CHAPTER 2

Relationship between clinical features and inflammation related monocyte gene expression in bipolar disorder

Towards a better understanding of psychoimmunological interactions
Abstract

Objectives
Existing and previously published datasets were examined for associations between illness and treatment characteristics and monocyte pro-inflammatory gene expression in bipolar disorder (BD) patients. We a priori hypothesized that increased monocyte pro-inflammatory gene expression would be found more frequently in patients with a lifetime history of psychotic symptoms.

Methods
Monocyte QPCR and symptom data of 64 BD patients were collected from three Dutch studies. Regression analyses were performed to analyze the various associations of which feature-expression heat maps were drawn.

Results
Symptoms
No associations were found with lifetime psychotic symptoms, while a positive association was identified between sub-cluster 2 genes and manic symptoms.

Age at onset / duration of illness
For several sub-cluster 1a genes a negative association was found with age at onset. For most sub-cluster 2 genes a positive association was found with the duration of illness.

Medication
Current use of antidepressants and of anti-epileptics were associated with sub-cluster 2 gene expression, and current use of lithium and antipsychotics with sub-cluster 1a gene expression.

Conclusions
Our hypothesis that lifetime psychotic features would be associated with the pro-inflammatory monocyte gene expression was not confirmed. In an explorative analysis we found: (1) a possible relation between pro-inflammatory gene expression and manic symptomatology, (2) a differential immune activation related to age at onset and duration of illness, and (3) support for the concept of an immune suppressive action of some of the mood regulating medications.
Introduction

The pathophysiology of bipolar disorder (BD) is complex. While there is no doubt that both genetic predisposition and environmental factors play a role, it remains important to further investigate their neurobiological underpinnings and their interaction. As both the stress system and the immune system interact with the brain and are influenced by the environment, they can be regarded as a linking pin. The “monocyte-T-cell theory of mood disorders” considers an activated inflammatory response system (IRS) in mood disorders as a driving force behind the illness. IRS activation can be regarded as a disbalance in immune regulatory processes. Pro-inflammatory cytokines are capable of destabilizing brain function, which makes the brain vulnerable to stress and possibly other yet unknown factors with mood disturbances as the consequence.

Padmos et al. described a sensitive quantitative polymerase chain reaction (Q-PCR) assay system to detect the pro-inflammatory state of circulating monocytes of naturally treated patients with BD and detected in the monocytes a coherent, mutually correlating set of 19 aberrantly expressed inflammatory genes (‘a pro-inflammatory signature‘), supporting the concept of an activated IRS in mood disorders. We expect the IRS to be an intermediate process between genotype and phenotype (figure 1). The pro-inflammatory signature occurred in 55% of BD patients versus 18% in healthy controls.

![FIGURE 1](image_url)

**FIGURE 1**
**Schematic presentation of the monocyte-T-cell theory of mood disorders in BD**
In a subsequent study, Drexhage et al. found nine inflammation-related schizophrenia (SZ) genes using the same method, indicating that monocytes of SZ and BD patients partly overlap, but also differ in inflammatory gene expression. Combined with six specific auto-immune genes, related to BD, this resulted in a set of 34 genes (table 1). Moreover, via cluster analysis they identified the expression of sub-clusters 1a and 1b within the signature genes, relating to core inflammation and transcription, and sub-cluster 2, relating to adhesion, motility, and chemotaxis (figure 2).

Several of these 34 genes were also investigated in other studies in BD. Savitz et al. recently demonstrated an increased expression of TNF and eleven other genes in monocytes of a combined sample of depressed BD patients and major depressive disorder patients compared to healthy controls. On the genomic level CCL2 was found to be weakly overexpressed in BD patients in a Nordic genome-wide association study. PDE4B, IL6, IL1, TNF, EGR3, CCL2, and FABP were investigated in gene polymorphism studies.

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Name of corresponding protein</th>
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<tr>
<td><strong>Inflammation</strong></td>
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</tr>
<tr>
<td>DUSP2</td>
<td>Dual specificity protein phosphatase 2</td>
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<tr>
<td>ATF3</td>
<td>Cyclic AMP-dependent transcription factor 3</td>
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<tr>
<td>PDE4B</td>
<td>cAMP-specific 3’,5’-cyclic phosphodiesterase 4B</td>
</tr>
<tr>
<td>IL6</td>
<td>Interleukin 6</td>
</tr>
<tr>
<td>IL1</td>
<td>Interleukin 1</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>TNFAIP3</td>
<td>Tumor necrosis factor, alpha-induced protein 3</td>
</tr>
<tr>
<td>BCL2A1</td>
<td>B-cell lymphoma-2-related protein A1</td>
</tr>
<tr>
<td>PTX3</td>
<td>Pentraxin-related protein 3</td>
</tr>
<tr>
<td>PTGS</td>
<td>Prostaglandin G/H synthase (cyclooxygenase)</td>
</tr>
<tr>
<td>CCL20</td>
<td>C-C chemokine ligand 20</td>
</tr>
<tr>
<td>CXCL2</td>
<td>C-X-C chemokine ligand 2</td>
</tr>
<tr>
<td>EREG</td>
<td>Epiregulin</td>
</tr>
<tr>
<td>CXCL3</td>
<td>C-X-C chemokine ligand 3</td>
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<tr>
<td><strong>Transcription</strong></td>
<td></td>
</tr>
<tr>
<td>MXD</td>
<td>MAD protein</td>
</tr>
<tr>
<td>EGR3</td>
<td>Early growth response protein 3</td>
</tr>
<tr>
<td>F3</td>
<td>Tissue factor 3</td>
</tr>
<tr>
<td>MAFF</td>
<td>Musculoaponeurotic fibrosarcoma oncogene homolog F</td>
</tr>
<tr>
<td>THBS</td>
<td>Thrombospondin 1</td>
</tr>
<tr>
<td>SERPINB2</td>
<td>Plasminogen activator inhibitor-2</td>
</tr>
<tr>
<td>RGC32</td>
<td>Response gene to complement 32 protein</td>
</tr>
<tr>
<td><strong>Adhesion / motility / chemotaxis</strong></td>
<td></td>
</tr>
<tr>
<td>PTPN</td>
<td>Protein tyrosine phosphatase, non-receptor type 7</td>
</tr>
<tr>
<td>NAB2</td>
<td>Nerve growth factor-induced protein A binding protein 2</td>
</tr>
<tr>
<td>MAPK6</td>
<td>Mitogen-activated protein kinase 6</td>
</tr>
<tr>
<td>EMP1</td>
<td>Epithelial membrane protein 1</td>
</tr>
<tr>
<td>STX1</td>
<td>Syntaxin-1A</td>
</tr>
<tr>
<td>DHRS3</td>
<td>Short-chain dehydrogenase/reductase 3</td>
</tr>
<tr>
<td>CCL2</td>
<td>C-C chemokine ligand 2</td>
</tr>
<tr>
<td>CCL7</td>
<td>C-C chemokine ligand 7</td>
</tr>
<tr>
<td>CDC42</td>
<td>Cell division control protein 42 homolog</td>
</tr>
<tr>
<td><strong>Other</strong></td>
<td></td>
</tr>
<tr>
<td>FABP</td>
<td>Fatty acid-binding protein 5</td>
</tr>
<tr>
<td>CD9</td>
<td>Cluster of differentiation 9 antigen</td>
</tr>
<tr>
<td>HSPA1</td>
<td>Heat shock 70 kDa protein 1</td>
</tr>
<tr>
<td>CCR2</td>
<td>C-C chemokine receptor type 2</td>
</tr>
</tbody>
</table>
Although in the studies by Padmos and Drexhage the BD patients as a group were characterized by a positive pro-inflammatory signature, a proportion of BD patients did not show a positive signature. This led to the subsequent question whether specific clinical characteristics in BD patients would be associated with the pro-inflammatory gene expression. In view of the aforementioned overlap of pro-inflammatory gene expression between BD and SZ we a priori hypothesized that the pro-inflammatory gene expression would be found more frequently in BD patients with a lifetime history of psychotic symptoms.

Secondly, we explored the associations with the individual manic and depressive symptoms of BD, extending on previous findings relating the mood state to specific...
pro-inflammatory gene expression changes⁴.

Thirdly, we elucidated the course of the pro-inflammatory gene expression by investigating the relation between the pro-inflammatory gene expression and age at onset and duration of illness in more detail. We presumed that patients with an earlier age at onset would have a biologically more severe form of the illness, therefore demonstrating an increased pro-inflammatory gene expression compared to patients with a later age at onset. Furthermore, we expected the pathogenic processes to cause progressing derailment of the illness, demonstrating a positive association between pro-inflammatory gene expression and duration of illness irrespective of age at onset.

Finally, we examined the association between current use of psychotropic medication and monocyte pro-inflammatory gene expression, as (part of) these medications have been reported to be immune suppressive in nature⁶,¹⁸–²¹.

Patients and Methods

Participants

Study participants comprised of patients and healthy controls from three different studies⁴,⁶,²²–²⁶. All patients (n=64) had a DSM-IV BD I or II disorder, confirmed by the Structured Clinical Interview for DSM-IV axis I disorders (SCID-I)²⁶ (table 2 and figure 3). For this extension analysis we selected all participants (64 outpatients, age range 16–61 years, 24 (38%) male) who were included in the original studies and of whom Q-PCR and questionnaire data were available. Regarding the twin study, only data from the index twin were used. There was no family relation between any of the participants. Patients needed to be free of any additional severe psychiatric disorders and of any relevant medical illness that might affect inflammation status at least 2 weeks before blood withdrawal.

Healthy controls (n=63) were also recruited within the three aforementioned studies and comprised of laboratory or medical staff, medical students and high school students. All controls were free of any lifetime psychiatric disorder and of a history of these disorders in first-degree family members (self report). Controls did not use any psychotropic or other medication and were also free of any relevant medical illness that might affect inflammation status at least 2 weeks before blood withdrawal.

Ethical considerations

The Medical Ethical Review Committee of the University Medical Center Utrecht approved the original studies, which were performed in accordance with the Helsinki Declaration of 1975. Written informed consent was obtained from all participants.
Relationship between clinical features and inflammation related monocyte gene expression in bipolar disorder

Assessments
Clinical features used in the analyses were extracted from the interviews held, which included the SCID-I, the Young Mania Rating Scale (YMRS), the Inventory of Depressive Symptoms (IDS) and a demographic questionnaire. The lifetime history of psychotic symptoms was derived from the SCID-I. The YMRS is an eleven-item,
multiple-choice questionnaire to assess manic symptoms. The IDS is a thirty-item, multiple-choice questionnaire to assess depressive symptoms of all symptom domains of depression. Individual YMRS and IDS item scores were used to qualify the presence of specific mood symptoms. The scores of the individual questionnaire items were transformed to a 0-1 scale to facilitate mutual comparison. Age at onset was defined as the age when the first mood episode occurred. Current medication use was dichotomously described as being present or absent.

Laboratory methods
Blood (drawn in the morning and within three days of psychiatric assessment in all cases) was collected in clotting tubes for serum preparation and stored at -80°C, and in sodium-heparin tubes for immune cell preparation. From the heparinized blood, peripheral blood mono-nuclear cell (PBMC) suspensions were prepared in the afternoon by low-density gradient centrifugation within 8 hours to avoid activation of the monocytes. To obtain cDNA for Q-PCR, 1 mg RNA was reversed-transcribed using the cDNA high-capacity cDNA Reverse Transcription kit (Applied Biosystems, USA). Q-PCR was performed on thirty-four genes selected by whole genome profiling, previously described by R. Drexhage et al., using a method described by Staal et al.

Preprocessing
The quantitative value obtained from Q-PCR is a cycle threshold (Ct) that was used to calculate household-gene (Abl) normalized Ct values (ΔCt = Ct gene – Ct house-keeping gene) via the ΔΔCt method. By subtracting the ΔCt with the mean ΔCt for the healthy control group, the relative gene expression (ΔΔCt) was determined. The healthy controls were solely used for this purpose and were not needed in the other analyses. Z-score transformation of the relative gene expression was applied to facilitate mutual comparison. The relative gene expression can consequently be expressed as fold change after transformation via the ΔΔCt method (fold change = 2^{-\Delta\Delta C_t}).

Statistical analyses
Regression methods were used to determine size and statistical significance of the association between gene expression and clinical features. Analyses were performed using individual immunologic details and individual psychiatric details. These regarded associations between specific mRNA gene expressions e.g. PDE4B, ATF3 and specific psychiatric symptoms, e.g. auditory hallucinations, depressed mood. The associations between pro-inflammatory gene expression and lifetime history of psychotic symptoms were analyzed using ordinal regression, taking lifetime history of psychotic symptoms as dependent variable, in accordance with our pathophysiological model (figure 1).
Associations of pro-inflammatory gene expression with current manic symptoms and with current depressive symptoms were analyzed using ordinal regression, taking symptoms as dependent variable.

Associations between age at onset and pro-inflammatory gene expression and between duration of illness and pro-inflammatory gene expression were analyzed using linear regression. This was done separately in univariable and in combined multivariable models without and with an interaction term for effect of age at onset on the duration of illness. These analyses were repeated while correcting for gender. Pro-inflammatory gene expression was the dependent variable.

Associations between pro-inflammatory gene expression and medications were analyzed with linear regression using the pro-inflammatory gene expression as dependent variable.

Continuous outcome measures (relative gene expression, duration of illness) were checked for normal distribution by graphical inspection and using the skewness and kurtosis normality test. Univariable and multivariable linear regression were used for continuous, normally distributed outcome variables (disease duration, gene expression in the medication analyses).

Because of the multiple individual statistical tests in the analyses, there is an increased risk of a wrongful rejection of the null hypothesis (type I error). To control for this problem, we applied correction for the false discovery rate (FDR), as described by Benjamini-Hochberg32, in most analyses and only considered clustering groups of associations to be of importance.

Statistical analyses were performed using Stata Statistical Software, release 11 (StataCorp. 2009, College Station, TX).

**Feature-expression heat maps**

To display the associations we have drawn adapted heat maps, visualizing the independent gene expression variable on the vertical y-axis and the dependent clinical symptom variables on the horizontal x-axis. In these feature-expression heat maps the associations between independent and dependent variables are represented by circles in each respective compartment. The regression coefficients indicating effect size are represented by the type (red=positive, blue=negative) and intensity of the color, whereas the statistical significance of the analyses is represented by the radius of the circles.

To facilitate the visual identification of meaningful clusters of association both axes were ordered into functional categories and sequences. Monocyte gene expression was ordered on the sub-clusters 1a (inflammation), 1b (transcription) and 2 (adhesion, motility and chemotaxis) categories, based on a cluster analyses performed by R.C. Drexhage5. Clinical symptoms were ordered into symptom categories. For psychotic symptoms a division into delusions, hallucinations and psychomotor symptoms
is used. Depressive symptoms were divided into general symptoms, melancholic symptoms and atypical symptoms. Manic symptoms were not subdivided into categories. Furthermore, symptoms were ordered into a phenomenological sequence for mood symptoms (core mood symptoms, thought symptoms, psychosomatic symptoms, motor symptoms, food intake symptoms, sleep symptoms, higher functional symptoms) and psychotic symptoms.

The heat maps were drawn with the corrplot package, by T. Wei, on R, release 2.14.1 (R Development Core Team 2011, Vienna, Austria). Centered dots were added to the compartments that complied with a FDR below 0.2, thus allowing 1/5 to be false positive. See chapter 3 for a more detailed description of the feature-expression heat map method.

Results

Associations between lifetime psychotic symptoms and monocyte pro-inflammatory gene expression

The associations between the psychotic symptoms and the expression of genes belonging to each subcluster were depicted in the psychosis feature-expression heat map (figure 4). This heat map demonstrated a clustering group of associations between the sub-cluster 2 genes and the psychomotor symptoms. Clustering groups of associations with delusional or hallucinatory symptoms could not be demonstrated. None of the individual analyses were statistically significant after FDR correction. Although not fitting into a clustering group of associations, the negative association between thought withdrawal and sub-cluster 1 gene expression, and the positive association between olfactory hallucinations and sub-cluster 1 gene expression are conspicuous.

Associations between current mood symptoms and monocyte pro-inflammatory gene expression

The manic feature-expression heat map (figure 5) was created of the associations between manic symptoms and the expression of genes belonging to each subcluster. This feature-expression heat map showed a clustering group of associations between the sub-cluster 2 genes and manic symptoms. Sixteen of these associations were significant after FDR correction, especially in the associations with the symptoms increased speech and increased motor activity.

The associations between depressive symptoms and the expression of genes belonging to each subcluster were drawn up in the depression feature-expression heat map (figure 6). With regard to this feature-expression heat map, clustering groups of associations could not be identified and none of the individual analyses were significant.
FIGURE 4
Association between lifetime psychotic symptoms and monocyte pro-inflammatory gene expression

Heat map depicting the regression coefficient of the association between lifetime psychotic symptoms and gene expression. Psychotic symptoms were ordinally measured. Gene expression was expressed as z-transformed $\Delta\Delta$Ct. Statistical analysis was performed using ordered logistic regression. Contrast was set to 3. Blank compartments either represent a small regression coefficient and statistical probability or an analysis with insufficient observations. None of the analyses were significant below the 0.2 false discovery rate (FDR) threshold for multiple testing.
FIGURE 5
Association between manic symptoms and monocyte pro-inflammatory gene expression

Heat map depicting the regression coefficient of the association between actual manic symptoms (within three days of blood sampling) and gene expression. Depressive symptoms were ordinally measured. Gene expression was expressed as z-transformed \( -\Delta \Delta C_t \). Statistical analysis was performed using ordered logistic regression. Contrast was set to 3. Blank compartments either represent a small regression coefficient and statistical probability or an analysis with insufficient observations. Dotted circles represent significance below the 0.2 false discovery rate (FDR) threshold for multiple testing.
Heat map depicting the regression coefficient of the association between actual depressive symptoms (within three days of blood sampling) and gene expression. Depressive symptoms were ordinally measured. Gene expression was expressed as z-transformed $-\Delta \Delta Ct$.

Statistical analysis was performed using ordered logistic regression. Contrast was set to 3. Blank compartments either represent a small regression coefficient and statistical probability or an analysis with insufficient observations. None of the analyses were significant below the 0.2 false discovery rate (FDR) threshold for multiple testing.
after FDR correction. Although not fitting into clustering groups of associations, some associations e.g. with a decreased appetite, with sympathetic arousal and with sad and irritable mood are notable. No noticeable difference exists in the number of associations between the atypical, melancholic and general depressive symptoms as separate groups.

**Association between age at onset, duration of illness and monocyte pro-inflammatory gene expression**

The associations between age at onset, duration of illness and the expression of genes belonging to each subcluster are described in table 3. Using univariable analyses many sub-cluster 1a genes associated significantly with the age at onset in a negative manner. Some sub-cluster 1a genes associated significantly with duration of illness in a positive manner. When entered into multivariable models, PDE4B, IL6, TNFAIP3 and PTX3 gene expression associated significantly with the age at onset in a negative manner, where no concurrent associations were demonstrated with duration of illness (table 3). The genes ATF3 and IL1 associated significantly with duration of illness in a positive manner in these analyses, where concurrent associations with age at onset could not be demonstrated.

Associations between any of the sub-cluster 1b genes and age at onset or duration of illness could not be found.

Many sub-cluster 2 genes associated significantly with the duration of illness in a positive manner and some sub-cluster 2 genes associated significantly with age at onset in a negative manner (table 3). When entered into multivariable models, MAPK6, EMP1, STX1, DHRS3 and CCL2 gene expression associated significantly with duration of illness in a positive manner, where age at onset was found not to be associated in any of these models for sub-cluster 2 genes (table 3).

Addition of an interaction term or gender to the multivariable analyses did not alter the results markedly. Significant associations could not be demonstrated between individual gene expression and age, except for MAPK6.
<table>
<thead>
<tr>
<th>mRNA genes</th>
<th>Age at onset - RC (ci)</th>
<th>Univariable</th>
<th>Duration of illness - RC (ci)</th>
<th>Age at onset - RC (ci)</th>
<th>Multivariable</th>
<th>Duration of illness RC (ci)</th>
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</thead>
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<tr>
<td>DUSP2</td>
<td>-0.0716 (-0.128 - 0.052)</td>
<td>0.0476 (0.0124 - 0.108)</td>
<td>-0.0639 (-0.128 - 0.00227)</td>
<td>0.0172 (0.00490 - 0.00835)</td>
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<tr>
<td>ATF3</td>
<td>-0.0536 (-0.0955 - 0.0115)</td>
<td>0.0679 (0.0267 - 0.110)</td>
<td>-0.0294 (-0.0752 - 0.0163)</td>
<td>0.0537 (0.00641 - 0.0101)</td>
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<td>PDE4B</td>
<td>-0.0785 (-0.126 - 0.0306)</td>
<td>0.0669 (0.0161 - 0.118)</td>
<td>-0.0618 (-0.115 - 0.00381)</td>
<td>0.0373 (0.0180 - 0.0396)</td>
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<tr>
<td>IL6</td>
<td>-0.233 (-0.403 - 0.0626)</td>
<td>0.106 (0.126 - 0.330)</td>
<td>-0.232 (-0.417 - 0.0479)</td>
<td>0.0921 (0.230 - 0.236)</td>
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<tr>
<td>IL1</td>
<td>-0.0798 (-0.163 - 0.0370)</td>
<td>0.122 (0.0340 - 0.211)</td>
<td>-0.038 (-0.128 - 0.0922)</td>
<td>0.104 (0.0686 - 0.203)</td>
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<td>TNF</td>
<td>-0.0653 (-0.126 - 0.00512)</td>
<td>0.0383 (-0.0316 - 0.108)</td>
<td>-0.0623 (-0.130 - 0.00518)</td>
<td>0.0781 (-0.0681 - 0.0838)</td>
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<td>TNFAIP3</td>
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<td>0.0161 (-0.0283 - 0.151)</td>
<td>-0.0986 (-0.160 - 0.0166)</td>
<td>0.022 (-0.0696 - 0.113)</td>
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<tr>
<td>BCL2A1</td>
<td>-0.0514 (-0.107 - 0.0432)</td>
<td>0.0685 (0.0121 - 0.125)</td>
<td>-0.0263 (-0.088 - 0.0354)</td>
<td>0.0559 (0.00795 - 0.120)</td>
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<td>PTX3</td>
<td>-0.1000 (-0.177 - 0.0231)</td>
<td>0.0794 (-0.0227 - 0.181)</td>
<td>-0.0889 (-0.172 - 0.00623)</td>
<td>0.0398 (0.00647 - 0.144)</td>
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<tr>
<td>PTGS</td>
<td>-0.0714 (-0.146 - 0.00273)</td>
<td>0.0355 (0.0618 - 0.133)</td>
<td>-0.0702 (-0.150 - 0.0106)</td>
<td>0.00426 (0.00972 - 0.106)</td>
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<td>CCL20</td>
<td>-0.024 (-0.371 - 0.0368)</td>
<td>0.203 (-0.0139 - 0.420)</td>
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<td>0.128 (-0.0985 - 0.353)</td>
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<td>CCL2</td>
<td>-0.112 (-0.204 - 0.0206)</td>
<td>0.131 (0.0377 - 0.224)</td>
<td>-0.088 (-0.169 - 0.0330)</td>
<td>0.0983 (0.00613 - 0.203)</td>
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<td>EREG</td>
<td>-0.0931 (-0.185 - 0.00100)</td>
<td>0.0892 (-0.0118 - 0.190)</td>
<td>-0.0681 (-0.183 - 0.0471)</td>
<td>0.0458 (-0.0787 - 0.170)</td>
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<tr>
<td>CXCL3</td>
<td>-0.086 (-0.229 - 0.0568)</td>
<td>0.149 (0.00770 - 0.297)</td>
<td>-0.0711 (-0.180 - 0.166)</td>
<td>0.144 (-0.0426 - 0.331)</td>
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</table>

Z-score transformation of the relative gene expression was applied to facilitate mutual comparison. Statistical analysis was performed using linear regression. Multivariable regression analyses consisted of age at onset and duration of illness as dependent variables. Correction for gender or the addition of an interaction term in the multivariable analyses did not alter results significantly. Results are reported as regression coefficients (RC) with 95% confidence intervals (ci). Significance is reported at the 10% (p=0.1), 5% (p=0.05)** and 1% (p=0.01)** level.
Association between monocyte pro-inflammatory gene expression and current medication use

The associations between the expression of genes belonging to each subcluster and current medication use were drawn up in the medication feature-expression heat map (figure 7). This feature-expression heat map showed a clustering group of negative associations between current use of an antidepressant and sub-cluster 2 genes. Furthermore, a clustering group of weaker positive associations was found between current use of anti-epileptics, especially carbamazepine, and sub-cluster 2 genes. Also noticeable were relative weak, statistical non-significant associations between current use of lithium and some sub-cluster 1a genes, and between current use of antipsychotics and sub-cluster 1a genes.

**FIGURE 7**

Association between current medication use and monocyte pro-inflammatory gene expression

Heat map depicting the regression coefficient of the association between medication and gene expression. Medication was qualitatively measured. gene expression was expressed as z-transformed $-\Delta\Delta$Ct. Statistical analysis was performed using linear regression. Contrast was set to 1.5. Blank compartments represent a small regression coefficient and statistical probability. The dotted circle represents significance below the 0.2 false discovery rate (FDR) threshold for multiple testing.
Discussion

To our knowledge this is the first study on the associations between an extensive set of clinical features and monocyte gene expression in BD and it can be regarded as a next step in the converging approach between immunology and psychopathology in unraveling the complex pathophysiological mechanisms of BD. Our a priori formulated hypothesis that lifetime psychotic features would be associated with the pro-inflammatory monocyte gene expression could not be confirmed. However, our method visualized the following interesting findings in BD patients: (1) a possible relation between pro-inflammatory gene expression and manic symptomatology, (2) a differential immune activation related to an earlier age at onset, (3) an increased immune system dysregulation during the course of the disorder and (4) support for the concept of an immune suppressive action of some of the mood regulating medications.

The association between immune activation and manic / psychomotor symptoms is evident in both the analyses with the lifetime psychotic symptoms and in the analyses with the manic symptoms, in other words irrespective of the questionnaire used.

This association between immune activation and manic symptoms is supported by a previous finding by Dickerson et al., who described the YMRS and individual manic symptoms (speech, appearance, irritability, language–thought disorder, thought content and increased motor activity/energy) to be related to an increased serum C-reactive protein (CRP)\(^3^4\). Findings by Brietzke et al. who described pro-inflammatory cytokines to be increased in manic BD patients\(^3^5\) and Cunha et al. who found CRP to be increased in manic BD patients, but not in euthymic and depressed ones, are also compatible with this association.

Where CRP levels are known to be induced by cytokines\(^3^6\), it must be noted that a discrepancy between cytokine gene expression and protein synthesis is known to exist\(^4\).

In general, the association between the pro-inflammatory gene expression and depressive symptoms is less outspoken than it is with manic symptoms. However, some associations seem to exist with individual symptoms, e.g. decreased appetite, sympathetic arousal and sad and irritable mood. These associations seem to have some analogy with what is known as sickness behavior during the course of an infection\(^3^7\)–\(^3^9\).

We could not find indications for a specific relation between atypical or melancholic symptoms and monocyte activation. To our knowledge this is the first study investigating an association between the subtypes of depression and biological parameters in BD. In unipolar major depressive disorder a differential role of HPA-axis function and inflammation, including CRP and cytokine levels, has been reported in melancholic versus atypical depression\(^4^0\).
The association between age at onset and monocyte activation supports the notion that patients with an earlier age at onset have a biologically more severe form of the illness. Several authors have demonstrated increased morbidity in BD patients with an earlier age at onset\textsuperscript{41-43}, which is in agreement with this observation. On the biological side, genetic differences between BD patients with earlier and later age at onset have been described\textsuperscript{44,45}. Manenschijn, Spijker et al. demonstrated an increase in cortisol in patients with a later age at onset, suggesting BD beginning on an earlier age to be less associated with HPA-axis disturbances\textsuperscript{46}. Additionally, the present study supports an association with the duration of illness and the theory that the pathogenic mechanisms cause further impairment in immune system regulatory mechanisms with the progression of the illness. This association is consistent with the findings from Drexhage et al.\textsuperscript{5}, partly based on the same dataset, and from Soreca et al., who described an increased medical burden, e.g. more cardiovascular, endocrine and metabolic disease, in BD patients with a longer duration of illness\textsuperscript{47}.

The observation of a differential immune activation related to an earlier age of onset and an increased immune dysregulation during the course of the disorder can also be interpreted in view of the staging hypothesis of BD. A staging model founded on neurobiological correlates of distinct stages of BD could potentially predict clinical care needs and assist in refining treatment options\textsuperscript{48}. Kauer-Sant’Anna et al. described an increased TNF and decreased IL6 cytokine levels in late-stage versus earlier-stage BD patients, all levels being elevated when compared to healthy controls\textsuperscript{49}. In the present study TNF and IL6 gene expression was not found to be associated with longer duration of illness, but increased gene expression of these molecules was related to an earlier age at onset. In an attempt to explain the known discrepancy between cytokine gene expression and protein synthesis\textsuperscript{4} it could be argued that earlier-stage patients in the Kauer-Sant’Anna study potentially had a biologically more severe form of the illness. That would be consistent with the earlier age at onset of this group and may explain the differentiated cytokine expression between the early-stage and late-stage groups. Based on the present study additional research is warranted on the ATF3, IL1, MAPK6, STX1, DHR53, CCL2 gene expression as potential neurobiological staging markers of the progression of the illness, whereas PDE4b, IL6, TNFAIP3, PTX3 gene expression could perhaps predict a more immune mediated profile in the earlier stages of the disorder.

In addition to previous findings by Padmos and Drexhage\textsuperscript{4,5}, who described relations between current use of lithium and antipsychotics and pro-inflammatory gene expression, we found associations between current use of antidepressants (negative), and of anti-epileptics (positive) and pro-inflammatory gene expression. Our study results support the theory that the effects of lithium and antipsychotics mainly concern down regulation of some genes and this could also be the case for antide-
pressants. Based on the relation between monocyte activation and manic symptoms, it can be argued that the addition of anti-inflammatory medication to standard anti-mania treatment could be a beneficial addition treatment strategy for manic episodes in addition to depressive episodes\(^6\). Although treatment studies have thus far not been performed in this regard, low dose (30–80mg/day) acetylsalicylic acid was found to produce a statistically significant duration-independent reduction in the relative risk of clinical deterioration in subjects on lithium, in a large pharmacoepidemiological study\(^4\) and further research into this treatment possibility is warranted.

The present study has several limitations. Firstly, the present study focuses on the pro-inflammatory gene expression of monocytes, which is a select part of the complex immune system, and generalized statements should be considered in that regard. Secondly, all patients were naturalistically treated and none of them was ‘medication naive’. The positive association between monocyte activation and current use of anti-epileptic leaves room for argumentation that medication is a causal factor for the increased monocyte pro-inflammatory gene expression in BD. Thirdly, an effect of age or gender cannot completely be ruled out. A fourth limitation is contained within the original selection design of the analyzed genes, where only highly over- and under-expressed genes, which were clearly involved in inflammatory processes, where selected, possibly ruling out important genes. The cross-sectional design of the study being suboptimal for analyses with regard to disease progression is a specific fifth limitation concerning the age at onset and duration of illness analyses. The final and in our opinion most important limitation concerns the multiple testing in the analyses leading to an increased risk of type I errors despite the application of FDR correction, combined with relatively small sample size. Nevertheless, we consider it as important for hypothesis forming and testing in further studies. Because of the relatively small sample size of BD-II patients no separate sub-group analyzes were performed comparing patients with BD-I or BD-II disorder.

The nature of the associations between psychiatric symptoms and pro-inflammatory monocyte activation is as yet unknown. With the current state of knowledge about these interactions we are unable to clarify why and how activation of sub-clusters of genes, or even specific gene activation, is related to a specific (group) of symptoms. However, we regard them as intermediary phenomenon in the neuroinflammation theory of BD, positioned between the activated inflammatory response system and the phenotypical expression (symptoms), as depicted in figure 1.

A theoretical model of action originates in activation of the mononuclear phagocyte system at the level of the brain (microglia), the circulation (monocytes), and the tissues (macrophages) as a key element in the pathogenesis of major psychiatric disorders. Whether there exists a direct migration of activated monocytes to the brain in psychiatric disease needs further exploration. Also, the role of inflammatory cytokine
exchange among the various compartments (circulation, brain, and peripheral tissues, such as adipose tissue and lymphoid tissue) needs clarification. Several routes have been proposed for cytokines to enter and act on the brain, e.g. by altering the blood-brain barrier and by affecting neuronal afferents such as the vagus nerve. Indeed, the vagus nerve is known to be essential for balancing anti- and pro-inflammation during sepsis. Supported by multiple observations of mania in vagus nerve stimulation, a treatment for refractory epilepsy thought to have its effect on the limbic system and frontal cortex, it is tempting to question the importance of the role of the vagus nerve in the association between monocyte activation and the psychiatric symptoms in BD.

In our opinion, especially the association between manic symptoms and immune activation deserves verification in further studies. The psychoimmunological model in BD does not stand on its own, but concerns other psychiatric disorders as well. This makes it interesting to study our findings also in other disorders, like SZ where motor function symptoms also play an important role.

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