Assessment of the use of immunosuppressants combined with cord blood for severe aplastic anemia

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Summary

Background: **The objective of this study was two-fold: 1) to investigate the changes of cytokines concentration in relation to severe aplastic anemia (SAA) when treated with immunosuppressants combined with cord blood (IS + CBI). and 2) to assess the curative effect of umbilical cord blood chimerism engraftment.**

Methods: **We selected 43 patients with SAA all treated with IS + CBI (newly diagnosed group). Among them, a total of 33 patients were treated effectively (effective group) while 10 cases were treated invalidly (invalid group). An additional 20 healthy individuals were selected as control (control group). The expression levels of IL-17, IL-22 and other cytokines in each group were detected by ELISA. The engraftment of cord blood stem cells was detected by using short tandem repeat-polymerase chain reaction (STR-PCR).**

Results: **1. IL-17, IL-22 and other cytokines expressions in the newly diagnosed group were significantly higher than in the control group. 2. After six months, the levels in the effective group were significantly lower than pre-therapy levels ($P < 0.05$). The levels in the invalid group did not differ to those observed prior treatment. 3. After one and three months of treatment, a small amount of engraftment was found in the effective group. However, after six months, transplant rejection was observed in all patients. No effective engraftment was observed in the invalid group.**

Conclusion: **1) Th17 and Th22 producing cells in SAA patients significantly increased indicating a positive correlation between these biomarkers and the progression of SAA. 2) During the IS + CBI treatment the maintenance of a normal hematopoietic function depended on immunesup-pressants. Early umbilical cord blood chimerism engraftment may promote hematopoietic recovery.** ***(Asian Pac J Allergy Immunol 2015;33:245-52)***

Keywords: *aplastic anemia, cord blood, cytokine, interleukin-17, interleukin-22*

Introduction

Aplastic anemia (AA) is a kind of acquired bone marrow failure syndrome characterized by anemia, infection and bleeding, which is caused by pancytopenia.[1] Especially in case of severe aplastic anemia (SAA) with rapid symptoms progression, the mortality rate is high.[2] So far, the main pathogenesis of AA is considered to be related to the 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 $CD34^+$ towards 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 with predominance of IL-22.[6] It is known that Th22 cells are highly expressed in chronic skin inflammation, asthma and other chronic respiratory inflammations, which 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, due to the implementation of the "one-child" policy in

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China there are decreasig chances to find identical siblings and consequently transplantation success is usually limited. 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. As a consequence, 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]

Papadopoulos et al.[13] proved that cord blood contained abundant 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 cells in bone marrow. Taking advantage of this particularity of cord blood, our treatment center have used immunosuppressants combined with cord blood infusion (IS + CBI) for the treatment of SAA for 10 years now. 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 successful hematopoietic reconstitution.

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

Methods

Patients

We recruited 43 SAA affected patients who were admitted to the Jinan Military General Hospital between Jan., 2003 and Jan., 2011. SAA diagnostics were consistent with the Guidelines for the Diagnosis of Aplastic Anaemia published by the British Committee in 2010.[14] All the patients, including 22 males and 21 of female, aged 2 to 28 years (median 12 years), were exposed to immunosuppressants combined with cord blood infusion (IS + CBI) treatment. Among them, 33 patients were treated effectively (effective group), while 10 cases were treated invalidly (invalid group). One case was a sibling donor with a completely matchingHLA6/6. There were 42 unrelated donors cases, including 4 cases with HLA 6/6 match, 8 cases with 5/6 match, 23 cases 4/6 match, 7 cases with 3/6 match. There were 27 cases with ABO blood types match, 6 cases ABO-incompatible in primary side, 10 cases ABO-incompatible in secondary side. In the control group we used20 cases of blood samples collected from healthy individuals, 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 and with approval from the Ethics Committee of Jinan Military General Hospital. Written informed consents were obtained from all participants (Table 1).

Specimen extraction

Patients were asked to fast for 12 hours before collection of 3 ml of heparinized peripheral blood that was stored at -80°C for subsequent examination. Another 20 healthy individuals were selected as normal controls.

Treatment protocols

Cord blood: cord blood collected from the qualified patients 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 who 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) 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 the day of cord blood transfusion, these patients were intravenously administrated with CsA in a dose of 1.5 mg-3 mg/(kg/d), and received an additional oral dose of 6-8 mg/(kg/d) twice per day after the remission of gastrointestinal symptoms. The doses administered were adjusted according to the CsA blood concentration (to maintain it at 150-400 μg/ml), assessed for 12-18 months.

Therapeutic effects were evaluated according to established standards of Camitta in 1976. Effective patients were characterized by complete remission (all blood cells recovered normal levels) and partial remission (patients did not need blood transfusion, blood routine examination was improved, and both of blood and bone marrow smear did not accord with standard of severe aplastic anemia diagnosis). Invalid patients were those who presented symptoms

Table 1. Basic information of patients with severe aplastic anemia treated using IS+CBI.

No.	Donor	HLA	Blood type of receptor	Treatment	Transplantation	Efficiency
1	Unrelated	5	Complete match	CTX+ALG	3.1	CR
2	Unrelated	5	Incompatible in primary side	CTX+ALG	2.8	CR
3	Unrelated	3	Incompatible in secondary side	CTX+ALG	2.9	CR
4	Unrelated	5	Incompatible in secondary side	CTX+ALG	3.2	CR
5	Unrelated	5	Complete match	CTX+ALG	5.6	CR
6	Unrelated	5	Complete match	CTX+ATG	7.5	CR
7	Unrelated	3	Complete match	CTX+ATG	2.9	CR
8	Unrelated	6	Incompatible in secondary side	CTX+ALG	8.7	CR
9	Unrelated	5	Incompatible in primary side	CTX+ATG	5.4	CR
10	Unrelated	6	Incompatible in secondary side	CTX+ATG	0	NR died
11	Unrelated	3	Complete match	CTX+ATG	0	NR died
12	Unrelated	4	Complete match	CTX+ATG	4.1	CR
13	Unrelated	4	Complete match	CTX+ATG	3.6	CR
14	Unrelated	4	Complete match	CTX+ATG	3.3	CR
15	Sister	6	Complete match	CTX+ATG	0	NR
16	Unrelated	5	Complete match	CTX+ALG	3.9	CR
17	Unrelated	3	Incompatible in primary side	CTX+ATG	4.8	CR
18	Unrelated	4	Incompatible in secondary side	CTX+ATG	0	NR died
19	Unrelated	4	Incompatible in secondary side	CTX+ALG	0	NR died
20	Unrelated	4	Complete match	CTX+ATG	4.7	CR
21	Unrelated	4	Incompatible in secondary side	CTX+ATG	3.4	CR
22	Unrelated	4	Complete match	CTX+ATG	3.5	CR
23	Unrelated	5	Complete match	CTX+ATG	0	NR died
24	Unrelated	4	Incompatible in primary side	CTX+ATG	3.8	CR
25	Unrelated	3	Incompatible in secondary side	CTX+ATG	2.9	PR
26	Unrelated	4	Complete match	CTX+ATG	4.6	CR
27	Unrelated	4	Complete match	CTX+ATG	6.3	CR
28	Unrelated	4	Incompatible in secondary side	CTX+ATG	3.7	CR
29	Unrelated	6	Complete match	CTX+ATG	7.7	CR
30	Unrelated	4	Incompatible in secondary side	CTX+ATG	3.9	CR
31	Unrelated	4	Complete match	CTX+ATG	0	NR died
32	Unrelated	3	Complete match	CTX+ATG	0	NR died
33	Unrelated	4	Incompatible in primary side	CTX+ATG	3.6	CR
34	Unrelated	4	Complete match	CTX+ATG	3.4	CR
35	Unrelated	3	Complete match	CTX+ATG	3.6	CR
36	Unrelated	4	Complete match	CTX+ATG	0	NR died
37	Unrelated	6	Complete match	CTX+ATG	8.9	CR
38	Unrelated	4	Complete match	CTX+ATG	3.4	PR
39	Unrelated	4	Complete match	CTX+ATG	2.8	CR
40	Unrelated	6	Complete match	CTX+ATG	0	NR died
41	Unrelated	4	Complete match	CTX+ATG	4.8	CR
42	Unrelated	4	Complete match	CTX+ATG	4.5	CR
43	Unrelated	4	Incompatible in primary side	CTX+ATG	3.3	CR

and blood picture indicating no partial remission after adequate therapy.

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

Targeted biomarkers (IL-17, IL-22, IL-21, TNF-α and IFN-γ) were detected from blood samples by ELISA assay (according to kit instructions) prior treatment, 3 months after treatment, 6 months after treatment, and 1 year after treatment.

Implantation detection

To detect the implantation rate of donor stem cells we used DNA short tandem repeat-polymerase chain reaction (STR-PCR), 1 month, 3 months, 6 months, 1 year and 2 years after IS + CBI treatment.

Statistical analysis

Statistical analyses were performed using SPSS19.0. Data were presented as mean ± standard deviation. Differences between treatment groups were assessed using LSD-t test. Student's t-test and one-way ANOVA were used to compare before and after treatment effects within one group and differences among 3 groups respectively. 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

The 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 as compared with pretherapy levels, 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 compared to 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 no different from pretherapy levels (P >0.05, Table 2).

Efficiency

The total effective rate was 76.74%: CR (31 cases) + PR (2 case)/ 43cases, NR (10 cases), included 9 invalid patients who died. Time to death was 1.5-13 months. One invalid case currently relies on regular blood transfusions for life sustenance. While no difference of HLA matches in terms of consistency (P > 0.05) and therapeutic schedule (P > 0.05) were oberved, blood type for the recipients and curative effect (P < 0.05, Table 3) had a significant effect.

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 theinvalid group.

Table 2. 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, $\bar{x}$ ±s).

Groups	Cases	IL-17	IL-22	IL-21	TNF-α	IFN-γ
Newly diagnosed group	43	337.32±41.24*	113.35±9.78*	285.37±21.83*	394.75±32.31*	360.41±28.79*
Effective group	33					
Pretherapy		342.18±32.21*	124.87±8.68*	284.27±17.45*	402.83±29.59*	365.28±25.56*
Three months		313.62±21.19*	98.46±10.41*	269.53±14.66*	376.66±29.44*	336.42±29.13*
Six months		247.48±36.17▲	74.59±11.28▲	224.07±12.20▲	285.73±22.53▲	267.67±12.37▲
One year		239.34±38.13▲	70.65±13.08▲	212.73±12.87▲	278.49±27.97▲	238.92±19.14▲
Invalid group	10					
Pretherapy		351.83±34.16*	118.24±8.96*	288.06±12.83*	396.46±31.13*	372.09±28.99*
Three months		345.64±42.16*	97.42±11.84*	288.54±14.11*	373.24±28.43*	363.75±27.67*
Six months		333.15±21.42*	108.23±7.81*	280.17±14.31*	385.38±23.23*	360.01±25.59*
One year		348.23±48.21*	117.86±8.73*	297.43±24.77*	408.18±24.63*	364.36±21.95*
Control group	20	251.13±31.32	63.26±10.21	201.23±18.97	297.43±26.46	255.38±17.68

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

Table 3. Single factor analysis of therapeutic effect using IS+CBI.

Index	Effective group	Invalid group	*P* value (χ^2 or *t* value)
Therapeutic schedule (such as ATG+CTX:ALG+CTX)	26:7	9:1	0.897 (0.017)
HLA match (such as 0:1:2:5)	3:7:18:5	2:1:5:2	0.695 (1.444)
Blood type consistency (such as, incompatible in primary or secondary side)	27:3:3	4:3:3	0.036 (6.671)

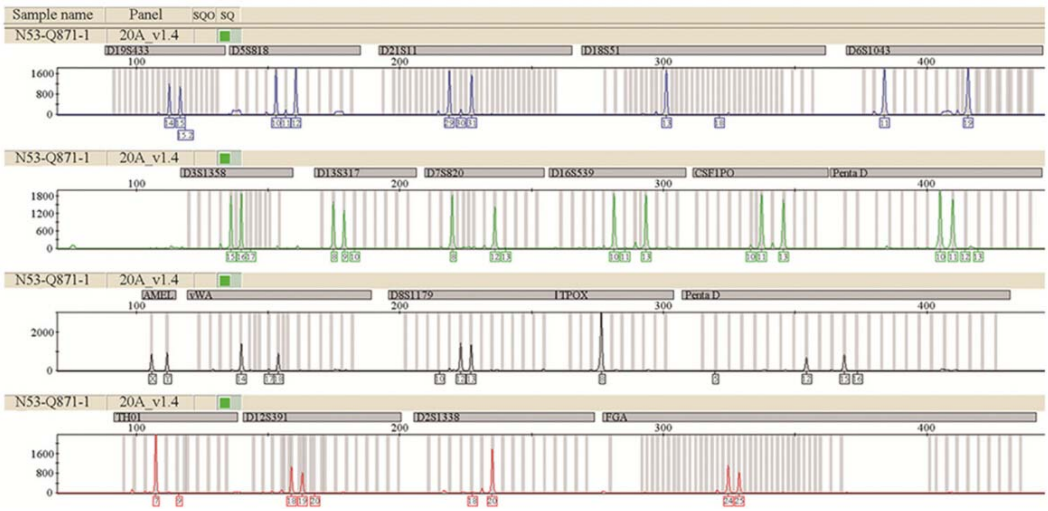
Discussion

In recent years, the description of the new Th cell subsets Th17 and Th22 cells, stimulated novel approaches and better understanding of aplastic anemia.[9,10] Harrington in a study of *Borrelia burgdorferi* in 2005[15] began to realize that Th cells which produced IL-17 were a CD_4 T cell subset that differed from Th1, Th2 and 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.[16] The hemopoietic negative regulatory factors in plasma were found to induce macrophages secretion of high levels of IL-17, IL-6, IL-8 and TNF-α which inhibited the hematopoiesis of bone marrow through direct and indirect effects. Th22 cells were another. Similarly, related researches reported that Th22 cells, Th cells independent from Th1, Th2 and Th17, could express CCR6, CCR4 and CCR10, as well as secrete IL-22, IL-10, TNF-α and other cytokines. Additionaly, Th22 also influenced autoimmune diseases, infectious diseases and tumors.[17] In a recent study, the number of Th22 cells was shown to be elevated in patients with aplastic anemia[10] which corroborate our findings were 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. Together these observations suggested that the SAA patients have disorders. The elevated level of Th17 and Th22 subsets resulted in immunologic injury of bone marrow hematopoietic stem cells and hematopoietic microenvironment which may have been one of the reasons for the onset of SAA.

At St. Louis Hospital in Paris, Gluckman et al.[18] 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 represents the third source of hematopoietic stem cells after the bone marrow and peripheral blood have become the research focus in the field of hematopoietic stem cell transplantation for nearly three decades. However, the slow hematopoietic reconstitution and bad implantation seriously affects the long-term survival after surgery, representing major obstacles in the wide application of adult unrelated umbilical cord blood transplantation.[19]

Using the characteristics of umbilical cord blood, we adapted a IS + CBI protocol 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 exposed, while 76.74% of the patients could achieve a complete remission and long-term survival by mean of successful hematopoietic reconstitution. The present study followed two angles of implementation, immunity and implantation and we observed that 1) IL-17, IL-22, IL-21, TNF-α, and IFN-γ levels decreased in effective group after 3 months of IS + CBI treatment, but that there was no significant difference compared with the newly diagnosed group. IL-17, IL-22, IL-21, TNF-α, and IFN-γ levels were 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 Th17 and Th22-mediated immune disorders of hematopoietic stem cell injury. 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 did not differ with those of the newly diagnosed group suggesting that the main causes of remission absence in invalid patients may be the abnormality of hematopoietic stem cells or hematopoietic microenvironment. Consequently, without effective hematopoietic stem cell engraftment, immunoregulation only could not correct the abnormal hematopoiesis. 3) 2.8%-8.9% of trace mixed chimerism was detected by STR-PCR detection in 24 patients with effective treatment after 1 and 3 months of treatment. No

A



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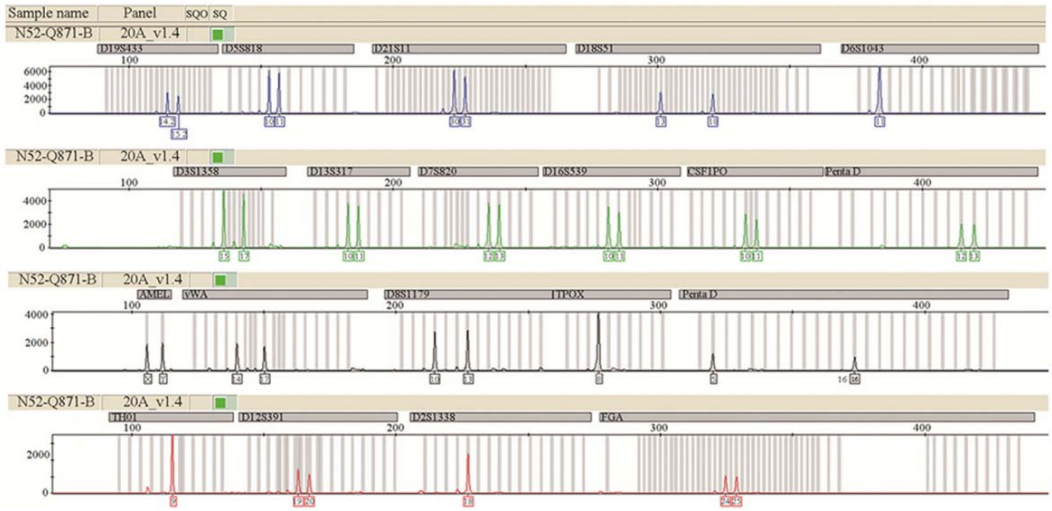

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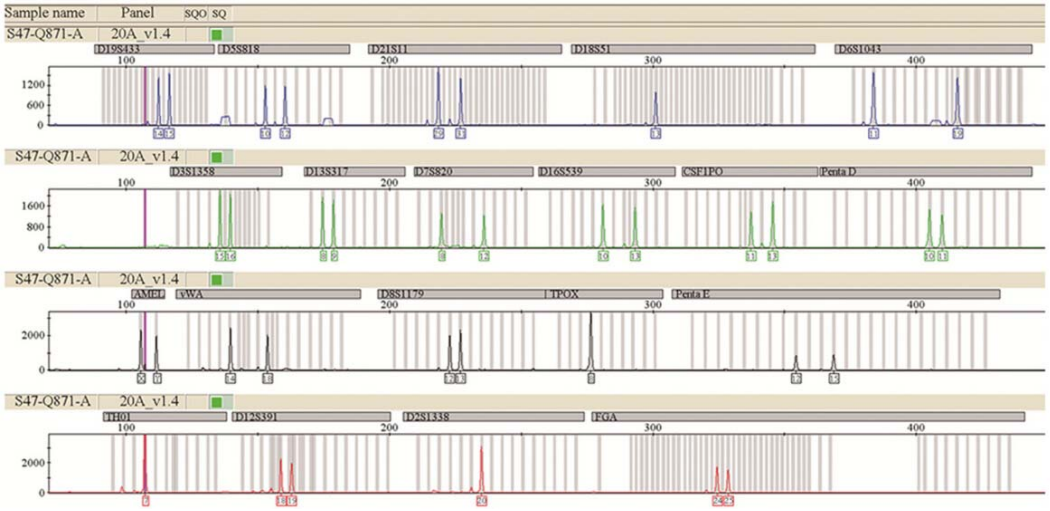

Figure 1. A 5 years old male child who was diagnosed as SAA, treated with immunosuppressants combined with cord blood infusion and used unrelated umbilical cord blood with HLA 4/6 matched.

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%.

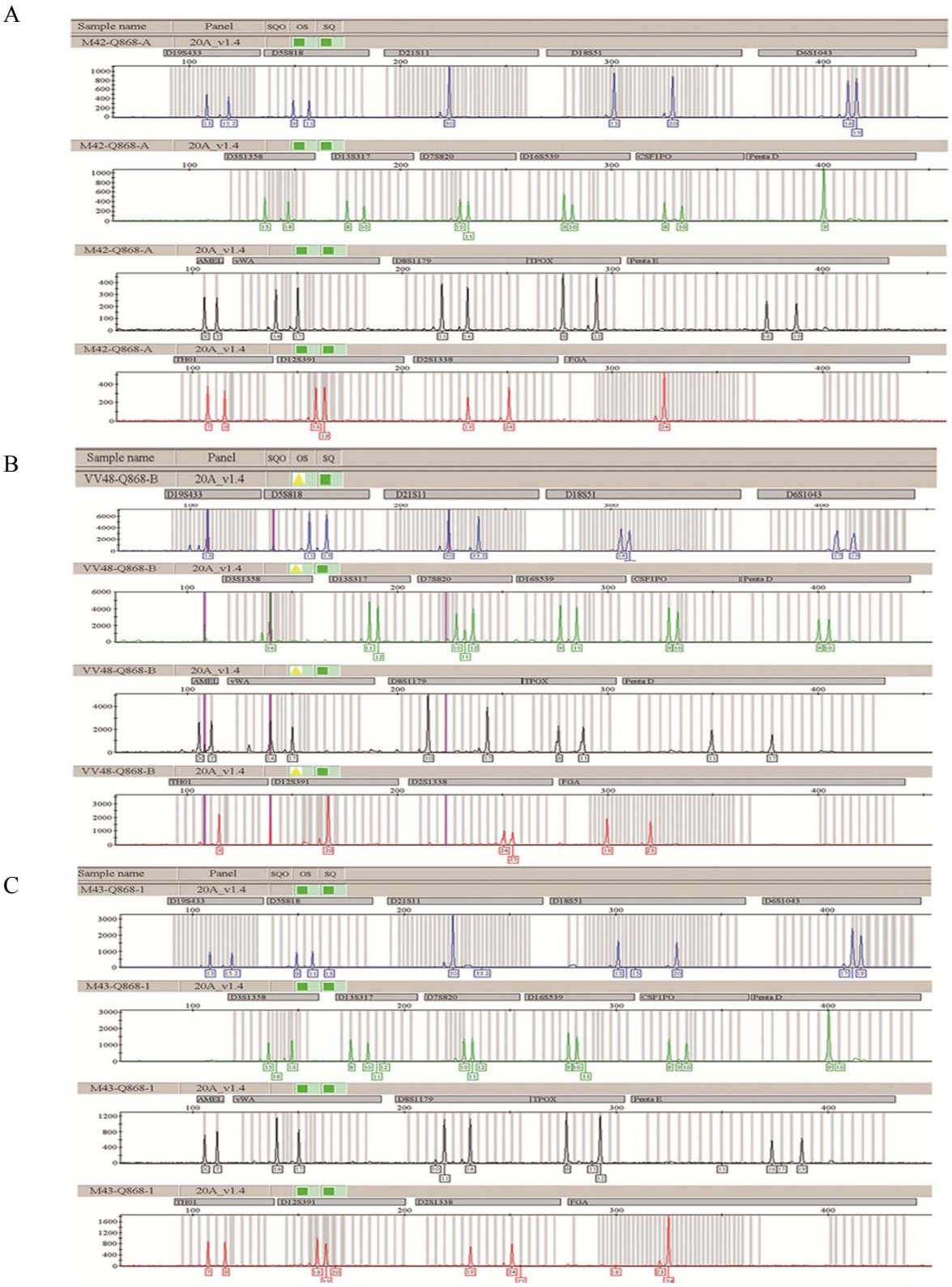

Figure 2. A 21 years old male who was diagnosed as SAA, treated with immunosuppressants combined with cord blood infusion and used unrelated umbilical cord blood with HLA 3/6 matched.

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%.

donor cells were detected after 6 months, 1 and 2 years and no implantation was detected in invalid patients. A strong dose of immunosuppressant plays a major role in treatment, and umbilical cord blood has a small amount of chimerism engraftment in early stage.

From clinical and laboratory research, we draw the following conclusions: 1) The new Th subsets Th17, Th22 play a significant role in immune disorders associated with the pathogenesis of SAA. These disorders can be corrected by IS + CBI treatment. 2) The maintenance of a normal hematopoietic function depends on immune-suppressants during IS + CBI treatment. Early chimerism engraftment in umbilical cord blood may promote hematopoietic recovery. However, because of the small sample size, more clinical samples and large-scale clinical trials are needed to confirm our results and further support our conclusions.

Conflict of interest

All authors have no conflict of interest regarding this paper.

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