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Impairments in peripheral blood T effector and T regulatory lymphocytes in bipolar disorder are associated with staging of illness and anti-cytomegalovirus IgG levels.

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Abstract

There is now evidence that, based on cytokine profiles, bipolar disorder (BD) is accompanied by simultaneous activation of the immune-inflammatory response system (IRS) and the compensatory immune-regulatory system (CIRS), and that both components may be associated with the staging of illness. Nevertheless, no BD studies have evaluated the IRS/CIRS ratio using CD (cluster of differentiation) molecules expressed by peripheral blood activated T effector (Teff) and T regulatory (Treg) subpopulations. This study examined T cell subsets both before and after *ex vivo* anti CD3/CD28 stimulation using flow cytometric immunophenotyping in 25 euthymic BD patients and 21 healthy controls as well as human cytomegalovirus (HCMV)-specific IgG antibodies. BD is associated with a significantly lowered frequency of baseline (unstimulated) CD3+CD8+CD71+ and CD4+CD25+FOXP3 and increased CD4+CD25+FOXP3+CD152+ frequencies and with lowered stimulated frequencies of CD3+CD8+CD71+, CD4+CD25+FOXP3+CD152+ and CD4+CD25+FOXP3+GARP cells and, consequently, by an increased stimulated Teff/Treg ratio. Moreover, the number of manic, but not hypomanic or depressive episodes, is significantly and negatively associated with the stimulated proportions of CD3+CD4+CD154+, and CD69+ and CD71+ expression on CD4+ and CD8+ cells, while duration of illness (≥ 10 years) is accompanied by a depleted frequency of stimulated CD152+ Treg, and CD154+ and CD71+ CD4+ T cells. BD and anti-human cytomegalovirus (HCMV) IgG levels significantly interact to decrease the expression of CD4+CD25+FOXP+GARP T phenotypes. In conclusion, BD is characterized by deficits in immune-regulatory functions while the staging of illness is characterized by additional impairments is Teff and Treg activation. HCMV seropositivity may contribute to an immune-risk phenotype associated with BD.

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Introduction

Bipolar disorder (BD) is a neuroprogressive disorder with recurrent manic, hypomanic and depressive episodes which alternate with euthymic phases, whereby the number of mood episodes is associated with increasing severity of symptoms, increased risk of future episodes, functional decline in the performance of daily tasks and deficits in memory and executive functions [1-7]. Based on staging characteristics including recurrence of episodes and past suicidal behaviors, BD patients may be classified into three stages: (a) an early stage when patients show only a few recurrent episodes and increased suicidal ideation; (b) the relapse-regression stage with increased recurrent episodes, more disabilities, lower health-related quality of life and socioeconomic status, and cognitive deficits in memory and executive functions; and (c) the suicidal-regression stage with all hallmarks of stage 2 combined with highly increased suicidal behaviors [7].

There is now also evidence that BD is accompanied by activation of the immune-inflammatory response system (IRS) as well as the compensatory immune-regulatory system (CIRS) [8]. Based on IRS cytokine profiles, we reviewed that BD is characterized by activated M1 macrophage cells as indicated by elevated levels of interleukin (IL)-1β, IL-6 and tumor necrosis factor-α (TNFα); T helper-1 (Th-1) cells with increased interferon-γ (IFN-γ) and IL-2 levels; and activated Th-17 cells with increased IL-17 [7]. Activated CIRS profiles are indicated by activated Th-2 cells, including increased IL-4 and IL-5, and T regulatory cells including increased IL-10 and transforming growth factor-β (TGF-β) production [7]. A recent meta-analysis shows that increased levels of immune-regulatory Th-2 (IL-4) and Treg (IL-10) cytokines play a role in BD and, additionally, that cytokine receptors with immune-regulatory functions are involved including increased levels of the soluble IL-1 receptor antagonist (indicating increased IL-1 signaling), sTNFR (indicating M1 activation), and sIL-2R (indicating Th-1 cell activation) [9]. The IRS-CIRS

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theory of BD considers that (a) activated M1, Th-1, Th-2 and Th-17 pathways may mediate the clinical and neurocognitive symptoms of the illness, and that (b) increased CIRS functions through IL-4 and IL-10, but also sIL-1RA, sIL-2R and sTNFR, may attenuate M1 and Th-1 effector functions thereby attenuating the primary IRS [8]. The IRS/CIRS ratio is somewhat higher in BD depression versus unipolar depression while there is no evidence whether IRS or CIRS functions prevail in the different phases of BD [10].

Immune-inflammatory and related nitro-oxidative stress pathways become more sensitized with increasing numbers of episodes and suicidal behaviors that accompany staging of illness. For example, Sowa-Kucma et al. [11] reported that in BD patients, the number of previous suicide attempts and previous episodes or hospitalizations the year prior to blood sampling are associated with indicants of cell-mediated immunity, CIRS (sIL-1RA levels) and related oxidative stress pathways (increased TBARS levels). Furthermore, a staging index computed with machine learning techniques in patients with BD was significantly associated with nitro-oxidative stress and lowered activity of the lipid-associated antioxidant enzyme paraoxonase 1 (PON1) [7]. There are also some preliminary results showing that inflammatory responses may be more pronounced in the later stages of the illness [12-14]. Nevertheless, these conclusions were mainly derived from results on cytokine/cytokine receptor profiles.

Another approach is to define IRS and CIRS immune cell phenotypes using surface markers or CD (cluster of differentiation) molecules expressed by activated T cells or soluble CD markers released in the plasma [15]. Already in the 1990s, it was established that patients with BD depression and mania show signs of T cell activation as measured with increased sIL-2R plasma levels while patients with unipolar and bipolar depression show increased soluble transferrin receptor (sTfR) concentrations [16,17]. Measuring CD markers with flow cytometric

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immunophenotyping methods allows to delineate the frequencies of activated T cells including (a) activated T effector (CD69+ and CD154+) subpopulations; (b) replication CD markers on T cells (e.g. CD71+ or TfR); and (c) activated Treg cells as defined by CD4+CD25+FOXP3+ expressing CD152+ or GARP [15,17-21]. **Table 1** summarizes the functional characterization of those immune subsets and CD markers. Nevertheless, only a few papers examined T cell activation markers in BD. For example, proportions of activated CD4+CD25+ T cells, as well as increased p-ERK signaling, were observed in peripheral T cell subsets of patients with BD, while CD4+CD25+FOXP3+ cells are lowered or increased [22-25]. Treg cells not only regulate and suppress inappropriate peripheral immune responses, thereby participating in CIRS functions, but also participate in immune surveillance in the brain, thereby mediating neuro-immune cross-talks [26]. T cells regulate hippocampal neurogenesis including neurocognitive functions and as such aberrations in the ratio between Teff and Treg cells may play an important role in affective disorders through direct effects and the production of IRS/CIRS cytokines [26]. Nevertheless, no studies have examined the ratio between the proportions of activated Teff and activated Treg cells in BD.

Human cytomegalovirus (HCMV) infection may be associated with the pathophysiology of BD and with changes in the expression of CD markers and immune cell functions. A recent study showed that IgG antibodies to HCMV are associated with BD and with elevated manic, but not depressed, mood [27]. One hypothesis is that herpes viruses and especially HCMV may facilitate exacerbations of mental disorders via interactions with susceptibility genes and through alterations in the production of neurotoxic cytokines [27]. Upon primary infection, HCMV remains persistent and may be accompanied by increased expression of late-differentiated memory T-cells in the CD4+ and CD8+ lineages, by a shift towards $CD8^+$ T-cell production with increased levels

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of M1 and Th-1 cytokines, and large populations of HCMV-specific CD4+ and CD8+ T-cells [28,29]. As such, HCMV latency may be accompanied by dysfunctional T-cell populations that could contribute to loss of T cell functions and, thus, an immune-risk phenotype that could contribute to the pathophysiology of illness including BD. Nevertheless, there are no studies that examined the impact of HCMV seropositivity on immune subsets in patients with BD.

Hence, this study was performed to examine whether (a) the ratio between activated Teff and Treg cells as assayed with flow cytometric immunophenotyping is increased in patients with BD and whether this balance is affected by staging of illness; and (b) whether HCMV IgG seropositivity is associated with aberrations in immune subsets thereby aggravating T cell impairments in BD.

Subjects and methods

Participants

This study recruited 25 euthymic BD patients with type I BD and 21 healthy volunteers of both sexes and aged 18 to 65 years. The BD patients attended the Department of Psychiatry, Federal University of Sao Paulo (UNIFESP/EPM), Sao Paulo, Brazil. Controls were recruited by word of mouth from the same catchment area. The diagnosis of BD was made according to DSM-IV-TR diagnostic criteria and using the Structured Clinical Interview for DSM-IV-Axis-I (SCID-I). The severity of depression and mania were assessed using the Hamilton Depression Rating Scale-17 (HAM-D) items and the Young Mania Rating Scale (YMRS), respectively [30,31]. BD patients were divided into two groups according to the duration of illness, namely those in an early phase of illness (≤4 years, n = 9) and those with a longer duration of illness (≥10 years, n = 15). The Global Assessment of Functioning (GAF) symptom scale was measured in all subjects [32].

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Inclusion criteria for patients were (a) being in a euthymic state for at least 2 months; (b) not fulfilling any DSM-IV-TR criteria for current mood episodes; (c) a HAM-D rating scale score < 8; (d) a YMRS score < 8; and (e) being under a stable medication treatment regimen for at least 2 months. Exclusion criteria for normal controls were any lifetime or current axis-I diagnosis and positive family history for affective disorders and suicide. Exclusion criteria for patients and controls were: (a) a current and lifetime treatment with immunomodulatory drugs including glucocorticoids; (b) use of any anti-inflammatory drugs two weeks before inclusion in this study; (c) pregnant and lactating women; and (d) medical illness including (auto)immune disorders such as psoriasis, inflammatory bowel disease, chronic kidney disease, rheumatoid arthritis, HIV infection, asthma, and neuroinflammatory disorders including Parkinson's disease and multiple sclerosis. Some of the BD patients used mood stabilizers (n = 6), lithium (n = 9), antipsychotics (n = 15), antidepressants (n = 5), and benzodiazepines (n = 9). Body mass index (BMI) was computed as body weight (in kg) divided by height (in m)^2. This study was approved by the National Commission for Ethics in Research (CONEP) and the local Institutional Research Board (no: 57938516.4.0000.5505). All participants provided written informed consent prior to their inclusion.

Assays

Blood Collection and Cell Isolation

After an overnight fast (12 h), 15 mL of blood was sampled between 08:00 and 10:00 a.m. and collected in BD Vacutainer® EDTA tube (10 mL) and BD Vacutainer® SST™ tube (5 mL) (BD Biosciences, Franklin Lakes, NJ, USA). The blood was kept at room temperature (30 min) to allow clotting and to isolate serum. Both tubes were centrifuged at 1100 ×g (4°C, 10 min) and the

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supernatant was aliquoted and kept at -80°C. Consequently, we isolated PBMCs by density gradient centrifugation (30 min at 900 ×g; Ficoll® Paque Plus, GE Healthcare Life Sciences, Pittsburgh, PA, USA). Cells were counted by means of microscopy and viability was confirmed to exceed 95% as assessed from their ability to exclude Trypan Blue (Sigma-Aldrich Corporation, St. Louis, MO, USA).

Immunophenotyping without stimulation

To examine specific lymphocyte subsets, 2 × 10 PBMCs were stained directly for 30 min with combinations of anti-CD3 FITC, -CD4 APCCy7, -CD8 BV510, -CD71 PE, -CD69 PeCy7, -CD25 BV510, and -CD152 PECy5 monoclonal antibodies (BD Biosciences). For intracellular FoxP3 staining, we used the Human FoxP3 Buffer Set (BD Biosciences) which includes cell fixation, permeabilization, and staining with the monoclonal antibody anti-FoxP3 Alexa647. Cells were analyzed by flow cytometry immediately after staining. A minimum of 50,000 lymphocytes was identified by size (forward scatter, FSC) and granularity (side scatter, SSC) and they were acquired with a FACSCantoII flow cytometer (BD Biosciences). Isotype controls for all fluorophores were used as negative controls. Data were analyzed using the FlowJo 7.2.5 software (Tree Star Inc., Ashland, OR, USA).

PBMC stimulation and activation CD marker immunophenotyping

Ninety-six wells plates were coated with 5 µg/mL anti-human CD3 antibody (OKT3, eBioscience). PBMCs (1 x 10^5 cells) were added to each well with anti-human CD28 antibody (CD28.2, eBioscience) in a final concentration of 5 µg/ml. Subsequently, PBMCs were cultivated in RPMI 1640 culture medium supplemented with L-glutamine, 10% fetal bovine serum,

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antimycotics and antibiotics (Thermo Fischer Scientific, Waltham, MA, USA). The cells were allowed to grow for 3 days at 37°C in a 5% CO_2 atmosphere. The experiments comprised a negative control (PBMC without anti-CD3 and -CD28 cultivated for 3 days). After 3 days, the same CD markers as described above were counted and stained and analyzed by flow cytometry as described above.

Human Cytomegalovirus (HCMV) serology

HCMV titers were measured in serum using commercial ELISA kits (EUROIMMUN AG, Luebeck, Germany) according to the manufacturer's instructions. Anti-HCMV IgG was used at a cut-off value of 20 UI/mL. All subjects included in this study showed negative IgM antibody titers against HCMV. All assays in all subjects were measured in the same run and on the same day in order to reduce analytical variation.

Statistics

Analysis of contingency tables (χ^2 test) was used to check associations between nominal variables and analysis of variance (ANOVA) to check differences in scale variables between diagnostic groups. Binary logistic regression analysis was used to examine the best predictors of BD as the dependent variable and controls as a reference group. The odds ratio with 95% confidence intervals (CI) were computed. We used Generalized Estimating Equation (GEE) analysis, repeated measures, to assess the effects of time (unstimulated versus stimulated), diagnosis (e.g. BD versus controls and /or IgG HCMV positive versus negative subjects) and two or three-way interactions between diagnostic groups × positive IgG HCMV antibodies while adjusting for possible effects of age and sex. Protected pairwise posthoc analyses were employed

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to examine the significant two-or three-way interaction patterns. The effects of BMI, smoking and the drug state of the patients on the frequency of the immune cell populations were examined by entering these data in the same GEE analyses. Tests were 2-tailed and a p-value of 0.05 was considered for statistical significance. All statistical analyses were performed using IBM SPSS for Windows, version 25 (IBM Corporation, Armonk, NY, USA).

The immune subset frequencies were processed in z transformations, and some Tables show the z transformations in order to evaluate the differences between the groups in standard deviations. We also computed z unit-weighted composite scores reflecting (activation of) T effector (Teff) and Treg (Treg) functions: Teff was computed as z % CD3+CD4+CD154+ + z % CD3+CD4+CD69+ (%); Treg was computed as z % CD4+CD25+FOXP3+CD152+ + z % CD4+CD25+FOXP3+GARP; and the Teff/Treg ratio computed as zTeff – zTreg.

Results

Sociodemographic data

Table 2 shows the sociodemographic data of the patients and healthy controls in this study. There were no significant differences in age, sex ratio, years of education, BMI, ethnicity, and marital status between the study groups. There were somewhat more smokers and unemployed people in the study group of BD patients. Patients with BD showed marginally increased HAM-D and YMRS scores as compared with controls, while both GAF symptom and function scores were significantly lower in patients than in controls.

Association between BD and T cell subpopulations

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In order to examine the differences in T cell subsets between BD and controls, both in baseline conditions and after *ex vivo* polyclonal stimulation, we have performed GEE analysis, which included effects of groups (diagnosis), time (basal versus stimulation), and the [time × group] interaction. The same analyses also included effects of age, sex, IgG HCMV positive versus negative samples (HCMV), and [time × HCMV] and [group × time × HMCV] interactions. We also examined the effects of BMI, smoking, the drug state (mood stabilizers, lithium, antipsychotics, antidepressants, benzodiazepines), and when significant, we adjusted the results for these variables. **Table 3** shows the results of those GEE analyses and shows the effects of time, BD (versus controls), and the [time × diagnosis] interaction. There were significant effects of time on all immune subpopulations. Significant [time × diagnosis] interactions were observed for CD3+CD4+CD69+, CD3+CD8+CD69+, CD3+CD4+CD71+, CD3+CD8+CD71+, CD4+CD25+FOXP3+CD152+, and Treg and Teff/Treg ratio. Consequently, we examined posthoc pairwise comparisons between the groups in order to explain the interactions. Thus, the basal CD3+CD4+CD69+ and CD3+CD8+CD69+ proportions were significantly lower in BD while the stimulated proportions did not differ between the groups. While there were no inter-group differences in basal CD3+CD4+CD71+ and CD3+CD8+CD71+ proportions, and the Treg score, their stimulated values were significantly lower in BD than in controls. While the proportion of baseline CD4+CD25+FOXP3+CD152+ was significantly higher in BD than in controls, their stimulated frequencies were significantly lower in BD than in controls. The stimulated Teff/Treg ratio was significantly higher in BD than in controls while its unstimulated values showed a trend towards lower values in BD.

Table 4 shows the outcome of two binary logistic regression analyses with BD as the dependent variable (controls as the reference group) and the proportions of the unstimulated or

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stimulated immune subpopulations as explanatory variables, while also allowing for effects of age and sex. The first regression analysis shows that lowered basal CD3+CD8+CD71+ and CD+CD25+FOXP3+ significantly predicted BD (χ^2 = 26.77, df = 2, p <0.001, Nagelkerke = 0.590) with an accuracy of 84.8%, a sensitivity of 84.0% and a specificity of 85.7%. The second logistic regression shows that lowered proportions of the stimulated CD3+CD8+CD71+ and CD+CD25+FOXP3+GARP proportions significantly predicted BD (χ^2 = 40.68, df = 2, p <0.001, Nagelkerke = 0.785) with an accuracy of 89.1%, a sensitivity of 88.0% and a specificity of 90.5%.

Effects of background variables

In the GEE analysis reported in **Table 2,** we always entered age and sex and did not detect significant effects of these two variables. Also, BMI and smoking did not show any significant effects. Entering the drug state of the patients in GEE analyses showed that the drug state of the patients has a significant effect on three immune subpopulations only, namely CD3+CD4+CD69+, CD3+CD8+CD69+, and CD3+CD25+FOXP3+CD152+ T cells. The **Electronic supplementary File (ESF) Table 1** shows that, after *p*-correction for false discovery rate, antidepressants, lithium, mood stabilizers, and benzodiazepines increased the proportions of the CD3+CD4+CD69+ T cell subset. After *p*-correction for false discovery rate, antidepressants showed a significant enhancing effect on the frequency of the CD3+CD8+CD69+ T cell population. Antidepressants increased the proportion of CD3+CD25+FOXP3+CD152+ T cells, while mood stabilizers, lithium and benzodiazepines significantly lowered the proportion of this subset. All data in **Table 3** concerning those three subsets were adjusted for those drug effects.

Association between the staging of BD and immune subsets

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Table 5 shows the results of GEE analyses, which considered the same variables as described in **Table 2** except that we considered three subgroups, namely normal controls and patients divided into those in an early and a late stage. In 4 different immune subpopulations, we found significant [time × group] interactions with significantly different patterns between the two staging groups. Thus, both the unstimulated and stimulated CD3+CD4+CD154+ T cell proportions were significantly lower in late-stage BP, as compared with controls and early-stage BD. The unstimulated CD3+CD4+CD71+ proportions were significantly lower in late-stage BD than in controls. The stimulated frequencies of CD3+CD8+CD71+ and CD4+2 CD25+FOXP3+CD152+ subpopulations were significantly different between the three study groups with the lowest values in late-stage BD.

Consequently, we have computed Spearman's rank-order correlation coefficients between the stimulated T cell proportions and the number of episodes. **Table 6** shows that the proportions of CD3+CD4+CD154+, CD3+CD4+CD69+, CD3+CD8+CD69+, CD3+CD4+CD71+, and CD3+CD8+CD71+ were significantly and negatively associated with the total number of episodes, as well as with the number of mania episodes, but not with the number of hypomanic or depressive episodes.

Effects of HCMV IgG seropositivity

ESF Table 2 shows the differences in the T cell subsets between subjects with and without positive IgG antibodies to HCMV. These results were derived from the GEE analyses, repeated measures, shown in **Table 2** which also considered the effects of HCMV groups and [time × HCMV] groups. We also examined the [time × diagnostic group × HCMV group] interaction and found a significant three-way interaction for two T cell subsets. These are shown in **ESF Table 3**.

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ESF Table 2 shows that the stimulated proportions of CD3+CD4+CD69+, CD3+CD8+CD69+, CD3+CD4+FOXP3+GARP, and Teff/Treg ratio, and the basal proportion of CD3+CD4+FOXP3+GARP were significantly lower in patients with increased IgG to HCMV than those with negative antibody levels. In contrast, the proportions of stimulated CD3+CD4+FOFP3+CD152+ and basal Teff/Treg ratio were higher in subjects who showed increased IgG HCMV antibodies as compared with those without.

ESF Table 3 shows the significant three-way interactions, namely for CD4+CD25+FOXP3+ and CD4+CD25+FOXP3+GARP. In IgG HCMV positive subjects the stimulated CD4+CD25+FOXP3+ proportion was significantly higher in BD patients than in controls, whereas in HCMV negative subjects there was a trend towards a lowered proportion in BD than in controls. In HCMV positive subjects the stimulated CD4+CD25+FOXP3+GARP proportion was significantly lower in BD patients than in controls, whereas in HCMV negative subjects there was a trend towards a higher proportion in BD patients than in controls.

Discussion

The first major finding of this study is that BD is characterized by a lowered baseline frequency of CD3+CD4+CD69+, CD3+CD8+CD69+, CD3+CD8+CD71+, and CD4+CD25+FOXP3 T lymphocytes and that the unstimulated frequency of CD4+CD25+FOXP3+CD152+ is increased in BD. These findings indicate that in baseline conditions there is a disbalance in T lymphocyte functions with a lowered expression of early (CD69+) and later (CD71+ and CD25+) [15] activation markers, while the proportion of activated CD152+ Treg cells is increased. The comparison of these data with previous results is limited because only a few papers examined CD markers in mood disorders [22-25]. Early findings in the

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acute phase of a major depressive episode and melancholia showed increased expression of T lymphocyte activation markers including CD4+CD45RA (T memory), CD25+ and HLA-DR+ (very late activation marker) cells [33,34]. Moreover, both unipolar depression and melancholia are qualitatively distinct classes (using machine learning techniques) with respect to those T cell activation markers as compared with minor depression and healthy controls [34,35]. The acute phases of BD, both depression and mania, are also accompanied by signs of T cell activation as indicated by increased levels of sIL-2R (Maes et al., 1995b) and by indicants of M1, Th-1, Th-2 and Treg activation [8]. Nevertheless, in the present study, we included BD type 1 patients who were in the euthymic phase of the illness. As such, it appears that the acute phase of BD is accompanied by T cell activation, whereas the euthymic phase is characterized by inhibition of proliferation (CD71+) and immune induction (CD69+) coupled with activation of some Treg phenotypes.

The second major finding of this study is that there are highly significant deficits in the expression of polyclonal-stimulated CD152+ and GARP+ Treg cells in BD patients coupled with the depletion of CD71+ bearing CD4+ and CD8+ cells. In the present study, T lymphocytes were activated with CD3/CD28 antibodies and, therefore, the results reflect the *in vivo* immune response following immune injury. As such, our results suggest that following infections or other injuries, BD patients display marked impairments in both T cell proliferation and Treg functions as compared with controls.

In the 1980s there were some studies that major depression is accompanied by lowered mitogen-induced T lymphocyte proliferative responses [36], although, to the best of our knowledge, no such results were reported in euthymic BD patients. Our results may suggest that deficits in polyclonal-stimulated lymphoproliferative responses could be explained by deficits in

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the uptake of the iron-transferrin complex and transport of iron into proliferating cells [15]. It is noteworthy that plasma levels of sTfR are significantly increased during the acute phase of BD, either in depression or mania [16,17]. Increased plasma levels of sTfR not only reflect alterations in iron status, but also immune activation including IL-2-related mechanisms [37]. Thus, it appears that the increased shedding of TfR during the acute phase of BD [16] is accompanied by longer-lasting deficits in the expression of surface CD71+ and, therefore, by deficits in T cell proliferation. Since CD71-related mechanisms positively mediate T and B cell functions, deficits in CD71 surface expression may at least in part underpin the impairments in T lymphocyte proliferation.

Treg cells play a key role in the resolution of inflammation and immune homeostasis, thereby preventing excessive inflammation and autoimmune responses, while deficits in Treg functions may impair healing and tissue remodeling [18,19,21. Treg cells mediate and regulate regeneration via different mechanisms including inhibition of M1 macrophages and increased Teff activities [38]. Moreover, Treg cells play a key role in tissue repair by interacting with neutrophils and macrophages and they are additionally involved in repair mechanisms in the central nervous system by promoting remyelination and differentiation of oligodendrocyte progenitor cells [38]. Treg cells have also neuroprotective effects and depletion of Treg cells may induce neurocognitive deficits [39] and may be accompanied by increased infiltration of T cells into the brain as well as reactive astrogliosis [40].

By inference, our unstimulated flow cytometric results may indicate that the euthymic phase of BD is characterized by increased CIRS signaling and that, consequently, IRS signaling pathways are more adequately suppressed. Moreover, it should be underscored that the euthymic phase of BD type 1 and a major depressive episode is characterized by increased toxicity due to oxidative and nitrosative stress, as indicated by hypernitrosylation and increased levels of

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malondialdehyde and advanced oxidation protein products, as well as by signs of increased bacterial translocation [41-43]. Therefore, it is safe to posit that the euthymic phase of BD is accompanied by enhanced CIRS signaling, which attempts to repair the damage by nitro-oxidative and immune-inflammatory stressors.

Our stimulated flow cytometric results reveal that, following immune injuries, patients with BD will exhibit a strongly suppressed regulatory CIRS signaling leading to greater activation of positive IRS signaling through M1, Th-1 and Th-2-like cytokine production and Teff cell differentiation. Such mechanisms could have dire effects during the acute phase of BD, because lowered Treg activities may lead to less resilience against host-commensal interactions, enhanced IRS and autoimmune responses, metabolic inflammation and increased damage by oxidative and nitrosative stress pathways [8,18,19,21,38,44,45]. As such, the deficits in Treg functions in BD may play a key role in the immune pathophysiology of BD.

The third major finding of this study is that staging of illness, either duration of illness or number of episodes, has a highly significant suppressant effect on the expression of activated Teff (CD154+ and CD69+) and Treg (CD152+) surface molecules, as well as the proportion of CD71+ cells, indicating that staging of illness is accompanied by deficits in Treg and Teff functions and lymphoproliferative mechanisms. As described in the introduction, increased staging in BD (i.e. higher numbers of suicide attempts and mood disorder episodes prior to blood sampling) is associated with increased levels of sTNFR80, sIL-1RA, TBARS and a composite score comprising assessments of superoxide dismutase, lipid peroxidation, nitric oxide metabolites and advanced oxidation protein products (AOPP) and lowered levels of paraoxonase 1 (PON1) enzymatic activity [7,11]. Other results show that immune-inflammatory responses are more pronounced in the later stages of the illness [12,14]. Furthermore, in major depression, the number of prior

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depressive episodes is accompanied by increased levels of plasma IL-1, sIL-1RA, IL-6, TNF-α and neopterin [46-48], suggesting that staging of illness is characterized by M1 and Th-1 activation. Previously, the relationship between staging and immune pathways was explained [46,47] by the knowledge that pro-inflammatory signals including IFN-γ and LPS may modulate or even induce sensitization in the immune system [49-51]. While this is a plausible mechanism, the results of the present study indicate that also staging-associated gradual deficits in Treg functions may underpin the greater immune-inflammatory and oxidative stress responses during the acute phase of the illness. Nevertheless, in late-stage BD, there are also profound deficits in the stimulated CD71+ expression indicating that the CIRS, as well as lymphoproliferative responses, are grossly compromised in BD possibly leading to dire consequences when facing immune injuries.

It is also important to note that manic (and not the hypomanic or depressive) episodes of BD account for the impact of the number of episodes on the Teff and Treg surface markers. This is interesting because there are highly significant differences between BD type 1 versus type 2 in that oxidative damage to lipids and proteins and upregulation of the nitrosylome are present in type 1 but not type 2 BD [41,42], and that patients with type 1 BD show higher IgA mediated responses to Gram-negative bacteria as compared with major depressed subjects [43]. Therefore, one hypothesis is that these pathways may play a role in the downregulation of Teff and Treg surface markers. Another possibility is that increased T cell exhaustion coupled with increased induction of cell death pathways including TIM-3 and PD-1 expression [52], following repeated activation with increased levels of pro-inflammatory cytokines or chronic antigen exposure (e.g. LPS from Gram-negative bacteria) are involved.

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The fourth major finding of this study is that Teff and Treg surface markers were modified by HCMV seropositivity and the use of psychotropic drugs. We found that IgG antibodies to HCMV were somewhat albeit significantly higher in BD patients than in controls, findings that are in agreement with those of Prossin et al. [27]. Infected persons will continuously harbor HCMV, which will coexist lifelong with the host through latency or chronic virus infection [28,29]. Nevertheless, all patients included in this study showed negative IgM responses to HCMV suggesting that patients with increased IgG to HCMV underwent past exposure and show HCMV latency rather than active or reactivated infections. Those subjects maintain large proportions of HCMV T cells while T cell-related surveillance plays a key role in latency [28,29]. Likewise, persistent HCVM infection may alter the expression of different T cell surface molecules as reviewed in the introduction [28,29]. However, in the current study, we detected that HCMV seropositivity is accompanied by a lowered stimulated expression of the CD69+ marker on CD4+ and CD8+ cells and an increased frequency of CD4+CD25+FOXP3+CD152+ cells, suggesting that HCMV latency may interfere with the balance between the Treg and Teff activation ratio. We also detected that HCMV seropositivity may aggravate the depleted proportion of CD4+CD25+FOXP3+GARP Treg cells in BD. GARP or LRRC32, a leucine-rich repeat molecule that is co-expressed with latent TGF-beta in platelets and activated Treg membrane surfaces, is critical for tethering TGF-beta to the cell surface [53]. Thus, HCMV seropositivity and BD interact to induce a lowered expression of the GARP surface marker on activated Treg cells, suggesting that the combination of both conditions is accompanied by a more profound loss of inhibitory signals mediated by TGF-β [18,53]. As such, HCMV latency could contribute to an immune-risk phenotype associated with BD.

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The current study also showed that psychotropic drugs impact selected T cell phenotypes, namely CD69+ bearing CD4+ and CD8+ T cells and CD152+ activated Treg cells. Thus, all psychotropic drugs significantly enhanced expression of CD69+ surface molecule, while antidepressants increase the frequency of CD3+CD25+FOXP3+CD152+ cells. Lithium, antipsychotics, and benzodiazepines, on the other hand, attenuated the frequency of the CD152+ Treg cells and, therefore, these psychotropic drugs may further inhibit Treg functions in BD patients. Previously, it was found that lithium may enhance the stimulated production of TNF-α and IFN-γ and also sIL-1RA and IL-10 mediated Treg activities [54]. On the other hand, antidepressants could have more beneficial effects because they appear to enhance the proportion of CD152+ bearing Treg cells, which are known to downregulate immune responses [19,55]. Previously it was shown that antidepressants of different classes have immune-regulatory effects by increasing the production of IL-10 [8].

Conclusions

In conclusion, the euthymic phase of BD is characterized by a suppression of early activation markers and proliferative capacity of Teff cells coupled with activation of Treg cells. Polyclonal stimulation shows that Treg functions will be suppressed in BD patients following immune injuries probably leading to lowered resilience against host-commensal interactions, (auto)immune responses, and nitro-oxidative stress. Moreover, the staging of illness, either duration of illness (≥ 10 years) or an increasing number of manic episodes, is accompanied by impairments in Teff and Treg cells. The presence of latent HCMV infections may contribute to the pathophysiology of BD by depleting the frequency of activated GARP bearing Treg cells. These multiple disorders in adaptive immunity in BD may play a role in the pathophysiology of this

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disorder through changes in the production of IRS and CIRS cytokines or direct cell-cell interactions within the CNS.

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Conflicts of interest

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Authorships

All the contributing authors have participated in the manuscript. All authors contributed to the interpretation of the data and writing of the manuscript. All authors approved the final version of the manuscript.

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Table 1 Description of the immune CD markers and T cell phenotypes examined in the present study.

Immune subsets (CD markers)	Alternative name(s)	Ligands	Marker and functional characterization
CD3+	T3, T cell marker	TCR	Mediates T cell signal transduction
CD4+	T4, T cell marker	MHC Class II	T helper/inducers
CD8+	Leu2, T cell marker	MHCI	Suppressors/killers; T cell-mediated killing
CD25+	Tac antigen IL-2R	Interleukin-2	Receptor for interleukin-2, activation marker
CD69+	AIM, EA		Early activation marker expressed on T effector cells, mediating lymphocyte proliferation and natural killer cell signal transmission
CD3+CD4+CD69+			CD69+ coexpressing CD3 CD4 cells, adaptive immune cells
CD3+CD8+CD69+			CD69+ coexpressing CD3 CD8 cells
CD71+	Transferrin receptor	Transferrin	Uptake of the iron-transferrin complex and transport of iron into proliferating cells; positively regulates T and B cell functions
CD3+CD4+CD71+			Indicates replicating/proliferating CD3+CD4+ cells
CD3+CD8+CD71+			Indicates replicating/proliferating CD3+CD8+ cells
CD154+	CD40L (member of the tumor necrosis factor family)		T effector cell activation marker mediating B cell proliferation and immunoglobulin switching
CD3+CD4+CD154+			Antigen-specific activated CD3+CD4+ T cells

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FOXP3	forkhead box 3 or scurfin		Key regulatory component of Treg cell development
CD4+CD25+FOXP3+			T reluatory cells important for maintaining immune tolerance
CD152+	CTLA-4 or cytotoxic T-lymphocyte-associated protein 4	Cd80, cd86	Activated T cells T cell inhibition, downregulation of immune responses
CD4+CD25+FOXP3+CD152+			Activated T regulatory cells
GARP	glycoprotein A repetitions predominant		Maintaining regulatory functions through secretion of Transforming growth factor-β (TGF-β), mediate inhibitory signals
CD4+CD25+FOXP3+GARP			Activated T regulatory cells releasing TGF-β

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Table 2: Demographic, clinical and biomarker data of healthy controls (HC) and bipolar disorder (BD) participants in the present study

Variables	HC [A] (n=21)	BD [B] (n=25)	F/X^2	df	p
Age (years)	36.1 (13.1)	41.5 (12.4)	2.01	1/44	0.164
Sex (Female/Male)	7/14	7/18	0.15	1	0.695
BMI (kg/m^2)	26.4 (4.9)	28.9 (5.7) [C]	2.36	1/38	0.133
Smoking (No/Yes)	1/20	8/17		-	0.020
Employment (No/Yes)	4/17	13/12	5.32	1	0.021
Education (years)	12.3 (3.4)	10.6 (3.7)	2.68	1/43	0.109
Married (No/Yes)	9/12	10/15	0.04	1	0.845
Caucasian (No/Yes)	7/14	14/11	0.55	1	0.460
HAM-D	1.5 (2.0)	3.1 (2.9)	4.20	1/43	0.047
YMRS	0.2 (0.8)	1.33 (1.93)	5.95	1/43	0.019
GAF-S	86.9 (7.5)	77.4 (10.9)	11.99	1/41	<0.001
GAF-F	86.9 (4.0)	75.0 (11.5)	19.99	1/41	<0.001

Results are shown as mean (SD).
BMI: body mass index; HAM-D: Hamilton Depression rating Scale; YMRS: Young mania Rating Scale; GAF: Global Assessment of Functioning (GAF) symptom (GAF-S) and functioning (GAF-F) scales

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Table 3: Results of generalized estimating equation (GEE) analysis, repeated measures, with activated T cell subsets as dependent variables. Here we examine the differences in unstimulated (basal) and stimulated proportions of immune subpopulations between healthy controls (HC) and bipolar depressed patients (BD)

T cell subsets	Time	Mean values in % (SE)		Time		Time X group		Group	
		HC [A]	BD [B]	Wald	p	Wald	p	Wald	p
CD3+CD4+CD154+ (%)	Basal Stimulation	0.01 (0.09) 7.57 (0.77)	0.03 (0.21) 6.38 (1.21)	108.07	<0.001	0.71	0.401	0.61	0.433
CD3+CD8+CD154+ (%)	Basal Stimulation	0.04 (0.02) 1.34 (0.12)	0.07 (0.05) 1.47 (0.19)	149.84	<0.001	0.18	0.668	0.52	0.473
CD3+CD4+CD69+ (%)*	Basal Stimulation	17.96 (3.80) [B] 66.78 (4.51)	11.35 (1.86) [A] 72.58 (1.66)	1122.87	<0.001	14.39	<0.001	0.01	0.91
CD3+CD8+CD69+ (%)*	Basal Stimulation	18.33 (12.04) [B] 65.50 (5.81)	10.46 (1.14) [A] 73.75 (2.03)	1057.14	<0.001	20.40	<0.001	0.04	0.834
CD3+CD4+CD71+ (%)	Basal Stimulation	3.05 (0.50) 87.79 (1.92) [B]	1.52 (0.92) 49.39 (5.18) [A]	656.45	<0.001	49.45	<0.001	44.14	<0.001
CD3+CD8+CD71+ (%)	Basal Stimulation	1.27 (0.53) 83.16 (2.25) [B]	0.17 (0.86) 47.65 (4.74) [A]	660.83	<0.001	43.89	<0.001	43.38	<0.001
CD4+CD25+FOXP3+ (%)	Basal Stimulation	1.06 (0.12) 17.62 (3.65)	0.60 (0.05) 22.58 (3.51)	50.46	<0.001	1.41	0.235	0.47	0.494
CD4+CD25+FOXP3+CD152+ (%)*	Basal Stimulation	1.96 (0.28) [B] 39.35 (4.66) [B]	5.33 (1.21) [A] 18.56 (2.51) [A]	138.97	<0.001	36.47	<0.001	2.63	0.105

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CD4+CD25+FOXP3+GARP (%)	Basal Stimulation	6.35 (1.84) 13.82 (2.18)	10.43 (2.11) 13.94 (1.83)	14.89	<0.001	1.92	0.166	0.70	0.402
Teff (z scores)	Basal Stimulation	-0.90 (0.25) 0.85 (0.12)	-0.91 (0.04) 0.93 (0.15)	484.39	<0.001	0.30	0.584	0.11	0.741
Treg (z scores)	Basal Stimulation	-0.75 (0.12) 1.10 (0.18) [B]	-0.38 (0.21) 0.28 (0.14) [A]	104.72	<0.001	19.91	<0.001	1.32	0.250
Teff/Treg (z scores)	Basal Stimulation	0.09 (0.19) -0.41 (0.31) [B]	-0.47 (0.20) 0.29 (1.90) [A]	0.20	0.651	17.43	<0.001	0.202	0.653

All results are shown as estimated marginal mean (SE) values obtained after GEE analysis, repeated measures. Shown are the effects of time (basal versus stimulation), time X group (BD versus HC) and group differences, while adjusting for the effects of age and sex.

*These data are adjusted for the drug state of the patients. No effects of the drug state were established for the other subsets.

Teff: index of T effector functions, computed as z % CD3+CD4+CD154+ + z % CD3+CD4+CD69+ (%)

Treg: index of T regulatory functions, computed as z % CD4+CD25+FOXP3+CD152+ + z CD4+CD25+FOXP3+GARP

Teff/Treg: computed as z (aTeffect – aTreg)

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Table 4: Results of two different binary logistic regression analyses with the diagnosis of bipolar disorder as dependent variable (and controls as reference group)

Time	Explanatory Varriables	B	SE	Wald	df	p	OR	95% CI
Unstimulated	**CD3+CD8+CD71+**	-1.04	0.34	9.20	1	0.002	0.36	0.18-0.69
	CD4+CD25+FOXP3+	-4.84	1.46	11.07	1	0.001	0.01	0.00-0.14
Stimulated	**CD3+CD8+CD71+**	-0.208	0.068	9.26	1	0.002	0.81	0.71-0.93
	CD3+CD25+FOXP3+GARP	-0.228	0.106	4.63	1	0.032	0.80	0.65-0.98

This table shows the results of 2 different binary logistic regression analysis. In the first we entered the unstimulated immune subpopulation frequencies and in the second the stimulated.
OR: Odds ratio; 95% CI: 95% confidence intervals.

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Table 5: Results of generalized estimating equation (GEE) analysis, repeated measures, with activated T cell subsets as dependent variables. Here we examine the differences in immune subpopulations between three diagnostic groups, namely healthy controls (HC), and early and late stage bipolar disorder (BD).

T cell subsets	Time	Mean values in % (SE)			Time X group		Group	
		HC [A]	Early BD (≤3 years) [B]	Late BD (≥ 10 years) [C]	Wald	p	Wald	p
CD3+CD4+CD154+ (z scores)	Basal Stimulation	-0.70 (0.58) [C] 0.86 (0.15) [C]	-0.58 (0.09) [C] 0.63 (1.21) [C]	-0.95 (0.09) [A,B] 0.20 (0.23) [A,B]	1.83	0.401	13.94	0.001
CD3+CD4+CD71+ (z scores)	Basal Stimulation	-0.85 (0.02) [C] 1.41 (0.05) [B,C]	-0.87 (0.03) 0.78 (0.15) [A]	-0.95 (0.05) [A] 0.09 (0.14) [A,B]	72.26	<0.001	89.30	<0.001
CD4+CD8+CD71+ (z scores)	Basal Stimulation	-0.86 (0.02) 1.38 (0.06) [B,C]	-0.87 (0.03) 0.78 (0.15) [A,C]	-0.95 (0.05) 0.09 (0.14) [A,B]	51.12	<0.001	68.35	<0.001
CD4+CD25+FOXP3+CD152+ (z scores)*	Basal Stimulation	-0.82 (0.03) 1.42 (0.17) [B,C]	0.51 (0.15) 0.41 (0.15) [A,C]	-0.65 (0.11) -0.13 (0.15) [A,B]	45.81	<0.001	26.55	<0.001

All results are shown as estimated marginal mean (SE) values obtained after GEE analysis, repeated measures. Shown are the effects of time (basal versus stimulation), time X group (early BD versus late BD versus HC) and group differences, while adjusting for the effects of age and sex.

*These data are adjusted for the drug state of the patients. No effects of the drug state were established for the other subsets.

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Table 6. Spearman's rank order correlation coefficient between the frequencies of stimulated T cell subpopulations and the number of previous episodes in 25 patients with bipolar disorder

T cell subsets	Total number of episodes	Manic episodes	Hypomanic episodes	Depressive episodes
CD3+CD4+CD154+	-0.511 (0.011)	-0.450 (0.027)	0.006 (0.977)	-0.370 (0.082)
CD3+CD4+CD69+	-0.493 (0.014)	-0.548 (0.006)	-0.122 (0.565)	-0.274 (0.206)
CD3+CD8+CD69+	-0.569 (0.004)	-0.648 (0.001)	-0.140 (0.515)	-0.404 (0.056)
CD3+CD4+CD71+	-0.549 (0.005)	-0.422 (0.040)	-0.056 (0.794)	-0.399 (0.059)
CD3+CD8+CD71+	-0.510 (0.010)	-0.441 (0.031)	0.048 (0.825)	-0.397 (0.061)

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Electronic Supplementary File (ESF)

Impairments in the expression of peripheral blood activated T effector and T regulatory CD markers in bipolar disorder are associated with staging of illness and IgG Cytomegalovirus positivity.

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ESF Table 1. Effects of psychotropic drugs on the frequency of immune cell populations

Phenotypes (%)	Treatment drugs	No use drugs (%)	Use (%)	Wald (df=1)	p-value
CD3+CD4+CD69+	**Antidepressants**	42.0 (2.2)	49.53 (3.4)	5.71	0.017
	Lithium	41.4 (1.2)	50.2 3.3	17.22	<0.001
	Mood stabilizers	42.0 (1.3)	49.5 (3.5)	10.07	0.002
	Benzodiazpines	43.2 (1.4)	48.4 (3.1)	5.10	0.024
CD3+CD8+CD69+	**Antidepressants**	41.8 (2.7)	51.7 (3.2)	17.82	<0.001
CD4+CD25+FOXP3+CD152+	**Antidepressants**	12.1 (2.0)	18.4 (3.3)	7.42	0.006
	Lithium	18.6 (1.7)	11.9 (3.5)	6.32	0.012
	Mood stabilizers	18.3 (2.0)	12.2 (3.5)	4.52	0.003
	Benzodiazepines	19.2 (1.9)	11.3 (3.3)	12.32	<0.001

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ESF Table 2 Results of generalized estimating equation (GEE) analysis, repeated measures, with activated T cell subsets as dependent variables while examining the differences between subjects with and without human cytomegalovirus (HCMV) IgG seropositivity.

T cell subsets	Time	HCMV IgG		Time X HCMV		HCMV Groups	
		HCMV negative [A]	HCMB positive [B]	Wald	p	Wald	p
CD3+CD4+69+ (z scores)	Basal Stimulation	-0.78 (0.10) 1.27 (0.12) [B]	-0.75 (0.08) 0.94 (0.09) [A]	10.62	0.001	3.27	0.070
CD3+CD8+CD69+ (z scores)	Basal Stimulation	-0.86 (0.12) 1.16 (0.14) [B]	-0.78 (0.11) 0.90 (0.13) [A]	9.26	0.002	0.88	0.348
CD4+CD25+FOXP3+CD152+ (z scores)*	Basal Stimulation	-0.78 (0.16) 0.35 (0.20) [B]	-0.89 (0.14) 0.87 (0.17) [A]	5.59	0.018	3.73	0.054
CD4+CD25+FOXP3+GARP (z scores)	Basal Stimulation	0.13 (0.23) [B] 0.48 (0.23) [B]	-0.51 (0.07) [A] 0.22 (0.15) [A]	2.06	0.151	3.94	0.047
Teff/Treg	Basal Stimulation	-0.55 (0.28) [B] 0.03 (0.22) [B]	0.06 (0.12) [A] -1.45 (0.17) [A]	6.56	0.010	0.86	0.353

All results are shown as estimated marginal mean (SE) values of the z transformed data obtained after GEE analysis, repeated measures. Shown are the effects of HCMV groups (that is subjects with positive versus negative HCMV IgG antibodies) and HCMV groups X time (that is unstimulated versus stimulated condition) interactions while adjusting for the effects of diagnosis (bipolar disorder versus controls), age and sex.

*These data are adjusted for the drug state of the patients. No effects of the drug state were established for the other subsets.

Teff: index of T effector functions, computed as z % CD3+CD4+CD154+ + z % CD3+CD4+CD69+ (%)

Treg: index of T regulatory functions, computed as z % CD4+CD25+FOXP3+CD152+ + z CD4+CD25+FOXP3+GARP

Teff/Treg: computed as z (Teff – Treg)

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ESF Table 3. Significant three-way interactions among time (unstimulated versus stimulated condition), and two groups, namely bipolar disorder (BD) versus healthy controls (HC), and groups divided according to IgG human cytomegalovirus (IgG HCMV) seropositivity.

Group	IgG HCMV	Time	CD3+CD25+FOXP3+	CD4+CD25+FOXP3+GARP
HC	**Negative**	**Unstimulated**	0.58 (0.03)	-0.02 (0.32)
		Stimulated	0.66 (0.29)	0.21 (0.33)
	Positive	**Unstimulated**	-0.55 (0.02)	-0.78 (0.06)
		Stimulated	-0.05 (0.25)	0.57 (0.23)
BD	**Negative**	**Unstimulated**	-0.63 (0.06)	0.10 (0.34)
		Stimulated	-0.00 (0.21)	0.92 (0.27)
	Positive	**Unstimulated**	-0.59 (0.02)	-0.38 (0.10)
		Stimulated	0.99 (0.29)	0.01 (0.18)
Wald, df, p for the three-way interaction			**Wald=10.58, df=1, p=0.001**	**Wald=13.35, df=1, p<0.001**

All results are shown as estimated marginal mean (SE) values after z transformations and obtained after GEE analysis, repeated measures. Shown are the effects of time X diagnostic groups (BD versus controls) x IgG HCMV groups while adjusting for the effects of age and sex.