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Original Article

Clinical significance of Vδ1 T cell detection in sepsis patients

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Abstract: This study is to investigate changes of the number of PB Vδ1 T cells and their function in patients with sepsis, analyze the clinical significance of Vδ1 T cell detection and validate the effects of Vδ1 T cells on the onset of sepsis. Forty patients with sepsis were included into this study together with forty healthy subjects who received the physical examination in the hospital during the same period, and the clinical data of all subgroups of patients with sepsis were recorded. In the morning, the fasting peripheral venous blood at 10 ml was sampled from patients with sepsis and subjects in the Health Control (HC) group. The flow cytometry (FCM) was used to measure the percentage of Vδ1 T cells in PB, analyze the correlation between the percentage of Vδ1 T cells, APACHEII score and blood lactic acid level in patients with sepsis, and detect the expression level of Foxp3 on the surface of Vδ1 T cells. CFSE staining method was used to detect the influence of Vδ1 T cells on the proliferation capacity of naïve CD4 T cells. Compared with the HC group, the number of Vδ1 T cells in PB was significantly increased in patients with sepsis ($P<0.01$), and the sepsis shock group exhibited the highest expression level of Vδ1 T cells, followed by the severe sepsis group and the sepsis group. The percentage of Vδ1 T cells in patients with sepsis was positively correlated with APACHEII score and the lactic acid level, respectively. Compared with the HC group, the expression level of Foxp3 on the surface of Vδ1 T cells in PB was significantly increased in patients with sepsis ($P<0.01$), with the highest expression level of Foxp3 in the sepsis shock group, followed by the severe sepsis group and the sepsis group. In the HC group and patients with sepsis, the proliferation rate of naïve CD4 T cells co-incubated by Vδ1 T cells and naïve CD4 T cells in PB was (66.94±8.91)% and (47.24±9.86)%, respectively. Vδ1 T cells in PB of patients with sepsis exhibited obviously increased inhibition on the proliferation of naïve CD4 T cells, indicating that PB Vδ1 T cells in patients with sepsis presented stronger immunosuppressive functions than those in the HC group ($P<0.01$). The percentage of PB Vδ1 T cells in patients with sepsis was increased and the immunosuppressive function was increased, so that immune function of patients with sepsis was inhibited and then sepsis occurred. This indicated that Vδ1 T cells in PB might play important roles in the immune pathogenesis of sepsis and provide clinical values in the evaluation of prognosis.

Keywords: Sepsis, peripheral blood (PB), Vδ1 T cells, Foxp3, proliferation

Introduction

Sepsis refers to the systemic inflammatory response syndrome (SIRS) induced by infections. It has been clinically proved that bacteria or highly suspected infection lesions exist. Sepsis is an important cause for human death [1-5]. Patients with sepsis are generally accompanied by organ dysfunction, including tissue hypo-perfusion and hypoxia, lactic acidosis, oliguria or brain function changes [6, 7]. There were wide studies on sepsis over past twenty years, but sepsis is one of main causes for intensive care units (ICU) death [8]. Through studies on the pathogenesis of sepsis, we can further understand this complex syndrome and find effective therapies.

It has been proved that decreased immune response blocks the effective clearance of bacteria, which is one of main causes for sepsis aggravation [9]. The immunosuppressive state of patients with sepsis seems to explain why the application of anti-inflammatory therapies cannot improve the prognosis of sepsis in most clinical trials [10]. This means that the correc-

tion of immunosuppression may be the key to treating sepsis [11]. Regulatory T cells (Treg) are a cluster of cells with immunosuppressive functions, and an increase in the percentage of Treg cells or functional enhancement will decrease human immune functions and impair the immune balance. There are great differences between different Treg cell subgroups and functions, and also in the mechanism that Treg cells with different phenotypes and functions mediate the immunosuppression. CD4+CD25+ regulatory T cells (Treg) are important cells to maintain self-tolerance and regulate physiological and pathological immune responses [12, 13]. To date, many literatures have proved that CD4 Treg closely correlate with the onset of sepsis [14, 15]. Chen et al. [14] suggested that the proportion of CD4+CD25+ regulatory T cells in sepsis patients was closely related to the prognosis of patients with sepsis in ICU. Zu et al. [15] found that the increased proportions of CD4+CD25+ regulatory T cells led to the decreased ratios of CD3 (+) CD4 (+)/CD3 (+) CD8 (+), so that the body's immune function was in a state of inhibition, which were involved in the pathogenesis of sepsis.

However, in addition to CD4 Treg, there also exist other cells with immunosuppressive functions in PB, such as regulatory B cells, regulatory DC cells and Vδ1 T cells [16-18]. Pan X et al. [19] found that the proportion of CD19+CD24hiCD38hi regulatory B cells was significantly increased in neonatal sepsis patients, and the incidence of increased CD19+CD24hiCD38hi regulatory B cells may be involved in the pathogenesis of sepsis by suppressing the immune response. Vδ1 T cell is a T cell subgroup with immunosuppressive functions newly discovered in recent years [18]. To date, there have been nearly no literatures on the effects of Vδ1 T cells in sepsis. Therefore, the changes in the number of Vδ1 T cells in PB for patients with sepsis were firstly detected in this study, and the correlation analysis was performed to analyze the correlation between the number of Vδ1 T cells in PB for patients with sepsis and the conditions of patients with sepsis. Furthermore, this study detected the changes in PB Vδ1 T cell functions for patients with sepsis through functional experiments. Thus, this study investigated the effects of Vδ1 T cells on the onset of sepsis. The results are reported as follows.

Materials and methods

Clinical data

Forty patients with sepsis admitted to ICU in the hospital from March 2015 to June 2016 were enrolled into this study together with forty healthy subjects who received the physical examination in the hospital during the same period. All the patients conformed to the diagnostic criteria of sepsis prepared by the International Sepsis Definitions Conference in Washington, U.S.A. in December 2001 [6], and these patients were divided into three groups in accordance with the classification standards (the severe sepsis: sepsis accompanied by organ dysfunction, tissue hypoperfusion or hypotension; the sepsis shock: persistent hypotension that cannot be corrected after patients with severe sepsis were revived with sufficient liquids, and also considered as one special type of severe sepsis): The sepsis group (n=16), including 9 males and 7 females, at the age of (60.13±18.92); The severe sepsis group (n=14), including 8 males and 6 females, at the age of (61.22±21.46); The sepsis shock group (n=10), including 6 males and 4 females, at the age of (60.34±20.12). The following patients were excluded: Patients with autoimmune diseases, acute stroke, myocardial infarction, and viral hepatitis or HIV infection; patients who received hormones or immunosuppressors within 3 months after admission. Meanwhile, forty healthy subjects, who received the physical examination in the hospital during the same period, were used as the Healthy Control group (male: 23, female: 17), with the average age (60.74± 18.31).

Main agents

Bovine serum albumin (BSA) was purchased from Sigma Corporation; RPMI 1640 medium, fetal bovine serum (FBS) and phosphate buffer saline (PBS) were purchased from Gibco Corporation; Lymphocyte separation medium was purchased from Tianjin Haoyang Biological Products Technology Co., Ltd.; PE-anti-CD3 antibody, PEcy5-anti-CD4 antibody, FITC-anti-TCR V1 antibody, APC-anti-Foxp3 antibody and Foxp3 staining kit were purchased from Biolegend Corporation; Purified anti-TCR V1 antibody used for amplification was purchased from Beckman Corporation; Naïve CD4 T cell separation kit was purchased from MiltenyiBiotec Corporat-

ion; Purified anti-CD3 antibody and Purified anti-CD28 antibody for amplification were purchased from BD Corporation; CFSE dye solution was purchased from Thermo Corporation.

Separation of peripheral blood mononuclear cell (PBMC)

Fasting peripheral venous blood at 10 mL was sampled from patients with sepsis and the health control (HC) group in the morning. A 50 mL centrifuge tube was used and added with 10 mL of lymphocyte separation medium; then blood sample was absorbed by the sucker and placed on the fluid level of the separation liquid (make sure blood was placed on the surface of the lymphocyte separation medium). Afterwards 800×g solution was centrifuged for 18 min; at the end of centrifugation, the centrifuge tube was divided into four layers from the top to the bottom (Layer I: plasma; Layer II: circular milk-white lymphocyte; Layer III: transparent separation medium; Layer IV: red blood cell). A sucker was used to carefully draw the circular milk-white lymphocyte layer into another 15 mL centrifuge tube, and the centrifuge tube was added with 10 mL of serum-free RPMI 1640 medium cleaning liquid, evenly mixed and then 4000×g solution was centrifuged for 10 min; the supernatant was removed. Cell sediments were resuspended by 10 mL of serum-free RPMI 1640 medium, and then 250×g solutions was centrifuged for 8 min; then, 1 mL RPMI-1640 complete medium containing 10% fetal bovine serum was used to resuspend cell sediments, and then prepared to 2×10^6/mL cell suspension after trypan blue staining and counting.

Determination of the percentage of Vδ1 T cells in PB

The above PBMC at 1×10^6 units was added into the Eppendorf tube, and then 1 mL PBS solution containing 1% BSA was added into this tube. Then, 250×g solutions were centrifuged for 8 min, and the supernatant was removed. Finally, the above operations were repeated. δAfterwards, cells were resuspended by 0.1 ml PBS containing 1% BSA, PE-anti-CD3 antibodies and FITC-anti-TCR V1 antibodies were added into the solution, and then incubated at 4°C for 30 min in dark places; δAfter the solution was rinsed by PBS containing 1% BSA twice, cells were resuspended into 0.1 ml 1% paraformaldehyde fixed liquid and then detected by the flow cytometry (FCM).

Detection of the expression level of Foxp3 on the surface of Vδ1 T cell

After the above PBMC received surface molecular staining as described in 1.4, 0.5 ml cytomembrane fixed permeabilization solution in Foxp3 staining kit was added, and then the solution was placed at room temperature for 30 min in dark places; δAfterwards, after cells were washed twice by the permeabilization solution in Foxp3 staining kit, APC-anti-Foxp3 antibodies were added into the solution, and then placed at room temperature for 30 min in dark places; Next, permeabilization solution was used to rinse cells twice, and cells were resuspended in 0.2 ml 1% paraformaldehyde fixed solution for detection by the flow cytometer.

Amplification for Vδ1 T cells

Each well of 48-well plastic culture plate was added with 0.2 ml RPMI-1640 medium containing 0.125 μg anti-TCR Vδ1 monoclonal antibody, and then incubated at 37°C for 2 h in CO_2 incubator. PBMC suspension resuspended by complete medium was added into a 48-well plate coated by anti-TCR Vδ1 monoclonal antibody (1.0 ml for each well). The culture plate was cultivated at 37°C in 5% CO_2 saturated wet environment. The liquid was changed or wells were separated every 1~3 days based on the cell growth state. After the cells were incubated for 2W, the flow cytometry was used to separate and obtain Vδ1 T cells with the purification above 90%.

Separation of naïve CD4 T cells

As described in Naïve CD4 T cell separation kit, operations were performed as follows: Firstly the number of PBMC to be separated was determined, and then 300×g solution was centrifuged for 10 min; The supernatant was removed, and 1×10^7 cells were resuspended by 40 μL buffer solution; 1×10^7 cells were added into 10 μL Naive $CD4^+$ T Cell Biotin-Antibody Cocktail II in Naïve CD4 T cell separation kit; The solution was evenly mixed and then incubated at 2-8°C for 5 min; 1×10^7 cells were resuspended by 30 μL buffer solution; 1×10^7 cells were added into 20 μL Naive $CD4^+$ T Cell MicroBead Cocktail II in Naïve CD4 T cell separation kit; The solution was evenly mixed and then incubated at 2-8°C for 10 min; Incubated

Table 1. Comparison of clinical data among three subgroups

Parameter	The sepsis group (n=16)	The severe sepsis group (n=14)	The sepsis shock group (n=10)	*P value*
Age (years of age)	60.13±18.92	61.22±21.46	60.34±20.12	0.95
M/F	9/7	8/6	6/4	0.91
Blood lactic acid level (mmol/L)	1.78±0.74	2.85±0.77[a]	4.68±0.76[a,b]	0.00
APACHE II score	9.56±2.07	18.32±3.19[a]	28.37±4.22[a,b]	0.00
Oxygenation index (kPa)	38.83±7.91	27.54±7.76[a]	15.11±6.95[a,b]	0.00

Notes: Compared with the sepsis group, [a]P<0.01; Compared with the severe sepsis group, [b]P<0.01.

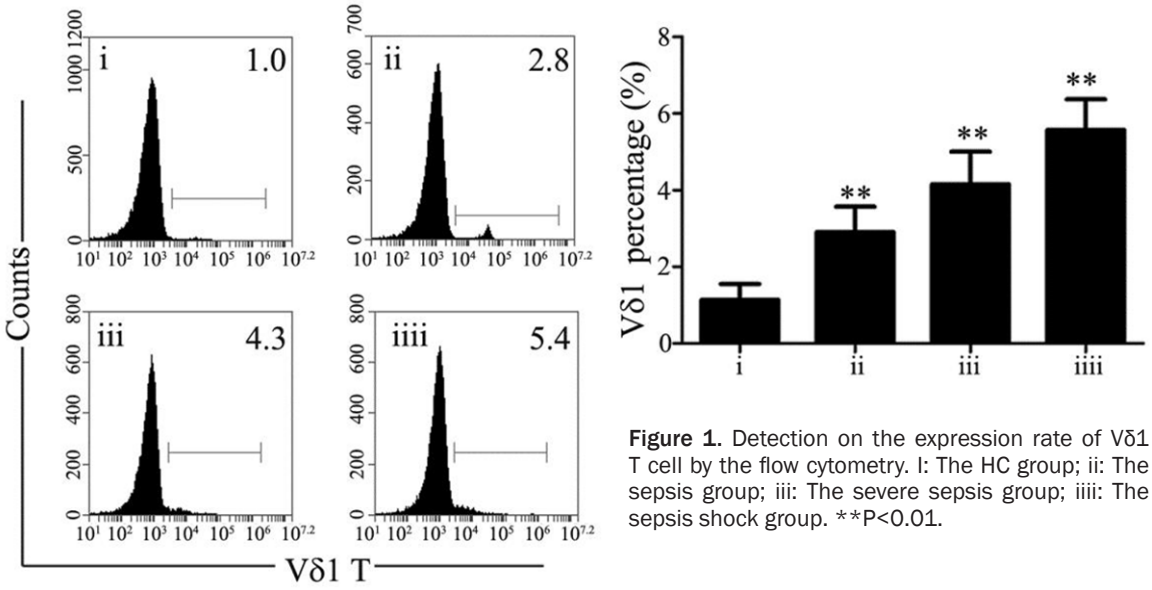

Figure 1. Detection on the expression rate of Vδ1 T cell by the flow cytometry. I: The HC group; ii: The sepsis group; iii: The severe sepsis group; iiii: The sepsis shock group. **P<0.01.

cells passed through the cell separation column to separate and obtain Naïve CD4 T cells with the purification above 90%.

Detection on the proliferation of CFSE cells

Naïve CD4 T cells were rinsed once by 10 ml serum-free RPMI 1640 medium, CFSE dyeing solution with the final concentration of 5 mmol/L was added into, and then incubated at 37°C in 5% CO_2 incubator for 10 min in dark places. Afterwards, 5 ml pre-cooled CFSE dyeing terminated liquid (RPMI 1640 medium containing 5% FBS) was added into cells and then placed on ice for 5 min to terminate dyeing. Then, 400×g solution was centrifuged once for 8 min and washed by 10 ml RPMI-1640 medium once. After cells were resuspended by RPMI-1640 complete medium Vδ1 T cells and the above Naïve CD4 T cells were added into the 48-well culture plate coated by 1 g/ml CD3 antibodies and 2 g/ml CD 28 antibodies at the ratio of 1:1, and then incubated for 5 d. Finally, cells were collected and detected by the flow cytometry.

Statistical methods

Data in this study were expressed by mean ± SD. The analyses were conducted by using SPSS 16.0 statistical software. Two Way ANOVA analysis was used for multi-group data comparison. Two-sided t-test was used for data comparison between two groups. Spearman correlation analysis was performed for correlation analysis.

Results

Comparison of clinical data between all subgroups of patients with sepsis

As shown in **Table 1**, there were differences in the comparison of clinical data, such as the age and sex composition, between the sepsis group, severe sepsis group and sepsis shock group, but these differences were not statisti-

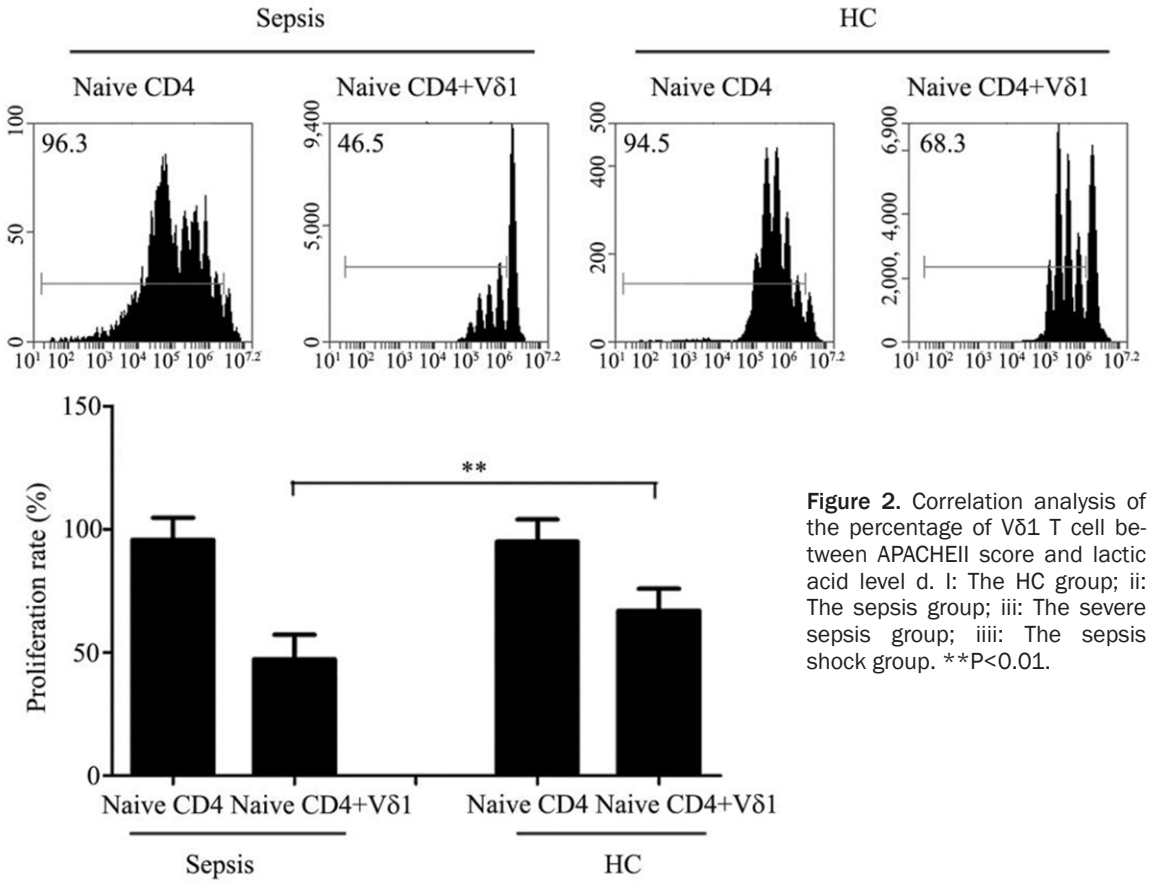

Figure 2. Correlation analysis of the percentage of Vδ1 T cell between APACHEII score and lactic acid level d. I: The HC group; ii: The sepsis group; iii: The severe sepsis group; iiii: The sepsis shock group. **P<0.01.

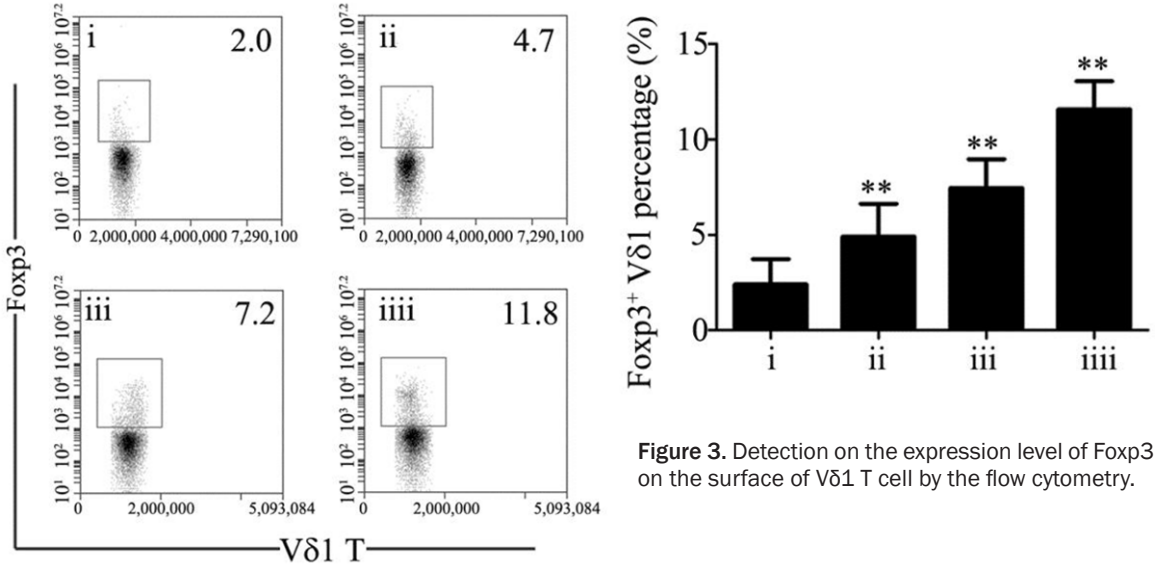

Figure 3. Detection on the expression level of Foxp3 on the surface of Vδ1 T cell by the flow cytometry.

cally significant (P>0.05). There were differences in the blood lactic acid level, APACHE II score and oxygenation index between the sepsis group, severe sepsis group and sepsis shock group, and these differences were statistically significant (P<0.01).

Changes in the percentage of PB Vδ1 T cells in patients with sepsis and the HC group

As shown in **Figure 1**, the percentage of PB Vδ1 T cells in the HC group was (1.14±0.41)%, and the percentage of PB Vδ1 T cells in the sepsis

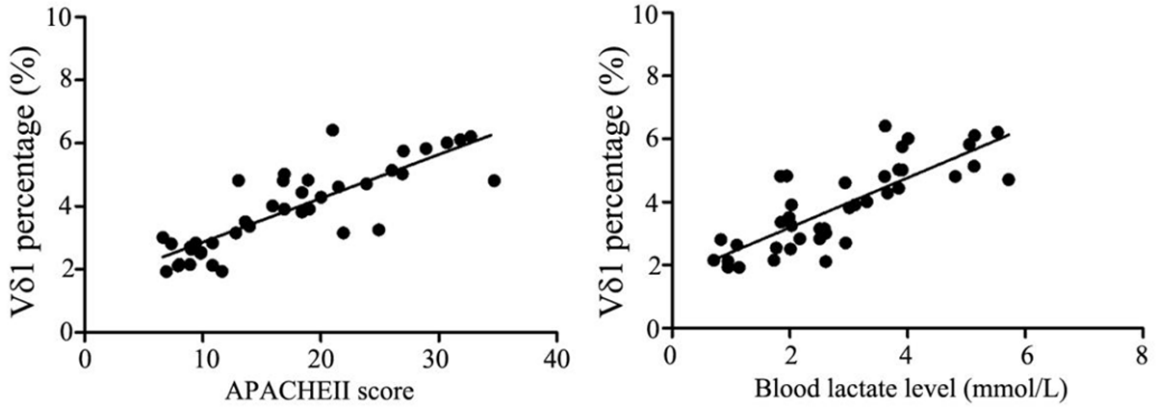

Figure 4. Detection on the inhibition of Vδ1 T cell on the proliferation rate of Naïve CD4 T cell.

group, severe sepsis group and sepsis shock group were (2.55±0.46)%, (4.17±0.61)% and (5.60±0.62)% respectively. The expression rate of Vδ1 T cell in the sepsis group was significantly higher than that in the HC group (P<0.01), and the sepsis shock group exhibited the highest expression rate of Vδ1 T cell, followed by the severe sepsis group and sepsis group.

Correlation analysis of the percentage of Vδ1 T cells between both APACHEII score and lactic acid level in patients with sepsis

As shown in **Figure 2**, the percentage of Vδ1 T cell in patients with sepsis was positively correlated with APACHEII score (r=0.8394, P<0.01) and lactic acid level (r=0.7879, P<0.01).

Detection on the expression level of Foxp3 on the surface of Vδ1 T cell in patients with sepsis and the HC group

As shown in **Figure 3**, the expression level of Foxp3 on the surface of PB Vδ1 T cell in the HC group was (2.41±1.32)%, and the expression levels of Foxp3 on the surface of PB Vδ1 T cell in the sepsis group, severe sepsis group and sepsis shock group were (4.91±1.72)%, (7.46±1.51)% and (11.58±1.47)%, respectively. Compared with the HC group, the expression level of Foxp3 on the surface of PB Vδ1 T cell in patients with sepsis was significantly increased (P<0.01), with the highest expression level of Foxp3 in the sepsis shock group, followed by the severe sepsis group and the sepsis group.

Detection of the inhibition of Vδ1 T cell on the proliferation of Naïve CD4 T cells

As shown in **Figure 4**, the proliferation rate of single PB Naïve CD4 T cell in patients with sepsis was (95.71±8.92)%, while the proliferation rate of Naïve CD4 T cell co-incubated by Vδ1 T and Naïve CD4 T cells was (47.24±9.86)%; The proliferation rate of single PB Naïve CD4 T cell in the HC group was (94.94±9.01)%, while the proliferation rate of Naïve CD4 T cell co-incubated by Vδ1 T and Naïve CD4 T cells was (66.94±8.91)%. Vδ1 T cell in PB for patients with sepsis exhibited significantly increased inhibition function (P<0.01).

Discussion

Sepsis is considered as a death competition between pathogenic microorganisms and the immune system. Whether to maintain the appropriate balance between pro-inflammatory and anti-inflammatory factors decides the survival of patients with sepsis. Patients with sepsis are always in the immunosuppressive state, which typically features that defects exist in the congenital and adaptive immune responses and can lead to increased secondary infection rate and mortalities [20, 21]. Treg cell is able to inhibit the congenital and adaptive immune responses, and is considered as the mark of prognosis and recurrence for some diseases [22, 23]. The study of Wan et al. [24] indicated that at the initial stage of sepsis, the percentage of CD4 Treg was significantly increased and the immunosuppressive function was also obviously increased. Such changes in the percentage and function of CD4 Treg cell may be correlated with a higher mortality of sepsis.

Huang et al. [25] found that the percentage of PB CD39+ Treg in patients with sepsis was significantly increased, and this percentage was related to conditions of such patients. A higher percentage of PB CD39+ Treg in patients with

sepsis indicated poor prognosis in such patients. Shao et al. [26] indicated that the percentage of CD4+CD25+ regulatory T cell in PB was significantly increased for patients with sepsis, and an increase in the percentage of CD4+CD25+ regulatory T cell can reduce CD3+, CD4+ and CD4+/CD8+ level. On this basis, the immune function is in the inhibitory state and thus participates in the onset of sepsis. In addition, Pagel J et al. [27] Confirmed that the proportion of CD4+CD25+ regulatory T cells was significantly elevated in premature infants with early-onset sepsis. Gupta DL et al. [28] have confirmed that the increase of the proportion of CD4+CD25+ regulatory T cells in patients with post-traumatic sepsis led to the imbalance of Treg/Th17, which was closely related to the pathogenesis of sepsis after trauma.

However, in addition to CD4 Treg, there also exist other cells with immunosuppressive functions in PB, such as regulatory B cells, regulatory DC cells and Vδ1 T cells [16-18]. δHerein Vδ1 T cell is a cell subgroup of human δ T cells in PB. δ T cell is mainly classified into two cell subgroups based on different expressions of T cell receptors (TCR), i.e., T cell and δ T cell [29]. αβγγδ T cell only accounts for a small proportion of CD3 cells with the percentage of about 1%-10% [30]. δ T cell can be further classified into two cell subgroups: Vδ1 T cell (mainly distributing in epithelial lymphoid tissues) and Vδ2 T cell (mainly distributing in PB) [31, 32]. Vδ1 T cell and Vδ2 T cell have different functions. Vδ2 T cell mainly participates in inflammatory responses, while Vδ1 T cell mainly presents the immunoregulatory function [33]. δVδ 1 T cell expresses Foxp3, and literatures report that the number of Vδ1 T cell is decreased in the autoimmune disease-systemic lupus erythematosus (SLE) [18, 34]. To date, there have been nearly no literatures on the effects of Vδ1 T cells in sepsis.

This study firstly detected changes in the number of PB Vδ1 T cells in patients with sepsis, and the results revealed that the percentage of PB Vδ1 T cells in the HC group was (1.14±0.41)%, and the percentage of PB Vδ1 T cells in the sepsis group, severe sepsis group and sepsis shock group were (2.55±0.46)%, (4.17±0.61)% and (5.60±0.62)% respectively. The expression rate of Vδ1 T cell in the sepsis group was significantly higher than that in the HC group (P<0.01), and the sepsis shock group exhibited the highest expression rate of Vδ1 T cell, followed by the severe sepsis group and sepsis group. At the same time, the percentage of Vδ1 T cell in patients with sepsis was positively correlated with APACHEII score (r=0.8394, P<0.01) and lactic acid level (r=0.7879, P<0.01), respectively. This result indicated that the number of PB Vδ1 T cell in patients with sepsis was closely correlated with conditions of such patients. Subsequently, in this study the flow cytometry was used to measure the expression level of Foxp3 on the surface of Vδ1 T cell. The results indicated that compared with the HC group, the expression level of Foxp3 on the surface of PB Vδ1 T cell in patients with sepsis was significantly increased (P<0.01), with the highest expression level of Foxp3 in the sepsis shock group, followed by the severe sepsis group and the sepsis group. δThe results from further functional experiments showed that the inhibition function of PB Vδ1 T cell on the proliferation of Naïve CD4 T cell was significantly increased for patients with sepsis, indicating that PB Vδ1 T cell exhibited stronger immunosuppressive function in patients with sepsis than that in the HC group (P<0.01).

In conclusion, the results of this study indicated that the percentage and functions of PB Vδ1 T cell in patients with sepsis were significantly increased, so that the immune function of patients with sepsis was inhibited. Such changes in the immune function of Vδ1 T cell in patients with sepsis may be correlated with the development of sepsis.

Disclosure of conflict of interest

None.

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