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Research paper

Dysregulation of cytokine coding genes in peripheral blood of bipolar patients

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ABSTRACT

Background: The role of immune response dysregulation has been previously noticed in the pathogenesis of bipolar disorder (BD).
Methods: In the current investigation, we compared expression levels of eight cytokines and a chemokine (*CXCL8*) in the peripheral blood of BD patients and healthy subjects. All BD patients were in euthymic phase.
Results: We found higher expression of *IL-1B, IL-10, IFN-G, TNF-a, TGF-B* and *IL-2* in male patients compared with male controls (ExR = 3.44, $P < 0.0001$, ExR = 2.54, $P < 0.0001$; ExR = 2.39, $P < 0.0001$; ExR = 2.74, $P < 0.0001$; ExR = 2.32, $P < 0.0001$; ExR = 1.87, $P = 0.04$ respectively). For these cytokines, no significant differences were found between female patients and female controls. While expression of *IL-6* was higher in male patients compared with male controls (ExR = 2.07, $P = 0.006$), in female subjects the opposite trend was detected (ExR = 0.44, $P = 0.02$). However, no significant difference was detected between female subjects. Expression levels of *IL-17* were not different between patients and controls or between any subgroups of them. We found significant correlations between expression of *IFN-G* and age at disease onset ($R = 0.25$, $P = 0.04$) as well as expression of *CXCL8* and both age of patients and age at disease onset ($R = 0.26$, $P = 0.03$; $R = 0.25$, $P = 0.04$). Moreover, inverse correlation was detected between expression of *TNF-a* and age in control group ($R = -0.34$, $P = 0.008$).
Conclusion: Combination of transcript levels of six genes could differentiate BD patients from healthy subjects with diagnostic power of 0.85 (Sensitivity = 78%, Specificity = 80% and $P < 0.0001$). The current investigation highlights the role of cytokine coding genes in the pathogenesis of BD and potentiates them as diagnostic biomarkers.

1. Introduction

Cytokines are produced by different cells in the immune system including the M1 and M2 macrophages of innate immune system and T helper (Th) cells. T helper cells are categorized into IFN-γ producing Th1 cells; IL-4, IL-5, IL-10 and IL-13 producing Th2 cells; TGF-β producing Th3 cells and IL-17 producing Th17 cells (Roberts et al., 2015; Weiner, 2001). The pro-inflammatory cytokines IL-1β, IFN-γ, IL-6, TNF-α and IL-17 have been implicated in the development of organ specific autoimmune diseases such as rheumatoid arthritis, psoriasis, and multiple sclerosis (MS). On the other hand, the anti-inflammatory cytokines IL-10 and TGF-β have been suggested to ameliorate the inflammatory events and protect against these diseases (Hugh and Weinberg, 2018; Chen et al., 2019; Wang et al., 2018). On the contrary, dysregulated production of IL-10 seems to play a role in systemic lupus erythematosus (Barcellini et al., 1996). Notably, irrespective of the pathogenic predominance of a cytokine subset vs. another, these autoimmune diseases are often associated with mood disorders (Amanat et al., 2018; Martin-Subero et al., 2016; Tisseverasinghe et al., 2018). Consequently, cytokines might be regarded as a functional link between mood disturbances and the autoimmune event. Moreover, certain medications used for the above-mentioned conditions modify the levels of cytokines. For instance, the levels of IL-1 receptor antagonist and IL-1 receptor type II has been elevated in sera MS patients after corticosteroid therapy (Dujmovic et al., 2009). Moreover, TGF-β1 concentrations have been increased in MS patients following IFN-β1b therapy (Nicoletti et al., 1998). Based on these evidences, cytokines might contribute in the pathogenesis of mood disorders.

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Bipolar disorder (BD) is a complex neuropsychiatric condition characterized by the occurrence of symptoms of mania or depression (Merikangas and Tohen, 2011). The role of immune system dysregulation has been noticed in the pathogenesis of BD (Anderson and Maes, 2015). Induction of inflammatory responses has been detected in both bipolar depression and mania (Anderson and Maes, 2015). For instance, Maes et al. have detected higher soluble interlekin-6 receptor (sIL-6R) and soluble interlekin-2 receptor (sIL-2R) levels in manic patients compared with healthy subjects. They also reported no significant changes in these immune-related molecules after treatment with valproate in these patients (Maes et al., 1995). In addition, the authors detected higher plasma levels of IL-6, sIL-6R, sIL-2R and transferrin receptor (TfR) in major depressed patients compared with healthy individuals. Moreover, plasma concentrations of these proteins have not been affected by fluoxetine or tricyclic antidepressants (Maes et al., 1995). Wadee et al. have demonstrated higher levels of complement proteins C3, C6 and Factor B in patients experiencing acute manic episodes compared with controls and suggested associations between acute mania and acute phase immunological factors (Wadee et al., 2002). More recently, do Prado et al. have shown decreased frequencies natural T regulatory cells and higher cytokine release in BD patients compared with healthy controls. They also detected a robust bias toward Th1 responses (do Prado et al., 2013). Taken together, several lines of evidences suggest existence of immune dysfunction in BD patients irrespective of disease phase or drug administration. In the present investigation, we compared transcript levels of eight cytokines (*IL1-B, IL-2, IL-6, IL-10, IL-17, IFN-G, TNF-α* and *TGF-B*) and a chemokine (*CXCL8*) in the peripheral blood of BD patients and healthy subjects to assess their contribution in the pathogenesis of BD and their potential role as disease biomarker.

2. Material and methods

2.1. Study participants

The current project was performed on the peripheral blood samples obtained from 50 BD patients and 50 age- and sex-matched healthy individuals enrolled in the control group. The diagnosis of patients was confirmed based on the Diagnostic and Statistical Manual of Mental Disorders-5 (DSM-5) (Association, 2013). Individuals enrolled in the control group had no prior signs of psychiatric or neurodegenerative diseases as confirmed by the Mini International Neuropsychiatric Interview (Sheehan et al., 1998), mental retardation, cancer or infection. They were non-smokers and did not take any drug in a period of one month prior sampling. All BD patients were under treatment with Carbamazepine and were in euthymic phase (as defined by Hamilton Depression Rating Scale (HDRS) and the Young Mania Rating Scale (YMRS) scores < 8 (Clark et al., 2005) and had type I BD. The study protocol was approved by ethical committee of Shahid Beheshti University of Medical Sciences (IR.SBMU.RETECH.REC.1397.872). Written consent forms were obtained from all study participants or their guardians. The exclusion criteria for patients were identification of schizophrenia or schizoaffective disorder in any time, signs of primary anxiety disorder when being enrolled in the project, substance dependence, pregnancy or breast-feeding. No individual left the study after primary recruitment. Blood samples were gathered from patients and controls at comparable time during the day to avoid circadian variations of the cytokine. Sampling was not conducted in fasting conditions.

2.2. Expression assessments

Expression assays were conducted after extraction of total RNA from blood specimens using Hybrid-R Blood RNA (GeneAll Biotech, Korea) and subsequent cDNA production using PrimeScript 1st strand cDNA Synthesis Kit (Clontech, Japan). Experiments were implemented in real-time PCR system (Rotor Gene 6000, Corbett Research, Australia) using the primer and probes being summarized in Table 1 and the RealQ Plus Master Mix (Ampliqon, Denmark). The *HPRT1* gene was used for normalization of expression data in both study groups.

Table 1
The nucleotide sequences of primers and probes used for expression analysis.

Gene name	Primer and probe sequence	Primer and probe length	Product length
HPRT1	F: AGCCTAAGATGAGAGTTC	18	88
	R: CACAGAACTAGAACATTGATA	21	
	FAM -CATCTGGAGTCCTATTGACATCGC-TAMRA	24	
IL-1β	F: ATAGCCTGGACTTTCCTGTTGTC	23	163
	R: GTGAGTAGGAGAGGTGAGAGAGG	23	
	FAM- ACACCAATGCCCAACTGCCTGCCT-TAMRA	24	
IL-2	F: GGGATCTGAAACAACATTCATGTG	24	109
	R: AGTCAGTGTTGAGATGATGCTTTG	24	
	FAM -TGATGAGACAGCAACCA -TAMRA	17	
IL-4	F: TGCTGCCTCCAAGAACACAAC	21	88
	R:GTCCTTCTCATGGTGGCTGTAG	22	
	FAM- CCGGAGCACAGTCGCAGCCCT-TAMRA	21	
IL-6	F: ATGCAATAACCACCCCTGACC	23	160
	R: CCATGCTACATTTGCCGAAGAG	21	
	FAM- ACCACAAATGCCAGCCTGCTGACG-TAMRA	22	
CXCL8	F:CGGAAGGAACCATCTCACTGTG	22	77
	R:AGAAATCAGGAAGGCTGCCAAG	22	
	FAM- TGACTTCCAAGCTGGCCGTGGCTC-TAMRA	24	
IL-17	F: CAGCAAGAGATCCTGGTCCTG	21	176
	R: GGTCGGCTCTCCATAGTCTAAC	22	
	FAM-AGCCTCCACACTGCCCCAACTCCT-TAMRA	24	
IFN-γ	F: GGCAAGGCTATGTGATTACAAGG	23	96
	R: CATCAAGTGAAATAAACACACAACCC	26	
	FAM- AGGGGCCAACTAGGCAGCCAACCT -TAMRA	24	
TGF-β	F: GCTCCACGGAGAAGAACTGC	20	101
	R: GTTGGCATGGTAGCCCTTGG	20	
	FAM- CCACTTCCAGCCGAGGTCCTTGCG -TAMRA	24	
TNF-α	F: TCCACCCATGTGCTCCTCAC	20	97
	R: TCTGGCAGGGGCTCTTGATG	20	
	FAM- CTACCGAGTCCGTGTCTACCA -TAMRA	21	

2.3. Statistical methods

Statistical assessments were executed using SPSS 22.0 (IBM, Chicago, IL, USA) and MedCalc statistical softwares. Expression of cytokine genes were compared between two study groups using independent T test. The correlation between relative expression of cytokine coding genes and patients' features were judged using regression model. $P < 0.05$ was considered as significant. The suitability of transcript levels of genes for differentiation of disease status was evaluated by depicting the receiver operating characteristic (ROC) curves and quantifying the area under curve (AUC).

3. Results

3.1. Features of study participants

The study population was consisted of 50 BD patients (35 males and 15 females) and 50 healthy subjects (35 males and 15 females) whose general information is summarized in Table 2. None of study participants had metabolic syndrome or autoimmune disorders.

Table 2
General information of study participants.

Study groups	Parameters	Values
Cases	Age	36.5 ± 9.32 (17–56)
	Age at onset	32.64 ± 8.04 (15–48)
	Disease duration	3.86 ± 2.66 (1–14)
Controls	Age	33.62 ± 8.59 (14–52)

3.2. Expression analyses

We found higher expression of *IL-1B, IL-10, IFN-G, TNF-a, TGF-B* and *IL-2* in male patients compared with male controls (ExR = 3.44, $P < 0.0001$, ExR = 2.54, $P < 0.0001$; ExR = 2.39, $P < 0.0001$; ExR = 2.74, $P < 0.0001$; ExR = 2.32, $P < 0.0001$; ExR = 1.87, $P = 0.04$ respectively). For these cytokines, no significant differences were found between female patients and female controls. While expression of *IL-6* was higher in male patients compared with male controls (ExR = 2.07, $P = 0.006$), in female subjects the opposite trend was detected (ExR = 0.44, $P = 0.02$). Expression of *CXCL8* was higher in total patients, male patients and female patients compared with the corresponding control groups (ExR = 2.51, $P = 0.001$; ExR = 2.45, $P = 0.01$; ExR = 2.57, $P = 0.03$ respectively) (Table 3). Expression levels of *IL-17* were not different between patients and controls or between subgroups of them. Fig. 1 shows the relative expression of genes in cases and controls.

3.3. Correlation between transcript levels of cytokine coding genes and clinical data

We found significant correlations between expression of *IFN-G* and age at disease onset ($R = 0.25$, $P = 0.04$) as well as expression of *CXCL8* and both age of patients and age at disease onset ($R = 0.26$, $P = 0.03$; $R = 0.25$, $P = 0.04$). Moreover, inverse correlation was detected between expression of *TNF-a* and age in control group ($R = -0.34$, $P = 0.008$). Table 4 shows partial correlation between expression of genes and patients data.

3.4. Correlations between expressions of cytokine coding genes

Pairwise correlations were identified between transcript levels of cytokine coding genes. No difference was detected between cases and controls except for three situations. Correlations between *TGF-B* and *IL-1B* or between *IL-2* and *IL-17* were detected only in patients. On the contrary, correlation between *TNF-a* and *CXCL8* was detected only in controls (Table 5).

Table 3
Relative expression of genes in patients compared with controls.

Genes	Parameters	Total patients vs. total controls (50 vs. 50)	Male patients vs. male controls (35 vs. 35)	Female patients vs. female controls (15 vs. 15)
IL1-B	Expression ratio	2.82	3.44	1.66
	P-value	< 0.0001	< 0.0001	0.11
IL-2	Expression ratio	1.23	1.87	0.46
	P-value	0.44	0.04	0.14
IL-6	Expression ratio	1.3	2.07	0.44
	P-value	0.22	0.006	0.02
IL-10	Expression ratio	1.63	2.54	0.57
	P-value	0.02	< 0.0001	0.09
IL-17	Expression ratio	1.16	1.62	0.52
	P-value	0.56	0.12	0.08
IFN-G	Expression ratio	1.79	2.39	0.94
	P-value	< 0.0001	< 0.0001	0.75
TNF-a	Expression ratio	2.16	2.74	1.27
	P-value	< 0.001	< 0.0001	0.15
TGF-B	Expression ratio	1.54	2.32	0.6
	P-value	0.02	< 0.0001	0.04
CXCL8	Expression ratio	2.51	2.45	2.57
	P-value	0.001	0.01	0.03

3.5. ROC curve analysis

Expression of *IL1-B, IL-10, IFN-G, TNF-a, TGF-B* and *CXCL8* could differentiate BD patients from healthy subjects with different specificity and sensitivity values being summarized in Table 6. Based on the AUC values, *TNF-a* had the best performance (AUC value = 0.8). Combination of these six genes improved the sensitivity and specificity values to 78% and 80% respectively (AUC = 0.85) (Fig. 2).

4. Discussion

In the present investigation, we compared peripheral expression of nine immune-related transcripts between BP patients and healthy individuals. Except for *IL-17*, expressions of other genes were different among at least one subgroup of BD patients compared with matched healthy subjects. In an *in vitro* assessment of peripheral blood mononuclear cells (PBMCs) from 27 euthymic female subjects with BD type I and 24 age- and sex-matched healthy subjects, do Prado et al. have reported higher production of IL-2, IL-4, IL-5, IL-10, IL-17, IFN-g and TNF-a in patients compared with controls (do Prado et al., 2013). In the current study, we could not detect any significant difference in peripheral expression levels of *IL-2, IL-10, IL-17, IFN-G* and *TNF-a* between female cases and female controls. Such discrepancy might be related to different experiments methods (*in vitro* stimulation of cytokine production vs. assessment of expression in clinical samples).

Brietzke et al. have shown higher levels of IL-2, IL-4 and IL-6 during mania and elevated levels of IL-6 in depressive episode when comparing these levels with those of normal subjects thus suggested the presence of a pro-inflammatory profile in mania, and to some degree in depression. However, IL-4 was the only cytokine with different concentrations between patients in remission and healthy controls (Brietzke et al., 2009). We found higher expression of *IL-1B, IL-10, IFN-G, TNF-a, TGF-B* in total patients compared with total controls and in male patients compared with male controls. These cytokines have distinct roles in immune responses. While IL-1B promotes inflammatory Th2 differentiation (Stephan et al., al.), TGF-β and IL-10 (Th2 cytokines) suppress the production of IFN-γ and TNF-α (Th1 cytokines) (Cavalcanti et al., 2012). However, this functional definition does not apply to the assessed patients in the current study as we detected simultaneously elevated levels of IFN-γ, IL-10 and TGF-β. Contrary to do Prado et al. who demonstrated a robust bias toward Th1 responses (do Prado et al., 2013), our study revealed a complex dysregulation of cytokine genes expressions in peripheral blood of BD patients.

Besides, we detected a sex-based dimorphism in expression of *IL-6*. While expression of this cytokine was higher in male patients compared with male controls, in female subjects the opposite trend was identified. A previous meta-analysis have shown no significant difference in IL-6 serum levels between depressed, manic, euthymic patients and healthy controls (Munkholm et al., 2013). However, the mentioned meta-analysis did not assess sex parameter in evaluation of cytokine levels. The sex-based dimorphism in IL-6 production has been noticed previously. Sperry et al. have shown disproportionate IL-6 production after serious injury in male subjects (Sperry et al., 2008). On the other hand, Jankord et al. reported that females have greater stalk median eminence (SME) level of IL-6 and higher hypothalamo-pituitary-adrenocortical reaction to stress (Jankord et al., 2007). Recently, Da Pozzo et al. demonstrated that in normal situations, male cells produce greater amounts of IL-6 than female cells. However, after cortisol exposure, production of IL-6 has been augmented in male cells, while it was not changed in female cells (Da Pozzo et al., 2018). Taken together, IL-6 levels might be different in males and females based on the physiological or pathological conditions.

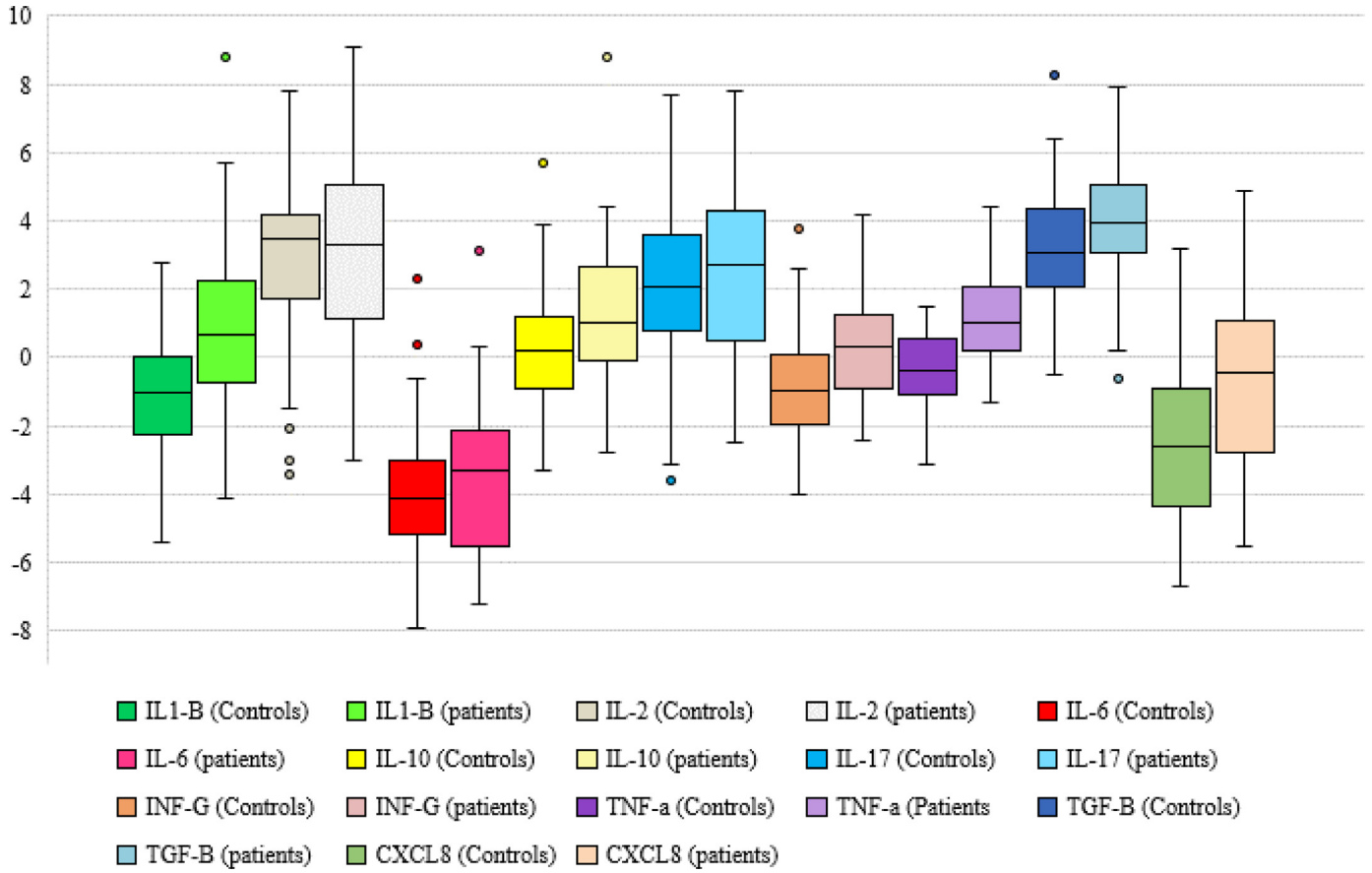

Fig. 1. Relative expression of cytokine coding genes in BD patients and healthy individuals as shown by –delta CT values ($CT_{\text{housekeeping gene}}$-$CT_{\text{target gene}}$).

The sex-biased production has been described for another cytokine implicated in the neuropsychiatric diseases namely macrophage migration inhibitory factor (MIF). Benedek et al. have shown higher levels of this cytokine in males with progressive MS compared with female MS patients and suggested MIF as a sex-specific disease modifier (Benedek et al., 2017). This cytokine has both pro- and anti-depressant functions (Bloom and Al-Abed, 2014) and is recognized to have sex-specific functions in inflammation, wound healing and response to ischemic stroke (Houdeau et al., 2007; Turtzo et al., 2013).

Notably, we demonstrated higher expression of *CXCL8* in total patients, male patients and female patients compared with the corresponding control groups. Our results are in discordance with Barbosa et al. study which demonstrated lower levels of this chemokine in both euthymic and manic patients compared with healthy subjects (Barbosa et al., 2013). However, a previous systematic review about the role of chemokines in psychiatric disorders stated that there is inadequate information about CXCL8 to determine whether it might be altered in BD (Stuart and Baune, 2014).

We also demonstrated higher levels of *IL-2* expression in male patients compared with male controls. However, this difference was not detected in female subjects. IL-2 and IFN-γ induce the enzymatic function of indolamine 2,3-dioxygenase (IDO), thus stimulating the conversion of tryptophan to depressogenic tryptophan metabolites (Wang and Dunn, 1998). Meanwhile, IL-6 and TNF-α induce conversion of serotonine to 5-hydroxyindoleacetic acid (Wang and Dunn, 1998). Both conditions can directly harm mood and cognitive activities (Arango et al., 2002).

All patients in the current study were euthymic at the time of venipuncture. Consequently, the observed gene dysregulation has not been related with mood episodes. We found significant correlations between expression of *IFN-G* and age at disease onset as well as expression of *CXCL8* and both age of patients and age at disease onset. Such findings further highlight the role of these immune-related molecules in the pathogenic course of BD. Moreover, inverse correlation was detected between expression of *TNF-α* and age in control group. Although previous studies have shown over-expression of TNF-a in aged people (Bruunsgaard et al., 2003), based on the difference in age range between the current study participants and mentioned studies, it is not possible to compare these results.

We also detected significant correlations between transcript levels

Table 4
Partial correlation between expression of genes and patients data (controlled for sex).

		IL1-B		*IL-2*		*IL-6*		*IL-10*		*IL-17*		*IFN-G*		*TNF-α*		*TGF-B*		*CXCL8*	
		R	P value	R	P value	R	P value	R	P value	R	P value	R	P value	R	P value	R	P value	R	P value
Case	Age	0.13	0.18	0.07	0.32	0.2	0.09	- 0.01	0.46	- 0.09	0.27	0.23	0.05	0.09	0.26	0.2	0.09	0.26	0.03
	Age at onset	0.12	0.19	0.14	0.17	0.21	0.07	0.02	0.45	- 0.04	0.39	0.25	0.04	0.12	0.21	0.2	0.08	0.25	0.04
	Disease duration	0.09	0.28	- 0.17	0.11	0.04	0.4	- 0.1	0.25	- 0.19	0. 1	0.06	0.35	- 0.04	0.39	0.08	0.29	0.15	0.15
Control	Age	- 0.2	0.09	- 0.1	0.25	0.004	0.49	- 0.08	0.3	- 0.01	0.47	- 0.14	0.16	- 0.34	0.008	- 0.14	0.16	- 0.17	0.12

Table 5
Pairwise correlations between expression levels of cytokine coding genes (R^2 values are shown).

		CXCL8	TGF-B	TNF-a	IFN-G	IL-17	IL-10	IL-6	IL-2
IL1-B	Patients	0.32**	0.12*	0.04	0.01	0.002	0.0001	0.06	0.003
	Controls	0.46**	0.08	0.19*	0.06	0.01	0.12*	0.12*	0.11*
IL-2	Patients	0.001	0.14*	0.13*	0.15*	0.22*	0.16*	0.13*	
	Controls	0.03	0.11*	0.13*	0.12*	0.07	0.11*	0.21*	
IL-6	Patients	0.07	0.37**	0.38**	0.54**	0.36**	0.33**		
	Controls	0.005	0.59**	0.26**	0.46**	0.34**	0.49**		
IL-10	Patients	0.001	0.35**	0.37**	0.29**	0.43**			
	Controls	0.002	0.38**	0.2*	0.32**	0.47**			
IL-17	Patients	0.0001	0.28**	0.36**	0.33**				
	Controls	0.06	0.26**	0.12*	0.18*				
IFN-G	Patients	0.01	0.37**	0.45**					
	Controls	0.005	0.5**	0.29**					
TNF-a	Patients	0.008	0.36**						
	Controls	0.09*	0.19*						
TGF-B	Patients	0.03							
	Controls	0.003							

Table 6
The results of ROC curve analysis (a: Youden index, b: Significance level P (Area = 0.5), Estimate criterion: optimal cut-off point for gene expression).

	Estimate criterion	AUC	J[a]	Sensitivity	Specificity	P-value[b]
IL1-B	≤ 0.2	0.73	0.38	66	72	< 0.0001
IL-10	≤ −1	0.63	0.28	58	70	0.02
IFN-G	≤ 1.1	0.7	0.32	84	48	0.0001
TNF-a	≤ −0.2	0.8	0.44	76	68	< 0.0001
TGF-B	≤ −3.3	0.63	0.26	72	54	0.02
CXCL8	≤ 1.5	0.68	0.34	68	66	0.0008
Combination of all genes	> 0.52	0.85	0.58	78	80	< 0.0001

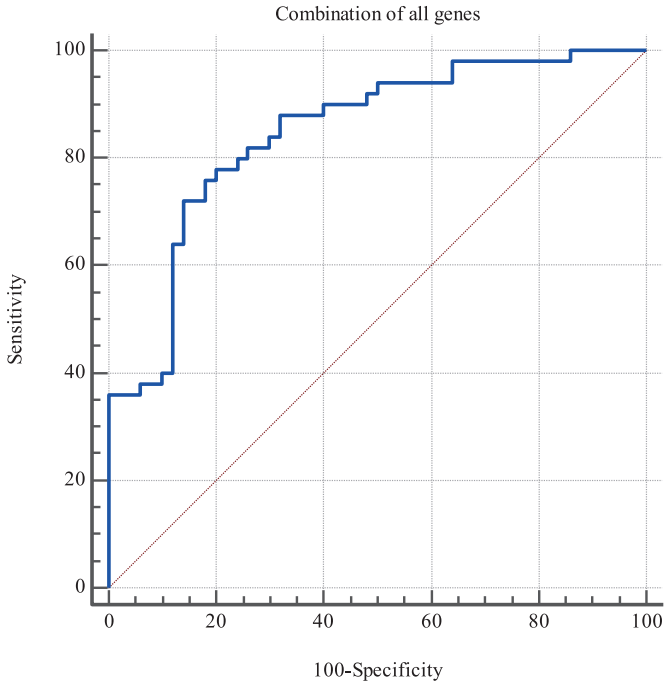

Fig. 2. ROC curve of analysis of diagnostic power of six cytokine coding genes in BD.

of mentioned cytokines. The pattern of correlation was similar between cases and controls except for three situations. Maes et al. have previously reported significant correlations between plasma levels of IL-6 and sIL-6R, IL-6 and sIL-2R, IL-6 and TfR, and between sIL-2R and TfR only in major depressed patients and not in healthy individuals (Maes et al., 1995). The difference between their results and ours might be due to the differences in the assessed cytokines or in the level of expression assessment (protein vs. mRNA level).

Finally, we have shown that expression levels of *IL1-B, IL-10, IFN-G, TNF-a, TGF-B* and *CXCL8* could differentiate BD patients from healthy subjects. Based on the AUC values, *TNF-a* had the best performance, meanwhile combination of all genes improved the diagnostic power. Although the clinical significance of altered expression levels of cytokines in BD needs to be explored, transcript levels of these genes are suggested as putative biomarkers for differentiation of BD from healthy subjects. However, as expression of these genes might be altered in several other pathologic conditions, these putative biomarkers are not intended to replace the current diagnostic criteria but facilitate diagnosis in complicated situations.

Taken together, we demonstrated altered cytokine profile in BD compared with healthy situation. The observed dysregulation of immune response might reflect the role of this system in the pathogenesis of BD. Moreover, based on the role of certain cytokines such as TNF-α, IL-2 and IL-6 in direct modification of monoamine concentrations (Capuron et al., 2003), they can affect disease course through several mechanisms. It is worth mentioning that MIF and other cytokines whose expressions have not been evaluated in the current study (for instance: IL-12, IL-18, IL-23, IL-22 and IL-9) might act in concert with these cytokines in the pathogenesis (or ultimately in the modulation) of BD. Moreover, as we assessed expression of genes in euthymic patients, the detected cytokine profile in these patients may either represent a protective factor against manic/ depressive episodes or represent prodromal pathogenic factors. Longitudinal assessment of cytokine levels in BD patients in association with disease episodes is needed to answer this question. Our study has a limitation regarding lack of data of body mass index (BMI) of study participants.

Contributors

MDO and SGH wrote the manuscript and supervised the study. VKO analyzed the data. MT performed the Tests.

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Conflict of interest

The authors declare they have no conflict of interest.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jad.2019.06.028.

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