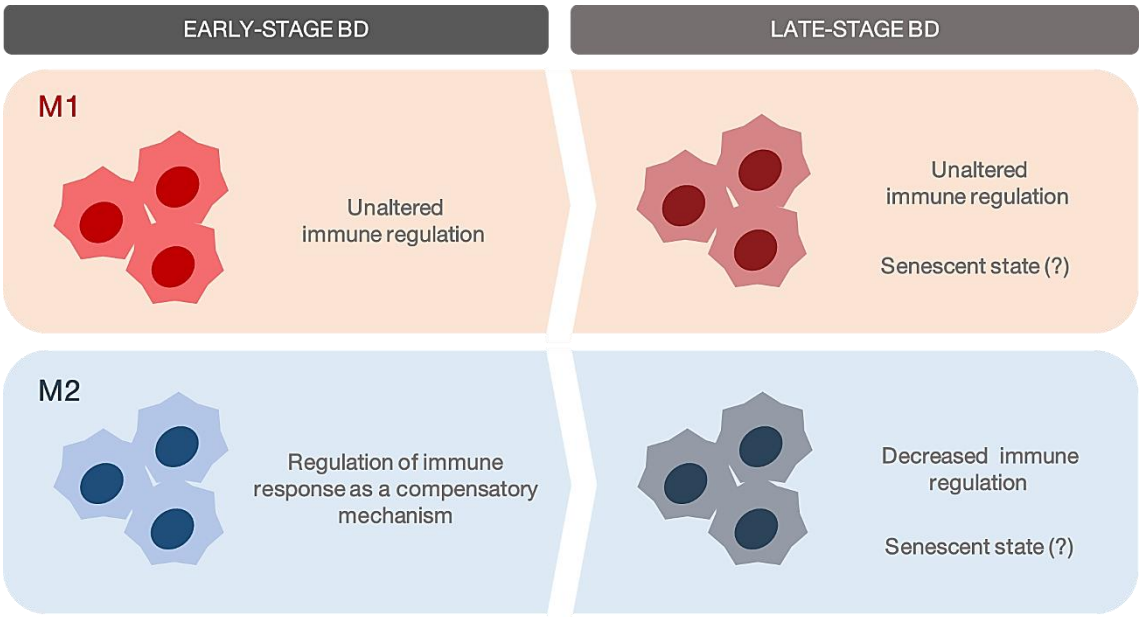

Figure 3. Macrophage function at early and late stages of BD. Evidence from our work suggests that macrophages from patients with BD display different response patterns depending on the stage of the disorder. We hypothesize, according to our previous and current study, that a chronic low-grade inflammatory state observed in BD overstimulates macrophages resulting in impaired function at late stages of BD at both M1 or proinflammatory and M2 macrophages or anti-inflammatory – represented by M(IFNγ+LPS) and M(IL-4), respectively. While M1 function might decline due to accelerated aging (i.e., senescence) or other associated mechanism, M2 function impairment at late stage of BD is associated with immune regulation. Thus, different mechanisms might underlie the impaired function of each macrophage phenotype in distinct stages of BD. Early apoptosis and accelerated again have been reported in BD, but senescence markers were not evaluated in this study.

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