Original Article

Flow cytometric analysis of peripheral blood related immune cells and cytokines in patients with depression

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Abstract: Purposes: Profiling the peripheral blood lymphocyte subpopulations (PBLS), regulatory T cells, immune checkpoints and Th1/Th2/IL-17A cytokines to investigate the immunological variations in patients with depression. Methods: A total of 51 patients diagnosed with depression and 50 healthy persons as normal control were recruited. The profiles of PBLS, regulatory T cells, immune checkpoints (PD-1, TIM-3, LAG-3) on T cells were labeled with specific antibodies, and detected by flow cytometry. Th1/Th2/IL-17A cytokines were determined by the CBA assay. Results: Compared to that of the control group, the percentages of CD3+/CD4+ T cells, CD3+/CD8+/CD45+ T cells and CD19+/CD45+ B cells in patients with depression had no significant differences (P>0.05), however, the percentage of CD16+56+/CD45+ NK cells was significantly lower in patients with depression (P<0.05). Moreover, the percentage of CD4+CD25+FoxP3+ Treg cells was very significantly lower in patients with depression (P<0.01). As compared to that of the control group, the percentages of CD4+PD-1+ and CD8+PD-1+ T cells were very significantly higher (P<0.01), CD4+TIM-3+ T cells were significantly higher (P<0.05) and CD4+LAG-3+ T cells were very significantly lower (P<0.01) in patients with depression, however, there were no significant differences in percentages of CD8+TIM-3+ and CD8+LAG-3+ T cells (P>0.05). The levels of IL-6 and TNF in patients with depression were significantly higher than that in the control group (P<0.05), but there were no significant differences in IL-2, IL-4, IL-10, IL-17A and IFN-γ concentrations (P>0.05). Conclusion: Our study showed the potential influences of immunological factors in the development of depression, which could facilitate its diagnosis and treatment.

Keywords: Flow cytometry, depression, lymphocyte subpopulations, treg, checkpoint

Introduction

Depression is a common affective disorder characterized by persistent mood depression. The continuous and rapid development of the society, has resulted in intense competition and various pressures, thereby increase the incidence of depression year by year. The incidence of deaths and disabilities caused by depression has also risen, which resulting in low life quality and causing serious psychological and economic burden to families and society. The World Health Organization predicts that by 2020, depression will be the second largest disease burden after ischemic heart disease worldwide [1]. In recent years, studies have found that patients with depression have obvious abnormal immune function [2, 3], mainly reflected in the cell and immune imbalance, abnormal cytokine levels [4, 5], immunoglobulin level anomaly, lymphocyte subset changes (including Th1/Th2 imbalance, cytotoxic T lymphocyte increase and regulatory T cells loss, etc.) and other aspects [3, 6]. However, the etiology and pathogenesis of depression remains unknown, and its diagnosis mainly relies on psychological counseling. In this study, we profiled the lymphocyte subsets, Treg cells, immune checkpoints (PD-1, TIM-3, LAG-3) and Th1/Th2/IL-17A cytokines to facilitate effective diagnosis and clinical treatment of depression.

Materials and methods

Participants

A total of 51 cases of depression who visited the Second Affiliated Hospital of Xinxiang Medical College as outpatients and inpatients from
Flow cytometric analysis of the peripheral blood in depression patients

Table 1. Demographic and clinical characteristics of the study subjects (Σ±x)

<table>
<thead>
<tr>
<th></th>
<th>Healthy controls (n=50)</th>
<th>Depression (n=51)</th>
<th>Statistics</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>37.16±5.38</td>
<td>33.26±5.21</td>
<td>t=1.262</td>
<td>0.215</td>
</tr>
<tr>
<td>Education (years)</td>
<td>9.51±3.03</td>
<td>9.46±3.22</td>
<td>t=0.059</td>
<td>0.972</td>
</tr>
<tr>
<td>Sex, Male%</td>
<td>47%</td>
<td>51%</td>
<td>χ²=2.112</td>
<td>0.137</td>
</tr>
<tr>
<td>Marriage, married%</td>
<td>68%</td>
<td>72%</td>
<td>χ²=2.326</td>
<td>0.229</td>
</tr>
<tr>
<td>Duration (years)</td>
<td>-</td>
<td>7.06±0.96</td>
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</table>

October 2015 to August 2016, aged 21-55 years, average age (33.26±5.21), duration 180d-5 years, the average duration (7.06±0.96) months were included in this study. Inclusion criteria: in line with the “Chinese classification and diagnostic criteria for mental disorders (CCMD-3)” single phase depression diagnostic criteria, two deputy chief physicians above the rank of psychiatric specialist performed the diagnosis. Exclusion criteria: patients who did not meet the inclusion criteria or were unwilling to cooperate; patients who took antidepressants, nerve blocks and hormone drugs, contraceptives, or any drugs that inhibit the immune function in the past two weeks; other psychiatric diagnosis; diagnosed with other serious diseases, such as malignant tumor, cardiovascular, liver, kidney and hematopoietic system diseases.

The control group included 50 healthy normal people, aged 22-59 years, with an average age of (37.16±5.38) years. There was no difference in age and education level between the depression group and the control group by the t-test and chi-square analysis of the two mean scores (Table 1).

This study was approved by the medical ethics committee of the First Affiliated Hospital of Xinxiang Medical College (batch number: 2015-074). All participants or their families signed informed consent forms.

Specimen collection and determination

Two ml venous blood was collected from all participants in EDTA anticoagulant tubes, stored at 4°C, and treated within 2 h.

Detection of lymphocyte subsets by flow cytometry

100 μl anticoagulant whole blood was added to two flow tubes, marked A and B, respectively, followed by 10 μl CD3/CD4/CD8/CD45 antibody (BD Multitest™ IMK Kit) to the A tube and 10 μl CD3/CD16+56/CD19 antibody (BD Multitest™ IMK Kit) to the B tube. The samples were mixed at room temperature in the dark and incubated for 20 min. Then, 500 μl hemolysin was added to each tube, mixed, incubated at room temperature in the dark for 10 min, and detected within 2 h. The percentage of CD3+/CD45+, CD3+CD4+/CD45+, CD3+CD8+/CD45+, CD16+56+/CD45+ and CD19+/CD45+ were counted (accounted for CD45+ cell) and compared between the healthy normal group and depression group.

Detection of treg cells by flow cytometry

Three flow tubes were marked as CD4+ 'A' tube, CD4+CD25+ 'B' tube, CD4+CD25+FoxP3+ 'C' tube, respectively. 100 μl anticoagulant whole blood was added to each tube followed by 10 μl of the corresponding labeled antibody [BD Pharmingen™ anti-human (CD4-FITC/CD25-APC/FoxP3-PE)]. In the C tube, the cells were ruptured (eBioscience FoxP3 Staining Buffer Set) before adding 10 μl FoxP3-PE antibody. The tubes were incubated at room temperature in the dark for 20 min. Then, 500 μl hemolysin was added to each tube, mixed, and incubated at room temperature in the dark for 10 min. The tubes were incubated for 30 min at room temperature in the dark and detected on flow cytometry within 2 h. The percentage of CD4+CD25+FoxP3+ positive cells were counted (accounted for CD4+ T cell) and compared between the healthy normal group and depression group using independent sample t-test.

Detection the expression levels of PD-1, TIM-3 and LAG-3 in CD4+ T cells by flow cytometry

Four flow tubes were marked CD4+, CD4+PD-1+, CD4+TIM-3+, and CD4+LAG-3+, respectively. 100 μl anticoagulant blood was added to each tube, followed by 10 μl of the corresponding labeled antibody [Biolegend anti-human (CD4-APC/CD4-FITC/TIM-3-PerCP/LAG-3-PE)], mixed, incubated for 20 min at room temperature. Then, 500 μl hemolysin was added to each tube, mixed, incubated at room temperature in the dark for another 10 min, and detected on flow cytometry within 2 h. The percent-
Flow cytometric analysis of the peripheral blood in depression patients

Age of CD4\(^+\)PD-1\(^+\), CD4\(^+\)TIM-3\(^+\), and CD4\(^+\)LAG-3\(^+\) positive cells (accounted for CD4\(^+\) T cell) were counted and compared between the healthy normal group and depression group using independent sample \(t\)-test.

Detection of expression levels of PD-1, TIM-3 and LAG-3 in CD8\(^+\) T cells by flow cytometry

Four flow tubes were marked CD8\(^+\), CD8\(^+\)PD-1\(^+\), CD8\(^+\)TIM-3\(^+\), CD8\(^+\)LAG-3\(^+\), respectively. 100 \(\mu\)l anticoagulant blood was added to each tube, followed by 10 \(\mu\)l of the corresponding labeled antibody [Biolegend anti-human (CD8-APC/PD-1-FITC/TIM-3-PerCP/LAG-3-PE)], mixed, and incubated at room temperature in the dark for 20 min. Then, 500 \(\mu\)l hemolysin was added to each tube, mixed, incubated at room temperature in the dark for 10 min, and detected on flow cytometry within 2 h. The percentage of CD8\(^+\)PD-1\(^+\), CD8\(^+\)TIM-3\(^+\), and CD8\(^+\)LAG-3\(^+\) positive cells (accounted for CD8\(^+\) T cell) were counted and compared between the healthy normal group and depression group using independent sample \(t\)-test.

Detection of related cytokines by flow cytometry

The samples were processed according to the instructions provided by the Cytometric Bead Array (CBA) human Th1/Th2/Th17 cytokine kit of BD Company, and then IL-2, IL-4, IL-6, IL-10, IL-17A, TNF and IFN-\(\gamma\) were detected and analyzed. The concentration of cytokines was calculated according to the protocol of CBA kit, which was then compared between the normal control group and depression group.

Statistical analysis

Flowjo 7.6.1 software was used to analyze the flow chart, and SPSS 20 software was used for statistical analysis. All measurement data were expressed by \(\bar{x}\pm s\). The two groups were compared using independent sample \(t\)-test, and the difference was considered as statistically significant when \(P<0.05\).

Results

Detection of lymphocyte subsets in the depression and control groups

As compared to that of the control group, the percentages of CD3\(^+\)/CD4\(^+\) T cells, CD3\(^+\)CD4\(^+\)
Flow cytometric analysis of the peripheral blood in depression patients

**Figure 2.** Flow cytometry of peripheral blood Treg cells. First, the single staining of CD4 antibody was used to define the quadrant of CD4⁺CD25⁺, then the CD4CD25 antibody double staining was used to define the quadrant of CD4⁺CD25⁺Foxp3⁺, finally the CD4CD25Foxp3 antibody triple staining was used to detect the percentage of CD4⁺CD25⁺Foxp3⁺ positive cell accounting for the CD4 positive T cells.
Table 3. Comparison of peripheral blood Treg cells between the two groups (x±s)

<table>
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<th>Healthy controls (n=50)</th>
<th>Depression (n=51)</th>
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<tbody>
<tr>
<td>CD4⁺CD25⁺FoxP3⁺ (%)</td>
<td>3.45±0.33</td>
<td>2.23±0.31**</td>
</tr>
</tbody>
</table>

Note: **P<0.01.

CD4⁺ T cells, CD3⁺CD8⁺/CD45⁺ T cells and CD19⁺/CD45⁺ B cells in patients with depression had no significant difference (P>0.05). However, the percentage of CD16⁺/56⁺/CD45⁺ NK cells in patients with depression was significantly lower than that in the control group (t=10.124, P<0.05) (Figure 1 and Table 2).

Detection of Treg cells in the depression and control groups

The percentage of CD4⁺CD25⁺FoxP3⁺ Treg cells was very significantly lower in the patients with depression than that in the control group (t=6.121, P<0.01) (Figure 2 and Table 3).

Detection of PD-1, TIM-3 and LAG-3 in peripheral blood CD4⁺ T and CD8⁺ T cells in the healthy normal group and depression group

As compared to that in the control group, the percentages of CD4⁺PD-1⁺ and CD8⁺PD-1⁺ T cells were very significantly higher (t=12.306 and 5.059, P<0.01, respectively), CD4⁺TIM-3⁺ T cells was significantly higher (t=2.968, P<0.05) and CD4⁺LAG-3⁺ T cells was very significantly lower (t=-9.460, P<0.01) in the patients with depression. However, there were no significant differences in the percentages of CD8⁺TIM-3⁺ and CD8⁺LAG-3⁺ T cells between the two groups (P>0.05) (Figure 3 and Table 4).

Detection of related cytokines

The levels of IL-6 and TNF in patients with depression were significantly higher than that in the control group (t=3.057 and 5.836, P<0.05, respectively). There were no significant differences in IL2, IL-4, IL-10, IL-17A and IFN-γ concentrations between the two groups (P>0.05) (Figure 4).

Discussion

Depression was resulted by many factors. Its etiology and pathogenesis are complex and still unclear. The immune system plays an important role in the development and treatment of depression [7]. Based on the surface markers and different functions, lymphocytes can be divided into T lymphocytes, B lymphocytes and NK cells. T lymphocytes are mainly involved in the cellular immunity, B lymphocytes in humoral immunity, and NK cells in independent spontaneous antigen stimulation to exert cytotoxic effects. These three types of lymphocytes maintain homeostasis of the human body. Based on the cell surface markers and different functions, T lymphocytes can be divided into CD4 T and CD8 T subsets, CD8 T are the cytotoxic T lymphocytes, responsible for the removal of target cells, while CD4 T are the helper T cells (Th cells) that can be divided into Th1, Th2, Th17 and regulatory T cells (Treg cells) based on the function and secreted factors [8]. Patients with depression have broad immunosuppression. CD4⁺CD25⁺FoxP3⁺ regulatory T cells are a crucial subset of T cells, and regulatory T cells suppress effector T cell function by various ways, such as the secretion of inhibitory cytokines and cell contact, in order to maintain the body’s immune balance [9]. Li Y et al showed that patients with depression had no significant difference in the number of peripheral blood lymphocyte subsets [10], but their regulatory T cells were significantly lower than those in the control group. Excessive suppression of regulatory T cell function can cause enhanced immune response, as seen in autoimmune diseases, tumors, inflammatory diseases and other diseases related to regulatory T cells. This study used flow cytometry assay to detect the peripheral blood lymphocyte subsets and CD4⁺CD25⁺FoxP3⁺ regulatory T cells in depression patients, and found that the percentage of regulatory T cells was significantly decreased in the depression group as compared to healthy controls. However, there were no significant difference between the two group among the percentages of lymphocyte subsets, CD3 cells, helper T cells (CD3⁺CD4⁺), cytotoxic T cells (CD3⁺CD8⁺), and the B cell ratio. But the NK cell ratio was significantly decreased in the depression group as compared to the control group.

Cytokines are heterogeneous proteins secreted by various cells (Th1, Th2, Th17, macrophages and B cells), and are divided into pro-inflammatory cytokines (IL-1, IL-2, IL-6, IL-12, TNF-α, TNF-β, IFN-α, IFN-γ etc.) and anti-inflammatory proteins.
Flow cytometric analysis of the peripheral blood in depression patients

Figure 3. Flow cytometry of PD-1, TIM-3 and LAG-3 in peripheral blood CD4^+ T and CD8^+ T cells. A. Control group using CD4 or CD8 antibody single staining. B. Healthy control group using CD4 or CD8 antibody combined with PD-1, Tim-3 or LAG-3 double staining. C. Depression group with CD4 or CD8 antibody single staining. D. Depression group which was CD4 or CD8 antibody combined with PD-1, Tim-3 or LAG-3 double staining.
Flow cytometric analysis of the peripheral blood in depression patients

Table 4. Percentage of PD-1, TIM-3 and LAG-3 positive cells in peripheral blood CD4+ T and CD8+ T cells in the two groups (x̄±s)

<table>
<thead>
<tr>
<th></th>
<th>Healthy controls (n=50)</th>
<th>Depression (n=51)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4+PD-1+ (%)</td>
<td>40.68±3.32</td>
<td>82.82±6.90**</td>
</tr>
<tr>
<td>CD4+TIM-3+ (%)</td>
<td>1.67±0.26</td>
<td>2.30±0.40*</td>
</tr>
<tr>
<td>CD4+LAG-3+ (%)</td>
<td>73.36±3.22</td>
<td>23.28±4.77**</td>
</tr>
<tr>
<td>CD8+PD-1+ (%)</td>
<td>35.30±4.46</td>
<td>54.72±7.33**</td>
</tr>
<tr>
<td>CD8+TIM-3+ (%)</td>
<td>0.30±0.05</td>
<td>0.31±0.08</td>
</tr>
<tr>
<td>CD8+LAG-3+ (%)</td>
<td>32.18±8.59</td>
<td>36.24±5.51</td>
</tr>
</tbody>
</table>

Note: *P<0.05, **P<0.01.

Recent studies have shown that immune checkpoint plays an important role in the regulation of T cells [17-19]. This

matory cytokines (IL-4, IL-10, IL-13). In 1991, Smith proposed that the excessive release of some pro-inflammatory cytokines can cause abnormal activities related to depression [11]. The results of this study showed that the serum levels of IL-6 and TNF were significantly higher in patients with depression than in the control group. Maes suggested that depression is an inflammatory disease, and pro-inflammatory cytokine levels are increased in the etiology of depression index [12]. Gupta R et al found that the serum levels of TNF-α were significantly decreased in depression patients after treatment [13]. Li B et al have shown that TNF-α is involved in the development of depression in SD rats [14]. Liu CX et al found that the plasma levels of pro-inflammatory cytokines were significantly higher in patients with depression than in the normal subjects, but the levels of these cytokines decreased after treatment with antidepressants [15]. This study found that patients with depression had increased pro-inflammatory cytokine levels, which is in agreement with several domestic and international studies [14-16]. However, the levels of IL-2, IL-4, IL-10, IL-17A and IFN-γ were comparable between the two groups, perhaps due to the pathogenesis of depression, complexity of the human immune system, or differences within the group, diagnostic tools, demographic data.

Recent studies have shown that immune checkpoint plays an important role in the regulation of T cells [17-19]. This
Flow cytometric analysis of the peripheral blood in depression patients

study examined the expression of PD-1, TIM-3 and LAG-3 in CD4 T and CD8 T cells, and found that the percentages of CD4\(^+\)PD-1\(^+\), CD8\(^+\)PD-1\(^+\) T cells and CD4\(^+\)TIM-3\(^+\) T cells in peripheral blood was significantly higher in the depression patients than in the healthy control group. Huang YH et al found that after the suppression of PD-1 and TIM-3 with specific antibodies, the tumor killing ability of CTL was enhanced [17]. Zhang L et al showed that the expressions of PD-1, TIM-3 and LAG-3 in cultured CIK were increased by varying degrees, and they were involved in the negative regulation of T cell function [19]. However, this study showed that the percentage of CD4\(^+\)LAG-3\(^+\) T cells in peripheral blood was significantly lower in the depression patients than the healthy control group, while the CD8\(^+\)TIM-3\(^+\) and CD8\(^+\)LAG-3\(^+\) percentage of T cells were comparable between the two groups. These results indicated that in patients with depression, PD-1 may play a role in the negative regulation of CD4 T and CD8 T, TIM-3 mainly through the role of CD4 T, while LAG-3 may or may not play a role in depression. However, due to the complexity of the immune system, testing methods adopted, instruments used and other factors, these results need further validation.

In summary, our results indicated the close relationship between depression and imbalance of immune function. This study examined the total lymphocytes, T cells and B cells in patients with depression. Although the absolute numbers of total lymphocytes, T cells and B cells were similar, the percentage of CD16\(^+\)56\(^+\)NK cells and CD4\(^+\)CD25\(^+\)FoxP3\(^+\) regulatory T cells were significantly decreased in patients with depression; the serum levels of IL-6, TNF were significantly increased, the expression of PD-1 on the surface of CD4\(^+\) T cells was significantly increased, TIM-3 was significantly increased, while only PD-1 expression was significantly increased on the surface of CD8\(^+\) T cells. The expression of TIM-3 and LAG-3 did not change significantly, indicating that CD4\(^+\) T cells may play a more important role in the development of depression than CD8\(^+\) T cells. Recently, Baruch K reported that PD-1 antibodies could delay the onset of Alzheimer’s disease in mice and improve their memory [20]. Combined with the findings in this study, we hypothesized that PD-1 may play an important role in the development of depression, and PD-1 antibodies may be useful in the treatment of depression. These results could facilitate clinical diagnosis and potential treatment strategies for depression.

Acknowledgements

The authors thank Dr. Dong-jun Zhang for the selfless help in the evaluation and screening of patients with depression. This work was supported by the Disciplinary group of Psychology and Neuroscience, Xinxiang Medical University (2016PN-KFKT-12); Henan Key Laboratory of Neural Regeneration and Repairment (HNS-JXF-2016-006; HNSJXF-2016-012 ); the Young scientist project in the First Affiliated Hospital of Xinxiang Medical University (QN-2017-A010); Key Scientific Research Projects for Higher Education of Henan Province (grant No. 17A310022; 15A320063).

Disclosure of conflict of interest

None

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