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ORIGINAL ARTICLE

The Role of Cytokines in the Peripheral Blood of Major Depressive Patients

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SUMMARY

Background: **Recent technological advances offer an opportunity to further elucidate the complex cytokine network in Major Depressive Disorder (MDD). The objective of this study was to investigate the role of pro-inflammatory and anti-inflammatory cytokines in the mechanism of depressive disorders. Given the activating role of cytokines on the hypothalamic-pituitary-adrenal (HPA) axis, and the relevance of its regulation in MDD, we also analyzed the relationships between several cytokines and cortisol levels.**
Methods: **Twenty-five unipolar depressive patients and 20 healthy controls were recruited in this study. Flow cytometric bead array system (FCM-CBA) was used to examine the concentration of cytokines (IL-2, IL-4, IL-6, IL-10, TNF, INF-α) in peripheral blood. Plasma Adrenocorticotropic Hormone (ACTH) and serum cortisol concentrations were detected.**
Results: **Compared with the controls, depressive patients had a significant increase in concentration of IL-2, TNF, serum cortisol, and TNF/IL-4 ($p < 0.05$). There was a significant positive correlation between serum cortisol and IL-2, as well as ACTH and IL-2 ($p < 0.05$) in depressive patients. There was a significant positive correlation between IL-2 and the Hamilton depression rating scale (HAMD) total scores in depressive patients, and also with TNF ($p < 0.05$). There was no significant difference in concentration of IL-4, IL-6, IL-10, and INF-α between two groups ($p > 0.05$).**
Conclusions: **The present results suggest that depressive patients had an increase in concentration of some pro-inflammatory cytokines. Both IL-2 and TNF play important roles in the development of depressive disorders, and their concentration in peripheral blood may be used to evaluate the severity of depressive disorders.**
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INTRODUCTION

In the research field of psychoneuroimmunology, accumulating evidence has indicated the existence of reciprocal communication pathways between nervous, endocrine, and immune systems [1-3]. Specifically, hypothalamic-pituitary-adrenal (HPA) axis is the most important signal transduction pathway in the neuro-endocrine-immunology (NEI), which exerts significant regulation

on the immune system including humoral immunity and cellular immunity by glucocorticoid (GC) effects [4-5]. Mounting data indicate that the HPA axis is the main hormonal system involved in major depression, but the mechanisms underlying its abnormalities in these patients are still unclear. One of the most reliably reported neurobiological alterations in major depression is both HPA axis hyperactivity and impaired HPA axis glucocorticoid feedback sensitivity [6-8]. Depressed patients have been shown to exhibit increased concentrations of the HPA axis hormone, cortisol, in plasma, urine, and cerebrospinal fluid (CSF) [9,10]. Of note, increases in HPA axis activity are especially apparent in individuals who are older and/or who are more severely depressed [11]. This phenomenon is referred to as glucocorticoid resistance. The presence of glucocorticoid resistance in mood disorders is supported by evidence of cortisol non-suppression to dexamethasone in the dexamethasone suppression test (DST) and the more recently developed dexamethasone-CRH (DEX-CRH) test [12]. Glucocorticoids play a fundamental role in restraining inflammatory and neuroendocrine responses to a variety of challenges including pathogen exposure and stress [13]. Failure of glucocorticoids to inhibit inflammatory and neuroendocrine responses to challenges may contribute to disease development [14,15]. Accumulating evidence suggests that major depressive disorder (MDD) is associated with immune system dysregulation [16,17]. Research shows that depressive patients display an increasing risk for development of chronic inflammatory diseases or autoimmune diseases [16,18]. Data indicate that major depression is associated with immune activation as reflected by increased plasma and CSF concentrations of a variety of cytokines and their receptors, including IL-1, IL-2, IL-6, and TNF-a, as well as increases in plasma concentrations of acute phase reactants (which reflect the effects of pro-inflammatory cytokines on the liver), chemokines, and cellular adhesion molecules [19]. Probably the most reproducible finding of immune activation in patients with depression is increased plasma levels of IL-6 and its downstream product from the liver, C-reactive protein (CRP) [16]. Specially, immune activation has been associated with treatment responses in patients with major depression, and there is preliminary evidence that polymorphisms in relevant cytokine genes may predict antidepressant treatment responsiveness [20].

In the preliminary work done by our team we found that the MDD group was positive for serum antinuclear antibody (ANA) more frequently than the healthy control group and had significantly higher Th17/Treg ratios in depressed subjects compared to controls observed in the same work [21]. These findings revealed the fact that immune system disarrangement does exist in patients with major depression. As technology and medical knowledge of the complex system of pro/anti-inflammatory balance develops, there is an opportunity to further elucidate cytokine profiles and specific cytokines perturbed in MDD beyond those previously identified. We measured the pro-inflammatory cytokine profiles using high-sensitivity assays, hypothesizing that hyperactivity of the HPA axis in patients with depression would correlate with increased levels of circulating pro-inflammatory cytokines. We present a panel of 6 cytokines measured simultaneously in well-characterized individuals with MDD and matched controls.

MATERIALS AND METHODS

Subjects

Unmedicated major depression patients were recruited from the Psychiatric Institute of Mental Health Hospital (Guangzhou, Guangdong, China). Inclusion criteria for the depressed subjects included: (1) DSM-IV criteria for unipolar major depression based on the Structured Clinical Interview for DSM Disorders-non-patient version (SCID-1/NP), (2) diagnosis of major depression for the first time, (3) 24-item Hamilton Depression Rating Scale scores $\geq$ 18 (HAMD), (4) clinical labs (complete blood count, electrolytes, liver, thyroid and renal function tests) with no clinically significant abnormalities, (5) age greater than 18 but less than 65 years and (6) ability to provide informed consent. Exclusion criteria included: (1) comorbid psychiatric disorders such as bipolar, schizophrenia, etc. (2) medical illnesses (e.g., autoimmune diseases, diabetes, HIV, endocrine disorders, hepatitis, cancer, or chronic infections) or medications (e.g., steroid medications, antioxidants, corticosteroids (oral, injected, inhaled and/or topical), immunotherapy, antibiotics that could affect the immune system, (3) psychotropic medication use (including antidepressants, mood stabilizers, anti-anxiety medications or antipsychotics) within previous 6 weeks, and (4) females in lactation or in gestational period. Twenty-five patients were recruited for the present study.

Twenty healthy, non-psychiatric volunteers were included in the study as controls, group-matched to subjects who had major depression according to age, gender, and ethnicity. Each control was given a non-structured clinical interview. All control subjects showed normal laboratory findings in blood count, chemistry, renal, thyroid and liver function, and ECG. A clinical interview confirmed that members of the control group did not suffer from any mental disorder and their prior history was free from mental and autoimmune disorders and free of any medication for at least 6 weeks. Informed consent was obtained from all subjects. This study was approved by the Institutional Ethical Committee of the Technology Bureau of Guangzhou, China.

Procedures

Blood samples

Ulnar venous blood (5 mL) was drawn between 8 a.m. and 9 a.m. from each subject the day after the clinical assessment was finished. Serum was separated from the venous blood and stored at -80°C for the measurement of cytokines, adrenocorticotropic hormone (ACTH),

and serum cortisol.

FCM-CBA test for cytokines in serum
The concentrations of IL-2, IL-4, IL-6, IL-10, TNF, IFN-γ in peripheral serum of patients and controls were quantitatively determined by the cytometric bead array (CBA) kit - BD™ CBA Human Th1/Th2 Cytokine Kit II (BD Biosciences, San Jose, CA, USA) following the instructions. Briefly, the CBA technique was based on six bead populations with distinct fluorescence intensities that had been coated with capture antibodies specific for IL-2, IL-4, IL-6, IL-10, TNF, IFN-γ proteins. The fluorescent dye had a maximal emission wavelength of approximately 650 nm, which was detectable by flow cytometry. The cytokine capture beads were mixed with the phycoerythrin-conjugated detection antibodies and then incubated with recombinant standards or test samples to form sandwich complexes. Following the acquisition of sample data on a FACS Canto™ flow cytometer (Becton Dickinson, San Jose, CA, USA), the sample results were generated in graphical and tabular format using the BD CBA Software (BD Biosciences, San Jose, CA, USA). Six standard curves (range from 0 to 5000 pg/mL) were obtained from one set of calibrators and six results were obtained on one test sample.

Test for ACTH and serum cortisol
The concentrations of ACTH and serum cortisol in peripheral serum of patients and controls were tested using a chemiluminescence method and an electrochemiluminescence method, respectively.

Statistical analysis
Data are described using the mean and standard deviation (mean ± s). Statistical significance of the differences between the patient group and the control group was established mostly using the Wilcoxon rank sum test; Spearman's rank correlation analysis was used to evaluate the correlation between cytokines and HAMD total score; $p < 0.05$ was considered to be significant. The statistical package used for the analysis was SPSS 13.0.

RESULTS

Concentrations of cytokines in serum
Table 1, Figure 1.

Concentrations of ACTH and serum cortisol in serum
Table 2.

Correlation between cytokines and HAMD total score
Spearman's rank correlation analysis was used to investigate the correlation between IL-2, TNF, and HAMD total score. Positive linear trend was found from the scatter plot of both IL-2-HAMD and TNF-HAMD, $p < 0.05$.

Correlation between IL-2 and serum cortisol, IL-2 and ACTH
Spearman's rank correlation analysis was used to investigate the correlation between IL-2 and serum cortisol and between IL-2 and ACTH. A positive linear trend was found from the scatter plot of IL-2-serum cortisol, $p < 0.05$.

DISCUSSION

The relationship between the neuroendocrine and immune system has been analysed since the 1980s, opening new important horizons in the neuro-endocrine-immune field [22]. It has been known that the neuroendocrine system can both directly and indirectly influence the developmental and functional activity of the immune system, building up the feedback protection to prevent over self-impairment [1,3]. On the other hand, the immune system can collaborate in the regulation of neuro-endocrine system activity [23], which would recognize and arouse the defense mechanism. The nervous, endocrine, and immune systems orchestrate a complex and integrated response to stimuli through interaction of molecules to maintain the homeostasis [24].
Accumulating evidence suggests that the HPA axis not only regulates normal physiological functions, but also has a close correlation with inflammation and autoimmune diseases [13,25]. Specifically, the HPA axis is the most important signal transduction pathway in the NEI, which exerts a significant regulation on the immune system including humoral immunity and cellular immunity by glucocorticoid (GC) effects. It is believed that hyperactivity of the hypothalamic-pituitary-adrenal (HPA) axis and immune dysregulation are among the most consistent biological findings in major depression. With extensive pathogenetic studies on HPA-immune axis of depressive disorder, it was found that depression patients showed endogenous glucocorticoid resistance, which was in a close relationship with extensive immune abnormalities in depression patients [8,26]. Research shows that depressive patients display an increasing risk for development of chronic inflammatory diseases or autoimmune diseases. Meanwhile, depressive symptoms have been found at a high prevalence in autoimmune diseases [27,28]. Therefore, in a previous study, we presented a hypothesis that depression could be regarded as an autoimmune disease [29]. In addition, we proposed that there was an emerging tendency towards autoimmune processes in MDD patients from a novel insight of Th17 cells [21].
In the present study, by using high-sensitivity assays of FCM-CBA, we found that the concentrations of IL-2 and TNF-α were higher in major depression patients compared with the concentrations in controls, suggesting patients are in a pro-inflammatory state. On the other hand, there were no significant differences among de-

Table 1. Cytokines in MDD patients.

Groups	n	IL-2 *	IL-4	IL-6	IL-10	TNF *	IFN-γ	TNF/IL-4 *
MDD patients	25	12.35 ± 0.80	6.88 (1.74)	9.99 (12.18)	5.92 (1.35)	4.73 (10.86)	4.21 (1.24)	0.69 (1.46)
Controls	20	11.77 ± 1.03	6.66 (1.03)	9.09 (14.39)	4.95 (2.56)	3.68 (2.84)	4.26 (1.93)	0.57 (0.25)

* **- The concentrations of IL-2, TNF, and TNF/IL-4 were much higher in major depression patients compared with the concentrations in controls ($p < 0.05$).**

Table 2. ACTH and cortisol in MDD patients.

Groups	n	Cortisol *	ACTH
MDD Patients	25	21.30 (10.73)	7.13 (3.50)
Controls	20	11.00 (4.59)	5.38 (2.29)

* **- Remarkably higher concentrations of serum cortisol in major depression patients' serum were observed ($p < 0.05$).**

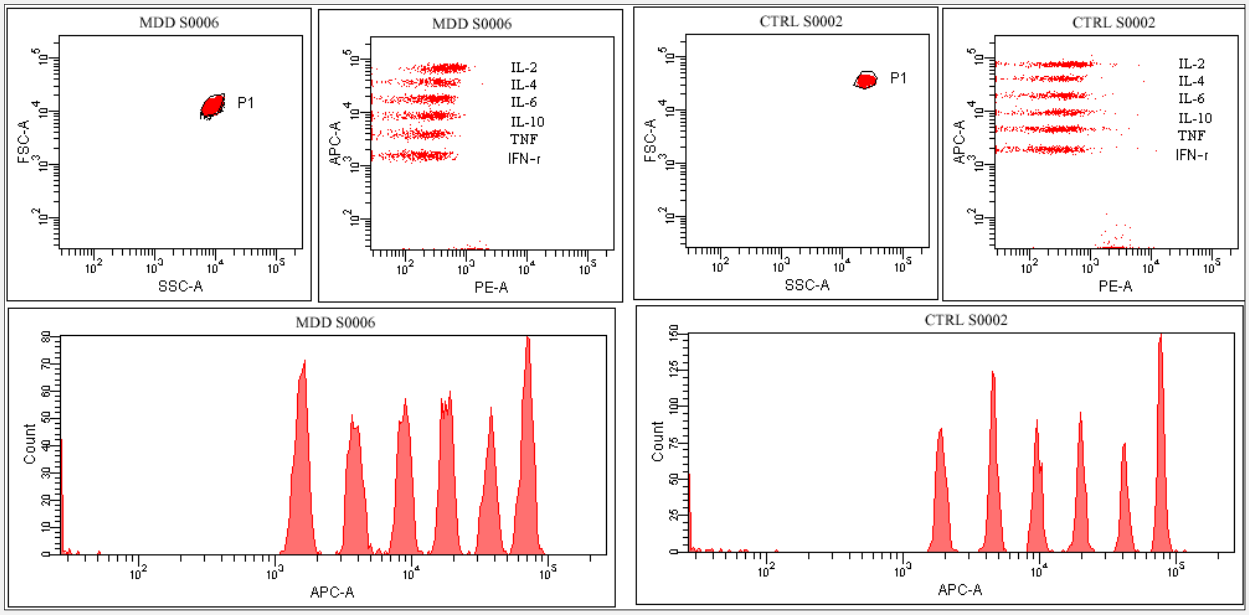

Figure 1. Th1/Th2 cytokine patterns in MDD patients (left) and controls (right).

pressed and nondepressed subjects for the other cytokines studied, including IL-4, IL-6, IL-10, IFN-γ. Our finding of an increased IL-2 and TNF-α is in keeping with the results of Sutcigil et al. [31]. This IL-2 overproduction could be integrated in the inflammatory response system, which is activated during depression and is consistent with the shift Th1/Th2 mechanism. Dysregulation of the functional activity of the immune system in depression is a phenomenon that has been demonstrated numerous times [18,19,30]. In 1999, Maes proposed the "IRS model of major depression", which implies that major depression is related to activation of the inflammatory response system (IRS). According to this model, major depression may be considered a psy-

choneuroimmunological disorder, in which peripheral immune activation, through the release of pro-inflammatory cytokines, is responsible for the variety of behavioural, neuroendocrine, and neurochemical alterations that are associated with this psychiatric condition. Of note, neurogenesis is believed to be affected by activation of the hypothalamic-pituitary-adrenal (HPA) axis due to the antineurogenic properties of glucocorticoids [8,14]. The stress response system is intricately linked with pro-inflammatory signaling. The stress response involves the release of IL-2 and TNF-α, which increases the release of corticotropin-releasing hormone (CRH), adrenocorticotropic hormone (ACTH), and cortisol b acting directly on hypothalamic and pituitary cells [31]. Moreover, the molecular mechanisms through which cytokines can influence the GR have been explored in previous studies [2,26,32]. They found that cytokines can exert their effects on GR function by one of two ways. First, through binding to specific membrane receptors and activation of secondary messenger cascades, they inhibit GR-mediated gene transcription, and furthermore, they inhibit GR translocation from cytoplasm to nucleus via possible interactions with heat-shock proteins, one of the constituents of the cytoplasmic GR complex. Wang et al. [33] demonstrated that pro-inflammatory cytokine inhibition of GR function is associated with activation of the p38 mitogen-activated protein kinase signal transduction pathway. TNF-a would bind to its receptor and result in activation of IκB kinase β (IKKβ), which phosphorylates IκB, allowing NF-κB to translocate to the nucleus. Through protein-protein interactions, activated NF-κB associates with GR, thus interfering with GR-DNA-binding. Of note, in our study, remarkably higher concentrations of serum cortisol in major depression patients' serum were observed, in agreement with relevant findings [8,10], indicating that this phenomenon was probably referred to as glucocorticoid resistance. Recent data suggested that glucocorticoid resistance may be a result secondary to chronic exposure to inflammatory cytokines as may occur during chronic medical illness or chronic stress [26].

However, we did not find an increased concentration of IL-6 in depressed patients, which was inconsistent with other major research studies. It is believed that high level of IL-6 plays a significant role in the pathogenic mechanism of depression. We assumed that different from most cytokines, which exert their actions primarily through paracrine and/or autocrine mechanisms, IL-6 is a circulating molecule with classic endocrine activity, whose plasma levels increase with age, are influenced by estrogens, and correlate with body mass index (BMI) [34,35]. In healthy individuals, IL-6 concentrations show a characteristic circadian pattern, with a zenith during sleep hours, between 0100 and 0500 hours, and a nadir during morning hours, between 0800 and 1000 hours; a secondary peak between 1700 and 1900 hours has also been reported [36,37]. Therefore, a single-time-point measurement of IL-6 makes it hard to reflect the physiological complexity in its secretion, ignoring the shift of its circadian rhythm and undermining important temporal considerations. In addition, one of the limitations in our present study is that we overlooked the contribution of potential confounders, such as age, gender, and body composition, to this biological abnormality. Furthermore, we established a relationship between TNF-a/IL-2 levels and HAMD score whereby the higher the concentration of circulating TNF-a/IL-2, the higher the score on HAMD, which indicated there was a positive correlation between the severity of the depressive episode and the level of pro-inflammatory cytokines, such as IL-2 and TNF-a.

We here report, for the first time, that the serum pro-inflammatory cytokines in major depression patients were measured by flow cytometric bead array system (FCM-CBA). The combined advantages of the broad dynamic range of fluorescence detection via flow cytometry, and the efficient capturing of analytes via suspended particles coated with distinct capture antibodies, enable CBA system to use fewer sample dilutions to determine analyte concentration in substantially less time (compared to conventional ELISA), showing a high sensitivity, reproducibility, and quality.

CONCLUSION

While the etiology of cytokine elevations in MDD remains unknown and significantly more work is needed, the growing understanding of cytokines in individuals with MDD suggests the intriguing possibility that early intervention, perhaps with anti-inflammatory agents and/or antioxidants, might reduce or prevent downstream medical consequences of MDD.

Declaration of Interest:
The authors declare no competing financial interest.

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