

Original Article

Abnormal quantity and function of Th17 cells in patients with severe aplastic anemia combined with infection

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Abstract: Severe aplastic anemia (SAA) is a hematologic disease characterized by peripheral pancytopenia with bone marrow failure. Fatal bacterial or invasive fungal infections are the most frequent cause of mortality in SAA. Th17 cells are a recently discovered CD4⁺ T-helper subset that secrete cytokines such as interleukin 17A (IL-17A) and subsequently provide protective immunity against infections and cancer. Further, Th17 cells are also associated with chronic inflammation and autoimmunity. In this study, we analyzed alterations and quantitative and functional changes in Th17 cells in the peripheral blood of patients with SAA who had severe infections, as compared with SAA patients with recovered infection after anti-infection therapy and normal controls. The results showed that the IL-17A level in the Th17 cells of peripheral blood lymphocytes was increased in patients with SAA who had severe infections. After anti-infection therapy, the level of IL-17A in the Th17 cells was dramatically decreased. The median expression of IL-17A mRNA in the Th17 cells was increased in patients with SAA who had severe infections, as compared with the remission group and normal controls. Therefore, we concluded that an increase in excessively activated Th17 cells might participate in the immune dysfunction and the development of inflammatory response in patients with SAA who had severe infections. The above mentioned findings indicate that Th17 could be a potential target for treatment and could help improve the prognosis of SAA patients.

Keywords: Severe aplastic anemia, Th17, infection

Introduction

Severe aplastic anemia (SAA) is a serious hematologic disease characterized by bone marrow hematopoietic failure with a high mortality rate. The pathogenesis of SAA is T-lymphocyte hyperfunction that leads to the apoptosis of hematopoietic cells through type I lymphokines and effector T cells [1-3]. Patients with SAA usually die for a series of complications such as infection, severe anemia, or hemorrhage. Neutropenia combined with the use of glucocorticoids, cyclosporin, and antithymocyte globulin (ATG) makes patients with SAA a high-risk group for infection. Fatal bacterial or invasive fungal infections are the most frequent cause of death in SAA [4-6].

Recent studies have found the presence of a new CD4 effector T cell, Th17 cells, which are differentiated from natural T-cell precursors. Th17 cells are characterized by expressing the chemokine receptor, CCR6; transcription fac-

tors; retinoic acid (RA)-related orphan receptor α (ROR α); and RA-related orphan receptor γ thymus (ROR γ T) that are critical for Th17 differentiation [7]. Th17 cells play an important role in autoimmune diseases, cancer, transplant rejection, and anti-infective therapy [8]. Transforming growth factor- β and inflammatory cytokines such as IL-6, IL-21, and IL-23 participate in the generation of Th17 cells. IL-17A, the canonical Th17 cytokine, is a proinflammatory cytokine [9]. IL-17A is secreted as a disulfide-linked homodimeric glycoprotein [10] that is thought to play an influential role in inflammation and tissue homeostasis. IL-17 also downregulates micro-RNA 23b, which negatively regulates inflammatory responses [11]. Animal studies have shown that activated Th17 cells recruit neutrophils to infection sites and facilitate tertiary lymphoid structure development in infected lungs [12]. Previous studies have implicated Th17 cells in the pathogenesis of inflammatory diseases including many autoimmune disorders [13-15].

Table 1. The primers sequences

Target	Sense and anti-sense sequences	Bp
IL-17	F: 5'-CTCAGGCTTCCTTTGGAGATTA-3' R: 5'-CTCCTTTCTGGGTTGTGTGGTG-3'	123 bp
̑-Actin	F: 5'-TTGCCGACAGGATGCAGAA-3' R: 5'-GCCGATCCACACGGAGTACT-3'	100 bp

Herein, we will show that patients with SAA who have infections have an increased number of circulating Th17 cells, as compared with healthy controls. Moreover, we will show that IL-17A is highly expressed in the Th17 cells of these patients. Therefore, Th17 cells might play a major role in the mechanism of infections in SAA and thus be a new target for anti-inflammation in SAA.

Material and methods

Subjects

Twenty-one newly diagnosed SAA patients with infections (13 males and 8 females; median age, 41 [range, 7-73 years]), 14 SAA patients convalescing from an infection (6 males and 8 females; median age, 50 [range 7-75 years]) and 10 uninfected SAA patients (4 males and 6 females; median age, 47 [range 9-64 years]) were enrolled in this study. All patients were diagnosed from March 2014 to March 2015 in the Hematology Department of General Hospital Tianjin Medical University using the International AA Study Group criteria. The disease was considered SAA if at least two of the following parameters were met: neutrophil count of $< 0.5 \times 10^9/L$; platelet count of $< 20 \times 10^9/L$; and a reticulocyte count of $< 20 \times 10^9/L$ with hypocellular bone marrow ($< 30\%$ cellularity). If the neutrophil count was $< 0.2 \times 10^9/L$, the AA was considered very severe (vSAA). Patients were excluded if they had congenital AA or other autoimmune diseases. Patients were screened for paroxysmal nocturnal hemoglobinuria (PNH) with flow cytometry using anti-CD55 and anti-CD59 antibodies, and no PNH clones had been found. All patients in the group had a fever after receiving IST (including the use of glucocorticoids, cyclosporin, and ATG) or were suspected of having a bacterial or invasive fungal infection according to microbiological or radiographic evidence and clinical criteria. During and after IST, a neutropenic fever in these patients was treated initially with empiric

broad-spectrum antibiotics, followed by empiric anti-fungal therapy within 48 h if the fever persisted. Patients received granulocyte transfusions and G-CSF treatment depending on medical necessity. Responses to anti-infective therapy were defined as improvement in microbiologic (resolution of bacteremia), radiographic (decrease in infiltrates or nodule size), and clinical criteria. The clinical criteria included defervescence or body temperature decrease of at least 1.5°C , hemodynamic stabilization, and improvement in symptoms such as dyspnea [16].

Eighteen healthy volunteers with a median age of 34 years (range, 19-45 years) were included as normal controls. The Ethics Committee of Tianjin Medical University approved the research program, and all subjects provided informed consent.

Bacteriological examination

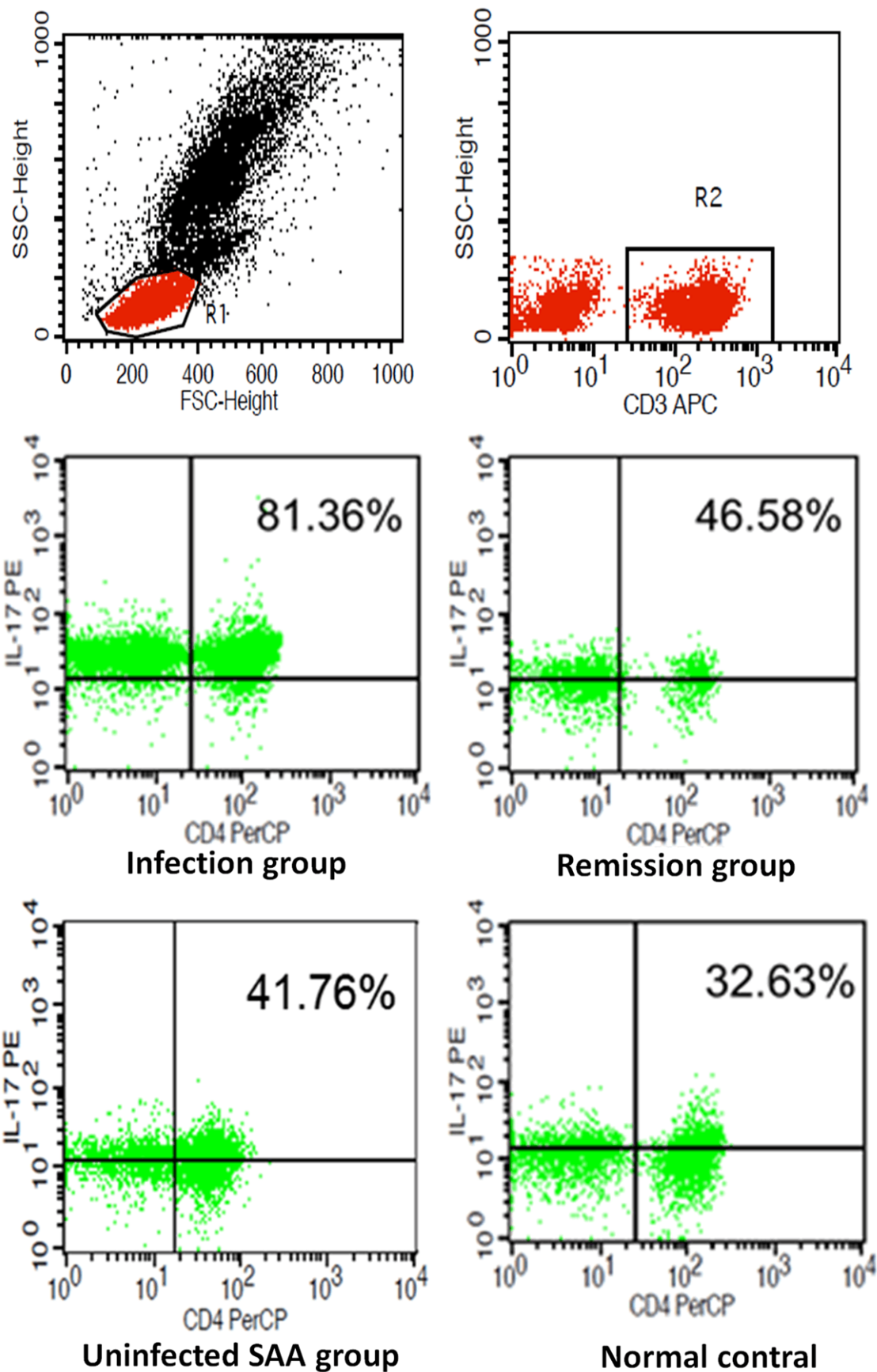
Bacteriological examination were assessed, including responses at different time points (prior to therapy and 1 and 4 weeks post-therapy), as well as bacteriological responses in different species and sites.

Monoclonal antibodies

APC-labeled mouse anti-human CD3 monoclonal antibody, PerCP-labeled mouse anti-human CD8 monoclonal antibody, PE-labeled mouse anti-human IL-17A monoclonal antibody, and their respective isotype controls were purchased from Becton Dickinson (BD, Franklin Lakes, NJ, USA). An FACS Calibur flow cytometer was used (Becton Dickinson, USA).

FCM analysis

Th17 cells (CD3+ CD4+ IL-17A+) were identified using flow cytometry. One hundred microliters of a whole blood sample was immunostained in control and test TruCount tubes (BD) in the dark at 4°C for 20 min, followed by red blood cell lysis in 2.0 mL of an erythrocyte lysis solution (BD Biosciences, San Jose, CA, USA) at room temperature for 8 min and mixing with 1.0 mL of an FACSTM permeabilization solution (BD) for 10 min. At least 10000-30000 cells were acquired using a fluorescence-activated cell sorter FACS Calibur flow cytometer (BD Biosciences, San Jose, CA, USA) and analyzed



Th17 cells in patients with severe aplastic anemia combined with infection

Figure 1. Flow cytometry (FCM) tests. The levels of IL-17A in Th17 cells in infection group, remission group and normal control.

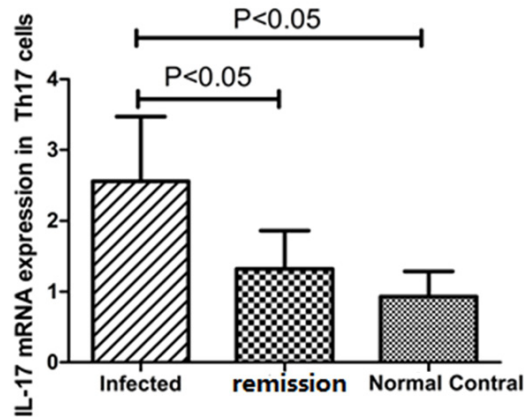


Figure 2. The IL-17 mRNA expression of Th17 cells in infected SAA patients, patients in remission group and normal controls.

using CellQuest software version 3.1 software (BD).

Isolation and purification of Th17 lymphocytes

Ten milliliters of fresh peripheral blood was obtained from the infected SAA group, remission group, and normal controls. Peripheral blood mononuclear cells (PBMCs) were isolated from the blood samples with density gradient centrifugation using a Ficoll-Paque Plus solution (Amersham Bioscience, Uppsala, Sweden). After staining with fluorophore-conjugated monoclonal antibodies as previously described, the Th17 cells were subsequently sorted and obtained using a FACS Aria flow cytometer (BD Biosciences). The sorted cells were analyzed using the Cell Quest software program (Version 3.1, Becton Dickinson), and the purity of Th17 cells was > 90%.

Quantitative real-time PCR

The total RNA for the Th17 cells from the infected SAA group, remission group, and normal controls was extracted using a TRIzol reagent (Invitrogen, USA) and then reverse-transcribed using a TIANScript RT Kit (TIANGEN, Beijing, China). β -actin was employed as a housekeeping gene to standardize the targeted mRNA expression. **Table 1** presents the sequences of primers specific to IL-17 and β -actin that were designed and synthesized by Sangon Biotech

(Shanghai, China). Quantitative real-time PCR was performed using the Bio-Rad iQ 5 Real-time System (Bio-Rad, Hercules, CA, USA). SYBR Green (TIANGEN, Beijing, China) was used as a double-strand DNA-specific dye. Amplification was performed using 40 cycles at 95°C for 5 s and 56°C for 34 s with extension at 72°C for 30 s. The IL-17 levels were calculated using the $2^{-\Delta\Delta C_t}$ method ((C_t , target gene- C_t , β -actin) sample - (C_t , target gene- C_t , β -actin)), after normalizing the data according to the β -actin mRNA expression.

Statistical analysis

The statistical analysis software SPSS 21.0 was used. Normally distributed numerical variables are presented as mean \pm standard deviation (SD). The difference between 2 groups was compared using the Student's t test. Skewed distributed data are reported as the median and were analyzed using the Wilcoxon test. Differences were considered statistically significant with a P value of < 0.05.

Results

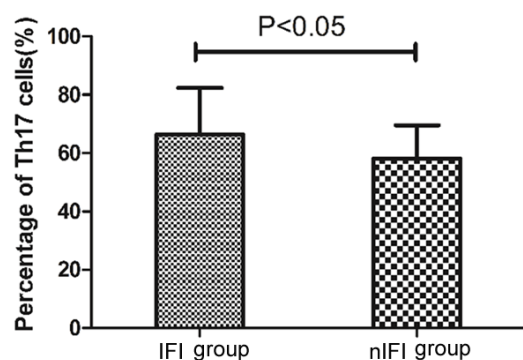
Percentage of Th17 cells increased in the peripheral blood of patients with SAA and an infection

We analyzed the quantity of circulating Th17 cells in uninfected SAA patients, normal controls, SAA patients with severe infections and SAA patients convalescing from infections using FCM. The IL-17A level in CD3⁺ CD4⁺ IL-17A⁺ cells from peripheral blood lymphocytes was increased in patients with SAA and severe infections (62.15 \pm 19.60%). After anti-infection therapy, the IL-17A level in the Th17 cells gradually decreased (47.94 \pm 18.71%). The IL-17A levels in the Th17 cells in the uninfected SAA group and normal control group was (42.96 \pm 10.11)% and (38.74 \pm 13.81)% respectively. The percentage of Th17 cells in patients with SAA and an infection was significantly higher than those of the remission group, uninfected SAA group and normal controls (P < 0.05). Moreover, The IL-17A levels in the Th17 cells in the uninfected SAA group was also higher than normal control group (**Figure 1**). There

Table 2. Characteristics of infections in SAA patients

	N. of patients	Blood	Lung	CNS
Bacterial	10			
Staphylococcus	4	4		
Stenotrophomonas maltophilia	2	1	2	
Escherichia coli	2	2		
Acinetobacter baumannii	1		1	
Klebsiella pneumoniae	1		1	
Fungal	11			
Aspergillus spp	4		4	
Candida albicans	4	2	3	
Candida tropicalis	2	1	1	
Cryptococcus neoformans	1			1

strain or fungal cause or a mixture of infections. Among them, 11 patients had invasive fungal infections, including 4 cases involving an *Aspergillus* infection in the lung. Other fungi included *Candida albicans* and *Candida tropicalis*. Among the 10 patients who had severe bacterial infections, 7 cases had bacteremia. *Stenotrophomonas maltophilia*, *Staphylococcus*, and *Escherichia coli* were the common pathogens found in the blood samples from these patients. In addition to blood infections, the lung was the most common site of infection (**Table 2**). However, the percentage of Th17 cells in patients with SAA was independent of body part ($P > 0.05$).

**Figure 3.** Th17 level in invasive fungal infections (IFI) group and noninvasive fungal infection (nIFI) group.

For the 11 patients who had invasive fungal infections (IFI), the percentage of Th17 cells was $(66.47 \pm 15.90)\%$ and was slightly higher than that of the noninvasive fungal infection group ($P < 0.05$) (**Figure 3**).

A trend towards higher Th17 levels in patients who had prior therapy for *Aspergillus* spp was seen ($P = 0.06$), as compared with other pathogens. There were no significant differences observed in the SAA patients with infections for any other pathogens at any time point.

was no significant difference in IL-17A between the remission group and uninfected SAA group.

IL-17 mRNA expression increased in the peripheral blood Th17 cells from patients with SAA and an infection

To determine the role of Th17 cells in SAA, IL-17 mRNA expression was evaluated in the peripheral blood Th17 cells from patients with SAA and an infection, the remission group, and normal controls. The mRNA expression for the relative expression of IL-17 mRNA was significantly increased in the peripheral blood Th17 cells from infected cases (2.65 ± 1.21), as compared with the remission group (1.63 ± 0.42) and normal controls (0.93 ± 0.36) ($P < 0.05$; **Figure 2**).

Bacteriological examination

Of the 21 identified patients with a severe infection, most patients had multiple microbial infections involving more than one bacterial

Patient outcomes by time point

After anti-infective therapy, the percentage of peripheral Th17 cells in patients with SAA and an infection at 1 week and 4 weeks was $58.03 \pm 11.91\%$ and $47.94 \pm 18.71\%$, respectively. The patient outcomes at 1 week and 4 weeks are shown in **Figure 4**. The percentage of peripheral Th17 cells was found to be significantly lower at 4 weeks post-therapy than 1 week post-therapy and in untreated patients with SAA. No significant difference was detected between the untreated SAA.

We also investigated the relationship between the duration of infection and the percentage of peripheral Th17 cells. With the increase of the duration of infection, the level of Th17 cells were significantly elevated (**Figure 5**).

Discussion

SAA is a hematological disease characterized by hematopoietic failure of the bone marrow and has a high mortality rate. Recent studies have demonstrated that enhanced immuno-

Th17 cells in patients with severe aplastic anemia combined with infection

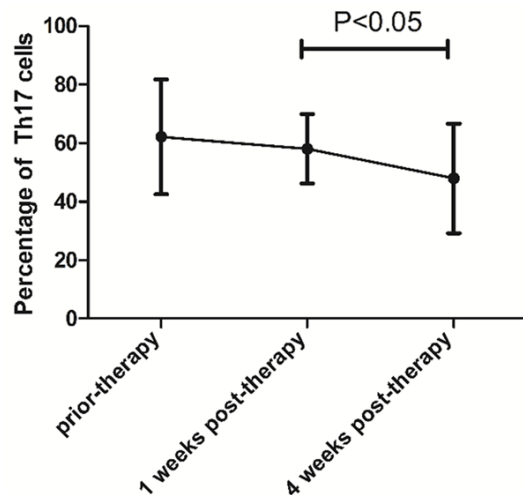


Figure 4. Percentage of peripheral Th17 cells in SAA patients with infections by timepoint.

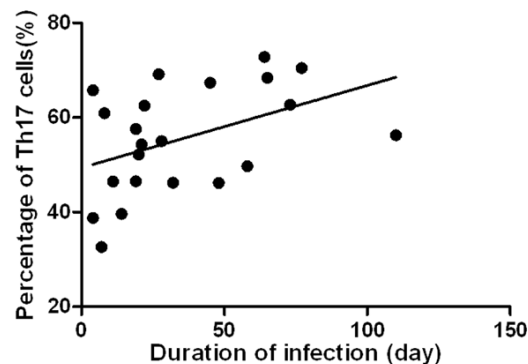


Figure 5. Correlation between the duration of infection and Th17 cells.

suppression combined with the promotion of hematopoiesis have improved the treatment efficacy of SAA to 70% in China. A higher mortality rate from infection is observed in patients who do not respond to treatment. The pathogenesis of SAA involves the abnormal function of T lymphocytes combined with severe agranulocytosis, resulting in a higher incidence of infection in patients because of impaired anti-infective capabilities. Once these patients develop an infection, the infections are usually more severe and do not respond to enhanced antibiotic treatments. Therefore, anti-infective therapy has become a critical issue for the treatment of SAA. Only effective anti-infection treatment can gain times for immunosuppressive drugs to take effect.

Th17 cells have become a “hotspot” in immunological studies in recent years; they were the third type of effector T cells that were identified after the Th1 and Th2 subtypes. For the secretion of IL-17, Th17 cells have been reported to play an important role in the pathogenesis of autoimmune diseases such as rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE). A study conducted by Lubberts et al. demonstrated the important role of Th17 in RA through symptom amelioration after administering an IL-17 inhibitor. In patients with SLE, the interaction of IL-17 with IL-21 induces B cells to secrete a large amount of autoantibodies, leading to disease progression. Meanwhile, recent studies found that Th17 cells also play a critical role in host defense against bacterial infections. IL-17 is an important cytokine in host defense against pathogens through the recruitment of neutrophils. Romagnani [17] and Annunziato [18] demonstrated that Th17 cells have proliferative abilities and cytotoxicity by causing B cells to produce IgG, IgM, or IgA, but not IgE. For the first time, Huang [19] confirmed the critical role of Th17 response in host defense against *Monilia* infections, as demonstrated by the increased sensitivity to *Monilia albicans* infections with the decreased release of IL-17RA. Furthermore, IL-17R-mediated inflammation leading to a *Monilia albicans* infection in mouse intestines has been reported. In addition, a higher sensitivity and obvious immunological and pathological changes to *Monilia* and *Aspergillus* infections were observed in TIR8-deficient (a negative regulatory factor of Th17) mice [20, 21]. All these observations indicate the importance of Th17 in host defense against *Monilia* infections. Meanwhile, IL-17 also plays an important role in *Pneumocystis carinii* infections.

We measured the number of Th17 cells in patients with SAA who had an infection (SAA-infection) and found a significantly higher number of cells were observed in patients with SAA than in patients posttreatment and normal controls, suggesting a close association with Th17 cells in SAA-infection. By conducting a correlation analysis, we also found a correlation with Th17 cells numbers and hematology recovery, along with the percentage of peripheral CD4+/CD8+, indicating that Th17 cells are associated with not only infection, but also humoral immu-

nity, leading to the pathogenesis of SAA-infection. Furthermore, we obtained the same results as a PCR analysis that indicated higher expressions of IL-17A in patients with SAA-infection, further supporting the abovementioned conclusion. Meanwhile, we observed higher numbers of Th17 cells in patients with a mycotic infection than those without, suggesting the importance of Th17 cells in mycotic infections. In addition, Th17 cell numbers were also associated with the amount of time patients had an infection, as in patients with infections lasting a longer time, higher numbers of Th17 cells were found.

In conclusion, the pathogenesis of SAA has been confirmed to be associated with immune abnormalities. Severe agranulocytosis usually leads to a higher incidence of infection-related mortality. As a cell population related to immunity and infection, the Th17 cell was found to be associated with SAA immunity and infection in our study, indicating it might be involved in the immunological and infectious pathogenesis of SAA that will be a focus of our future investigations.

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Disclosure of conflict of interest

None.

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