Original Article
Mechanism of immunosuppressants combined with cord blood for severe aplastic anemia

Zhe Yu, Fang Zhou, Lin-Fu Ge, Xi-Min Liu, Yuan Fang, Lin-Na Xie, Fan-Sheng Kong, Ning-Xia Song, Qing-Qing Yu

Department of Hematology, Jinan Military General Hospital, Jinan, Shandong 250031, China

Abstract: This study aims to explore the mechanism of immunosuppressants combined with cord blood (IS + CBI) for severe aplastic anemia. Selecting 30 patients with SAA and all treated with IS + CBI (newly diagnosed group). 23 patients who were treated effectively (effective group) while 7 cases were treated invalidly (invalid group). Another 20 healthy individuals were selected as control group. To detect the expression levels of IL-17, IL-22 and other cytokines by ELISA method in each group. To detect the engraftment of cord blood stem cells by using short tandem repeat-polymerase chain reaction (STR-PCR) method. 1. IL-17, IL-22 and other cytokines expressions in newly diagnosed group were significantly higher than in the control group. 2. After 6 months, the level in effective group was significantly lower than pretherapy ($P < 0.05$).The level in invalid group had no obvious difference than pretherapy. 3. After 1 month and 3 months of treatment, a small amount of engraftment was found in effective group. After 6 months, implant rejection was showed. No effective engraftment was observed in invalid group. 1. IL-17, IL-22 cells in SAA patients increased which might positively correlated with the progression of SAA. 2. During the treatment of IS + CBI, there is a bridging mechanism between the early stage of engraftment and the advanced stage of immunosuppressant adjustment. The first 3 months after treatment, it relies on the engraftment of cord blood stem cells to promote hematopoietic recovery and 3 months later, it relies on immunosuppressants to maintain normal hematopoietic function.

Keywords: Aplastic anemia, cord blood, cytokine, interleukin-17, interleukin-22

Introduction

Aplastic anemia (AA) is a kind of acquired bone marrow failure syndromes characterized by anemia, infection and bleeding, which is caused by pancytopenia [1]. Especially in severe aplastic anemia (SAA), rapid progression, the mortality rate is high [2]. So far, the main pathogenesis of AA is considered as abnormal activation of T cells [3]. Th17 is a newly discovered class of Th cell subsets, which can secrete IL-17, IL-21, IL-22 and other cytokines [4]. Pawel et al. found that IL-17 can promote the proliferation and differentiation of hematopoietic progenitor cell of CD_{34} + towards to neutrophils and can induce fibroblasts to produce IL-6, IL-8 and G-CSF to affect hematopoiesis [5]. Th22 cells are a newly discovered class of independent Th subsets, which can secrete IL-22, IL-10, tumor necrosis factor- α (TNF-α) and other cytokines, but mainly play their role by IL-22 [6]. At present, Th22 cells are highly expressed in chronic skin inflammation, asthma and other chronic respiratory inflammations, this may aggravate chronic inflammations of skin and respiratory system [7, 8]. Some studies found that AA patients had significantly increased peripheral blood Th17, Th22 cells, which was positively correlated with the development of disease [9, 10].

Allogeneic hematopoietic stem cell transplantation (Allo-HSCT) is an effective method to cure severe aplastic anemia [11]. However, there is less and less chances of identical siblings due to the implementation of the "one-child" policy in China. Although China marrow donor program (CMDP) has been greatly expanded in recent years, human leukocyte antigen (HLA) genes requires a high degree of consistency and a long process from matching to transplant. So we are still unable to meet the needs of all the patients. These problems spurred the seek for another source of hematopoietic stem cells (HSC) [12].

1974, Kundtzond et al. [13] firstly found that there were not only large amounts of hematopoietic stem cells/progenitor cells (HSC/HPC) in human cord blood, but also a lot of mesenchymal stem/progenitor cells. These two cells are the main functional cells to maintain hematopoiesis. Papadopoulos et al. [14] proved that cord blood contained abundant of hematopoietic stimulating factors and other hematopoietic growth factors, such as IL-1, IFN and TNF which have been confirmed to promote the proliferation of hematopoietic progenitor cell in bone marrow. With this feature of cord blood, our treatment center used immunosuppressants combined with cord blood infusion (IS + CBI) for the treatment of SAA since 10 years ago. After years of clinical studies, we found that some effective patients in the early stage of transplantation may form trace mixed chimerism. But eventually, the formation of transplantation rejection would cause invalid implantation, while 76.66% of the patients could achieve a long-term survival by successfully hematopoietic reconstitution.

By analyzing the expression level of the cytokines secreted by Th cells (IL-17, IL-22, IL-21, IFN-γ, TNF-α) before and after the IS + CBI treatment on patients with SAA and monitoring the implantation of umbilical cord blood stem cells in this study, we explored the therapeutic mechanism of IS + CBI on SAA patients.

Materials and methods

Patients

30 cases of SAA patients admitted into Jinan Military General Hospital from Jan., 2010 to Jan., 2013 were recruited, all of them were consistent with Guidelines for the Diagnosis and Management of Aplastic Anaemia published by British Committee in 2010 [15]. All the patients underwent immunosuppressants combined with cord blood infusion (IS + CBI) treatment. Including 13 cases of male and 17 of female, aged 4 to 55 years (median 24 years). There were 23 patients who were treated effectively (effective group) while 7 cases were treated invalidly (invalid group). The control group had 20 cases of blood samples which were taken from healthy blood donors, including 12 males and 8 females, aged 10 to 58 years (median 32 years). This study was conducted in accordance with the declaration of Helsinki. This study was

conducted with approval from the Ethics Committee of Jinan Military General Hospital. Written informed consent was obtained from all participants.

Specimen extraction

Patients were fasted 12 hours later, in the next morning 3 ml of fasting heparinized peripheral blood was collected to separate the plasma and be stored at -80°C for detection. Another 20 healthy individuals were selected as normal controls

Treatment protocols

Cord blood: Collected from the qualified samples in Shandong, Sichuan, Beijing Umbilical Cord Blood Bank, these patients were administrated via peripheral vein infusion. MNC: 2.25-15.1×10^7/Kg (for the patients received 2 units of umbilical cord blood, the mononuclear cells of these 2 units were added).

Regimen: Cyclophosphamide (CTX) 50 mg/(kg/d) × 2 d (d-3-d-2), Antilymphocyte globulin (ALG pigs) (Wuhan Institute of biological products, Wuhan, China) ALG 15 mg/(kg/d) or Anti thymocyte globulin (ATG rabbit) (French Sanofi products, French) 3 mg/(kg/d) × 5 d (day 4-day 8).

Sequential treatment of immunosuppressant after cord blood transfusion: Before a day of cord blood transfusion, these patients were intravenously administrated with CsA in a dose of 1.5 mg-3 mg/(kg/d), and turned to receive oral administration in a dose of 6-8 mg/(kg/d) twice per day after the remission of gastrointestinal symptoms, and their doses were adjusted according to the CsA blood concentration (to maintain it at 150-400 μg/ml), lasting for 12-18 months.

Detection of IL-17, IL-22, IL-21, TNF-α and IFN-r

3 ml of fasting heparinized peripheral blood was collected to separate the plasma and be stored at -80°C for detection. Then the samples were detected by ELISA assay (according to kit instructions). On the first visit, 3 months after treatment, 6 months after treatment, treatment after 1 year.

Implantation detection

To detect the implantation rate of donor stem cells by using DNA short tandem repeat-poly-

Table 1. The expression levels of IL-17, IL-22, IL-21, TNF-α and IFN-r before and after treatment in patients with SAA (ng/L, x̄±s)

Groups	Cases	IL-17	IL-22	IL-21	TNF-α	IFN-r
Newly diagnosed group	30	336.51±40.14*	112.23±9.79*	282.67±20.93*	396.83±34.33*	359.45±29.73*
Effective group	23					
Pretherapy		340.17±32.22*	123.85±8.98*	283.17±17.45*	401.83±29.49*	366.28±28.56*
Three months		312.64±22.16*	98.46±10.41*	269.53±13.66*	374.67±29.35*	338.42±30.13*
Six months		243.47±35.16▲	73.29±10.58▲	223.05±11.20▲	282.73±21.53▲	269.67±11.35▲
One year		239.24±37.53▲	70.85±12.08▲	210.73±13.87▲	276.49±28.97▲	248.92±19.11▲
Invalid group	7					
Pretherapy		350.83±36.18*	119.29±9.26*	290.06±12.81*	397.46±30.12*	371.09±29.39*
Three months		345.64±42.16*	96.42±12.84*	287.53±15.11*	372.21±29.43*	362.74±28.67*
Six months		334.15±21.41*	109.23±8.91*	279.17±15.39*	383.38±24.23*	359.05±23.53*
One year		349.21±50.61*	115.86±9.73*	298.44±24.79*	409.19±25.65*	363.33±20.95*
Control group	20	250.13±35.21	64.26±11.25	201.23±18.97	295.43±25.46	252.34±18.64

Note: *vs. Control group $P < 0.05$ ▲vs. Pretherapy $P < 0.05$.

Table 2. Efficiency analysis of IS + CBI (Cases, %)

Treatment	Cases	CR	PR	NR	Total effective rate (CR + PR)	Survival rate of 3 years
IS + CBI	30	22	1	7	23/30 (76.66)	23/30 (76.66)

Table 3. Hematopoietic recovery of IS + CBI (Days)

Treatment	Cases	Hematopoietic recovery days			
		WBC > 1.0 × 10^9/L	ANC > 0.5 × 10^9/L	PLT > 20 × 10^9/L	Hb > 60 g/L
Effective group	23	22.00 (12.00-31.50)	23.00 (12.50-28.50)	54.00 (31.00-74.75)	57.00 (39.50-73.00)
Invalid group	7	26.00 (15.00-33.00)	27.00 (12.00-32.00)		

WBC: White blood cells; ANC: Neutrophils; PLT: Platelet; Hb: Hemoglobin. All invalid patients' hemoglobin were not restored to the 60 g/L and platelets were not restored to 20 × 10^9/L.

merase chain reaction (STR-PCR) method after 1 month, 3 months, 6 months, 1 year and 2 years of IS + CBI treatment.

Statistical analysis

Statistical analyses were performed using SPSS19.0. Data were presented as mean ± standard deviation. Between the two groups were compared with LSD-t test, Student's t-test was used to compare before and after the treatment within one group and differences among 3 groups were compared with one-way ANOVA (a=0.05). A two-sided probability value < 0.05 was considered statistically significant.

Results

Levels of IL-17, IL-22, IL-21, TNF-α, and IFN-γ

IL-17, IL-22, IL-21, TNF-α, and IFN-γ levels in peripheral blood of newly diagnosed group were significantly higher than healthy control group ($P < 0.05$). IL-17, IL-22, IL-21, TNF-α, and IFN-γ levels decreased in effective group after 3 months of IS + CBI treatment compared with pretherapy, but there was no significant difference ($P > 0.05$). IL-17, IL-22, IL-21, TNF-α, and IFN-γ levels decreased to normal in effective group after 6 months and 1 year of IS + CBI treatment, and there were significant differences that compared with pretherapy ($P < 0.05$). IL-17, IL-22, IL-21, TNF-α, and IFN-γ levels in peripheral blood of invalid group after 3 months, 6 months and 1 year of IS + CBI treatment were with no significant difference compared with pretherapy ($P > 0.05$, **Table 1**).

Efficiency and hematopoietic recovery

The total effective rate was 76.66%: CR (22 cases) + PR (one case)/30 cases, survival rate of 3 years was 76.66% (23/30, **Table 2**). NR (7 cases), including 6 of 7 invalid patients died. Time of death (1.5-13 months). One invalid case currently rely on regular blood transfusions to maintain life (**Table 2**).
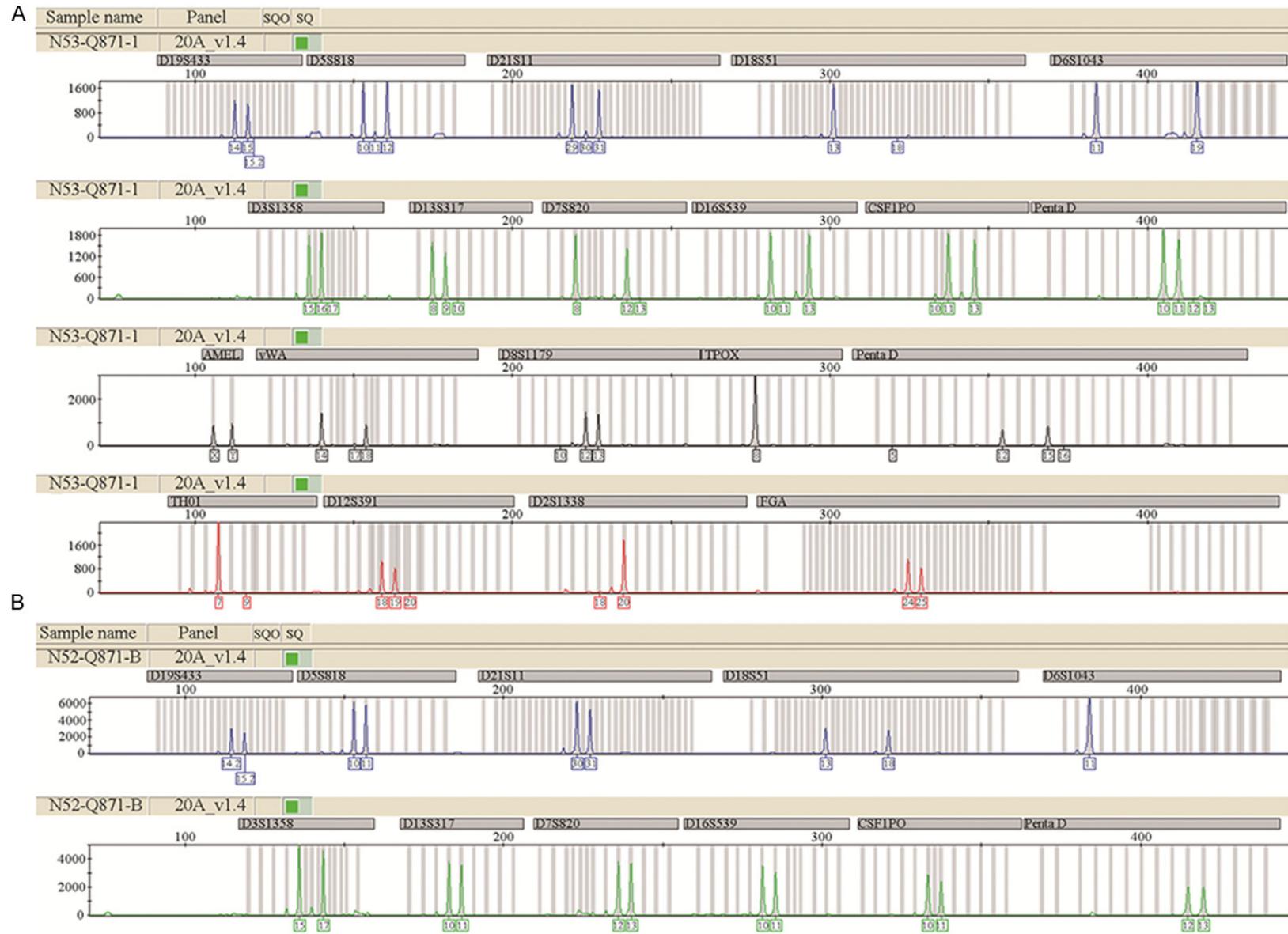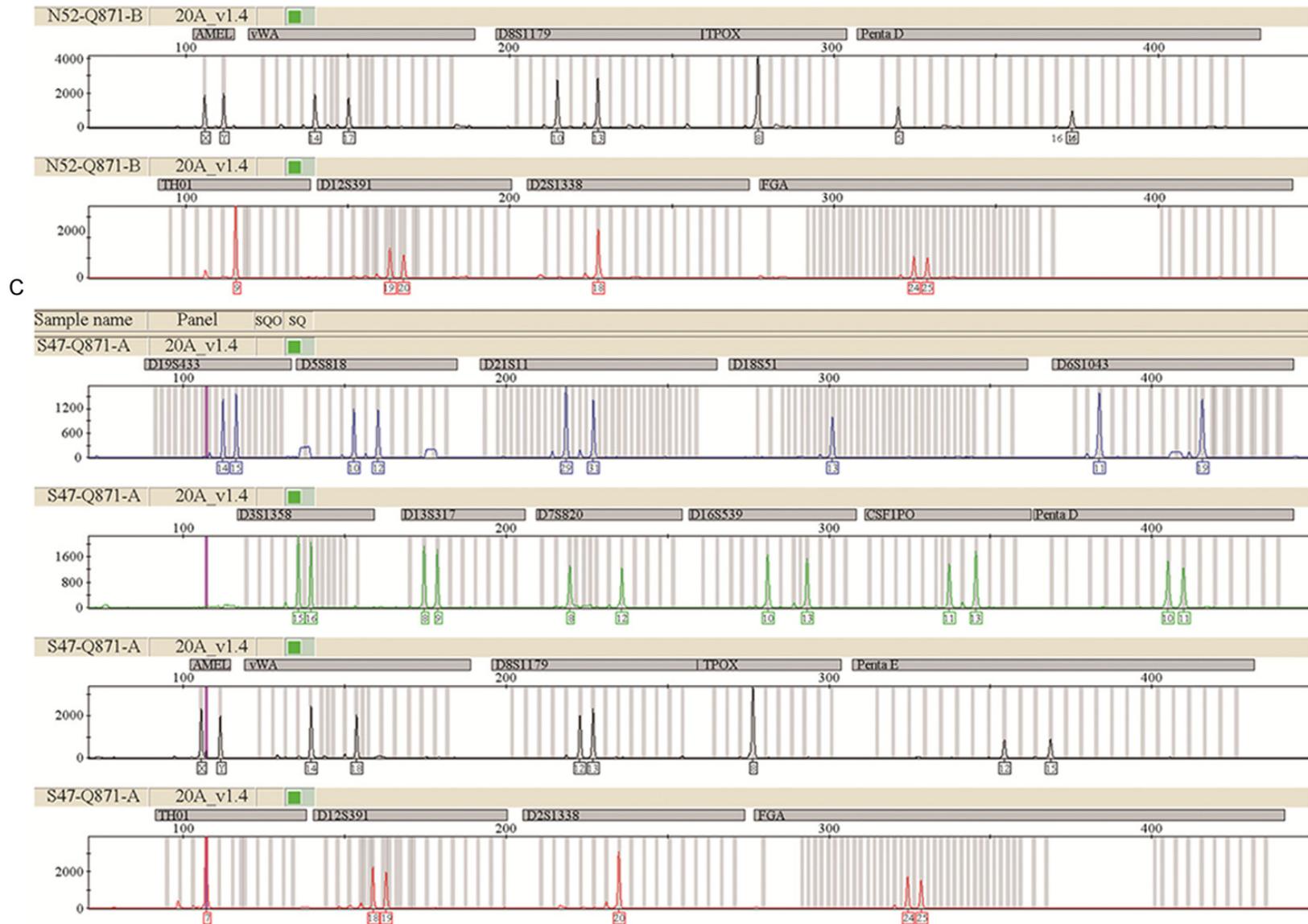
Figure 1. A 5 years old male child who was diagnosed as SAA, treated with immunosuppressants combined with cord blood infusion and used unrelated umbilical cord blood with HLA 4/6 matched. A: The peak map before the treatment. B: The peak map of the donors. C: The peak map of the patients after 1 month of treatment. Mixed chimerism showed in peripheral blood, and donor cells accounted for 3.4%.
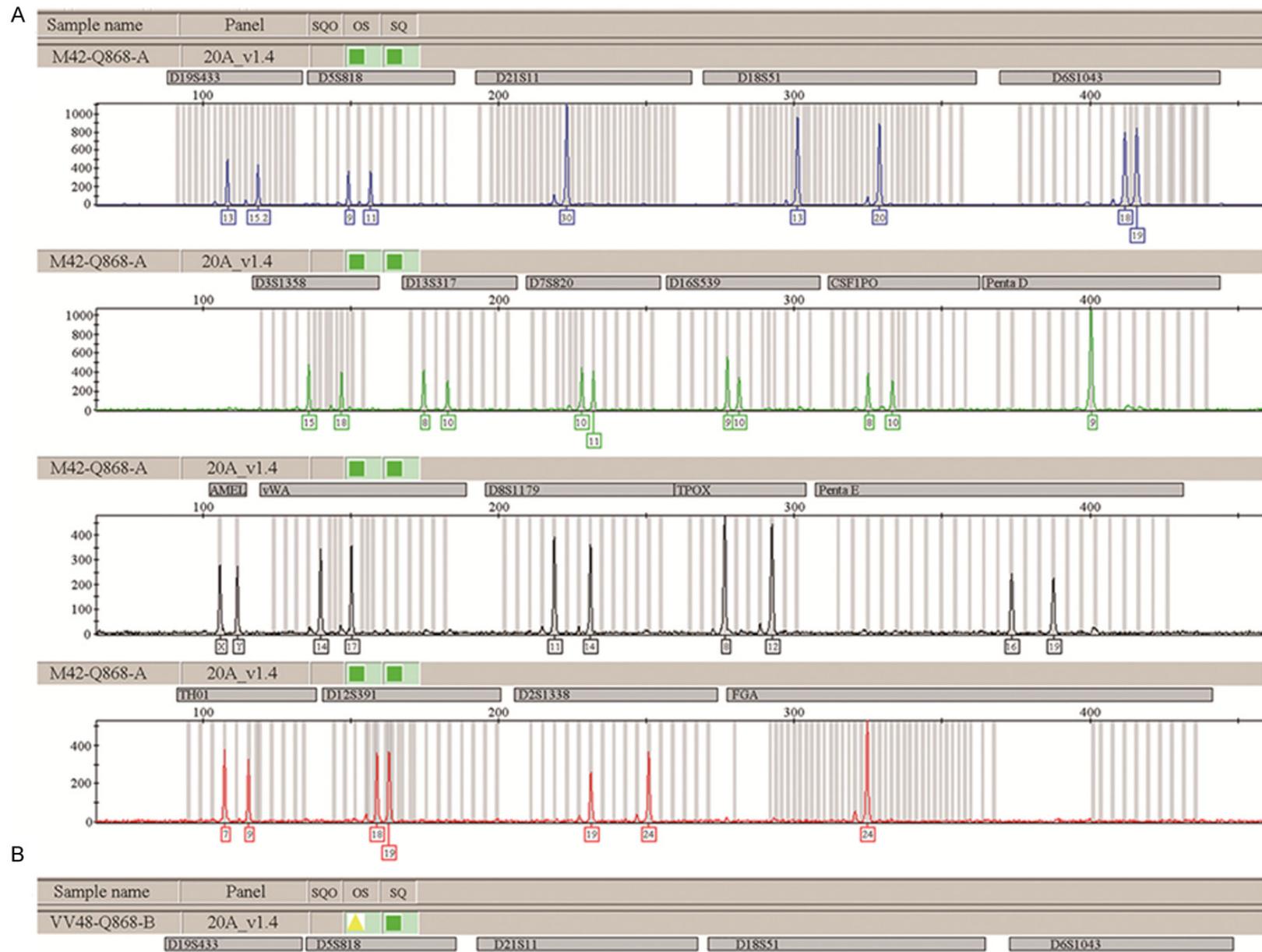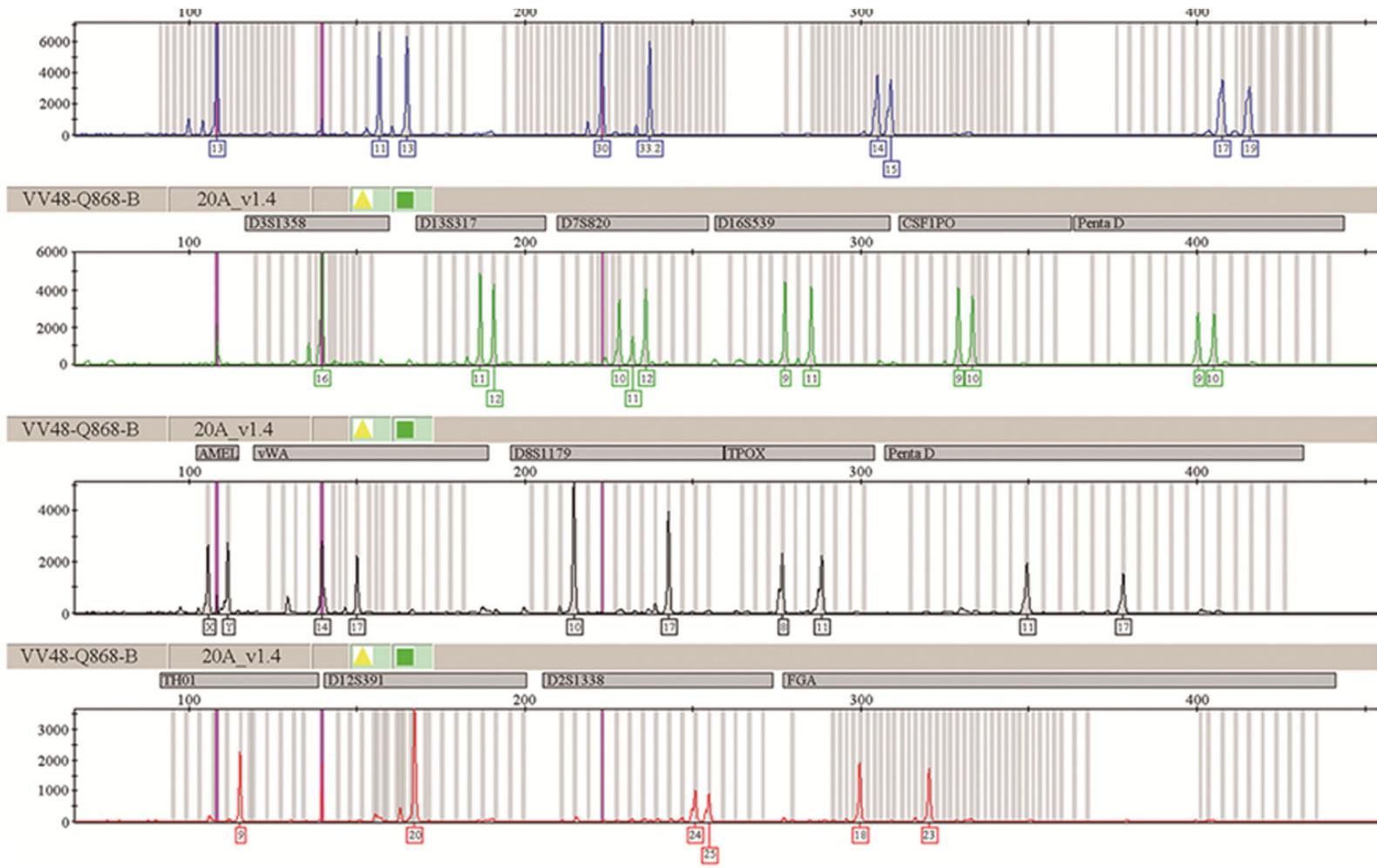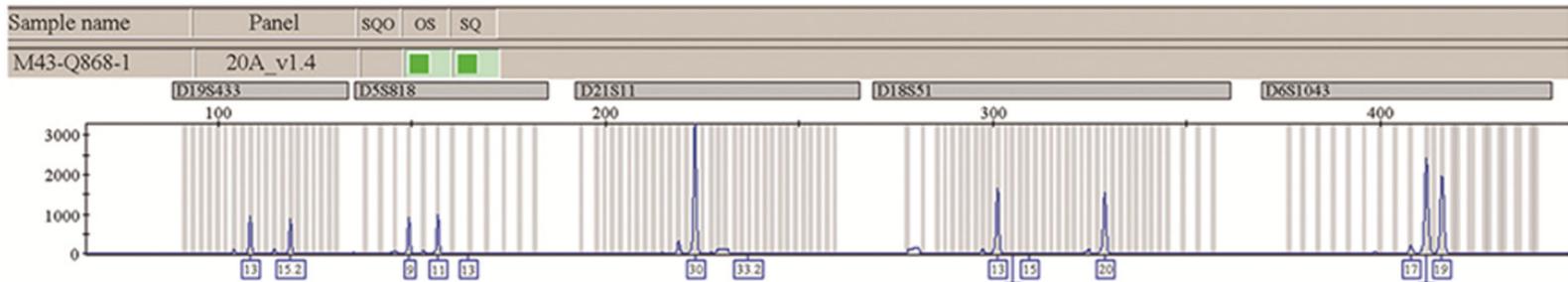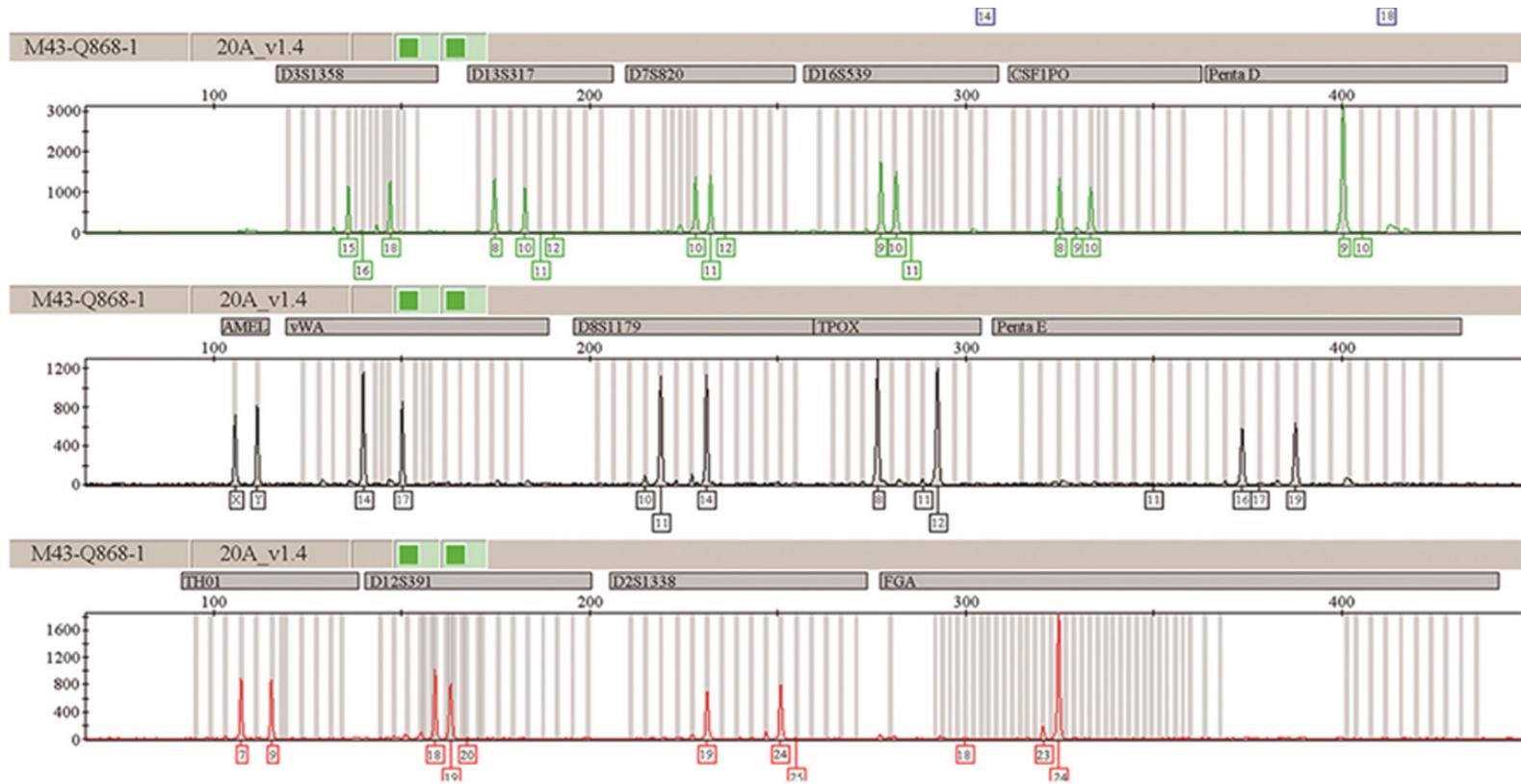
Figure 2. A 21 years old male who was diagnosed as SAA, treated with immunosuppressants combined with cord blood infusion and used unrelated umbilical cord blood with HLA 3/6 matched. A: The peak map before the treatment. B: The peak map of the donors. C: The peak map of the patients after 1 month of treatment. Mixed chimerism showed in peripheral blood, and donor cells accounted for 3.3%.

Hematopoietic recovery: The median recovery times of effective group: WBC > 1.0×10^9/L was 22 days. The median recovery times of Neutrophils > 0.5×10^9/L was 23 days. The median recovery times of PLT and Hb was 54 and 57 days. The median recovery times of invalid group: WBC > 1.0×10^9/L was 26 days. The median recovery times of Neutrophils > 0.5×10^9/L was 27 days. All invalid patients' hemoglobin were not restored to the 60 g/L and platelets were not restored to 20×10^9/L (**Table 3**).

Transplantation

By STR-PCR detection, 2.8%-8.9% of trace mixed chimerism was detected in 24 patients with effective treatment after 1 and 3 months of treatment (**Figures 1**, **2**). No donor cells were detected after 6 months, 1 and 2 years. No implantation was detected in invalid group.

Discussion

In recent years, the found of Th17 and Th22 cells which were the new Th cell subsets promoted people to have a new understanding of aplastic anemia [9, 10]. Harrington et al. in the study of Borrelia burgdorferi in 2005 [16] began to realize that Th cells which produced IL-17 were a CD_4 T cell subset that differed from Th1, Th2 but existed independently, characterized by high secretion of interleukin IL-17. Korthof et al. found that IL-17 mRNA expression in peripheral blood and bone marrow of aplastic anemia patients was higher than in normal subjects [17]. The hemopoietic negative regulatory factors in plasma which could induce macrophages to secrete high levels of IL-17, IL-6, IL-8 and TNF-α inhibited the hematopoiesis of bone marrow through direct and indirect effects. Th22 cells were another Th cells that were independent from Th1, Th2, Th17. Related researches reported that Th22 cells could express CCR6, CCR4 and CCR10, and secrete IL-22, IL-10, TNF-α and other cytokines, meanwhile, Th-22 also had double effects in autoimmune diseases, infectious diseases and tumors [18]. The study showed that [10] the number of Th22 cells increased in patients with aplastic anemia which might be positively associated with the occurrence and development of aplastic anemia. It was showed in our research that the average levels of IL-17, IL-22, IL-21, IFN-γ, and TNF-α in peripheral blood of newly diagnosed group were significantly higher than healthy control group which suggested that all the SAA patients had disorders of Th17 and Th22 subsets. It Resulted in immunologic injury of bone marrow hematopoietic stem cells and hematopoietic microenvironment which was one of the reasons for the onset of SAA.

At St. Louis Hospital in Paris, Frenchman Gluckman et al. [19] firstly successfully used HLA-compatible sibling umbilical cord blood transplantation (CBT) to cure Fanconi anemia which had created the precedent for human umbilical cord blood transplantation in 1988. Since then, cord blood as the third source of hematopoietic stem cells after the bone marrow and peripheral blood have been becoming the research focus in the field of hematopoietic stem cell transplantation for nearly 3 decades. However, the slow hematopoietic reconstitution and bad implantation seriously affected the long-term survival after surgery, and they were the major obstacles in the wide application of adult unrelated umbilical cord blood transplantation [20].

By using the advantages of umbilical cord blood, we adapted the IS + CBI project for the treatment of severe aplastic anemia. After years of clinical studies, the formation of transplantation rejection would cause invalid implantation in all the patients, while 76.66% of the patients could achieve a complete remission and long-term survival by successfully hematopoietic reconstitution. We conducted the research of its therapeutic mechanism for this phenomenon from the two perspectives which were immune and implantation: 1) To investigate whether immune disorders could be restored when good curative effects had achieved in clinic, we further observed the expression of the IL-17, IL-22, IL-21, IFN-γ and TNF-α levels after the IS + CBI treatment. We found that IL-17, IL-22, IL-21, TNF-α, and IFN-γ levels decreased in effective group after 3 months of IS + CBI treatment, but there was no significant difference compared with newly diagnosed group. IL-17, IL-22, IL-21, TNF-α, and IFN-γ levels back to normal in effective groups after 6 months and 1 year of IS + CBI treatment which suggested that IS + CBI could correct immune disorders of Th17 and Th22 in SAA effectively and improve the immune disorders of hematopoietic stem cell injury mediated by Th17 and Th22. 2) IL-17, IL-22, IL-21, TNF-α, and IFN-γ levels in invalid group after 3 months, 6 months and 1 year of IS + CBI treatment were with no significant difference compared with newly di-

agnosed group. It suggested that the main causes of such patients may be the abnormal of hematopoietic stem cells or hematopoietic microenvironment. So without effective hematopoietic stem cell engraftment, immunoregulation only could not correct the abnormal hematopoiesis. 3) By STR-PCR detection, 2.8%-8.9% of trace mixed chimerism was detected in 24 patients with effective treatment after 1 and 3 months of treatment. No donor cells were detected after 6 months, 1 and 2 years and no implantation was detected in invalid patients. It suggested that patients could rebuild its haematogenous of granulocytic series after 1 month of IS + CBI treatment and rebuild its haematogenous of erythrocytic and megakaryocytic series after 2 months. The short-term implantation of cord blood stem cells might have played a key role in these.

Through clinical and laboratory research, we get the following conclusions: 1) The new Th subsets Th17, Th22 play a certain role in immune disorders of the pathogenesis of SAA, and it can be corrected by IS + CBI treatment. 2) During the treatment of IS + CBI, there is a bridging mechanism between the early stage of umbilical cord blood stem cells engraftment and the middle and advanced stage of immunosuppressant adjustment, the hemogram of the early stage after the treatment and the recovery of hematopoiesis are related to the mixed chimerism of cord blood hematopoietic stem cells and a large number of hematopoietic stimulating factors in cord blood. However, cord blood stem cells are rejected after 3 months of treatment, while the immune disorders are effectively corrected by immunosuppressants. But because of the small sample size, more clinical samples with large-scale clinical trials are needed for further confirmation.

Disclosure of conflict of interest

None.

Address correspondence to: Fang Zhou, Department of Hematology, Jinan Military General Hospital, No. 25 Shifan Road Tianqiao District, Jinan 250031, Shandong, China. Tel: +86 15610104668; Fax: +86 0531 51666615; E-mail: yzdoccn@126.com

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