Regulatory T cells and CD20⁺ B cells in pediatric very severe aplastic anemia: possible clinical markers for evaluating the therapeutic efficacy and prognosis

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ABSTRACT

Objectives: To investigate the immune status of children with very severe aplastic anemia (VSAA), and evaluate the frequencies of CD20⁺ B cells and Regulatory T cells (Tregs) as potential markers for evaluating the therapeutic efficacy and prognosis.

Methods: We systematically analyzed CD20⁺ B cells and Tregs using Flow Cytometry in 36 children with VSAA (14 newly diagnosed cases and 22 cases in remission after therapy with HDIVIG + r-ATG + CSA).

Results: In newly diagnosed VSAA patients, the percentage of CD20⁺ B cells was higher than that in healthy children (P < .01), whereas the percentage of Tregs was lower than that in healthy children (P < .001). After treatment with HDIVIG + r-ATG + CSA, the percentage of CD20⁺ B cells in peripheral blood was decreased obviously, and the percentage of Tregs was significantly increased.

Conclusion: There is a moderate negative correlation between the percentage of Tregs and CD20⁺ B cells in our study. Our results shed light on the roles of Tregs and CD20⁺ B cells as therapeutic efficacy and prognostic markers of pediatric VSAA. Moreover, the mechanism underlying the decrease of blood Tregs and increase of CD20⁺ B cells in pediatric VSAA patients have been discussed, indicating that Tregs may suppress B cell responses.

KEYWORDS

Very severe aplastic anemia; pediatrics; CD20⁺ B cells; Tregs

Introduction

Severe aplastic anemia (SAA) is a refractory disease caused by an immune attack against hematopoietic stem and progenitor cells. This attack results in bone marrow failure characterized by signs of hypoplasia, pancytopenia and fatty bone marrow [1,2]. SAA in childhood remains life-threatening. Various mechanisms are concomitantly involved in this disorder. Moreover, SAA may have complicated pathological process, causing difficulties in treatment and poor prognosis. Particularly, patients with very severe aplastic anemia (VSAA) with extremely low neutrophils, platelets and overly low marrow hyperplasia have higher early death rate. Recent studies have shown that hematopoietic cells are destroyed by activated T cells in SAA [3–5]. However, the mechanism of T cell activation remains unclear. In 1995, Sakaguchi first defined Tregs as a special functional T cell subset [6]. It is vital in the maintenance of homeostasis and closely related to the occurrence of autoimmune diseases. Foxp3 is a specific marker in CD4⁺CD25⁺ Tregs and is essential for Tregs differentiation from naive CD4⁺ T cells [7]. Foxp3⁺ Treg is characterized by high expression of CD25 and low expression of CD127 [8]. With the progress in Tregs research, more and more scholars consider CD4⁺CD25⁺CD127dim T cells as Tregs.

CD20 is a specific marker expressed on the surface of almost all normal and abnormal B cells. CD20 plays an important role in B-cell differentiation and proliferation by regulating the flux of transmembrane calcium ions. Studies have shown that B cells might cause the abnormal activation of T cells by costimulatory molecules of Tregs, and subsequently lead to hematopoietic failure. CD20 is also the target for the most effective anti-cancer medicine call rituximab to date. It has now been used for treating autoimmune diseases, such as rheumatoid arthritis, lymphoma and systemic lupus erythematosus etc [9]. Several studies report that some patients with aplastic anemia have no response to CSA plus ATG. Instead, they achieve a complete remission after rituximab treatment [10–12].

Previous studies indicate Tregs function in the progression of some kind of autoimmune diseases like SAA [13–15]. To our knowledge, blood Tregs are significantly decreased in AA patients than their counterparts in normal people [16]. Furthermore, Tregs can also regulate the function of B cells and other non-T cells diametrically. In our study, we examined the changes
in abundance of blood Tregs and CD20+ B cells in untreated, recovered pediatric VSAA patients, and normal people. The possible mechanisms of pathogenesis of VSAA were also evaluated. Our study provides a theoretical foundation for designing a novel targeted pediatric VSAA therapy.

Materials and methods

Patients

The study was permitted by the Ethics Committee of the The Sun Yet-Sen University. Informed consent was received from all participants. A total of 36 (15 males, 21 females) patients were studied. The median age of VSAA patients was 8 years (ranging from 1–15 years). Among them, 14 were newly diagnosed untreated consecutive patients(U-VSAA) and 22 were in remission(R-VSAA) after therapy with intravenous administration of high-dose immunoglobulin (HDIVIG)+r-ATG + CSA. The SAA diagnostic criteria was based on the International AA Study Group Criteria [4]. VSAA patients with malignant disease, myelodysplastic syndromes, myelofibrosis, PNH or congenital AA like Fanconi’s anemia were excluded. AA was considered severe if at least two of the following parameters were reached: platelet count less than $20 \times 10^9$/L, neutrophil count less than $0.5 \times 10^9$/L, and a reticulocyte count less than $20 \times 10^9$/L with hypocellular bone marrow. The case was considered very severe if the neutrophil count was less than $0.2 \times 10^9$/L. All patients were measured for paroxysmal nocturnal hemoglobinuria (PNH) by anti-CD235a and fluorescent aerolysin (FLAER)-based FCM, and no PHN clones were found. The clinical features of patients in cohort are present in Table 1. Patients were considered to be in remission if: (1) there is no bleeding and anemia; (2) transfusion of blood products is not required; (3) WBC counts >3.5 × 10^9/L; (4) patients were in stable condition or had substantial improvement after a 3-month follow-up. Normal control group were determined by 23 age-matched healthy volunteers at 1–14 years old. The groups of age and condition were no significant difference ($P > 0.05$).

Samples and treatment

Peripheral blood of U-VSAA patients was collected 4 (2–5) weeks after onset of the disease. Blood of R-VSAA patients was collected 8 (6–12) months after treatment with HDIVIG + r-ATG + CSA. The treatment protocol was as follows: HDIVIG (0.4 g.kg$^{-1}$.d$^{-1}$) and r-ATG (2.5–3.5 mg.kg$^{-1}$.d$^{-1}$) were administered through intravenous infusion for five days. CSA (8 mg.kg$^{-1}$.d$^{-1}$) was given orally after 5 days of r-ATG treatment, and it reached the effective concentration (200–400 ng/L) 2 weeks later. The dosage of CSA was then adjusted according to its concentration in plasma, and was gradually decreased after patients reached remission for 10–12 months. Meanwhile, dexamethasone was given to prevent allergic reactions and serum sickness. The average time needed to achieve remission was 10 months. Among all U-VSAA patients, 12 reached a complete response (CR), and the other 10 had a partial response (PR).

Experimental methods

The fluorophore-conjugated monoclonal antibodies: CD20-ECD, CD4-FITC, CD127-PE, CD25-PC5, CD45-PerCp7, mouse IgG1-FITC, mouse IgG1-PE, mouse IgG1-ECD, mouse IgG1-PCS and erythrocyte lysis solution were purchased from Beckman Coulter(USA). Tregs and CD20+ B cells were identified with a Navios Flow Cytometer. Briefly, 100 µl of whole blood was immunostained in flow tubes (BC), followed by lysis in 0.5 ml of RBC lysis solution (BC). Each flow cytometry data was analyzed using the Kaluza software (BC).

Table 1. Clinical and demographic parameters of patients participating in the study.

| NC | U-VSAA | R-VSAA |
|----|-------|-------|
| Number | 23 | 14 | 22 |
| Age(years) | $5.73 \pm 4.21$ | $8.54 \pm 3.72$ | $7.68 \pm 3.31$ |
| Hb/(g/L) | $124.36 \pm 13.88$ | $50.7 \pm 10.64$ | $102.85 \pm 11.84$ |
| PLT($\times 10^9$/L) | $275.64 \pm 94.35$ | $13.13 \pm 5.97$ | $112.92 \pm 30.96$ |
| ANC($\times 10^9$/L) | $4.24 \pm 1.06$ | $0.12 \pm 0.08$ | $2.87 \pm 1.65$ |
| Ret% | $0.82 \pm 0.29$ | $0.27 \pm 0.22$ | $1.06 \pm 0.51$ |
| PHN clones | Negative | Negative | Negative |
| Therapy | None | Not previously treated except for transfusions | Treated with HDIVIG + r-ATG + CSA |

Statistical analysis

SPSS 22.0 software was used for all date analysis. Data were showed as mean ± standard deviation(SD). The One-way ANOVA was used to assess differences in variables between groups. The Pearson Correlation Coefficient was used to assess the correlation between variables. $P < 0.05$ was considered statistically significant.

Results

1. We analyzed the percentage of Tregs and CD20+ B cells in peripheral blood samples obtained from pediatric VSAA patients, patients in remission and normal volunteers (Figure 1). The percentage of...
blood Tregs was (2.63 ± 0.94)% in untreated VSAA patients, (4.70 ± 1.56)% in patients in remission and (4.83 ± 1.42)% in normal volunteers. Hence, the percentage of Tregs in untreated VSAA patients was lower than that in normal people (P < 0.01) or patients in remission (P < 0.01). The percentage of CD20+ B cells was (26.26 ± 10.53)% in untreated patients, (14.9 ± 3.48)% in patients in remission and (15.2 ± 3.7)% in normal volunteers. Hence, the percentage of CD20+ B cells in untreated patients was higher than that in normal people (P < 0.001) or patients in remission (P < 0.001). Notably, after therapy, the percentages of Tregs and CD20+ B cells almost returned to their normal levels (P > 0.05) (Figure 2).

2. We analyzed the correlation between Tregs and CD20+ B cells in our study. In VSAA and R-VSAA patients, the frequencies of Tregs was negatively correlated with the frequencies of CD20+ B cells (n = 36, r = −0.497, P = 0.005; Figure 3).

Discussion
The pathogenesis of SAA especially the VSAA is still not completely elucidated. However, great advances have been obtained in the acknowledge of its pathophysiology and in the treatment of pediatric SAA patients in the past decade. Many researchers have recently found one or more types of antibodies in SAA patients that prompt B cell-mediated humoral immunity. These antibodies may be involved in the pathogenesis of SAA [17,18]. It is suggested that abnormal B cell-mediated humoral immune response may contribute to the pathogenesis of SAA.

Tregs, a specialized T cell lineage, plays an important role in controlling immune responses to
autoantigens and immune responses to harmful foreign antigens [19]. Accumulated evidence has indicated that Tregs are indispensable for the maintenance of homeostasis and closely related to the occurrence of autoimmune diseases [20–24]. It has been reported that the decline of quantity and function with Tregs lead to an abnormal immune response to autoantigens, thus causing rheumatic diseases [25,26]. The etiology of SAA is still unclear, but most researchers have indicated that SAA is an immune-mediated disease with active damage of hematopoietic cells by T lymphocytes. Activated suppressor T lymphocytes produce interferon to participate in the pathogenesis of bone marrow failure. Declined of CD4+CD25+FOXP3+ Tregs in patients with AA, which may explain the development of increased autoreactive T cells and AA phenotypes [27]. Our results were consistent with the previous findings again. The imbalance of T cells and Tregs have a vital function in the pathogenesis of SAA.

B cells express a variety of cell surface molecules, and some of them are closely related to their functions. For instance, CD20 is expressed on all mature B cells [28,29]. It plays a crucial role in B cell proliferation and differentiation by regulating the flow of transmembrane calcium ions. CD20 exists on both resting and activated B cells. So CD20 is a selective B cell marker. Both B cells and Tregs are important players in the adaptive immune response. B cells exert their effects through antibody production, antigen presentation, and cytokine secretion. The major target of Tregs is autoreactive T cells. Recent studies show that B cells are important antigen presenting cells (APCs) for autoreactive T cells, and enhanced Treg function in the absence of B cells provides one explanation for the requirement for B cells in the development of autoimmune diseases [30]. Tregs can inhibit B cell-associated antibody production and B cell responses. Some researchers indicated that decrease of Tregs can cause imbalance antibody production in rodents [31]. Besides, a previous study shows that when anti-CD20 acts on B cells, it depletes and Tregs became about double times number [32]. However, whether Treg cells directly act on B cells is still unclear. In the classical theory, it is considered that Tregs directly affect T cells and subsequently act on B cells indirectly.

Some studies suggest that Tregs are inhibitory in vitro without APCs, demonstrating that their immunoregulatory function occurs through direct contact between Tregs and effector T cells [33]. The suppressive capacity of Treg cells also can mediate through secretory suppressive cytokines and bystander suppression. Besides, B cells are involved in the initiation of T cell activation during the early stage of diseases. Abnormal expression of co-stimulatory molecules on B cells such as CD86 causes excessive activation of T cells through B7-2-mediated co-stimulatory signaling. In SAA patients, B cells in the bone marrow express high co-stimulatory molecules, and they might subsequently induce abnormal activation of T cells to provoke hemopoiesis failure.

Our data demonstrate a significant decrease in the percentage of Tregs and an increase in the percentage of CD20+ B cells in peripheral blood of pediatric VSAA patients. After therapy with HDIVIG + ATG + CSA, the frequencies of these cells were restored to normal levels. In addition, there was a negative correlation between the percentage of Tregs and CD20+ B cells, suggesting that hyperfunctional autoimmune B cells reduced Treg abundance in pediatric SAA patients. It is likely that insufficient Tregs and increased CD20+ B cells failed to suppress the functions of DC and activated T cells. Consequently, highly functional DC and activated T cells impaired bone marrow cells.

Although our data were statistically significant, the small sample size in the study suggests that further research is needed to confirm our results. A thorough and systematic analysis will be helpful to acquire more accurate data and draw more convincing conclusions.

In conclusion, Tregs are decreased and CD20+ B cells are increased in peripheral blood of VSAA patients. These alterations might cause the bone marrow failure in pediatric VSAA patients. Our study suggests that blood Tregs and CD20+ B cells could be used as clinical indicators to evaluate the therapeutic efficacy and prognosis for pediatric VSAA patients. However, the mechanisms by which Tregs and CD20+ B cells are changed in pediatric VSAA patients need to be further studied. Whether Tregs directly suppress B cell responses also needs to be elucidated in future.

Disclosure Statement

No potential conflict of interest was reported by the authors.

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