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Original Article

Outcomes of Anti-CD19 CAR-T Treatment of Pediatric B-ALL with Bone Marrow and Extramedullary Relapse

Xinyu Wan[1], **Xiaomin Yang**[1], **Fan Yang**[2], **Tianyi Wang**[1], **Lixia Ding**[1], **Lili Song**[1], **Yan Miao**[1], **Xiang Wang**[1], **Yani Ma**[1], **Chengjuan Luo**[1], **Jingyan Tang**[1], **Longjun Gu**[1], **Jing Chen**[1], **Yanjing Tang**[1], **Jun Lu**[2], **Benshang Li**[1]

[1]*Department of Hematology and Oncology, Key Laboratory of Pediatric Hematology and Oncology Ministry of Health, Shanghai Children's Medical Center, Shanghai Jiao Tong University School of Medicine, Shanghai,* [2]*Department of Hematology/Oncology, Children's Hospital of Soochow University, Jiangsu, China*

Purpose Anti-CD19 chimeric antigen receptor T-cell (19CAR-T) immunotherapy has achieved impressive clinical results in adult and pediatric relapsed/refractory (r/r) B-lineage acute lymphoblastic leukemia (B-ALL). However, the application and effect of CAR-T therapy in B-ALL patients with extramedullary relapse are rarely issued even disqualified in some clinical trials. Here, we examined the efficacy of 19CAR-T in patients with both bone marrow and extramedullary involvement.

Materials and Methods CAR-T cells were generated by transfection of primary human T lymphocytes with a lentiviral vector expressing anti-CD19 single-chain antibody fragments with the cytoplasmic domains of 4-1BB and CD3ζ, and used to infuse patients diagnosed as having r/r B-ALL with extramedullary origination. Clinical responses were evaluated by the use of bone marrow aspiration, imaging, and flow cytometry.

Results Eight patients received 19CAR-T infusion and all attained complete remission (CR). Only one patient was bridged to hematopoietic stem cell transplantation (HSCT). Although three patients relapsed after infusion, they received 19/22CAR-T infusion sequentially and attained a second remission. To date, five patients are in continuous CR and all eight patients are still alive. The mean follow-up time was 21.9 months, while the 24-month estimated event-free survival is 51.4%.

Conclusion 19CAR-T therapy can lead to clinical remission for extramedullary relapsed pediatric B-ALL patients. However, the problem of $CD19^+$ relapses after CAR-T remained to be solved. For patients relapsing after CAR-T, a second CAR-T therapy creates another opportunity for remission for subsequent HSCT.

Key words B-ALL, Extramedullary relapse, CD19 CAR-T, Second CAR-T

Introduction

Acute lymphoblastic leukemia (ALL) is a leading hematological malignancy of children worldwide. Although the long-term survival rate for pediatric ALL approaches and even exceeds 90%, the 10%-15% of cases of ALL that relapse are considered one of the leading causes of cancer mortality in children [1,2]. Although relapse is mainly detected in bone marrow, occasionally extramedullary tissues are involved and these account for 15%-20% of all relapses [3]. Extramedullary relapse is most commonly identified as central nervous system (CNS) leukemia or testicular leukemia [4,5]. The natural history of extramedullary relapse generally correlates with poor prognosis and limited overall survival (OS) [6]. Conventional chemotherapy, radiation, or even allogeneic hematopoietic stem cell transplantation (allo-HSCT) tend to be unsuccessful in these patients, especially those with multiple relapses [7,8]. Therefore, the treatment of ALL patients with extramedullary disease remains a great challenge and new therapeutic strategies are urgently required.

Recently, chimeric antigen receptor T-cell (CAR-T) immunotherapy has been receiving wide attention and becoming an increasingly hot topic in the treatment of relapsed/refractory (r/r) B-lineage acute lymphoblastic leukemia (B-ALL). CAR-T cells were originally developed by Eshhar and colleagues in the late 1980s and early 1990s [9]. The approach harnesses the cytotoxicity of genetically modified T cells

Correspondence: Benshang Li
Department of Hematology and Oncology, Key Laboratory of Pediatric Hematology and Oncology Ministry of Health, Shanghai Children's Medical Center, Shanghai Jiao Tong University School of Medicine, Shanghai 200127, China
Tel: 86-18101893712 E-mail: libenshang@scmc.com.cn

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*Xinyu Wan, Xiaomin Yang, and Fan Yang contributed equally to this work.

Co-correspondence: Yanjing Tang
Department of Hematology and Oncology, Key Laboratory of Pediatric Hematology and Oncology Ministry of Health, Shanghai Children's Medical Center, Shanghai Jiao Tong University School of Medicine, Shanghai 200127, China
Tel: 86-18930872452 E-mail: tangyanjing@scmc.com.cn

Co-correspondence: Jun Lu
Department of Hematology/Oncology, Children's Hospital of Soochow University, Jiangsu 215025, China
Tel: 86-13962516534 E-mail: drlujun_sz@163.com

with high specificity for tumor cells. CAR-T cells contain an antigen-specific single-chain antibody fragment (scFv) linked to intracellular signaling domains independent of MHC restriction and antigen processing [10]. There is no doubt that CD19-targeted CAR-T (19CAR-T) therapy has achieved impressive clinical results in both adult and pediatric r/r B-ALL, with an amazing disease remission rate, and is emerging as a standard strategy for r/r B-ALL patients [11-14]. Nevertheless, the application and effect of CAR-T therapy in B-ALL patients with extramedullary relapses has rarely been described and such patients are sometimes excluded from some clinical trials [11,13].

Here we report our experiences using 19CAR-T cells to treat eight pediatric B-ALL patients with simultaneous extramedullary and bone marrow (BM) relapse.

Materials and Methods

1. Study design

Between September 2017 and July 2020, eight pediatric patients with $CD19^+$ B-ALL relapsed with both BM and extra-medullary sites and were enrolled in this study. The study was conducted in Shanghai Children's Medical Center, which is affiliated to Shanghai Jiao Tong University School of Medicine and Children's Hospital of Soochow University, and was registered at chictr.org.cn (ChiCTR2100041852) from November 2016. Informed consent forms were signed by the patients' guardians and were approved by the ethics committee of Shanghai Children's Medical Center and Children's Hospital of Soochow University.

2. Cell production

Peripheral blood (PB) was drawn directly from patients for T-cell harvesting using anti-CD3-coated beads (Invitrogen, Carlsbad, CA). For patients who relapsed after HSCT, PB from a donor was used. Anti-CD19 scFvs derived from FMC63 were ligated into a lentiviral vector, downstream of an EF1α promoter and in a backbone with the CD8A signal peptide, hinge, CD8 transmembrane domains, and the cytoplasmic domains of 4-1BB and CD3ζ to create a second-generation CAR construct. The manufacture of CAR-T cells usually took 7-10 days. Prior to CAR-T injection, all patients were given lymphodepleting chemotherapy: 500 mg/m^2 cyclophosphamide for 2 days (days –4 and –3) and 40 mg/m^2 fludarabine for 3 days (days –4, –3, and –2). On day 0, patients were infused with fresh CAR-T cells at a dosage of 1-15×10^6/kg. Patients who relapsed after the first exposure to CAR-T cells were allowed to receive a second CAR-T, and given 500 mg/m^2 cyclophosphamide and 50 mg/m^2 fludarabine per day for 3 days (days –4, –3, and –2) for lymphodepletion. For a combined CAR-T therapy, we combined 19CAR-T cells with anti-CD22 CAR-T cells whose scFv was derived from the 5/44 clone.

3. Patient evaluation

Before CAR-T administration, tumor burden was examined by flow cytometry of bone marrow, cerebrospinal fluid (CSF), or biopsy samples. Neurologic adverse events were graded according to the Common Terminology Criteria for Adverse Events (CTCAE) 4.03, and cytokine release syndrome (CRS) was graded as per the American Society for Transplant and Cellular Therapy grading criteria (ASTCT) [15]. A BM aspiration was done 7-14 days after CAR-T infusion, and disease burden assessment was performed on marrow samples. Disease-free marrow was defined as having less than 0.01% abnormal cells identified by multiparameter flow cytometry performed at the Key Laboratory of Hematology and Oncology Ministry of Health. Additionally, complete remission (CR) status was defined as no leukemic blasts detected in both BM and extramedullary sites. For CNS-involved patients, CSF was collected for analysis. For patients with subcutaneous and/or testicular tumors, changes in tumor size were observed and compared, and biopsies were performed for flow cytometry. Examinations of the relevant extramedullary sites were performed on days 7 and 14, and at 1 month, 2 months, 3 months, 6 months, 1 year, and 2 years following CAR-T cell infusion. CAR-T cell persistence in the PB or CSF of patients was measured using real-time quantitative polymerase chain reaction (qPCR) and copies per ug DNA were normalized to the single-copy gene CDKN1a.

4. Cytokine analysis

Quantification of PB and CSF soluble cytokines including interleukin (IL)-2, IL-4, IL-6, IL-10, tumor necrosis factor α, interferon γ (IFN-γ), and IL-17 was performed using a BD Cytometric Bead Array Human Th1/Th2/Th17 Cytokine Kit (Becton, Dickinson and Company, Franklin Lakes, NJ), according to the manufacturer's instructions.

5. Statistical analysis

For event-free survival (EFS), an event was defined as bridging to HSCT, or relapse, or death. Death was the event for analysis of OS. Kaplan-Meier curves were created for EFS and OS analysis and compared using the log-rank test. All statistics were performed as indicated, using Graph-Pad Prism 8 software for Windows, ver. 8.0.1 (GraphPad Software Inc., San Diego, CA). Statistically significant differences were defined at a $p < 0.05$.

Table 1. Patient characteristics and clinical responses

Patient ID	Sex	Age (yr)	Tissue involvement	Enrollment criteria	Time from initial diagnosis to CAR-T infusion (mo)	Genetic variation	Minimal residual disease before CAR-T (%)	Infusion dose of 19CAR-T ($\times 10^6$/kg)	CRS grade	CRES grade	IL-6 max (pg/mL)	CNS status at the time of CAR-T cell infusion	Disease status on day 28[a]
S001	M	4.3	BM, subcutaneous tumor	Relapse	7.50	TCF3-PBX1	95.83	6.9	1[b]	0	390.25	ND	MRD Neg, 30×21 mm[c]
S002	F	7.2	BM and CNS	2nd Relapse	44.07	-	0.27	10	1	1	100.39	ND	MRD Neg
S003	F	6.1	BM and CNS after HSCT	2nd Relapse	50.10	ETV6-RUNX1	31.35	12	3[b],[d]	0	60,618.45	ND	MRD Neg
S004	F	8.7	BM and CNS	2nd Relapse	72.87	ZCCHC7-CSF2RA	2.41	15	1[d]	1	1,272.27[e]	Mild Headache	MRD Neg
S005	M	9.3	BM and testis	Relapse	40.10	ETV6-RUNX1	1.00	15	3[b],[d]	2	1,445.5	Drowsiness and slow response to stimuli	-
S006	F	7.4	BM, subcutaneous tumor	Relapse	44.17	ND	0.46	10	0	0	96.4	ND	-
S007	M	7.1	BM, CNS, and testis	2nd Relapse	48.27	-	3.00	8.8	2[b]	2	13,116.22	Limb trembling and delirium	MRD Neg
S008	F	13	BM and CNS	2nd Relapse	116.83	ETV6: exon 2 del	32.40	1.8	2[b]	2	574.7[e]	Unconsciousness	MRD Neg

S004 received glucocorticoids by intrathecal injection. -, no follow-up; 19CAR-T, CD19 chimeric antigen receptor T-cell; BM, bone marrow; CAR-T, chimeric antigen receptor T-cell; CNS, central nervous system; CRES, CAR-T related encephalopathy syndrome; CRS, cytokine release syndrome; F, female; HSCT, hematopoietic stem cell transplantation; IL-6, interleukin 6; M, male; MRD, minimal residual disease; ND, not detected; Neg, negative. [a]MRD neg, undetectable BM and CNS MRD as measured by flow cytometry, [b]Tocilizumab, [c]According to the results of ultrasound, patient S001 was diagnosed with a 45×25 mm enlarged lymph node near an iliac vessel in the pelvis before CAR-T infusion; the dimensions decreased to 30×21 mm after 28 days, and after 3 months, only one 18×10 mm lymph node was found in the right inguinal region, [d]Corticosteroid, [e]Cerebrospinal fluid level.

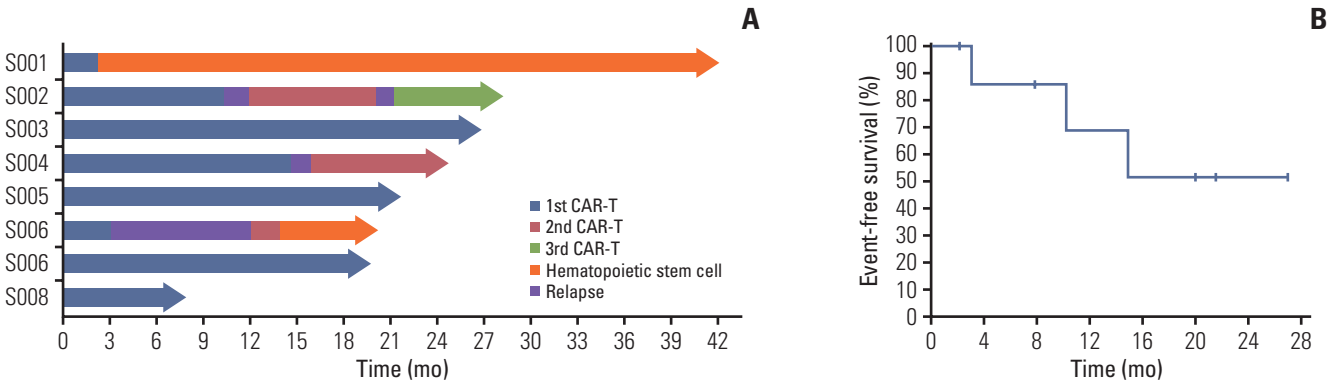

Fig. 1. Clinical outcomes of 19CAR-T cells. (A) Swimmer plot (n=8), in which each bar represents an individual patient as designated. (B) Kaplan-Meier graph of event-free survival in eight patients infused with 19CAR-T cells, demonstrating 68.57% event-free survival at 12 months and 51.43% at 24 months. 19CAR-T, anti-CD19 chimeric antigen receptor T-cell; CAR-T, chimeric antigen receptor T-cell.

Results

Patient characteristics are shown in Table 1. Briefly, a total of eight extramedullary relapsed childhood B-ALL patients with an average age of 7.9 years (range, 4.3 to 13.0 years) were enrolled in this study and treated at Shanghai Children's Medical Center (six patients) and Children's Hospital of Soochow University (two patients). There were four patients whose cancer infiltrated the CNS, one with a testis tumor, two with a subcutaneous tumor, and one with both CNS and testis involvement. All patients' disease had relapsed. The mean follow-up time was 21.9 months (range, 5.93 to 39.97 months). In addition, five of eight patients (39.4%) relapsed more than once, of whom only patient S003 relapsed after HSCT. Therefore, S003 was given donor-derived CAR-T cells from her 7/10 HLA-matched father, without developing Graft-versus-hostdisease. All patients received fresh CAR-T product and the average infusion dose was 9.9×10^6 per kilogram of body weight (range, 1.8×10^6 to 15.0×10^6).

Patient-level clinical response outcomes are exhibited in Fig. 1A. All eight patients achieved CR: 5 patients are now in continuous CR, and no CAR-T–related death occurred. Patient time to CR was usually 2-6 weeks. To date, only one patient (S006) chose bridging to HSCT after the second CAR-T. The estimated EFS of patients with 19CAR-T cells was approximately 68.57% at 12 months and decreased to 51.43% at 24 months (Fig. 1B).

Except patient S006, CRS was observed in all patients, and it was above grade 2 in three of eight patients. CAR-T related encephalopathy syndrome (CRES) was observed in five patients (62.5%), with no CRES above grade 2 identified. Four patients received tocilizumab, and two of these patients simultaneously received glucocorticoids. Only patient S004 received glucocorticoids alone, by intrathecal injection. No patient died of severe CRS or CRES (Table 1). IL-6 and IFN-γ concentrations are the main biomarkers of CRS severity. Similar patterns were observed in both IL-6 and IFN-γ (Fig. 2A); the highest level of IL-6 was 60618.45 pg/mL, detected in patient S003 (Table 1). On day 7, transient increases in levels of serum IL-6 and IFN-γ occurred in most patients during CRS after CAR-T administration. We also observed that a drop of IL-6 was always followed by an increase in lymphocyte numbers in the PB (Fig. 2B) that was temporally coincident with the resolution of CRS.

In four patients with subcutaneous or testicular infiltration, CRS initially presented as a painless soft swelling of the affected sites along with redness and fever, which usually would last for about 3 days after CAR-T infusion. After a peak in swelling at approximately day 7, the tissue would return to normal size. Finally, the clearance of leukemic cells in the site was confirmed in all by flow cytometry of cells from biopsies and BM.

Patients with CNS involvement had more severe symptoms of encephalopathy, manifested as high fever, headache, frequent vomiting, drowsiness, unconsciousness, hypotension or hypertension, limb trembling, and convulsions (Table 1). For the patient with convulsions, lumber puncture was required, and the CSF protein increased significantly; meanwhile, the CSF IL-6 concentration was much higher than that in PB. For example, on day 6, patient S004 suffered 1,272.27 pg/mL of IL-6 in the CSF but 26.62 pg/mL in the PB. We also detected a higher level of the CAR gene in CSF compared with PB. In patient S005, 465.026×10^3 copies of CAR per ug DNA were detected, over 10-fold the number detected in PB on day 3 (Fig. 2C). Additionally, CAR-T cell expansion determined using real-time qPCR revealed that the peak CAR-T expansion was variable in different extramedullary-relapsed

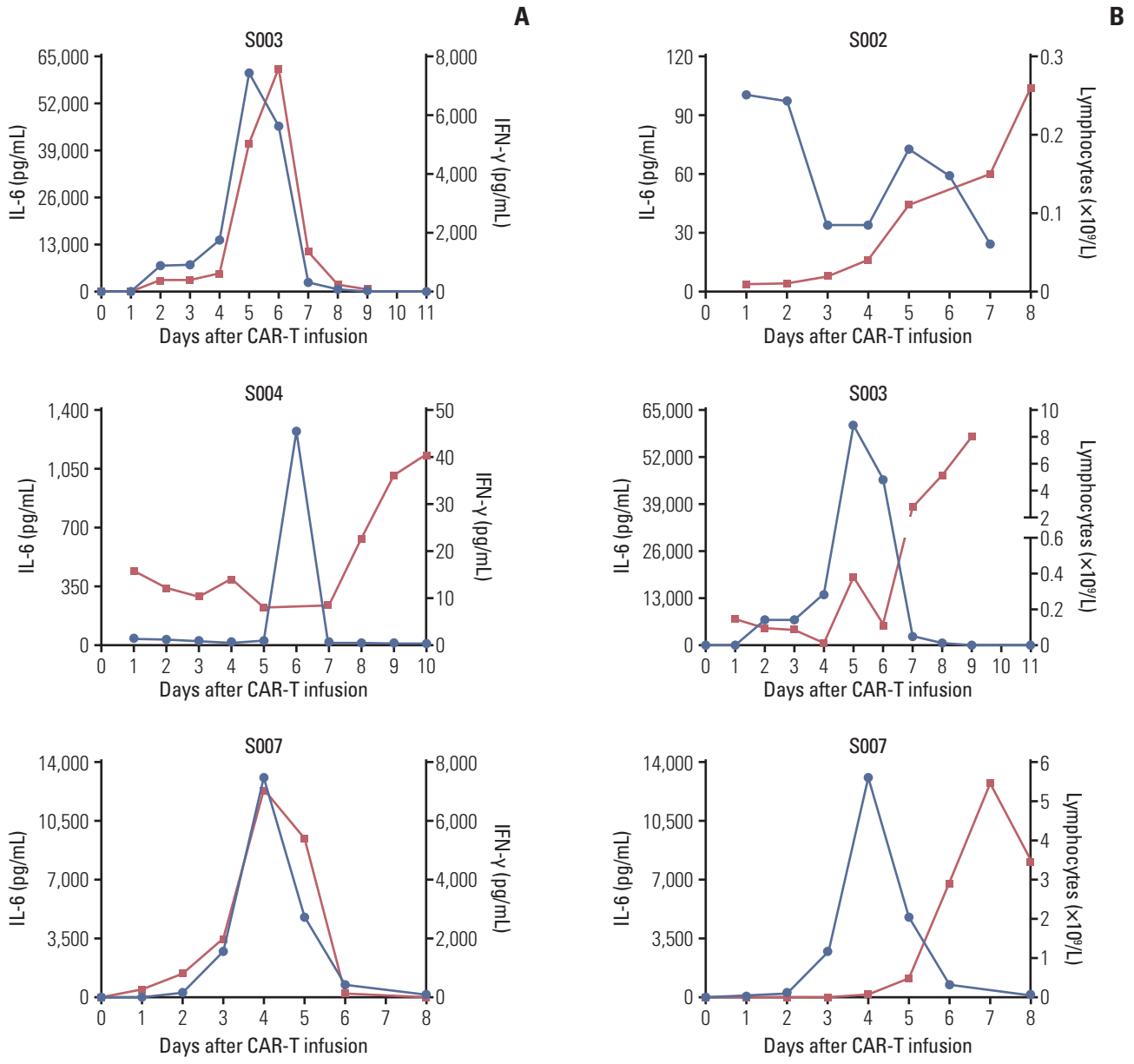

Fig. 2. Changes in patients' serum biomarkers in cytokine release syndrome. (A) The temporal relationship of interleukin 6 (IL-6) (blue line) and interferon γ (IFN-γ) (red line) from day 0 to day 11. (B) The temporal relationship of IL-6 (blue line) and lymphocyte numbers (red line) from day 0 to day 8. *(Continued to the next page)*

patients but mostly not observed after day 7 (Fig. 2D). About 2-3 weeks later, the absence of remaining leukemic cells was verified by flow cytometry of both bone marrow and CSF samples (Fig. 2F). During follow-up, the longest period of B-cell aplasia was found in patient S003, extending more than 200 days after CAR-T cell infusion; this was also indirectly confirmed using qPCR and Sanger sequencing for the existence of CAR-T cells (Fig. 2E).

Patients S002, S004, and S006 had relapsed after prior exposure to CAR-T cells. Their characteristics and responses are presented in Table 2. Patient S006 relapsed with an isolated subcutaneous tumor, while the other two patients had isolated BM relapses. Due to the high expression of CD19 and CD22 on the relapsed leukemic blasts, these patients accepted a combined CD19- and CD22-targeting second patient-derived CAR-T cell therapy. All of them then achieved CR again. All three patients experienced only grade 1 CRS along with fever. In patient S006, 23.45×10^3 copies of

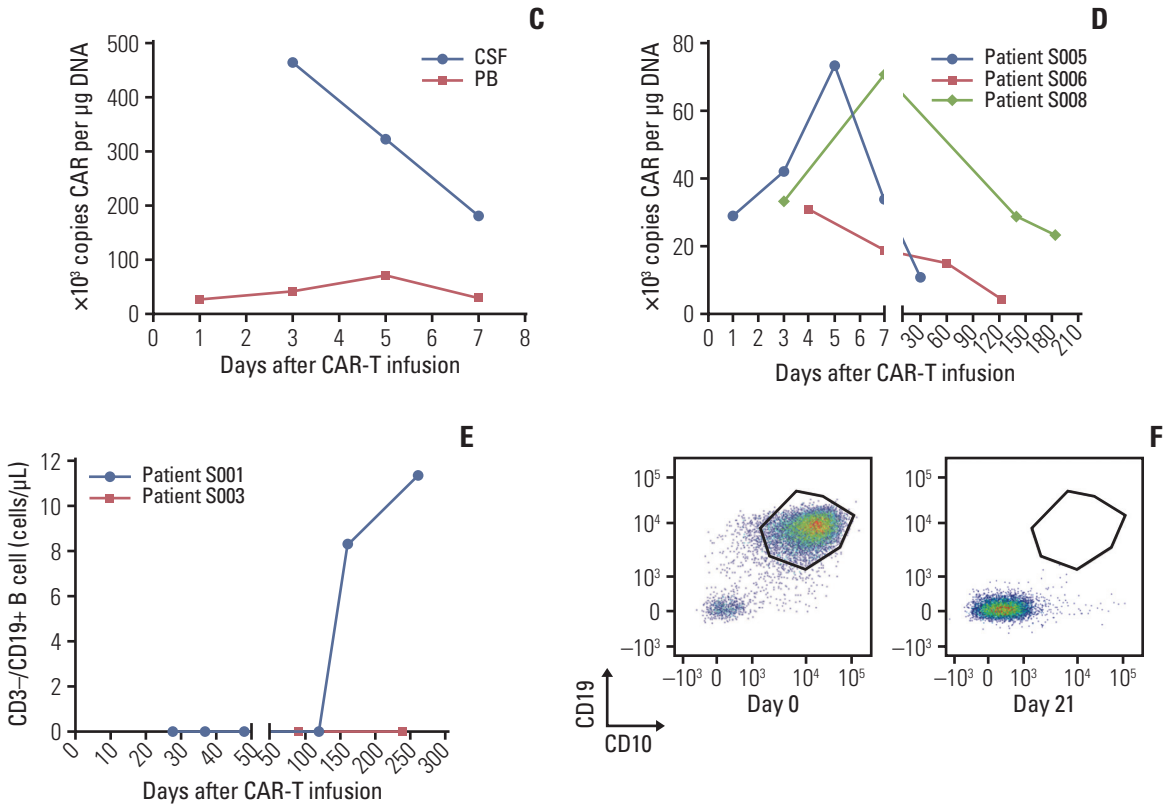

Fig. 2. *(Continued from the previous page)* (C) The number of copies of the chimeric antigen receptor (CAR) gene detected in Patient S005's peripheral blood (PB) and cerebrospinal fluid (CSF). (D) The persistence of circulating chimeric antigen receptor T-cell (CAR-T) cells identified by quantitative polymerase chain reaction (qPCR). (E) B-cell aplasia remained in patient S003 but not in S001, who had been bridged to hematopoietic stem cell transplantation. (F) There were 90.7% leukemic cells in bone marrow from patient S003 detected on day 0 (left) but 0.0% on day 21 (right).

CAR per ug DNA were detected on day 89 after the second CAR-T infusion, which was higher than the 14.93 copies of CAR per ug DNA on day 60 after her first 19CAR-T therapy. B-cell asplasia was observed in patient S002 on day 7 after CAR-T infusion. However, she rejected bridging to HSCT and then relapsed again after 9.3 months. At her parents' strong insistence, a third combined CAR-T therapy was conducted because of the remaining CD19- and CD22-expressing leukemic cells.

Discussion

There is no optimal defined protocol for treating extramedullary leukemia. Some patients experience multiple relapses even after receiving chemotherapy, allo-HSCT, and/or radiation [16,17]. Before off-the-shelf CAR-T therapy, blinatumomab had received regulatory approval and was widely available to treat relapsed or refractory B-cell precursor ALL patients [18]. However, extramedullary relapse is associated with a lower CR rate in patients treated with blinatumomab, which is a CD19/CD3 bispecific T-cell engager antibody that induces the death of $CD19^+$ leukemia cells by redirecting $CD3^+$ T cells [19]. Furthermore, many patients in whom blinatumomab therapy has failed still have $CD19^+$ leukemic cells [20]. Two studies have reported that CD19 CAR-T therapy can eradicate leukemic cells in B-ALL patients with extramedullary involvement. In a study of seven boys with testicular relapse, six of them remained in CR for a median of 14 months (5-23 months) [21]. The group from the Children's Hospital of Philadelphia demonstrated that 6 in 10 patients with various extramedullary sites of relapse were without recurrence of disease for 6-13 months after CAR-T infusion [22]. A clinical remission in extramedullary B-ALL patients with anti-CD19 CAR-T therapy was also noted in that study.

Extramedullary ALL presents a challenge similar to that of solid malignancies: the trafficking of targeted cells to the tumor sites. Some studies have found that CAR-T cells may migrate into and lead to partial tumor regression in extramedullary sites such as liver, lung, etc. [16,23,24]. Ho

Table 2. Patients with a second relapse after 19CAR-T

Patient ID	2nd relapse sites	CD19/CD22 expression after 2nd relapse (%)	Infusion dose of 19/22CAR-T ($\times 10^6$/kg)	19CAR-T in all (%)	CRS grade	CRES grade	IL-6 max (pg/mL)	Responses	B cell on day 7 after CAR-T infusion (cells/μL)	Bridge to HSCT
S002	BM	-	15	60.0	1	0	24.17	CR[a)]	0.0	N
S004	BM	98.3/99.7	4.26	51.3	1	0	-	CR	-	N
S006	Subcutaneous tumor	100/100	12	20.6	1	0	-	CR	-	Y

-, no follow-up; 19CAR-T, anti-CD19 chimeric antigen receptor T-cell; BM, bone marrow; CAR-T, chimeric antigen receptor T-cell; CR, complete remission; CRES, CAR-T related encephalopathy syndrome; CRS, cytokine release syndrome; HSCT, hematopoietic stem cell transplantation; IL-6, interleukin 6; N, no; Y, yes. [a)]Third relapsed after 2nd CAR-T infusion.

wever, the effect on testicular or CNS ALL remains unclear. For example, the absence of detectable donor cells in extramedullary lesions has been reported [25]. This suggests that intravenously injected CAR-T cells may not reach isolated extramedullary sites, and thus may have no effect. Intriguingly, the high rates of CR achieved with intravenous administration in this cohort are evidence that CAR-T cells can traffic to extramedullary sites and then eliminate tumor cells efficiently. However, there was a high incidence of immune effector cell–associated neurotoxicity syndrome, especially in B-ALL relapsed patients with CNS involvement. This may be associated with increases in CSF IL-6 levels (Table 1), which are thought to not be prevented by the blood-brain barrier [26]. The expression of IL-6 has been reported to be related to endothelial activation and blood-brain barrier dysfunction after CD19 CAR-T infusion [27].

The value of consolidative bone marrow transplantation (BMT) after CAR-T therapy remains controversial. On the one hand, Lee et al. [28] have suggested that BMT following the infusion of CAR-T cells enhances the survival of children and young adult patients. On the other hand, Park et al. [29] found that the survival improvement was not significant in adult patients. The costimulatory domain in the above two studies was CD28. Interestingly, a recent study showed that allo-HSCT achieved better EFS in patients aged over 18 years after treatment with CAR-T cells incorporating a 4-1BB costimulatory domain [30]. In this study, only patient S001, who was one of five non-relapsed patients, was followed with HSCT, but he gained the longest CR.

We note that 100% CR was achieved in the three relapsed patients in our study. This indicates that retreatment with CAR-T cell infusion may offer an opportunity for B-ALL pediatric patients with extramedullary relapse to wait and look for a bridge to effective HSCT. This result also suggests that combining CAR-T cells that target different antigens can be an effective solution to this kind of immune escape problem, which is associated with tumor-cell antigen switching.

This study reveals that 19CAR-T therapy can partially induce clinical remission in patients with both bone marrow and extramedullary relapses, while $CD19^+$ relapse remains the main problem. We suggest a second CAR-T cell treatment as a means to create another chance for survival in these patients.

Ethical Statement

The study was approved by the IRB of Shanghai Children's Medical Center affiliated to the Medical College of Shanghai Jiaotong University (IRB number: SCMCIRB-K2016067-1). Written informed consent for anti-CD19 CAR-T therapy was obtained from patients' guardians.

Author Contributions

Conceived and designed the analysis: Wan X, Chen J, Tang Y, Lu J, Li B.
Collected the data: Wan X, Yang X, Yang F, Wang T, Ding L, Song L, Miao Y, Wang X, Ma Y, Luo C, Tang J, Gu L, Chen J, Tang Y, Lu J, Li B.
Contributed data or analysis tools: Wan X, Yang F, Ding L, Song L, Miao Y, Wang X, Ma Y, Luo C, Tang J, Gu L, Lu J, Li B.
Performed the analysis: Wan X, Yang X, Wang T, Li B.
Wrote the paper: Wan X, Yang X, Li B.

ORCID iDs

Xinyu Wan: https://orcid.org/0000-0001-7538-0034
Xiaomin Yang: https://orcid.org/0000-0002-4032-358X
Fan Yang: https://orcid.org/0000-0001-8990-1443
Yanjing Tang: https://orcid.org/0000-0001-6790-7449
Jun Lu: https://orcid.org/0000-0002-6740-6828
Benshang Li: https://orcid.org/0000-0002-3726-7732

Conflicts of Interest

Conflict of interest relevant to this article was not reported.

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