Abnormalities of quantities and functions of CD56bright natural killer cells in non-severe aplastic Anemia

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ABSTRACT

Objectives: The mechanism of non-severe aplastic anemia (NSAA) is not clear. It may be different from severe aplastic anemia (SAA). CD56bright NK cells (regulatory NK cells) is a subgroup of NK cells that produce immunoregulatory cytokines and express high-affinity IL-2 receptor. To investigate CD56bright NK cells quantities and function in patients with NSAA and to explore how CD56bright NK cells participate in the progress of this disease.

Methods: In this study, we analyzed the quantitative and functional changes of CD56bright NK cells in peripheral blood of patients with NSAA by using Flow Cytometry (FCM) before and after immunosuppressive therapy (IST). The expressions of activating receptor (NKG2D, NKp46, NKp44), inhibitory receptor (NKG2A, CD158a, CD158b) and perforin and granzyme B were detected by FCM. IL-2 and IL-18 levels in serum were detected by ELISA. The correlation between these parameters and clinical indicators of patients were evaluated.

Results: We found that the percentage of CD56bright NK cells in newly diagnosed NSAA patients was higher than that in normal controls ($p = .011$, $p < .05$). The median expression of NKG2D in patients with NSAA was higher compared to that in normal controls ($p = .021$, $p < .05$), and the expression of CD158a was lower ($p = .047$, $p < .05$). The concentrations of IL-2 and IL-18 in the serum of patients with NSAA were higher than those in normal group.

Conclusion: These findings suggest that increased and activated CD56bright NK cells might play a protective role in the pathogenesis of NSAA.

1. Introduction

Aplastic anemia is classified as NSAA, SAA and very severe (VSAA) based on the degree of the peripheral blood cytopenia. The definitions and diagnosis of NSAA are not meet the criteria for SAA [1]. In the last two decades, a growing body of evidence had accumulated and supported the immune pathogenesis of AA characterized by abnormally activated T cells that damaged the hematopoietic stem cells [2]. But the clinical course of NSAA is quite variable with SAA. During the follow-up period, some patients progressed to SAA. A half to two thirds of patients with NSAA were expected to respond to oxymetholone, cyclosporine (CSA), cyclosporine alone or other agents. In our clinical practice, most NSAA patients had a good response to adrenocortical hormone (ACH) and/or high-dose intravenous immunoglobulin (IVIG) treatment [3,4]. There may also be a humoral immune mechanism involved in the pathogenesis of NSAA. Therefore, it is necessary and urgent to further study the pathogenesis of NSAA, which will further guide us to better clinical treatment. NK cells are a group of large granule lymphocytes different from T and B lymphocytes, which plays an important regulatory role in the innate immunity and adaptive immunity [5,6]. According to the expression level of CD56, NK cells can be divided into CD56dim CD16+ and CD56bright CD16- subgroup [7]. CD56bright NK cells were first considered ‘immunoregulatory’ by Cooper et al due to increased production of cytokines and reduced cytotoxicity compared to CD56dim NK cells [8,9]. The CD56dim CD16+ subgroup exerts the main killing function, while the CD56bright CD16-subunit can play an immunomodulatory role by secreting cytokines such as IFN-γ, TNF-β, IL-10 and so on [10,11]. The CD56bright NK cells play an important role in autoimmune diseases, therefore, more and more experts called it ‘Regulatory NK cell’ [6]. In our previous study, we have shown that the proportion of NK cells in patients with newly diagnosed patients with SAA compared with control group was decreased, the median expressions of CD158a, NKG2D and NKp46 on NK cells were higher in patients with SAA compared to that in normal controls [12]. However, the changes of CD56bright NK cells in NSAA was not analyzed. In this study, we will further analyze the changes in the quantity and function of CD56bright NK cells of peripheral blood in patients with NSAA and the concentrations of IL-2 and IL-18 in the serum, and then analyze
patients’ experimental data with the clinical and immunological index [8,13]. Finally, we will analyze the data to explore the CD56bright NK cells in the immune pathogenesis in patients with NSAA.

2. Materials and methods

2.1. Patients

Fifty NSAA patients who were successively diagnosed with NSAA were enrolled in Hematology Department of General Hospital from September 2016 to July 2017. The study was approved by the Ethics Committee of the Hospital China. Informed written consent was obtained from all patients or their guardians in accordance with the Declaration of Helsinki. Human blood samples were collected with a protocol approved by the Ethic Committee for the Hospital. Written consent was obtained from every individual according to the Ethic Committee for the Conduct of Human Research protocol. The diagnosis of NSAA was established according to Chinese expert consensus on the diagnosis and treatment of aplastic anemia (2017) [14]. The clinical characteristics of all subjects were shown in Table 1. Of which 25 cases were newly diagnosed NSAA patients, among 14 cases were male, 11 cases were female. The median age was 46 years (8–68 years). A total of 25 remission NSAA (R-NSAA) patients. After the immunosuppression and hematopoietic treatment, R-NSAA patients’ blood routine returned to the normal level [4]. There were 15 males and 10 females, with a median age of 40 (8–68) years. A total of 23 healthy volunteers served as the normal control group, with 12 males and 11 females, with a median age of 25 (23–55) years. Informed consent was obtained from patients and healthy volunteers and informed consent was signed.

Table 1. Clinical and laboratory indexes of newly diagnosed NSAA patients and remission NSAA patients.

| Clinical characteristics | NSAA (N = 25) | R-NSAA (N = 25) | P-value |
|-------------------------|---------------|-----------------|---------|
| Male/female             | 14/11         | 15/10           | .656    |
| Age                     | 46 (8–72)     | 40 (8–68)       | .158    |
| HB (g/L)                | 77.5 (52–131) | 106.5 (100–156) | .000*   |
| RBC (10¹²/L)            | 2.43 (1.33–3.90) | 3.35 (2.1–5.01) | .002*   |
| PLT (10⁹/L)             | 38.5 (13–80)  | 195.5 (110–300) | .000*   |
| WBC (10⁹/L)             | 3.52 (1.40–9.86) | 4.56 (2.19–17.58) | .109    |
| ANC (10⁹/L)             | 49.9 (17.2–90.4) | 50.8 (18.9–90.7) | .944    |
| Ret (%)                 | 1.97 (0.66–3.97) | 2.46 (0.72–6.37) | .205    |
| LYMPH (%)               | 38.85 (7.10–76.6) | 44.5 (5.20–72.2) | .901    |
| LDH (U/L)               | 202 (125–331) | 209 (157–378)   | .086    |
| IgG (g/L)               | 1350 (581–1800) | 1050 (702–1830) | .337    |
| IgM (g/L)               | 98.95 (47.5–260) | 117 (35.8–256)  | .735    |
| Complement C3 (g/L)     | 97.1 (46.4–131) | 101 (60.6–170)  | .257    |
| Complement C4 (g/L)     | 22.25 (9.02–35.6) | 22.25 (9.02–35.6) | .886    |
| CD4/CD8*                | 1.66 (0.5–5.57) | 1.42 (0.55–3.16) | .563    |
| CD5+CD19+               | 14.67 (0–39.28) | 6.98 (1.44–26.54) | .205    |

Notes: HB: Hemoglobin; RBC: Red blood cells; PLT: Platelet; WBC: white blood cell; ANC: Neutrophile granulocyte; Ret: Neutrophile granulocyte; LDH: Lactate dehydrogenase; *Compared with the remission group, p < .05. Enumeration data is represented by the median (minimum, maximum).

2.2. Flow cytometry

Each of the subjects was marked with seven fluid centrifuge tubes. Each tube was added to PerCP-CD3 (R&D Systems (Minneapolis, USA) 10 µl, PE-VIO770-CD56 10 µl, FITC-CD16 10 µl, PE-IgG 10 µl was added to the detection tube 1; After dark incubation, hemolysis, washing, CD3-CD16-CD56bright cells were detected by FCM. We accounted for the proportion of CD56bright NK cells of peripheral blood and the percentage of NKG2A, NKG2D, CD158a, CD158b, Nkp46, Nkp44 of CD3-CD16-CD56bright cells. Meanwhile, FCM was used to detect the expression of Perforin and Granzyme B of CD3-CD16-CD56bright cells.

2.3. Enzyme-linked immunosorbsent assay (ELISA)

Serum levels of IL-2 and IL-18 in NSAA patients and normal controls were measured by ELISA using reagent kits (SECOO88Hu and SEA063Hu; USCNLIFE, Wu Han, China) according to the manufacturer’s instruction. Diluted standards and patient serum (100 µl) were added in duplicate and incubated at 37°C for 1 h. After washing the plate 5 times, 100 µl of antibody was added to each well and incubated at room temperature for 90 min and HRP was added to each well. After incubation at 37°C for 30 min, the wells were washed 3 times. After that, TMB solution was added to each well, and the samples were incubated in the dark at room temperature for 20 min. Finally, the stop solution was added, and the OD was read at 450 nm within 15 min.

2.4. Statistical analysis

Using SPSS 24.0 statistical software analysis. The paired T test was used to measure the homogeneity of variance when the data between groups were tested, when the variance was not uniform, one factor analysis of variance (data is normal distribution) or nonparametric test was used (the data does not satisfy the normal distribution). Non-parametric tests were used for comparison among groups. Correlation analysis was performed by Pearson correlation test.

The normal distribution measurement data were expressed by mean standard deviation (x ± SD), and the non-normal distribution data was represented by median (four quantile interval). p < .05 had statistical significance.

2.5. Result

2.5.1. The percentage of CD56bright NK cells in patients of newly diagnosed NSAA was significantly increased. The percentage of CD56bright NK cells in patients of newly diagnosed NSAA (median:0.09%) was significantly higher than that in healthy controls
There was no significant difference between R-NSAA patients and normal controls (median 0.06% vs. 0.09%, \( p = .237, p > .05 \)).

2.5.2. The expression of CD56bright NK cells activated receptor in patients with NSAA was significantly higher. The expression of the activated receptor NKG2D in patients of newly diagnosed NSAA and the R-NSAA group was significantly higher than that in the normal controls (median 91.94% vs. 66.88%, \( p = .049, p < .05 \); median 90.91% vs. 66.88%, \( p = .021, p < .05 \)), and there was no significant difference between the newly diagnosed NSAA patients and the R-NSAA group (\( p = .713 \)) (Table 2). The expression of inhibitory receptor CD158a in patients of newly diagnosed NSAA was significantly lower than that in the R-NSAA patients and the normal controls (median 0.55% vs. 4.08%, \( p = .019, p < .05 \); 0.55% vs. 4.59%, \( p = .047, p < .05 \)), there was no significant difference between the R-NSAA group and the normal controls (\( p = .773, p > .05 \)) (Figure 2). The expression of CD56bright NK cells activated receptor NKP44 and NKP46 in patients of newly diagnosed NSAA was higher than that in normal controls (median 0.05% vs. 0.00%, \( p = .207, p > .05 \)).
2.5.3. The product of granzyme B of CD56bright NK cells percentage of peripheral blood in patients of new diagnosed NSAA group was significantly higher than that in normal controls (median 3.25% vs. 1.12%, \( p = .006, p < .01 \)) The product of perforin and CD56bright NK cells of peripheral blood in patients of NSAA patients was higher than in the normal controls (\( p > .05 \)) (Table 3, Figure 3).

2.5.4. The concentration of IL-2 in patients of newly diagnosed NSAA group and R-NSAA were significantly higher than the normal controls (median 433.21 vs. 189.34, \( p = .000, p < .01 \); 433.21 vs. 189.34, \( p = .000, p < .01 \)), the concentration of IL-18 in the treatment group was significantly higher than that in the remission group and the normal control group (269.72 ± 99.42 vs. 216.43 ± 65.26, \( p = .012, (269.72 ± 99.42 \text{ vs. } 188.67 ± 78.39, p < .05; p = .002, p < .01) \) (Table 4).

2.5.5. Correlation analysis of the proportion of CD56bright NK cells in peripheral blood of patients with NSAA with serum concentrations of IL-2 and IL-18 and clinical indicators. The correlation between the proportion of CD56bright NK cells and clinical index (Hb, RBC, PLT, WBC, ANC, Ret, IgG, IgM, complement3, complement4, LDH) and immune index (CD5+CDl9+/CDl9+, CD4+/CD8+) were analyzed. The proportion of CD56bright NK cells was negatively correlated with the HB of patients (\( r = -0.323, p = .042, p < .05 \)) (Figure 4). It is indicated that the more severe the anemia is, the higher the proportion of CD56bright NK cells inhibitory receptor NKG2A and CD158b in patients of new diagnosed NSAA was lower than that in normal controls (\( p > .05 \)).

Table 2. Expression levels of CD56bright NK cell activating receptors and inhibitory receptors in patients with non-severe aplastic anemia (%)

| Group  | Cases | NKG2D (%) | NKP44 (%) | NKP46 (%) | NKG2A (%) | CD158a (%) | CD158b (%) |
|--------|-------|-----------|-----------|-----------|-----------|------------|------------|
| NSAA   | 25    | 91.94 (38.53)a | 0.05 (0.02) | 80.49 (33.27) | 42.54 (56.88) | 0.55 (3.69)b | 10.73 (11.83) |
| R-NSAA | 25    | 90.91 (21.77)a | 0.00 (0.00) | 89.06 (34.95) | 34.15 (68.9) | 4.08 (16.67) | 16.46 (26.66) |
| Control| 23    | 66.86 (27.26) | 0.03 (0.01) | 69.23 (45.23) | 42.62 (61.05) | 4.59 (13.89) | 7.69 (25.74) |

Notes: Compared with the control group, and the non-normal distribution data was represented by median (four quantile interval). \( p < .05 \) had statistical significance. \( \text{a} p < .05; \text{b} p < .05 \); compared with the remission NSAA group. Data of uneven distribution and uneven variance are represented by median (four quantile interval).

Figure 2. Expression of CD158a, NKG2D in CD56bright NK cells of untreated NSAA, R-NSAA and normal controls. (A) The expressions of CD158a, NKG2D on CD56bright NK cells+ were detected by flow cytometry. (B) Median expression of NKG2D in CD56bright NK cells of untreated NSAA and R-NSAA was higher than that normal control group (\( p < .05 \)). (C) Median expression of CD158a in CD56bright NK cells of untreated NSAA was lower than that of R-NSAA (\( p < .05 \)).
NK cells in patients, and the proportion of CD56bright NK cells is related to the severity of the disease. IL-18 was positively correlated with IgM ($r = 0.40$, $p = .047$, $p < .05$). Granzyme B is positively correlated with the proportion of lymphocyte ($r = 0.344$, $p = .04$, $p < .05$). The results showed that the amount of Granzyme B secreted by CD56bright NK cells and IL-18 in serum were closely related to the immune status of the disease. The more proportion of lymphocyte and severe of the disease, the higher secretion of Granzyme B of CD56bright NK cells.

3. Discussion

NSAA belongs to one type of AA, which is one kind of chronic disease of the blood system that seriously affects the quality of life of patients, but the long duration of treatment and the cost is high [1,15]. Liu C et al. found that the proportion of CD56bright NK cells in SAA was decreased [12], but in this study, we found that the proportion of CD56bright NK cells was increased. The onset of NSAA is long, unlike that of SAA. Therefore, the number of CD56bright NK cells may increase in the course of the disease. This suggests that the pathogenesis of NSAA may be different from that of SAA.

Through years of research by domestic and foreign scholars, NSAA is recognized as an immune-related disease [16]. In our clinical work, we treated some patients which are diagnosed with NSAA by experimental administration of corticosteroids and/or high-dose gamma globulin, after three months of follow-up, all patients were found to have an obvious response. It was highly suggested that the incidence of this disease might be related to abnormal humoral immunity in patients [17,18]. An in-depth study of its pathogenic mechanism showed that immunorelated haemocytopenia (IRP, or BMMNC-Coombs test-positive haemocytopenia) is an autoimmune disease where autoantibodies target BM hemopoietic cells.

NK cells are the main carriers of innate immunity and the core regulatory cells of adaptive immunity. They play an important role in anti-infection, anti-tumor and elimination of alien cells. NK cells play an important supporting role in the activation of CTL, and it can also control the occurrence of certain autoimmune diseases by inhibiting CTL [19]. Recent studies have shown that NK cells have two subpopulations of CD56dimCD16+ and CD56brightCD16-subunits, the CD56dimCD16+ subgroup exerts the main

### Table 3. Expression levels of CD56bright NK cell activating receptors and inhibitory receptors in patients with non-severe aplastic anemia.

| Group | Cases | Perforin (%) | The product of Perforin and CD56bright NK cell percentage (%) | Granzyme B (%) | The product of Granzyme B and CD56bright NK cell percentage (%) |
|-------|-------|--------------|---------------------------------------------------------------|---------------|----------------------------------------------------------------|
| NSAA  | 25    | 92.43 (28.42)| 7.73 (20.71)                                                 | 50 (37.77)    | 3.25 (12.56)*                                                   |
| R-NSAA| 25    | 87.87 (35.27)| 4.53 (9.51)                                                 | 39.68 (49.17) | 2.59 (4.25)                                                    |
| Control | 23   | 84.69 (23.72)| 2.48 (4.19)                                                 | 29.82 (27.44) | 1.12 (2.27)                                                    |

Notes: $p < .05$ had statistical significance. *$p < .05$; compared with the control group. Data of uneven distribution and uneven variance are represented by median (four quantile interval).

### Table 4. Concentrations of IL-2 and IL-18 in serum of patients with NSAA.

| Group  | Cases | IL-2 (pg/ml) | IL-18 (pg/ml) |
|--------|-------|--------------|---------------|
| NSAA   | 32    | 433.21 (237.2)* | 269.72 ± 99.42* |
| R-NSAA | 32    | 269.64 (68.80)* | 216.43 ± 65.26 |
| Control | 16   | 189.34 (55.47) | 188.67 ± 78.39 |

Notes: Compared with the control group, *$p < .05$; compared with the remission NSAA group; $p < .05$. The normal distribution measurement data were expressed by mean standard deviation ($\bar{x} \pm SD$), the data of uneven distribution and uneven variance are represented by median (four quantile interval).
killing function, while the CD56brightCD16− subunit play an immunomodulatory role by secreting IFN-γ, IL-10, IL-13 and other cytokines [10]. CD56bright NK cells are the focus of interest, they are considered efficient cytokine producers endowed with immunoregulatory properties [20]. CD56bright NK cells play an important role in autoimmune diseases. Schepis et al. [21] had found an increased proportion of CD56bright NK cells in SLE, and they speculated that CD56bright cells play a major protective role in disease. Dalbeth et al. [22] found CD56bright NK cells are increased in synovial fluid of autoimmune diseases like Rheumatoid arthritis (RA), it could amplify the production of proinflammatory cytokines. Schierloh et al. [23] found in the pleural fluid of patients with tuberculosis, NK cell numbers are reduced; however, the CD56bright NK cells population predominates and is characterized by a high production of IFN-γ after interaction with M. bovis. In our study, NKG2D is the activating receptor of CD56bright NK cells, and its expression is increased in patients with NSAA. CD158a is the inhibitory receptor of CD56bright NK cells, and its expression decreases in patients with NSAA. It indicates that CD56bright NK cells are activated in patients with NSAA. CD56bright NK cells, which are involved in immunoregulation, account for the major advantages of producing large numbers of proinflammatory cytokines and participating in the pathogenesis [24]. These results suggest that CD56 bright NK cells play an important role in the human defense system and play an immunoregulatory effect in the immune system when the immune status changes. CD56bright NK cells play an important role in the hematopoietic system of patients with NSAA.

Interleukin 2 (IL-2) is a T cell growth factor that has been shown to modulate several in vitro immune responses. Hefeneider [25] found that administration of purified IL-2 to naive, non-antigen-challenged recipients resulted in substantial potentiation of NK cell activity. Fehniger [26] demonstrated that CD56bright NK cells endogenous T cell-derived IL-2, acting through the NK high-affinity IL-2 receptor, costimulating CD56 bright NK cells to secrete IFN-γ. IL-18 is a newly discovered proinflammatory cytokine, which activates NK cells closely related to its dose [27]. Shan found that the level of the IL-18 in the serum of AA was higher than that in NSAA and normal controls, the serious the disease, the higher the IL-18 [28]. Takeda [29] demonstrated that the importance of in vivo of IL-18 in NK lytic activity cell activity. Our result showed that IL-2 and IL-18 in the newly diagnosed NSAA group and the remission group were higher than that of the normal control group. A recent study showed that the effect of CD56bright NK cells in bacterial infections was demonstrated in vitro which in synergy with IL-2, exposure of NK cells to bacterial proteins through TLRs induces CD56bright NK cells to produce IFN-γ [30]. In our experiment, increased IL-2 may activate CD56 bright NK cells produced more granzyme B and promote the activation of the immune mechanism.

Recently, the CD56bright NK cell is often described as regulatory NK cells. Due to these cells can influence innate immunity through cytokine production. However, CD56bright NK cells also become cytotoxic after activated; cytokine-activated CD56bright NK cells are cytotoxic toward autologous activated CD4+ T lymphocytes [31]. This phenomenon might be therapeutically exploitable in the context of T cell–mediated autoimmune diseases [31]. Furthermore, Morandi et al. showed that CD56bright NK cells overexpress several ectoenzymes which lead to the production of the immunosuppressive molecule adenosine, which inhibits the proliferation of autologous CD4+ T cells in a way similar to regulatory T cells [32]. Th1 cells are CD4 positive cells which mainly secrete IL-2 and so on. Th1 cells are involved in regulating cellular immunity, assisting the differentiation of cytotoxic T cells and mediating a cellular immune response. In our experiment, the expression of IL-2 in patients with NSAA was higher than that in the normal group, which further indicates...
that TH1 plays an important role in the pathogenesis of NSAA.

All in all, the imbalance of immunity leads to the damage of hematopoietic function in NSAA. The exact cause is unknown, we guess due to some immune factors that chronic hematopoietic failure occurs. TH1 cells increased in vivo, and CD56 bright NK cells acted as regulatory cells, secreting immunomodulatory factors, together with TH1 cells to regulate the immune status of the body. We knew that CD56bright NK cells play an important role in the enhancement of immune regulation and prognosis of the disease through this experiment. Further, it suggests that we could isolate CD56brightNK cells in vitro and perform in vitro amplification and functional studies, which will provide new research ideas for the future treatment of NSAA.

In conclusion, the proportion of CD56bright NK cells in peripheral blood of NSAA patients increased, and activated regulatory NK cells might play an activated role in the pathogenesis of NSAA, which maybe the potential target of treatment in the future.

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