Potent anti-leukemia activities of chimeric antigen receptor modified T cells against CD19 in Chinese patients with relapsed/refractory acute lymphocytic leukemia

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DOI: 10.1158/1078-0432.CCR-16-1799 Published 30 December 2016
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Running title: Anti-leukemia activities of CART19s in Chinese patients

Word count: 4701, Figures/Tables: 6, Supplementary Figures/Tables: 11.

Funding: 973 Program, Zhejiang Provincial Natural Science Foundation of China, the Natural Science Foundation of China, Zhejiang Medical Technology & Education, Key Project of Science and Technology Department of Zhejiang Province.
Statement of translational relevance

Antitumor activities of chimeric antigen receptor modified T cells against CD19 (CART19s) have been observed in relapsed/refractory acute lymphocytic leukemia (ALL) patients at several institutions. However, data from systemic clinical trials in Asian populations have been limited. This report is the first trial to provide evidences that CART19s have potent anti-leukemia activities in Chinese patients. The data from this study demonstrate that CART19 therapy is a feasible approach for the majority of patients with relapsed/refractory CD19+ ALL and that CART19s have transient and tolerable toxicities. Moreover, patients with a high risk of relapse after CART19s might benefit from subsequent haploidentical hematopoietic stem cell transplantation. Our data reveals a high translational relevance, and it is definitely important to report these significant findings in a timely fashion.
Abstract

Purpose: Patients with relapsed/refractory acute lymphocytic leukemia (R/R ALL) have a poor prognosis. Chimeric antigen receptor modified T cells against CD19 (CART19s) have displayed anti-leukemia activities. However, data from systemic trials in Chinese patients are limited.

Study design: T cells transduced with CD19-directed CAR lentiviral vectors were infused in patients with R/R ALL under fludarabine- and cyclophosphamide-based lymphodepletion. The post-infusion responses, toxicities, expansion and persistence of CART19s in enrolled patients were observed and monitored.

Results: We enrolled 15 patients with R/R ALL. The median transduction efficiency of CART19s was 33%. In vitro cytotoxicity assays were conducted and showed prominent anti-leukemia activities with CART19s. The patients received CART19s infusion at doses of $1.1 \times 10^6$/kg to $9.8 \times 10^6$/kg. Twelve patients achieved complete remission 1 month after CART19s infusion. CART19s expanded and persisted in peripheral blood and bone marrow. At 150 days, the overall survival rate and leukemia-free survival rate were 65.5% and 37.8%, respectively. The cumulative incidence of relapse and the non-relapse mortality rate were 54.5% and 7.7%, respectively. Four patients underwent subsequent haploidentical hematopoietic stem cell transplantation. In this trial, ten patients experienced cytokine release syndrome (CRS). Grade 3 CRS developed in 40% of patients and was associated with a higher disease burden on day -1 and a higher number of previous relapses.

Conclusions: This trial demonstrated potent anti-leukemia activities of CART19s in Chinese patients with R/R ALL. Disease relapse remained the main obstacle. However, patients with a high risk of relapse after CART19s might benefit from subsequent haploidentical hematopoietic stem cell transplantation.
**Introduction**

Patients with relapsed/refractory acute lymphocytic leukemia (R/R ALL) have a very poor prognosis under current therapeutic modalities (1, 2). In the past two decades, advances in chemotherapy drugs, targeted agents and allogeneic hematopoietic stem cell transplantation (allo-HSCT) techniques have greatly improved clinical outcomes for these patients. However, limited progresses have been made in reshaping the overall complexion of ALL treatment considering the crucial fact that the complete remission (CR) rate is relatively low, with a median overall survival (OS) time of 3–6 months and a 5-year OS rate of less than 10% for patients with primary refractory disease, a short duration of first remission (<12 months), relapse after allo-HSCT, or disease progression after multiple courses of therapy (1, 3). Allo-HSCT has been recognized the only curative option for patients with R/R ALL (4), and salvage allo-HSCT is clinically available for these patients. However, according to data from recent clinical trials, the high relapse rate and poor OS in these patients remain great challenges (5, 6). Subsequently, re-induction remission is an urgent issue to address in order to improve the clinical outcomes of patients with R/R ALL.

Chimeric antigen receptor modified T cells against CD19 (CART19s) have shown promise as a novel therapy for R/R ALL patients in clinical trials (7-12). High anti-leukemia efficacies of CART19s have been consistently reported by independent trials at different institutions (13-16). Among these trials, CART19s prepared by each institution differ in several respects, including CAR design, T-cell activation and transduction methods. Costimulatory molecules such as CD28, 4-1BB, CD134, CD2, CD27, and ICOS are the integral CAR structural components, particularly CD28 and 4-1BB (17-18). The MSKCC team used a CAR construct containing the costimulatory domain CD28/CD3-ζ via γ-retrovirus transfer, whereas investigators from CHOP/UPenn used a CAR construct containing the costimulatory domain 4-1BB/CD3-ζ via lentivirus transfer (10, 12). According to current clinical trials, CD28/CD3-ζ co-stimulated CART19s demonstrate initially potent effector functions, but the in vivo persistence of these cells seems inferior to that of 4-1BB/CD3-ζ co-stimulated CART19s (13, 14).
Influence of CART19 doses, lymphodepleting chemotherapy regimens and patient populations are major parameters evaluated in clinical protocols (9-13). A broad range of CART19 doses, from $1 \times 10^5$/kg to $1 \times 10^8$/kg, were infused into patients with R/R ALL (13). Reports have suggested a correlation between the CART19 dose and the incidence as well as severity of cytokine release syndrome (CRS) (13). However, whether a higher dose is required for better efficacies remains uncertain. Updated lymphodepleting chemotherapies have been administered prior to CART19s infusion, including no chemotherapy, cyclophosphamide alone, and fludarabine combined with cyclophosphamide (13, 14), however, a well-recognized lymphodepleting regimen has not been acquired. By now, most clinical trials have been performed in Caucasians, Euro-Americans, Hispanic-Americans and Asian-Americans (13, 14), and data from systematic studies of Asian natives remain limited. To this end, validation trials are needed by confirming the safety and efficacy profiles of CART19s therapy in native Asian populations.

In the current study, we enrolled 15 consecutive patients with R/R ALL and administered individual lymphodepleting chemotherapy with 4-1BB/CD3-ζ co-stimulated CART19s. We evaluated the efficacy and safety profiles to pursue an optimal CART-based therapeutic strategy in these Chinese patients.

**Materials and methods**

**Clinical protocol design**

This clinical trial was designed to assess the safety and efficacy of infusing autologous T cells modified to express the CD19 specific CAR/4-1BB/CD3-ζ into Chinese patients with R/R ALL (Chictr.org number, ChiCTR-OCC-15007008) (Figure 1A). The inclusion criteria were as follows: 1) age less than 60 years; 2) relapsed or refractory CD19+ ALL; 3) relapsed allo-HSCT without evidence of graft versus host disease (GVHD) and not requiring immunosuppression therapy; and 4) measurable disease and adequate performance status and organ function. Patients with central nervous system leukemia (CNSL) were ineligible. The protocol was approved by the 1st Affiliated Hospital, School of Medicine, Zhejiang University Institutional
Review Board. All patients provided written, informed consent.

**Construct design and CART19s generation**

The single chain fragment variable (scFv) sequence specific for CD19 was derived from Clone FMC63 (19). The 4-1BB costimulatory domain and CD3ζ signaling domain were generated (20, 21, 22). CART19-4-1BB vectors harboring anti-CD19 scFv and the human 4-1BB and CD3ζ signaling domains were cloned into a lentiviral backbone as previously described (22). Lentivirus was produced by transfecting 293T cells with CAR lentiviral vectors and viral packaging plasmids which were frozen in -80°C and thawed immediately before transduction. The lentivirus supernatant was harvested. CD3+ T cells were isolated and activated as described (23). The cells were then cultured in X-VIVO 15 medium (Lonza) containing 100 U/ml interleukin-2 (IL-2) and transduced with lentivirus supernatant at high multiplicity of infection (MOI) from 5:1 to 10:1 within 24-48 hours. The CAR transduced T cells were cultured for 11 days. Three days before administration, fresh culture media were replaced. After that, no manipulation was conducted to the cells until transportation for infusion. The transduction efficiency was evaluated by flow cytometry (FACS) on day 5-7 after lentivirus transduction. The following anti-human antibodies were used: anti-hCD45 APC (BD Bioscience), anti-hCD3 FITC (BD Bioscience), biotin-labeled goat-anti-mouse IgG specific for F(ab’)2 fragment (Jackson immuno-Research, Cat# 115-065-072) and PE streptavidin (BD Bioscience). Data acquisition was performed using a CytoFLEX flow cytometer (Beckman).

**Quality control of CART19s prior to infusion**

Prior to CART19s infusion, FACS analysis of transduction efficiency and in vitro cytotoxicity assays of CART19s were performed for each patient as described in the supplementary materials. Additionally, CART19 cultures were checked twice for possible contaminations by fungus, bacteria, mycoplasma, chlamydia and endotoxin.

**CART19s treatment**

Peripheral blood mononuclear cells (PBMCs) were obtained from patients by leukapheresis for CART19s preparation on day -11, and the first day of CART19s
infusion was set as study day 0 (Figure 1B). Patients were given a conditioning treatment for lymphodepletion. Fludarabine- and cyclophosphamide-based conditioning treatment varied according to the tumor burden in the bone marrow (BM) and peripheral blood (PB) (Figure 1C). CART19s were transfused directly to patients in escalating doses over a period of 3 consecutive days without any premedication. Each day CART19s were transported to hospital, washed, counted, checked for viability and then prepared for administration to patients, who were then observed closely for at least 2 hours. CRS was graded according to a revised grading system (24). Other toxicities during and after therapy were assessed according to the National Institutes of Health Common Terminology Criteria for Adverse Events Version 4.0 (http://ctep.cancer.gov/). Therapy responses were assessed by flow cytometry and morphological analysis. When possible, patients were assessed by chimeric gene expression levels. The response type was defined as minimal residual disease (MRD) negative, complete response, complete response with incomplete count recovery, stable disease and progressive disease as described in the supplementary materials.

Assessment of CART19s expansion and persistence

Serial BM and PB samples after CART19s infusion were collected in K$_2$EDTA BD vacutainer tubes (BD). The persistence of CART19s from fresh PB and BM in patients was determined by FACS. Circulating CART19 numbers per μl were calculated on the basis of measured absolute CD3+ T lymphocyte counts. Simultaneously, CAR DNA copies were evaluated as another method of determining CART19s expansion and persistence. Genomic DNA was extracted using a QIAamp DNA Blood Mini Kit (Qiagen) from cryopreserved PB and BM. CAR DNA copies were assessed by quantitative real-time PCR as described in the supplementary materials.

Assessment of serum and CSF cytokine levels

The levels of cytokines IFN-γ, TNF-α, IL-4, IL-6, IL-10 and IL-17 in serum and CSF were measured in a multiplex format according to the manufacturer’s instructions as described in the supplementary materials.

Statistics
Comparisons of continuous variables and risk factors that may influence variations in grade 3 CRS development were compared using the Mann-Whitney U test for 2 groups. Fisher’s exact test was used to evaluate the influence of categorical variables on grade 3 CRS between 2 groups. Correlations were calculated using a rank-based Spearman test. Overall survival (OS) and leukemia-free survival (LFS) probabilities were determined by the Kaplan-Meier method using all enrolled patients to determine OS and those with MRD-negative responses for LFS. All quoted P values are two-sided, and P values less than 0.05 were considered statistically significant.

Results

Patient characteristics

A total of 22 patients with pathologically confirmed CD19+ ALL between July 2015 and April 2016 were recruited for this trial. Six patients were not eligible for the CART19 clinical trial owing to abnormal liver function (2 patients), heart failure (1 patient), renal dysfunction (1 patient) and lung infection (2 patients). One patient developed a severe infection during CART19s generation. These 7 patients were excluded from receiving lymphodepletion chemotherapy and CART19s (Figure 1A). Thus, 15 patients with CD19+ ALL aged between 7 and 57 years and with a median age of 32 years were enrolled in this trial (Supplementary Table 1). Of these patients, 4 had Ph+ ALL (ABL T315I mutation in 2 patients), 5 had prior allo-HSCT, and 8 had 2 or more times of relapse before receiving CART19s therapy. One patient (Patient No. 3) had never achieved MRD-negative remission despite 5 courses of intensive chemotherapy and TKI-targeted therapy including the 3rd-generation agent ponatinib. Of the 15 patients, 14 patients had detectable leukemic cells in BM at the time of CART19s infusion, and 1 had extramedullary relapse in the testes while the BM MRD status was negative. Four patients had previous CNSL, and 2 had previous extramedullary relapse in the testes. The median leukemia burden was 63.5% (ranging from 3% to 83%) of marrow blasts.

Generation, characterization and in vitro anti-leukemia activities of CART19s from patient PBMCs
After 11 days of culture, cells were released for infusion. The median transduction efficiency of the final products was 33%, with a range of 5% to 50% (Table 1). In vitro cytotoxicity assays of CART19s showed robust CART19s activation and prominent anti-leukemia activities of CD19+ leukemia cells (Supplementary Figure 1, Supplementary Figure 2 and Supplementary Table 2). A routine screening for fungus, bacteria, mycoplasma, chlamydia and endotoxin was negative in CART19 cultures prior to infusion. All 15 patients received CART19s infusion at doses of $1.1 \times 10^6$/kg to $9.8 \times 10^6$/kg, with a median dose of $3.7 \times 10^6$/kg, similar to the CART19 doses in previous studies (13, 14).

**Induction of remission after CART19s infusion**

Therapy responses were evaluated in 13 patients on day 7-10 and 12 patients on day 30 after CART19s infusion. Excluding Patient No. 14, who had a testicular relapse, of the 13 patients with BM results, 6 were MRD-negative and displayed a complete response with incomplete count recovery, 5 obtained a complete response with incomplete count recovery but were MRD-positive, 1 patient (Patient No. 10) had a stable disease, and 1 patient (Patient No. 12) had a progressive disease 7-10 days after CART19s infusion. On day 30 after CART19s infusion, the 12 patients remained MRD-negative (Table 1). Patient No. 13, who had an extramedullary leukemia relapse in the testes, also obtained CR 1 month after CART19s infusion, which was confirmed by ultrasonography and pathology examination (Supplementary Figure 3). These results demonstrate that CART19s induce CR rapidly and effectively in R/R ALL patients both in BM and in extramedullary sites.

**In vivo engraftment, expansion and persistence of CART19s in PB and BM**

Distribution profiles of CART19s in PB were assessed by means of FACS and qPCR assays at serial time points before and after CART19s infusion, respectively (Figure 2A, Supplementary Figure 4A). Of the 14 evaluable patients (Patient No. 4 was not included), the median peak CART counts were 342 per μl (95% CI 140-532) and 96 per μl (95% CI 61.5-132.8) in the grade 3 CRS group and in the non-CRS or grade 1 or 2 CRS group, respectively (P=0.002). The median peak CART DNA copies were $9.9 \times 10^5$ per μg (95% CI 61.5×10^6-132.8×10^6) and $2.2 \times 10^5$ per μg (95% CI
$1.5 \times 10^5$-$4.8 \times 10^5$) in the grade 3 CRS group and in the non-CRS or grade 1 or 2 CRS group, respectively (P=0.002 and 0.0047). During the 7-10 days after CART19s infusion, the median CAR DNA copies in BM were higher in the grade 3 CRS group than in the non-CRS or grade 1 or 2 CRS group ($2.5 \times 10^6$ per μg, 95% CI $-8.4 \times 10^5$-$8.3 \times 10^6$ vs $1.3 \times 10^5$ per μg, 95% CI $5.9 \times 10^4$-$4.1 \times 10^5$; P=0.002), while the peak CART/CD3+T cell percentages in BM were not significantly different between the 2 groups (Figure 2B). In 4 patients, the CAR DNA copies were higher in BM than in PB (Supplementary Figure 4B). Moreover, CART19s were detectable in the blood and marrow for up to 7 months (Figure 2A).

Paired measurements of CAR DNA copies in BM and PB on day 7 to 10 after CART19s infusion and the percentage of leukemia cells after FC chemotherapy showed strong correlations with the CAR DNA copy count (CAR DNA copies in BM vs PB, Spearman r=0.956, p<0.001; CAR DNA copies in BM vs percentages of leukemia cells after FC chemotherapy, Spearman r=0.699, p=0.011; CAR DNA copies in PB vs percentages of leukemia cells after FC chemotherapy, Spearman r=0.755, p=0.005) (Supplementary Figure 4C). Paired measurements of peak CAR DNA copies in BM or peak CART19 counts after CART19s infusion and CART19 dose showed no correlations (peak CAR DNA copies in BM vs CART19 dose, Spearman r=0.248, p=0.438; peak CART19 count in PB vs CART19 dose, Spearman r=0.172, p=0.594) (Supplementary Figure 4C).

**Prognosis**

Of the 12 patients with a median follow-up of 142 days (ranging from 30 to 281 days), 6 patients maintained CR. Six patients with CR at 1 month subsequently relapsed: 2 with CD19 (+) blasts (Patients No. 1 and 10, Supplementary Figure 5A), 2 with both CD19(-) and CD19(+) blasts (Patients No. 1 and 3, Supplementary Figure 5B), and 2 with CNS relapse while maintaining CR in BM (Patients No. 5 and 11). Two patients with only CNS relapse received therapeutic intrathecal injection with cytarabine and methotrexate before achieving CR again. Patients No. 2 and 3 underwent salvage haploidentical allo-HSCT. Patients No. 7 and 13 were MRD-positive but still maintained CR in BM and underwent a second allo-HSCT. All 4 patients maintained
CR after allo-HSCT (Figure 3A).

Of the enrolled 15 patients, 6 patients (40%) died: 3 (20%) owing to severe infection (2 died during the pancytopenia period, and 1 died during the follow-up period) and 3 (20%) owing to disease progression or relapse. At 150 days, the OS rate was 65.5%. The LFS rate was 37.8%. The cumulative incidence of relapse in BM or the CNS was 54.5%, whereas the cumulative incidence of relapse in BM only was 36.3% (Figure 3B, 3C). The non-relapse mortality (NRM) rate was 7.7% (Supplementary Figure 6). The median interval of LFS was 143 days (95% CI, 24 to 262 days).

Toxicities after CART19s infusion

Systemic toxicities—CRS

In this trial, 10 of 15 patients (66.7%) experienced CRS. Of these patients, three had grade 1 CRS, 1 had grade 2 CRS, and 6 had grade 3 CRS, as shown in Table 1 and Figure 4A. No grade 4 or 5 CRS occurred in this trial. CRS mostly occurred within a median of 2.5 days after infusion (range 1 to 10 days) and lasted for a mean of 5.9 days (range 2–9 days). The syndrome was fully reversible in all patients and was well managed with supportive care alone (n=4), supportive care plus the anti-interleukin-6 receptor monoclonal antibody tocilizumab (n=3), supportive care plus tocilizumab and corticosteroids (n=2), and supportive care plus corticosteroids (n=1) (Table 1).

All 10 patients experienced pyrexia. Patients with grade 3 CRS had pyrexia of higher temperatures that lasted for longer periods than those with grade 1 or 2 CRS (Figure 4B).

Serum levels of the cytokines IL-2, IL-6, IL-10, IFN-γ, IL-4, TNF-α and IL-17 as well as CRP, D-dimer and ferritin were evaluated. Serum levels of IL-6, IFN-γ, IL-10, CRP, D-dimer and ferritin were elevated during CRS (Supplementary Figure 7A), and the peak levels differed significantly between the grade 3 CRS group and the non-CRS or grade 1 or 2 CRS groups (Figure 4C, Supplementary Figure 7B). IL-6 is one of the most important biomarkers for CRS. Paired peak serum levels of IL-6, CRP, ferritin and D-dimer showed strong correlations (IL-6 vs CRP, Spearman $r=0.617$, $p=0.014$; IL-6 vs ferritin, Spearman $r=0.574$, $p=0.028$; IL-6 vs D-dimer,
Spearman $r=0.789$, $p<0.01$; Supplementary Figure 7C). Serum IL-2, IL-4, TNF-α and IL-17 levels were not associated with CRS (data not shown).

Risk factors associated with CRS were analyzed. Univariate analysis showed that the MRD after the conditioning regimen and the number of previous relapses were two factors associated with a high risk of grade 3 CRS ($P=0.026$ and 0.036, respectively; Table 2). That is, multiply relapse and the high tumor burden after the conditioning regimen were two factors associated with grade 3 CRS. Other risk factors including age, gender, previous therapy (chemotherapy or allo-HSCT), CART19 dose and MRD before the FC conditioning regimen were not associated with the risk of grade 3 CRS (Table 2). Multivariate analysis was not performed because of the small number of patients. Our results imply that the number of previous relapse and cases of MRD after the conditioning regimen might be novel predictors for grade 3 CRS.

Toxicities in specific organs

**Neurotoxicity** Reversible neurotoxicities were observed in 5 patients (Patients 2, 5, 8, 13 and 15). Patient 2 developed confusion and gait disturbances 7 days after CART infusion and dramatically improved after tocilizumab (4 mg/kg) therapy on day 9 after CART infusion. Patient 5 experienced headache and vomiting, recurrent right-sided facial and limb paresis, blurred vision and defective visual field with decreased myodynamia, high blood pressure, papilledema, and positive Babinski and Kernig signs since day 3, as we reported previously (22). The symptoms and signs were relieved by methylprednisolone and mannitol treatment. Patient No. 8 developed confusion followed by amnesia during CRS and recovered quickly once CRS was controlled. Patient No. 13 developed epilepsy once after 2 doses of tocilizumab (8 mg/kg) therapy and CRS recovery. Patient No. 15 developed transient muscle clonus in limbs at the time of CRS recovery after 2 doses of tocilizumab (8 mg/kg) therapy. Three patients (No. 2, 8 and 15) did not undergo cerebrospinal fluid (CSF) examination when neurotoxic symptoms occurred owing to pancytopenia. In patient No. 5, cytokine levels were much higher in CSF than in serum, with a serum IFN-γ concentration of 152 pg/ml versus a CSF concentration of 2977 pg/ml, and a serum IL-6 concentration of 46 pg/ml versus a CSF concentration of 8475 pg/ml.
Furthermore, qPCR analysis showed 3032265 CAR copies/μg DNA in CSF versus 988747 CAR copies/μg DNA in PB (22). In patient No. 14, no white blood cells (WBCs) were found in the CSF, but the CSF IL-6 level (935 pg/ml) was higher than that in serum (132 pg/ml).

The occurrence, management and outcomes in other specific organs are listed in Supplementary Table 3.

**Discussion**

In the current study, we utilized a CART19 strategy to treat Chinese R/R ALL patients. The clinical output of CART19 therapy in enrolled patients was encouraging. This trial demonstrated that CART19 therapy was a feasible approach for the majority of enrolled Chinese patients with R/R ALL, with an induction of MRD-negative CR at 1 month. Since progressing refractory disease is inevitable in patients not eligible for allo-HSCT, our data revealed that these patients would have a new opportunity to achieve CR quickly by choosing CART19 therapy as a transitional modality, increasing the opportunities for further therapies including, but not limited to, allo-HSCT. This study also established a comprehensive evaluation protocol covering multiple systems to monitor CART19-associated toxicities.

Patients enrolled in this study obtained a high CR rate in BM which confirmed a favorable outcome for CART19 therapy in R/R ALL. Our results also demonstrated the prominent therapeutic efficacy of CART9s in Chinese patients with R/R ALL. Recently, evidences for the efficacy of CART19 therapy in other patient populations for R/R ALL has been reported. Early in 2013, Grupp SA et al reported that 2 children with R/R ALL achieved CR after CART19s infusion (25). Brentjens R et al reported a 100% CR rate in 5 patients with R/R ALL and an 88% CR rate in 16 adult patients with R/R ALL (10, 26). Then, in 2014, a 90% CR rate in 30 pediatric and adult patients with R/R ALL was reported by Grupp SA et al (12). Other groups have reported similar results (13, 14). Lee et al reported a 66.7% CR rate in an NCI intent-to-treat analysis of children and young adults with ALL (9). Most of patients in these reports were not native Asian. In the current study of Chinese patients, the CR
rate was comparable or even superior to those in these previous studies. CART19s expansion and persistence was another indicator for evaluating efficacy. In a previous study, a shorter persistence duration of 1 to 6 months was reported following CART19s containing CD28/CD3-ζ infusion compared to that following CART19s containing 4-1BB/CD3-ζ infusion in adults with B-ALL, as assessed by FACS and qPCR (13, 14). Consistent with these results, robust expansion and long-term persistence of 4-1BB/CD3-ζ co-stimulated CART19s were observed in our trial with Chinese patients. Moreover, we observed the efficacy of CART19s in extramedullary relapse in the CNS and testes. This study is the first to report anti-leukemia activities of CART19s in testicular relapse. Trafficking of CART19s to several sites including the liver, lymph nodes, CNS and BM has been demonstrated previously (27). In this study, we noted that the CART19 count in PB was not significantly different from that in BM. We also observed that CART19s migrated to tumor sites and exerted anti-tumor effects in BM, CNS and testes. These results presented that CART19s were transported to leukemia sites and eradicated leukemia cells effectively and quickly in Chinese patients with R/R ALL.

In this study, we applied an FC-based lymphodepletion strategy before CART19s infusion. The pre-infusion chemotherapy facilitates CART19s engraftment by eliminating immune cells potentially competing for homeostatic cytokines (28). In addition, the chemotherapy exhibits direct anti-leukemia activities. However, a lymphodepletion regimen is not a standard option and has varied widely in different trials. Recent studies have demonstrated that lymphodepletion chemotherapy is associated with a better prognosis. Tengfei Zhang reported that the 6-month EFS for patients administered a lymphodepletion regimen before CART19s infusion was 94.6%, compared to 54.5% in patients without lymphodepletion (P <0.001) (29). Turtle et al observed that the addition of Flu to Cy lymphodepletion improved the persistence of CARTs and EFS time (30). We exploited FC chemotherapy before CART19s infusion in this study after considering the following disease-related factors: 1) relapsed or refractory leukemia cells in patients with high tumor burden usually have a high proliferation profile, which leads to a high risk of hyperleukocytosis; 2)
preparation of CARTs is a time-consuming procedure, and the allocated time for CART generation is approximately 10 to 14 days; 3) during the period of CART generation, patients are at a high risk of hyperleukocytosis, which may cause severe morbidity and mortality by inducing leukostasis and tumor lysis syndrome (31). Thus, the rationale for chemotherapy before CART19s in these patients is confirmed. We subsequently established a suitable FC-based regimen by considering the de facto tumor burden or leukemia cell proliferation activity in individual patients. In our study, no patient developed hyperleukocytosis-associated complications during CART19s preparation, despite the high tumor burden in most patients.

Serum levels of IL-6, IFN-γ and IL-10 were simultaneously increased during CRS, suggesting that these are causative cytokines. These results are consistent with those of previous studies (32-36). Additionally, we observed that serum ferritin, CRP and D-dimer levels correlated with the severity of CRS and declined in response to tocilizumab or corticosteroids, implying that serum CRP, D-dimer and ferritin levels might be CRS biomarkers for CRS diagnosis and grade. Severe grade 4 or fatal grade 5 CRS has been reported in other clinical trials. None of the 15 patients in this trial had grade 4 or 5 CRS, which may largely be attributable to the patients' immunological backgrounds, their overall status, and suitable intervention with tocilizumab or corticosteroid during the CART therapy. Previous reports have suggested that the severity of CRS may be associated with the disease burden at enrollment (before the lymphodepletion regimen) (9, 10, 13). In our study, the tumor burden was evaluated at 2 time points: the time of enrollment and when the FC lymphodepletion regimen was completed. We observed that the tumor burdens at the time of enrollment were not associated with the risk of grade 3 CRS; however, tumor burdens at the end of the FC regimen were statistically associated with grade 3 CRS, indicating that tumor burden after FC regimen is a more precise risk factor associated with grade 3 CRS. Interestingly, the number of previous relapses was another possible factor associated with grade 3 CRS. Multiply relapsed ALL usually indicates a high risk of grade 3 CRS. Leukemia relapse indicates aberrant selection for and emergence of increasingly malignant clones during progression and therapy (37). Leukemia cells
with multiple relapse experiences exhibit greater chemotherapy resistance and immune escape, and the eradication of these leukemia cells by indicated therapies is limited (38-39). Consequently, CART19s should be more rigorously activated to target these cells and simultaneously result in a more severe CRS. Our data demonstrate that, in addition to the tumor burden, the biological features of leukemia cell were associated with severe CRS. These results may further the understanding of post-CART CRS pathogenesis, and more data from clinical trials are warranted.

The disease relapse after CART19s therapy has been observed in previous studies (9, 13). During follow-up, in this limited-scale study, we observed that disease relapse remained the main obstacle for CART19s therapy. The following factors might be causative for relapse. 1) Our patients had a high tumor burden, with a median leukemia burden of 63.5%. 2) The enrolled patients were more refractory. Most patients had several experiences of relapse or primary refractory. Therefore, the genetic background and mutations in leukemia stem cells were unlikely to be eradicated completely. 3) Most patients had extramedullary relapses. Extramedullary leukemia can reside in a specialized stromal compartment that differs from the bone marrow microenvironment and facilitates tumor growth directly via paracrine secretion of growth factors and the provision of nutrients to permit escape from CART19s attack and BM relapse. Various treatment strategies have been applied for relapse after CART19s therapy, but consensus on an effective option has not been obtained. Several studies have attempted CARTs infusion to enhance anti-leukemia effects in those who had no CARTs persistence or low levels of CARTs, but the efficacy was less adequate. In our study, we observed that haploidentical HSCT after CART19s therapy remarkably reduced the relapse rate and improved OS. Haploidentical HSCT appears to be a promising strategy with a theoretically high donor availability of almost 100%. Based on our previous results, haploidentical HSCT is expected to trigger a more potent graft versus leukemia effect compared with HLA-identical transplants (40). Results from our another study showed that haploidentical HSCT improved outcomes in patients with high-risk leukemia (41). In this study, 4 patients who had received CART19s therapy then underwent
haploidentical HSCT, and no transplant-related deaths occurred, indicating that haploidentical HSCT is a favorable option for patients at high risk of relapse after CART19s therapy.

In conclusion, this study demonstrated that CART19s had potent anti-leukemic activities and improved clinical outcomes in Chinese patients with R/R ALL. Our data suggest that CART19s could provide a new therapeutic approach for patients with R/R ALL. CART19s therapy might be an effective transitional modality to bridge a brief remission with further interventional strategies, such as haploidentical HSCT and even a second allo-HSCT, to further improve overall clinical outcomes in patients at high risk of relapse after CART19s therapy.

ACKNOWLEDGMENTS

This work was supported by grants from the 973 Program (2015CB964900), the Zhejiang Provincial Natural Science Foundation of China (LY14H080002), the Natural Science Foundation of China (81230014, 81470341, 81520108002, 81500157), Zhejiang Medical Technology & Education Foundation of China (2014KYA064, 2014KYA066), and the Key Project of Science and Technology Department of Zhejiang Province (2015C03G2010091).

CONFLICT-OF-INTEREST DISCLOSURE:

Lei Xiao and Zhao Wu are co-founders of Innovative Cellular Therapeutics Co., Ltd, (formerly SiDanSai Biotechnology Co., Ltd), a biotechnology company focused on the research and development of cell therapy and stem cell technology. Chengfei Pu, Jing Jiang, and Jinping Wang are current employees of Innovative Cellular Therapeutics Co., Ltd. The other authors declare no conflicts of interest related to this work.

REFERENCES


Figure legends

**Figure 1 Study schema for the clinical trial of CART19s therapy.** A, Patient enrollment flow chart. B, Clinical treatment protocol. Patients underwent leukapheresis to obtain peripheral blood mononuclear cells (PBMCs) on day -11; the first day of CART19s infusion was set as study day 0. From day -11 to day 0, CART19s were transduced, cultured and expanded. CART19s were transfused in escalating doses over a period of 3 consecutive days (day 0, day +1 and day +2) after lymphodepletion chemotherapy. On day -1, +7 to +9 and every 30 days, bone marrow (BM) examinations were performed. C, Detailed FC–based lymphodepletion chemotherapy before CART19s infusion according to minimal residual disease (MRD) and leukemia cell proliferation activity. If MRD in bone marrow (BM) was less than or equal to 20%, the FC regimen consisted of fludarabine (FLU) 30 mg/m² on days -4 to -2 and cyclophosphamide (CTX) 750 mg/m² on day -2. If MRD in BM was higher than 20% and the white blood cell (WBC) count in peripheral blood (PB) was higher than 20×10⁹/L, the FC regimen consisted of FLU 25 mg/m² on days -7 to -3 and CTX...
750 mg/m² on days -2 to -1. Otherwise, the FC regimen consisted of FLU 30 mg/m² on days -5 to -3 and CTX 1000 mg/m² on days -2 to -1.

Figure 2 CART19s engraftment, expansion and persistence in vivo. A, Levels of CART19s in peripheral blood (PB) assessed by FACS at serial time points before and after infusion of CART19s, respectively. B, Peak levels of CART19s and CAR DNA copies in bone marrow (BM) and PB in patients who developed grade 3 CRS (n=5) compared with those without CRS or with 1 or 2 CRS (n=10, CAR DNA copies in BM in patient 13 could not be detected so n=9). The data represent the mean±SEM. The Mann-Whitney U test was used for statistical analysis.

Figure 3 Prognosis after CART19s therapy. A. Prognosis of patients who relapsed after CART19s therapy. B. Cumulative incidence of relapse. C. Leukemia-free survival (LFS) of 13 patients. The overall survival (OS) of all 15 patients treated in the study is shown. For NRM, LFS and OS, the survival fractions were calculated by the Kaplan-Meier method, and lines indicate censored patients.

Figure 4 CRS complication after CART19s therapy. A, CRS grade distribution in 15 patients. B, Fever developed in all CRS patients after infusion of CART19s. The maximum daily temperature of each CRS patient is shown. Patients with grade 3 CRS had a higher temperature that lasted a longer period than those with grade 1 or 2 CRS. C, Peak serum levels of IFN-γ, IL-6 and IL-10 in patients who developed grade 3 CRS (n=6) compared with those without CRS or with 1 or 2 CRS (n=10). The data represent the mean±SEM. The Mann-Whitney U test was used for statistical analysis. D, Peak serum levels of CRP, ferritin and D-dimer in patients who developed grade 3 CRS (n=6) compared with those without CRS or with 1 or 2 CRS (n=10). The data represent the mean±SEM. The Mann-Whitney U test was used for statistical analysis.
Table 1  Patient response, toxicities and prognosis after CART19 therapy.

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Flu dosage</th>
<th>CTX dosage</th>
<th>Condition</th>
<th>Infused CART19 cells</th>
<th>CRS Grade</th>
<th>BM leukemia cells (FACS)</th>
<th>Outcomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Flu 30 mg/m² d-6 to -4</td>
<td>CTX 1 g/m² d-3 to -2</td>
<td>50</td>
<td>5.16</td>
<td>2</td>
<td>N</td>
<td>0.05%</td>
</tr>
<tr>
<td>2</td>
<td>Flu 30 mg/m² d-6 to -4</td>
<td>CTX 1 g/m² d-3 to -2</td>
<td>40</td>
<td>9.4</td>
<td>3</td>
<td>Y, 4mg/kg</td>
<td>N</td>
</tr>
<tr>
<td>3</td>
<td>Flu 30 mg/m² d-6 to -4</td>
<td>CTX 1 g/m² d-3 to -2</td>
<td>40</td>
<td>5.5</td>
<td>0</td>
<td>N</td>
<td>0.95%</td>
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<tr>
<td>4</td>
<td>Flu 30 mg/m² d-6 to -4</td>
<td>CTX 750 mg/m² d-2</td>
<td>36</td>
<td>9.8</td>
<td>3</td>
<td>4mg/kg</td>
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<tr>
<td>5</td>
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<td>CTX 750 mg/m² d-2</td>
<td>22</td>
<td>6.7</td>
<td>3</td>
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<td>Y</td>
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<td>CTX 750 mg/m² d-2</td>
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<td>7.04</td>
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<td>CTX 1 g/m² d-3 to -2</td>
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<td>CTX 750 mg/m² d-3 to -2</td>
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<td>1.3</td>
<td>3</td>
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<td>9</td>
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<td>CTX 750 mg/m² d-3 to -2</td>
<td>35</td>
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<tr>
<td>11</td>
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<td>CTX 750 mg/m² d-2</td>
<td>15</td>
<td>3.5</td>
<td>0</td>
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<td>12</td>
<td>Flu 25 mg/m² d-8 to -4</td>
<td>CTX 750 mg/m² d-3 to -2</td>
<td>25</td>
<td>1.3</td>
<td>0</td>
<td>N</td>
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</tr>
<tr>
<td>13</td>
<td>Flu 30 mg/m² d-6 to -4</td>
<td>CTX 1 g/m² d-3 to -2</td>
<td>6</td>
<td>2.5</td>
<td>3</td>
<td>Y, 8mg/kg</td>
<td>N</td>
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<tr>
<td>14</td>
<td>Flu 30 mg/m² d-4 to -2</td>
<td>CTX 750 mg/m² d-2</td>
<td>22</td>
<td>4.7</td>
<td>0</td>
<td>N</td>
<td>N</td>
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<tr>
<td>15</td>
<td>Flu 25 mg/m² d-8 to -4</td>
<td>CTX 750 mg/m² d-3 to -2</td>
<td>33</td>
<td>3</td>
<td>3</td>
<td>Y, 8mg/kg</td>
<td>N</td>
</tr>
</tbody>
</table>

Flu=fludarabine; CTX=cyclophosphamide; CRS=cytokine release syndrome; CNSL=central
nervous system leukemia; Tocili=tocilizumab; BM=bone marrow; Y=yes; N=no; NA=not available; CR=complete remission; MRD=minimal residual disease; FACS=flow cytometry analysis; Haplo-HSCT=haploidentical hematopoietic stem cell transplantation.
Table 2. Univariate analysis of potential factors affecting grade 3 CRS after CART19 infusion

<table>
<thead>
<tr>
<th>Factors</th>
<th>Grade 3 CRS</th>
<th>P value</th>
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</thead>
<tbody>
<tr>
<td>Age</td>
<td>0.555</td>
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<tr>
<td>Gender</td>
<td>0.136</td>
<td></td>
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<tr>
<td>Previous therapy (chemotherapy or allo-HSCT)</td>
<td>0.608</td>
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<tr>
<td>Infused CART19 dose</td>
<td>0.443</td>
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<tr>
<td><strong>Number of previous relapses</strong></td>
<td><strong>0.036</strong></td>
<td></td>
</tr>
<tr>
<td>MRD before FC conditioning regimen</td>
<td>0.215</td>
<td></td>
</tr>
<tr>
<td><strong>MRD after FC conditioning regimen</strong></td>
<td><strong>0.026</strong></td>
<td></td>
</tr>
</tbody>
</table>
Figure 1

A

- Screen (n=22)
  - n=6 Excluded ineligible
  - n=1 Withdraw for severe infection

- PBMCs collection and CART19 preparation (n=16)

- FC-based conditioning regimen and CART19 infusion (n=15)

- Evaluation for toxicity (n=15)
  - Died of severe infection before primary disease evaluation
  - n=1

- Evaluation for efficacy (n=14)

B

- Day -11
- Day -8
- Day -2
- Day -1
- Day 0
- Day +1
- Day +7
- Day +10
- Follow up......

- PBMCs collection
- CART preparation

- FC based conditioning regimen
- CART infusion

- BM examination

C

- FLU 30mg/m² d-4 to -2 + CTX 750mg/m² d-2

- < or =20% MRD in BM
- >20% WBC count in PB

- < or =20x10⁹/L or leukemia cells with high proliferation activity

- FLU 30mg/m² d-5 to -3 + CTX 1000mg/m² d-2 to -1

- FLU 25mg/m² d-7 to -3 + CTX 750mg/m² d-2 to -1
Figure 2

A

Patients with grade 3 CRS

CART count in PB (Per μL)

Days after CART19 infusion

Patients with non-CRS or grade 1 or 2 CRS

CART count in PB (Per μL)

Days after CART19 infusion

B

Peak CART counts in PB (Per μL)

Grade 3 CRS Non-CRS or grade 1 or 2 CRS

P=0.002

Peak CAR DNA copies in PB (Per μg)

Grade 3 CRS Non-CRS or grade 1 or 2 CRS

P=0.0047

Peak CART percentage in BM (CAR/C3+ T cells)

Grade 3 CRS Non-CRS or grade 1 or 2 CRS

P=0.13

Peak CAR DNA copies in BM (Per μg)

Grade 3 CRS Non-CRS or grade 1 or 2 CRS

P=0.002
A

Peak serum IFN-γ level (pg/ml)

Grade 3 CRS
Non-CRS or Grade 1 or 2 CRS

*P=0.0008

Figure 4

B

Peak serum IL-6 level (pg/ml)

Grade 3 CRS
Non-CRS or Grade 1 or 2 CRS

*P=0.0016

C

Peak serum IL-10 level (pg/ml)

Grade 3 CRS
Non-CRS or Grade 1 or 2 CRS

*P=0.0076

No CRS

20.0% (3 patients) Grade 1 CRS

6.7% (1 patient) Grade 2 CRS

40.0% (6 patients) Grade 3 CRS

Total=15 patients

A

Temperature (°C)

Days after CART19 infusion

Patient 1
Patient 6
Patient 7
Patient 10

Patient 2
Patient 4
Patient 5
Patient 8
Patient 14
Patient 10

grade 1 and 2 CRS

grade 3 CRS