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Percentage of Peripheral CD19+CD24hiCD38hi Regulatory B Cells in Neonatal Sepsis Patients and Its Functional Implication

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Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Background: As a major cause of mortality in neonates, neonatal sepsis is often accompanied by immune dysfunctions, which are frequently caused by dysregulated T cell sub-populations. The role of regulatory B cells in neonatal sepsis, however, remains unknown. Therefore, this study investigated the percentage and functional variation of CD19+CD24hiCD38hi regulatory B cells in peripheral blood of neonatal sepsis patients in an attempt to elucidate the role of these regulatory B cells in pathogenesis of sepsis.





Material/Methods: Flow cytometry was used to quantify the percentage of CD19+CD24hiCD38hi regulatory B cells from peripheral blood samples. The correlation between B cell percentage and C reactive protein (CRP) level was analyzed. Secretion level of interleukin-10 (IL-10) and effects on the proliferation of naïve CD4+ T cells were further analyzed.

Results: The percentage of CD19+CD24hiCD38hi regulatory B cells in neonatal sepsis patients was significantly higher compared to healthy controls ($p < 0.05$), and was positively correlated with serum CRP level. The percentage of IL-10+ CD19+CD24hiCD38hi regulatory B cells was also higher in sepsis patients, and also had more potent inhibition on naïve CD4+ T cells ($p < 0.01$).

Conclusions: The elevation of CD19+CD24hiCD38hi regulatory B cells in neonatal sepsis can inhibit body immune function and thus may participate in the pathogenesis of sepsis.

MeSH Keywords: **COUP Transcription Factor II • Immunosuppression • Thrombocytopenia, Neonatal Alloimmune • Vestibular Neuronitis**

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Background

Sepsis is an acute systemic infection caused by the proliferation of pathogens and release of their toxins in the general blood circulation. Although standard protocols have been adopted in clinical treatment, sepsis still has a high mortality rate worldwide [1–5]. The symptoms of sepsis range from mild infection to severe infectious shock, DIC, and multiple organ failure. Neonatal sepsis is still one of leading causes of neonatal death [6]. The prognosis of sepsis is largely dependent on the immune status of the host, as the extreme inflammatory response and compromised immunity are major reasons for the failure of pathogen clearance [7]. Immune dysfunction is often accompanied with the progression of sepsis, due to the dysfunction of T cell sub-populations, including regulatory T cells (Treg) [8]. The activation of Treg-mediated immunosuppressant function can inhibit the excessive inflammatory response of the host body via activating TCR signaling, making Treg closely related to sepsis pathogenesis [9,10]. Besides classical Treg, other cells in the peripheral blood also have immunosuppressant functions, such as regulatory B cells, regulatory DC cells, and V δ 1 T cells [11–13]. Among those cells, the major surface markers of regulatory B cells are CD19+CD24hiCD38hi. These B cells exert their immunosuppressant function via secreting interleukin-10 (IL-10) and are termed regulator B cells (Breg) [14–16]. The difference between Breg and Treg is that Breg can only exert its immunosuppressive function by secreting IL-10, while Treg can either exert their immunosuppressive function by secreting IL-10 or by immune cell contact inhibition [8]. The role of CD19+CD24hiCD38hi Breg in neonatal sepsis is currently unknown. This study thus investigated the percentage and function of CD19+CD24hiCD38hi Breg in peripheral blood of sepsis patients in an attempt to elucidate the role of CD19+CD24hiCD38hi Breg in the pathogenesis of sepsis.

Material and Methods

Clinical information

From December 2014 to June 2015 a total of 30 neonatal sepsis patients (14 males and 16 females) in our hospital were recruited for this study. Most of these neonates were admitted with staph infections. The average age of all patients was 36.04 weeks. The body weights at birth were: 5 were 1000–1500 g, 15 were 1500–2500 g, and 10 were over 2500 g. All patients fit the diagnosis of neonatal sepsis [17]. The study protocol was approved by the Research Ethics Committee of our hospital.

Inclusion criteria were: (1) Presented as body temperature abnormality, jaundice, and weak response; (2) Laboratory results showed blood leukocytes larger than $20 \times 10^9/L$ or smaller than $5 \times 10^9/L$, rod nuclear cell ratio over 0.2, and/or C-reactive protein (CRP) above 8.0 mg/mL; and (3) Isolated pathogen from blood

culture or the same opportunistic pathogen by 2 consecutive cultures. All patients received standard treatment for sepsis.

Isolation of peripheral blood mononuclear cells (PBMCs)

A fasting blood sample with anti-coagulation treatment was added on top of an equal volume of lymphocyte isolation buffer, followed by centrifugation at 800 g for 18 min. The top white layer was gently transferred into a new tube containing 10 mL serum-free RPMI 1640 medium, which was then centrifuged at 600 g for 15 min. After discarding supernatants, cell precipitations were re-suspended in 10 mL serum-free RPMI 1640 medium, followed by 400-g centrifugation for 8 min. Collected cells were counted after trypan blue staining and were prepared in cell suspensions ($2 \times 10^6/mL$) for further use.

Determination of peripheral CD19+CD24hiCD38hi Breg

PBMCs were washed twice in PSB containing 5% bovine serum albumin (BSA). After discarding the supernatant, cells were incubated with antibody against APC-CD3, FITC-CD19, and APC-CD38 for 30 min. After washing in PBS containing 5% BSA, cells were re-suspended in 0.1 mL PBS for flow cytometry detection.

Serum CRP assay

CRP levels were determined by enzyme-linked immunosorbent assay (ELISA) using a test kit (Zhengbai Biotech, China) following the manufacturer's instructions. In brief, standard samples and serum samples were added into 96-well plates in triplicate. After 37°C incubation for 90 min, excess liquid was removed, followed by the addition of washing buffer (0.3 mL per well). After washing 3 times, biotin-labelled antibody working solution was added for 60-min incubation at 37°C. The enzyme-linked working solution was then applied for another 30-min incubation at 37°C. After gentle washing, chromogenic substrate (0.1 mL per well) was added for development in the dark at 37°C. The reaction was stopped and measured for optical density (OD) values at 450 nm in a microplate reader.

IL-10 secretion level

CD19+CD24hiCD38hi Breg were cultured in 96-well plates with 100X PMA+Ion. After 6 h, cells were collected for lysis in 0.5 mL lysis buffer, followed by incubation in the dark at room temperature. After washing twice, APC-IL-10 antibody was added for incubation. Cells were washed and re-suspended in 0.1 mL PBS for flow cytometry analysis.

CFSE cell proliferation assay

CD19+CD24hiCD38hi Breg and naïve CD4+ T cells were selected by flow cell sorter (FCS) method with purity greater than

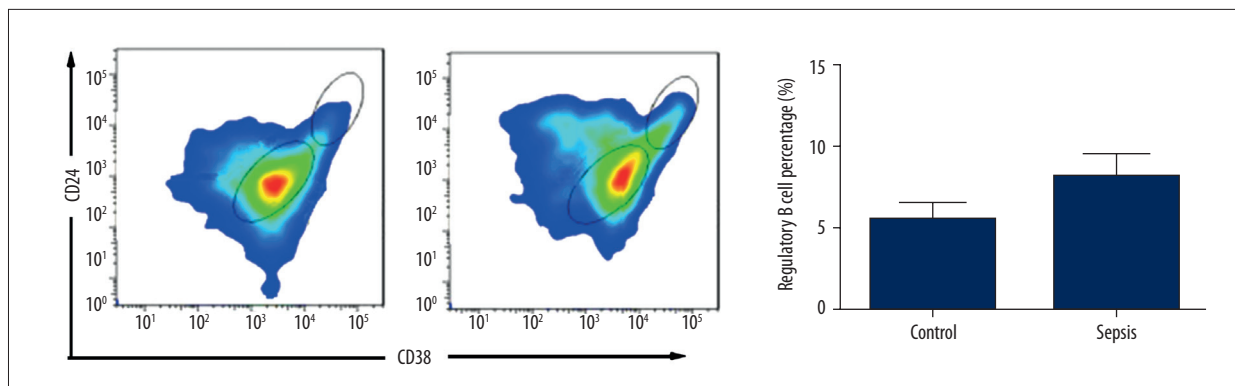


Figure 1. Percentage of CD19+CD24hiCD38hi Breg in control and sepsis neonates.

90%. We pre-coated a 96-well plate with 1 µg/mL CD3 antibody and 2 µg/mL CD28 antibody within 2-h incubation at 37°C. Naïve CD4+ T cells were stained by CFSE following the manual's instructions. After staining, cells were re-suspended in RPMI-1640 complete medium containing 10% fetal bovine serum (FBS) and added into the 96-well plate, which was tested for cell proliferation by flow cytometry after 5 days.

Statistical analysis

SPSS 16.0 software was used to process all collected data. The *t* test was used for comparing means between 2 groups. Correlation analysis was performed by Spearman test. Statistical significance was defined when $p < 0.05$.

Results

Percentage of peripheral blood CD19+CD24hiCD38hi Breg

Compared to healthy controls, sepsis patients had significantly higher percentage of CD19+CD24hiCD38hi Breg ($5.36 \pm 1.35\%$ vs. $8.04 \pm 1.58\%$, $p < 0.01$, Figure 1).

Correlation between CD19+CD24hiCD38hi Breg percentage and CRP level

Using the Spearman test, we found a significantly positive correlation between CD19+CD24hiCD38hi Breg percentage and serum CRP levels ($r = 0.4965$, $p < 0.01$, Figure 2).

IL-10 secretion level by CD19+CD24hiCD38hi Breg

As shown in Figure 3, the percentage of IL-10+CD19+CD24hiCD38hi Breg was $3.62 \pm 0.93\%$ in healthy control individuals, and $5.83 \pm 1.84\%$ in sepsis neonates. Compared to that in the control group, neonatal sepsis patients had significantly higher IL-10 levels ($p < 0.01$).

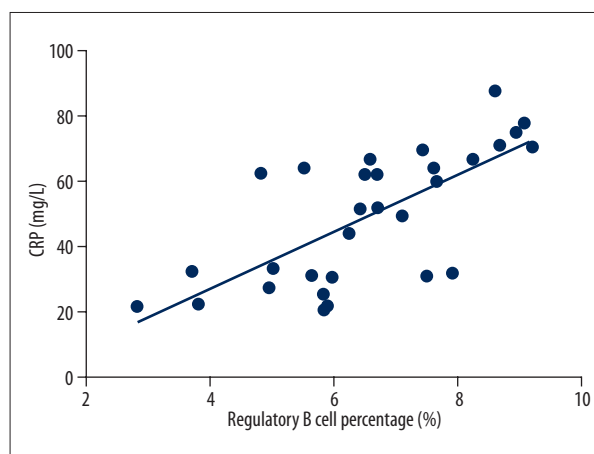


Figure 2. Correlation between CD19+CD24hiCD38hi Breg percentage and serum CRP level.

Immunosuppressant function by CD19+CD24hiCD38hi Breg

In healthy controls, the proliferative ability of naïve CD4+ T cells was $90.88 \pm 13.64\%$. After co-incubation with CD19+CD24hiCD38hi Breg, its proliferation percentage decreased to $53.33 \pm 10.52\%$. In septic neonates, the proliferative ability of naïve CD4+ T cells was $90.31 \pm 14.95\%$, and was $28.95 \pm 6.84\%$ after co-incubation. These results clearly suggest the significantly higher immunosuppressant function of CD19+CD24hiCD38hi Breg in septic patients ($p < 0.01$, Figure 4).

Discussion

As a common complication following severe infection, burns, trauma, major surgery, toxicity, and cardio-pulmonary resuscitation, sepsis has a mortality rate as high as 30~70% [1–5]. Recent findings suggest the important role of immunosuppressants, which may cause the inability to clear blood-borne pathogens, in the pathogenesis of sepsis [7]. As a group of immune cells with immunosuppressant function, Treg play a crucial role in maintaining body immune balance. Previous findings have

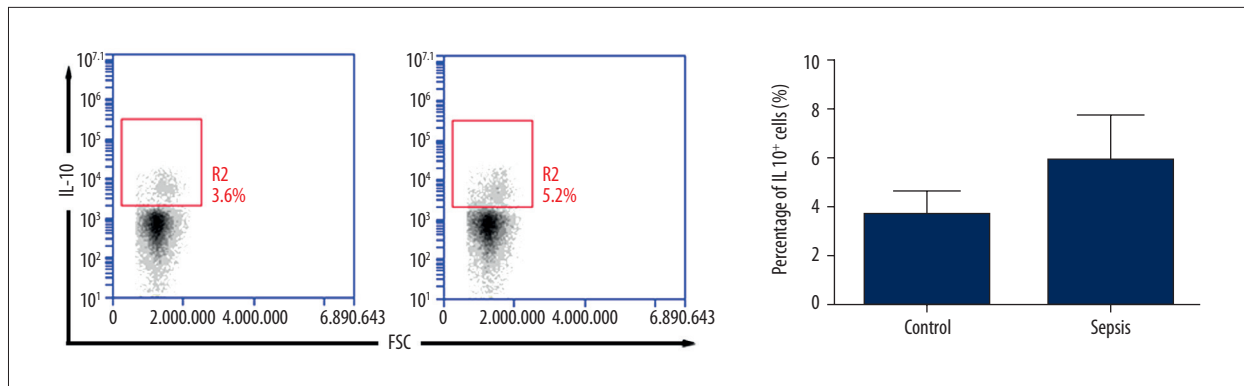


Figure 3. IL-10 secretion level of CD19+CD24hiCD38hi Breg.

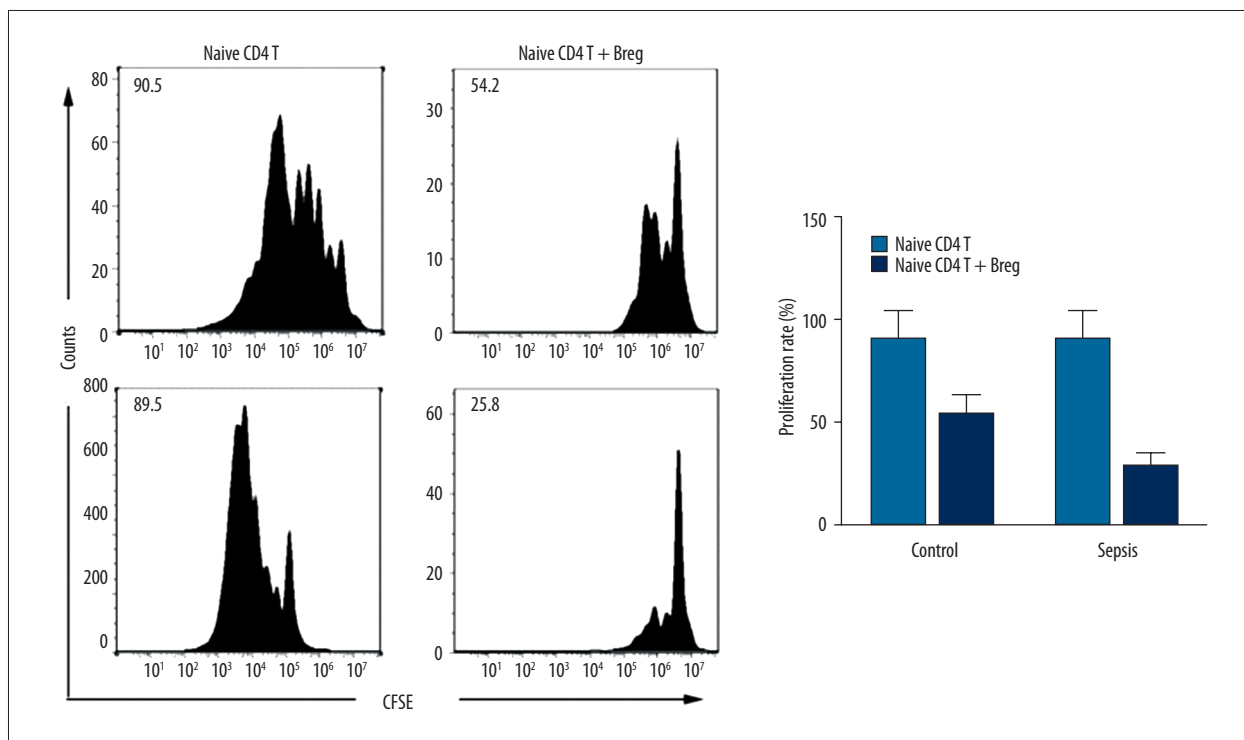


Figure 4. Immunosuppressant function of CD19+CD24hiCD38hi Breg.

suggested the value of CD4+CD25+ Treg in evaluating prognosis of severely septic patients [10]. The elevation of peripheral CD39+ Treg also indicated a lower survival rate of septic patients [18]. During the early stage of sepsis, the percentage of CD4+ Treg was significantly elevated, accompanied by potentiated immunosuppressant functions [19]. Therefore, most studies of sepsis-related immunosuppressant cells focus on T cell-related immune cells.

Traditional Treg cells mainly exert their immunosuppressive roles through 2 approaches: secretion of cytokines and contact inhibition [8]. By secreting IL-10, Treg cells can inhibit effector cells. In addition, Treg cells can also play immunosuppressive roles by binding its surface-expressed CTLA-4, TIM-3,

and GITR to the receptor of effector cells. Another population of B cells with major phenotype as CD19+CD24hiCD38hi, however, also exert certain immune regulatory functions and are termed Breg, in addition to classical Treg [14–16]. This study found a higher percentage of those CD19+CD24hiCD38hi Breg cells in peripheral blood of neonatal sepsis patients, in agreement with previous findings. We also found a positive relationship between CD19+CD24hiCD38hi Breg percentage and serum CRP level, which can be used as a key index reflecting inflammatory condition of septic patients [20]. These results suggest the close correlation between CD19+CD24hiCD38hi Breg and progression of neonatal sepsis. In addition to the role in sepsis, CD19+CD24hiCD38hi Breg are also known to have a critical function in the pathogenesis of rheumatoid

arthritis (RA), because a depressed cell percentage was found in peripheral blood of RA patients [21–24]. Cui et al. [23] have shown that Breg cells were significantly lower in patients with RA, and Breg proportion and DAS28 scores were negatively correlated. Kim et al. [24] reported that the proportion of Breg cells had a significant negative correlation with disease process. CD19+CD24hiCD38hi immunoregulatory B cells, unlike traditional Treg cells that can exert their immunosuppressive function by secreting IL-10 or by immune cell contact inhibition, exert their immunosuppressant function via secreting IL-10 cytokine. A previous study showed the elevated expression of CD19+CD24hiCD38hi Breg in hypercholesterolemia mice, and the inhibition of disease progression via IL-10 secretion [25]. We thus investigated the IL-10 secreting level from CD19+CD24hiCD38hi Breg in neonatal septic patients.

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Conclusions

This study found significant immunosuppression in neonatal sepsis, which was closely correlated with the elevation of CD19+CD24hiCD38hi Breg in those patients.