Vascular Endothelial Cells Activate Peripheral Natural Killer T Cells and Participate in Regulation of Downstream Immune Cascades in Patients with Sepsis

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Background: This study investigated the effect of supernatant of endothelial cells stimulated by peripheral blood serum from sepsis patients on phenotype and function of peripheral NKT cells.

Material/Methods: Twenty-one patients with sepsis and 21 healthy subjects were included. Peripheral blood (5 ml) was collected from all patients and healthy subjects. To isolate peripheral blood mononuclear cells (PBMCs), Ficoll lymphocyte separation solution was used. Flow cytometry was carried out to determine NKT cell ratio, activity, and cytokine secretion. Human umbilical vein endothelial cells were cultured with serum from sepsis patients for 48 h before changing to fresh medium, and supernatant was collected. The supernatant was used to co-culture PBMCs before analyzing NKT activity and cytokines.

Results: The ratios of CD3-CD56+NK cells and CD3+CD56+NKT cells were increased in peripheral blood from sepsis patients. Surface receptors p30, G2D, and p44 of CD3+CD56+NKT cells were elevated, while inhibitory receptors NKG2A and 158b were decreased. CD4+ NKT cells in peripheral blood from sepsis patients were enhanced. GranB, IFN-γ, IL-4, and IL-17 in NKT cells from sepsis patients were up-regulated. After co-culture with vascular endothelial cells treated with sepsis serum, expression of p30 and G2D in NKT cells was upregulated, and number of TCRVα24-positive cells was increased. In addition, ratio of CD4+NKT cells was increased, and intracellular expression of IL-4 and IFN-γ was elevated.

Conclusions: The study demonstrates that the level of NKT cells in peripheral blood from sepsis patients is increased, and their activity is enhanced. In addition, vascular endothelial cells from sepsis patients can regulate the activity of NKT cells.

MeSH Keywords: Endothelial Cells • Killer Cells, Natural • Sepsis

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Background

Sepsis is a clinically common infectious disease that has the highest mortality in intensive care units [1]. Sepsis is a systemic inflammatory response syndrome that is caused by serious damages to the internal environment of the body [1], mainly caused by invasion by bacteria and release of exogenous substances such as endotoxin and peptidoglycan into the circulation system [2]. Sepsis is often accompanied by clinical symptoms such as systemic fever, shortness of breath, and peripheral leukocytosis [3]. When sepsis is serious, organ dysfunction and tissue hypoperfusion are also present. If not treated promptly, patients often develop septic shock, or even acute multiple organ failure, leading to high mortality [4]. The clinical incidence of sepsis is increasing year by year. Sepsis is serious and developing rapidly, and there is no radical treatment plan for it. At present, treatment for sepsis is mainly limited to maintaining the stability of the body’s internal environment. Therefore, sepsis still has a high mortality rate, being a great threat to human health [5]. Although many studies have been carried out by medical sepsis researchers, its molecular mechanism remains unclear.

The main clinical symptom of sepsis is release of inflammatory mediators caused by infection by pathogenic microorganisms, which can initiate a cascade reaction in organs and form complex inflammatory networks, eventually causing cell damage [6]. The immune system plays an important role in the cascade of inflammatory factors induced by sepsis [7]. Innate immunity is the first barrier against infection. Natural killer T (NKT) cells are heterogeneous natural immune cells that have been receiving increasing research attention in recent years [8]. NKT cells have the characteristics of both natural killer (NK) cells and T cells, and they directly kill pathogens by regulating the balance of Th1 and Th2 cells [9]. According to the type of T cell receptor (TCR) and whether its function depends on CD1d, NKT is divided into 3 categories: type I, type II, and type III [10]. According to surface markers, NKT cells can be classified into CD3+CD56+NKT and CD3+Vα24+NKT (iNKT) [11]. After pathogens invade the body, as inherent immune cells, NKT cells respond quickly, synthesize and secrete a variety of cytokines such as IL6 and IFN-γ, initiate a downstream immune cascade reaction, and participate in tissue injury induced by sepsis [12,13]. Although NKT cells have important functions in immunoregulation and despite reports of abnormal activation of NKT cells in peripheral blood of sepsis patients, their functions and regulatory mechanisms are unclear and warrant further research.

Vascular injury is one of the basic pathological changes in patients with sepsis, including destruction of vascular endothelial structure, abnormal vasoconstriction, and dysfunction of vascular endothelial cells, and it plays important roles in tissue and organ injuries induced by mild and severe sepsis [14]. Studies show that substances released to the blood by pathogens such as endotoxins can directly kill vascular endothelial cells, cause destruction of the vascular endothelial barrier, and lead to disorder of secretory function of endothelial cells, aggravating inflammation, and causing ischemic injury in tissues and organs [15,16]. As a direct target of endotoxin, vascular endothelial cells are among the first cells to contact an antigen and have certain secretory functions. It has not been reported in the literature whether vascular endothelial cells can affect NKT cells. To provide an experimental basis for understanding the pathogenesis of sepsis, the present study investigated the effect of vascular endothelial cells from patients with sepsis on the phenotype and function of peripheral NKT cells.

Material and Methods

Patients

A total of 21 patients with sepsis who received treatments at our hospital between January 2016 and April 2017 were included in the present study. In addition, 21 healthy subjects were included in a control group. Peripheral blood (5 ml) was collected from all patients and healthy subjects. The first part of the peripheral blood (3 ml) was used for lymphocyte separation and flow cytometry, and the second part of the blood (2 ml) was used for serum separation. All procedures were approved by the Ethics Committee of Guangxi Medical University. Written informed consents were obtained from all patients or their families.

Cells

Human umbilical vein endothelial cells (HUVECs; Cell Bank, Chinese Academy of Sciences, Shanghai, China) were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS). When reaching 90% confluency, the cells were trypsinized and passaged. HUVECs in logarithmic growth were used for functional experiments.

To isolate peripheral blood mononuclear cells (PBMCs), peripheral blood (3 ml) from healthy subjects was treated with Ficoll lymphocyte separation solution (GE Healthcare, Chicago, IL, USA) according to the manufacturer’s manual. Then, PBMCs were mixed with 5 volumes of phosphate-buffered saline (PBS), and centrifuged at 1200 g for 6 min. After discarding supernatant, PBMCs were resuspended with PBS and mixed thoroughly.

DMEM (250 μl) containing 10% FBS was mixed with serum from healthy subjects (negative control (NC) group) or 4 severe sepsis patients (co-culture group) at a ratio of 1:1, and used for incubation of HUVECs for 48 h. When reaching 70–80% confluency,
the medium was replaced with fresh DMEM medium. After incubation for 24 h, culture supernatant was collected and centrifuged at 12 000 g and 4°C for 15 min. Then, the supernatant was added to HUVECs conditional medium, which was then mixed with RPMI-1640 medium containing 10% FBS at a ratio of 1:1. The peripheral lymphocyte group (negative control (NC) group) was cultured with 500 μl RPMI-1640 medium supplemented with 100 U/ml interleukin (IL)-2 and 10% FBS, while the co-culture group (HUVECs group) was treated with 500 μl HUVECs conditional medium containing 100 U/ml IL-2. The medium was replaced every 24 h, and the cells were cultured for 72 h before examinations.

Flow cytometry

NKT cell membrane and intracellular markers were detected by flow cytometry. The mononuclear lymphocyte population was identified by FSC/SSC, and the CD3\(^+\)CD56\(^+\) cell population was chosen for further analysis. Normal human PBMCs were cultured in vitro for 72 h before centrifugation at 1000 g for 5 min. CD3\(^+\)CD56\(^+\) NKT cells were collected and used for detection of the expression of NKGD2, NKGA2, NKP30, NKP44, CD158b, CD4, CD8, TCR\(\alpha\)24, IL-4, IFN-\(\gamma\), perforin, granzyme B (GranB), and Ki-67. Each experiment was repeated at least 3 times.

Statistical analysis

The results were analyzed using Graph Pad Prism 7.0 statistical software (GraphPad Software, La Jolla, CA, USA) and data are expressed as means ± standard deviations. Data were tested for normality. Multigroup measurement data were analyzed using one-way ANOVA. In case of homogeneity of variance, the least significant difference and Student-Newman-Keuls methods were used; in case of heterogeneity of variance, Tamhane’s T2 or Dunnett’s T3 method was used. Comparison between 2 groups was carried out using the \(t\) test. \(P<0.05\) indicated statistically significant differences.

Results

Numbers of NK and NKT cells in peripheral blood of sepsis patients were increased compared to those in healthy subjects, suggesting natural immune activation in patients with sepsis.

To determine the ratio of CD3\(^+\)CD56\(^+\)NKT cells in peripheral blood of sepsis patients, flow cytometry was used. The data showed that the percentage of NKT cells in peripheral blood of sepsis patients (6.82±0.19%) was significantly higher than that of healthy subjects (2.78±0.27%) (\(P<0.05\)). In addition, the ratio of CD3\(^+\)CD56\(^+\) NKT cells in peripheral blood of sepsis patients (21.6±0.72%) was significantly higher than that from healthy subjects (12.76±0.43%) (\(P<0.05\)) (Figure 1). The results suggest that the numbers of NK and NKT cells in peripheral blood of sepsis patients are higher than in healthy subjects, suggesting natural immune activation in patients with sepsis.

Expression of surface receptors on CD3\(^+\)CD56\(^+\) NKT cells in peripheral blood from sepsis patients was altered compared with that in healthy subjects.

To detect the expression of surface receptors of CD3\(^+\)CD56\(^+\) NKT cells in peripheral blood, flow cytometry was carried out. The data showed that the ratio of NKT cells with positive expression of p30 in sepsis patients (54.3±1.77%) was significantly higher than that in healthy subjects (31.6±1.24%) (\(P<0.05\)). The ratio of NKT cells with positive expression of NKG2D in sepsis patients (45.3±0.93%) was also significantly higher than that in healthy subjects (25.7±0.34%) (\(P<0.05\)). Similarly, the ratio of NKT cells with positive expression of p44 in sepsis patients (38.7±1.6%) was significantly higher than that in healthy subjects (16.7±0.83%) (\(P<0.05\)). By contrast, the ratio of NKT cells with positive expression of inhibitory receptor NKG2A in sepsis patients (13.7±0.34%) was significantly lower than that in healthy subjects (27.8±1.1%) (\(P<0.05\)). The ratio of NKT cells with positive expression of 158b in sepsis patients (41.8±1.9%) was significantly lower than that in healthy subjects (57.4±1.7%) (\(P<0.05\)). Moreover, the ratio of NKT cells with positive expression of TCR\(\alpha\)24 in sepsis patients (11.87±0.63%) was significantly higher than that in healthy subjects (6.32±0.71%) (\(P<0.05\)) (Figure 2). The results indicate that the expression of surface receptors on CD3\(^+\)CD56\(^+\) NKT cells in peripheral blood from sepsis patients is altered compared with that in healthy subjects.

Ratios of CD4\(^+\) and CD4–CD8– subtypes of NKT cells in sepsis patients were higher than those in normal subjects.

To examine the ratio of CD4\(^+\) and CD4–CD8– subtypes of NKT cells, flow cytometry was performed. The data showed that the ratio of CD4\(^+\) NKT cells in peripheral blood of sepsis patients (17.5±0.56%) was significantly higher than that in normal subjects (1.34±0.09%) (\(P<0.05\)). The ratio of CD8– NKT cells in peripheral blood of sepsis patients (36.78±1.59%) was not different from that in normal subjects (38.9±1.12%) (\(P>0.05\)). In addition, the ratio of CD4–CD8– NKT cells in peripheral blood of sepsis patients (61.98±2.1%) was significantly higher than that in normal subjects (50.2±1.70%) (\(P<0.05\)) (Figure 3). The results suggest that the ratios of CD4\(^+\) and CD4–CD8– subtypes of NKT cells in sepsis patients are higher than those in normal subjects.
Peripheral NKT cells in patients with sepsis were significantly activated and their secretion of cytokines was enhanced.

To study the expression of GranB, IFN-γ, IL-4, and IL-17 in NKT cells, flow cytometry was carried out. The data showed that the ratios of NKT cells with positive expression of GranB (87.56±2.67%), IFN-γ (56.7±2.30%), IL-4 (45.8±1.76%), and IL-17 (37.54±2.11%) in sepsis patients were significantly higher than those in normal subjects (72.5±2.26%, 27.3±0.95%, 15.7±0.83%, and 14.8±1.4%, respectively) (P<0.05) (Figure 4). The results indicate that peripheral NKT cells in patients with sepsis are significantly activated and their secretion of cytokines is enhanced.

Vascular endothelial cells co-cultured with the serum of sepsis patients enhanced the activity of NKT cells

To further test whether vascular endothelial cells play a role in the activation of NKT cells, PBMCs from normal subjects were co-cultured with the culture supernatant of HUVECs that were stimulated with serum from sepsis patients. The data showed that the percentages of CD3+CD56+NKT cells with positive expression of NKG2D and TCRvα24 in sepsis patients were significantly higher, while that for p30 was significantly lower, than those in negative control group (P<0.05). By contrast, the percentages of CD3+CD56+NKT cells with positive expression of G2A, 158b, and p44 in sepsis patients were not different from those in the negative control group (P>0.05) (Figure 5). The results suggest that vascular endothelial cells co-cultured with the serum of sepsis patients enhance the activity of NKT cells.
**Figure 2.** Percentages of NKT cells with positive expression of indicated surface markers. Flow cytometry was used to detect indicated surface markers. * P<0.05 compared with normal subjects.
Supernatant of HUVECs pretreated with serum from sepsis patients promoted the differentiation of CD3+CD56+ NKT cells from normal human into CD4+ subtype

To test the effect of vascular endothelial cells on CD4 and CD8 subtypes of NKT cells, flow cytometry was used. The data showed that the ratio of CD4+ subtype of NKT cells co-cultured with supernatant of HUVECs pretreated with serum of sepsis patients (11.25±0.62%) was significantly higher than that in negative control group (5.71±0.39%) (P<0.05). By contrast, the ratio of CD8+ subtype of NKT cells co-cultured with supernatant of HUVECs pretreated with serum of sepsis patients was not different from that in the negative control group (P>0.05) (Figure 6). The results indicate that supernatant of HUVECs pretreated with serum from sepsis patients promotes the differentiation of CD3+CD56+ NKT cells from normal humans into CD4+ subtype.

Vascular endothelial cells co-cultured with the serum of sepsis patients stimulated peripheral NKT cells to secrete IL-4 and IFN-γ, thereby activating downstream immune cascades

To study the effect of vascular endothelial cells on the expression of IL-4 and IFN-γ in NKT cells, flow cytometry was carried out. The data showed that the ratios of NKT cells with positive expression of IL-4 and IFN-γ in the co-culture group (36.75±1.36% and 31.98±1.5%, respectively) were significantly higher than those in the negative control group (22.5±0.83% and 18.3±0.97%, respectively) (P<0.05) (Figure 7). The results suggest that vascular endothelial cells co-cultured with the serum of sepsis patients can stimulate peripheral NKT cells to secrete IL-4 and IFN-γ, thereby activating downstream immune cascades.

Figure 3. Ratios of NKT cells with positive expression of CD4 and CD8. Flow cytometry was used to detect indicated subtypes. * P<0.05 compared with normal subjects.
Discussion

Sepsis is a clinically common acute and severe disease, and immune disorders caused by uncontrolled systemic inflammatory responses are important causes of the occurrence and development of sepsis [17]. Innate immunity is the earliest contact with foreign antigens in vivo and can react quickly. It plays an important role in eliminating foreign antigens and regulating innate immunity [18]. NKT cells are innate immune cells, with relatively lower ratio in peripheral blood. NKT cells have attracted attentions from researchers because of their ability to regulate immune homeostasis by secreting Th1 and Th2 cell factors [19]. As an important barrier of the vascular wall, vascular endothelial cells have strong endocrine function, which

Figure 4. Ratios of NKT cells with positive expression of effector factors and cytokines. Flow cytometry was used to detect indicated intracellular markers. * P<0.05 compared with normal subjects.
Figure 5. Effect of supernatant of HUVECs pretreated with serum from sepsis patients on the ratios of NKT cells with positive expression of surface receptors. Flow cytometry was used to detect indicated surface markers. * P<0.05 compared with negative control (NC).
is very important in regulating the circulatory system [20]. It is reported that NKT cells are one of a group of thymus-derived special heterogeneous cell populations that have CD1d molecular restriction when recognizing antigens [21]. NKT cells can express cell surface markers of NK cells and T cells simultaneously. NK and T cell combination markers such as CD3+CD56+ were often used to analyze NKT in clinical research. According to the constancy of TCR, NKT cells can be divided into type I and type II. In mice, TCR phenotype of type I NKT cells is mainly marked by V\textalpha 24-J\textalpha 18 coupled with V\textbeta 8.2, V\textbeta 7 or V\textbeta 2 [22]. In humans, TCR markers for type I NKT are mainly V\textalpha 24-J\textalpha 18 coupled with V\textbeta 11 [23]. Although NKT cells account for a small proportion of the peripheral blood, they have important functions in immune regulation. NKT cell activation not only promotes downstream immune response, but also inhibits some functions of the immune system, depending on NKT cell subtypes [24]. For example, human CD4+ NKT cells secrete Th1 cytokine IFN-\gamma and Th2 cytokines IL-4 and IL-13 [25]. CD4– NKT cells mainly produce Th1 cytokines, and CD4–CD8– NKT cells have strong IL-4 secretion ability [26]. When exogenous antigens enter the blood, NKT cells are activated rapidly, secreting cytokines and activating other immune cells such as NK, CD4+T, and CD8+T cells. However, the proportion and role of NKT cells in peripheral blood of sepsis patients have received scant research attention. The present study shows that the number of NK and NKT cells in peripheral blood of sepsis patients is significantly higher than that of normal persons. The proportions of CD3+CD56+ and CD3+CD V\textalpha 24+ NKT cells are increased significantly. Moreover, activating receptors on the surface of CD3+CD56+ NKT cells are increased, while inhibitory receptors are downregulated, suggesting that activity of peripheral NKT cells in patients with sepsis is upregulated. Further analysis shows that the ratio of CD3+CD56+CD4+ NKT cells in peripheral blood of sepsis patients is increased significantly, suggesting that the synthesis and release of Th1 and Th2 cytokines are increased. Intracellular cytokine analysis shows that the expression of GranB, IFNy, IL-4, and IL-17 is increased in peripheral NKT cells of sepsis patients. IL-4 can participate

Figure 6. Effect of supernatant of HUVECs pretreated with serum from sepsis patients on the ratios of NKT cells with CD4 or CD8 subtypes. Flow cytometry was used to detect indicated subtypes. * P<0.05 compared with negative control (NC).
in the differentiation and activation of downstream Th cells, while IL-17 can stimulate the occurrence of inflammation. Our results suggest that the killing of pathogenic microbes and the promotion of downstream immune cascade by sepsis-induced NKT cells are both enhanced.

Vascular injury plays an important role in promoting the occurrence and development of sepsis. The state of vascular endothelial cells is important in maintaining normal vasoactive function, and is also an important target for clinical treatment of sepsis [27]. Vascular endothelial cells are natural physical barriers between blood vessel walls and blood, and are the largest

**Figure 7.** Effect of supernatant of HUVECs pretreated with serum from sepsis patients on the ratios of NKT cells with positive expression of intracellular markers. Flow cytometry was used to detect indicated intracellular markers. * P<0.05 compared with negative control (NC).
secretory organ in the body [20]. It is reported that sepsis patients often have vascular injury, and their vascular endothelial cell barrier also has structural damage and dysfunction [13]. For example, disordered secretion of vasoactive substances by vascular endothelial cells can affect vasoconstriction and downstream inflammatory response [28]. After treatment with peripheral blood serum of sepsis patients, the culture supernatant of HUVECs upregulates the number of NKT cells with positive expression of p30, NKG2D, and TCRvα24, suggesting that HUVECs activate NKT cells in sepsis patients. Subtype analysis shows that, after co-culture, CD4+ subtypes among CD3+CD56+ NKT cells are increased, and CD4+ subtypes of NKT cells can secrete multiple Th2 cytokines, suggesting that vascular endothelial cells in sepsis patients are involved in NKT-mediated immune cascades. Cytokine analysis shows that, after co-culture, the synthesis of IL-4 and IFN-γ in NKT cells is significantly increased, suggesting that HUVECs promote the synthesis and release of NKT cytokines, and aggravate immune damage in sepsis patients. These results suggest that vascular endothelial cells in patients with sepsis are involved in the activation of peripheral NKT cells, thus promoting the immune cascades mediated by NKT cells and aggravating immune injury in the body. A limitation of the present study is that the analysis of NKT in total mononuclear lymphocytes cannot exclude the effect of other immune cells on NKT. In addition, the mechanism by which HUVECs regulate NKT is still unclear. These topics will be explored in future studies.

Conclusions

The present study demonstrates that the ratio of NKT cells in peripheral blood of patients with sepsis is upregulated and activated, while vascular endothelial cells can activate NKT cells and participate in the regulation of downstream immune cascades. Further studies on this mechanism will help find new targets for inhibiting immune injuries caused by sepsis.

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Conflict of interests

None.

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