ARTICLE

ACUTE MYELOID LEUKEMIA

Characteristics of anti-CLL1 based CAR-T therapy for children with relapsed or refractory acute myeloid leukemia: the multi-center efficacy and safety interim analysis

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C-type lectin-like molecule-1 (CLL1) is preferentially expressed on acute myeloid leukemia (AML) stem cells and AML blasts, which can be considered as AML-associated antigen. Anti-CLL1-based CAR-T cells exhibited effective tumor-killing capacity in vitro and in AML-bearing mouse model. In this report, eight children with relapsed or refractory AML (R/R-AML) were recruited for a phase 1/2 clinical trial of autologous anti-CLL1 CAR-T cell immunotherapy. The objectives of this clinical trial were to evaluate the safety and the preliminary efficacy of anti-CLL1 CAR-T cell treatment. Patients received one dose of autologous anti-CLL1 CAR-T cells after lymphodepletion conditioning. After CAR-T treatment, patients developed grade 1–2 cytokine release syndrome (CRS) but without any lethal events. 4 out of 8 patients achieved morphologic leukemia-free state (MLFS) and minimal residual disease (MRD) negativity, 1 patient with MLFS and MRD positivity, 1 patient achieved complete remission with incomplete hematologic recovery (CRi) but MRD positivity, 1 patient with partial remission (PR), and 1 patient remained at stable disease (SD) status but had CLL1-positive AML blast clearance. These results suggested that anti-CLL1-based CAR-T cell immunotherapy can be considered as a well-tolerated and effective option for treating children with R/R-AML.

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INTRODUCTION

Outcomes of children with acute myeloid leukemia (AML) remain lagged behind that of children with acute lymphoblastic leukemia (ALL), with 5-year disease-free survival (DFS) varying between 33.3% and 79.5%, worldwide, as reported by the CONCORD-2 study [1]. Though 50–70% of children with primary AML can be cured with conventional intensive therapy [2, 3], the cure rate is not improved much by the introduction of novel agents [4]. The relatively poor prognosis for childhood AML has been mostly attributed to less available targeted agents or therapies for those relapsed/refractory AML (R/R-AML) patients. Up to now, allogeneic hematopoietic stem cell transplantation (allo-HSCT) is generally considered as the best chance of cure for patients with high-risk or R/R-AML [5].

Impressively, the prognosis for patients with R/R-AML can be greatly enhanced if they are in a negative minimal residual disease (MRD) state even without receiving allo-HSCT [6]. Therefore, novel therapies are highly needed for further improvement in the R/R-AML therapy. Several novel agents have been currently introduced into pediatric R/R-AML therapies (i.e., sorafenib, gilteritinib, gemtuzumab, ozogamicin (GO), and venetoclax), however the responses remain unsatisfactory, with ~50% CR/CRi (complete remission/complete remission with incomplete hematologic recovery) rate [7–10]. The development of immunotherapy has given the clinicians with new opportunity for treating patients with hematological malignancies. In this regard, autologous chimeric antigen receptor (CAR) T cell therapy has been increasingly accepted as a highly effective regimen for relapsed/refractory hematological malignancies [11–13].

For example, anti-CD19 CAR-T cell immunotherapies are reported to be highly effective in R/R B-cell ALL (B-ALL) patients, with CR rates ranging from 70 to 94% [14–16], although near one third responding patients eventually experienced relapse due to multiple mechanisms [17]. However, the potential of CAR-T cell therapy in R/R-AML remains undetermined.

C-type lectin-like molecule-1 (CLL1) is identified as a novel AML stem cell associated antigen [18]. Intriguingly, CLL1 is highly expressed on AML leukemia stem cells (LSCs), majority of AML blasts and normal myeloid cells, but not on normal hematopoietic stem cells (HSCs) and lymphoid cells, suggesting that targeting CLL1 can be a novel AML therapy while not affecting normal hematopoiesis.

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hematopoiesis and lymphocyte-directed immune function. Several studies have successfully developed novel CLL1-directed therapies (i.e., antibody-based and cellular therapies) with definite efficacy on human AML with promising ex vivo and in vivo evidences [19–24]. Liu [25] and Zhang [26] have independently and successfully treated two secondary AML patients with anti-CLL1 based CAR-T cells, highlighting the potential of CAR-T cell therapy in R/R-AML.

To this end, we recruited 8 children with R/R-AML for a phase 1/2 clinical trial with autologous anti-CLL1 based CAR-T cell therapy to test its safety and preliminary efficacy among two independent institutions.

MATERIALS AND METHODS

Eligibility, ethics approval, and treatment schema

This study was approved by the Institutional Review Board of Guangzhou Women and Children’s Medical Center (2020-23) and Nanfang-Chunfu Children’s Institute of Hematology and Oncology, Dongguan Taixin Hospital (TXEC-2019-005). This clinical trial was registered at Chinese Clinical Trial Registry (www.chictr.org.cn) with registration number ChiCTR1900027684 and www.clinicaltrials.gov (NCT073222674). Detailed study design is included in supplementary file. Children with R/R AML patients who met criteria for this clinical trial were enrolled. Informed consent according to institutional guidelines and the Declaration of Helsinki was obtained from the parents or guardians. Briefly, all enrolled patients received lymphodepletion regimens cyclophosphamide and fludarabine for 3–5 days prior to CAR-T cell infusion [26]. A single dose (0.35–1 × 10^9/kg) of anti-CLL1 based CAR-T cells was infused through a peripherally inserted central venous catheter (PICC). The treatment response and CAR-T cell-related toxicities were systemically evaluated. Bone marrow morphologic and flow cytometric assessments for treatment response were performed every month for the first three months after CAR-T cell therapy, and every three months thereafter if not followed by allo-HSCT. Patients achieving CR/MFS were transferred for further allo-HSCT if their socioeconomic status and donor status allowed.

Treatment response evaluation

Bone marrow (BM) specimens were longitudinally collected prior to and after anti-CLL1 CAR-T cell infusion for experiments. The European LeukemiaNet (ELN) recommendations for diagnosis and management of AML (2017) were used to evaluate the treatment response [27]. Briefly, complete remission (CR) meets the following criteria: <5% blasts in the bone marrow; no blasts with Auer rods; normal maturation of all cellular components in the bone marrow; no extramedullary disease; neutrophils ≥1000/µL; platelets ≥100,000/µL; and transfusion independent. Complete remission with incomplete hematologic recovery (CRI) meets all the CR criteria except for residual neutropenia <1000/µL or thrombocytopenia <100,000/µL. Morphologic leukemia-free state (MLFS) is defined as bone marrow blasts <5%; absence of blasts with Auer rods; absence of extramedullary disease and there is no hematologic recovery required. The definition of partial remission (PR) meets all hematologic criteria of CR, but with decrease of bone marrow blast percentage to 5–25% and decrease of pretreatment bone marrow blast percentage by at least 50%. Stable disease (SD) is defined as absence of CR, CRI, MLFS, PR, and does not meet the criteria for PD. Progressive disease (PD) meets the following criteria: evidence for an increase in bone marrow blast percentage and/or increase of absolute blast counts in the blood; >50% increase in marrow blasts over baseline (a minimum 15% point increase is required in cases with <30% blasts at baseline); or persistent marrow blast percentage >70% over at least 3 months; without at least a 100% improvement in ANC to an absolute level >0.5 × 10^9/L, and/or platelet count to >50 × 10^9/L in non-transfused condition; or >50% increase in peripheral blasts (WBC x% blasts) to >25 × 10^9/L (in the absence of differentiation syndrome); or with new extramedullary disease. Relapse needs to meet: after CR, bone marrow blasts >5%; or reappearance of blasts in the blood; or development of extramedullary disease.

Safety and tolerability

The common terminology criteria for adverse events (CTCAE) 5.0 criteria was applied to systemically evaluate the safety and tolerability of anti-CLL1 CAR-T cells in this study. All patients were managed using guidelines from Mahadeo and Neelapu [28, 29].

Monitoring cytokine release

Novus Biologicals human IL-6 ELISA kit was used to determine the IL6 levels in patients’ plasma.

Statistical analyses

The safety, tolerability, side effects, and clinical response data of patients who received anti-CLL1 CAR-T cells were pooled for analysis using SAS® Version 9.2 or higher, and descriptive statistics were used to summarize the data.

Generation of clinical-grade anti-CLL1 CAR-T cells

All GMP viral vector production practices were following the regulatory guidelines. Lentiviral vector supernatant for the anti-CLL1-CAR was produced by transfecting of 293T cells (Takeda) with the corresponding CAR plasmid and 3 packaging plasmids: plP1, plP2, and plP/VSFG and the medium was changed 4 h after transfection. Forty-eight hours later, the cell supernatant was pooled and filtered with a 0.45μm filter, followed by Benzonase treatment (Merck) for 16 h. Then, the harvest was passed through a Mustang Q ion-exchange capsule ( Pall, Ann Arbor, MI). The Mustang Q membrane was washed using 50 mM Tris-HCl, pH 8.0 with 750 mM NaCl and then eluted in fractions using 50 mM Tris-HCL, pH 8.0 with 1.5 M NaCl, and diluted with phosphate buffer pH 7.2. The elution was further concentrated approximately 10-fold by 300 KD TFF column. The final concentrate was formulated with human serum albumin (HSA) to 2%, filtered with a 0.22 μm filter, aliquoted to 2 mL cryotubes, quick frozen on dry-ice, and stored at ~80°C.

Patients’ T cells from peripheral blood mononuclear cells (PBMCs) were enriched by CD3 magnetic beads (Miltenyi), and stimulated by anti-CD3/CD28 beads (Dynabeads, Human T Activator CD3/CD28, Life Technologies) at a 1:3 (beads: T cells) ratio, and then cultured in h3 medium (Takara) with 4% Human AB serum and 10 ng/mL recombinant human IL7 and IL15 (Miltenyi). Cells were exposed to lentivirus containing supernatant on days 2 and 3 with multiplicity of infection (MOI) of five, on Retronectin-coated non-tissue culture plates (Takara/Clonetech). Beads were magnetically removed on day 4 or 5, and cells were further expanded for 3–5 days in h3 media containing 10 ng/mL recombinant human IL7 and IL15 until use in vitro or in vivo. The cells were harvested and cryo-preserved. Once the standard operating procedure was completed, the cell product was shipped for clinical application under the clinical trial guideline.

The ex vivo and in vivo AML targeting capacity of anti-CLL1 based CAR-T cell therapy

In vitro cytotoxic assays were performed by co-culturing 50,000 CAR-T cells with 50,000 AML cells in complete media in a 96-well plate. The supernatant was collected after 24 h co-incubation. Human interferon-gamma (IFNγ) from cell culture supernatant were measured by enzyme-linked immunosorbent assay (ELISA) development kit (4A Biotech, Beijing) according to the manufacturer’s instructions.

Severe immune-deficient B-NDG mice were purchased from Jiangsu Biocytogen Co., Ltd. (Nantong, China), and anesthetized with 3% isoflurane (Minrad International, Buffalo, NY, USA) in an incubation chamber. Anesthesia (David Kopf Instruments, Tujunga, CA, USA) was maintained at 2% isoflurane delivered through a nose adapter. Half million AML HL60-luc cells were injected into 6–10 week-old B-NDG mice using a blunt-end needle through tail vein. Leukemia occurrence was serially monitored by bioluminescence in vivo imaging on an IVIS spectrum instrument (Caliper Life Science) and quantified with Living Image software (PerkinElmer, Waltham, MA, USA). With the establishment of HL60-luc AML B-NDG mice model, mice were randomized and treated with 3 million or 10 million anti-CLL1 CAR-T cells or an equivalent number of non-CAR T cells (matched for total T cell dose) intravenously by tail vein injection. Living image software was used to analyze the IVIS data.

RESULTS

We initiated a single-arm, Phase 1/2 clinical trial to test the feasibility and safety of the autologous T cells, expressing a CLL1-targeted CAR with 4–18B and CD3z endoplasmic domains, in patients with refractory/relapsed (R/R) AML. The anti-human CLL1
CAR-T extracellular scFv was derived from a high-affinity mouse CLL1 monoclonal antibody (clone 27H4, Supplementary Fig. 1A), which was generated by hybridoma technology. Membrane protein array was applied to confirm the CLL1 binding specificity of clone 27H4 as shown in Supplementary Fig. 1B. Then we proceeded to construct the second generation of CAR using the variable region derived from clone 27H4 targeting CCL1 (Supplementary Fig. 2A). The design, development, and generation of clinical grade anti-CLL1 based CAR-T cells were shown in Supplementary Fig. 2B. Firstly, we tested their anti-tumor efficacy by co-culturing anti-CLL1 CAR-T cells with Raji-CLL1 (B lymphoblastoid cell line ectopically expressing CLL1), or HL60 (CLL1-expressing AML cell line). After co-culturing, a significant IFN-γ release was observed when anti-CLL1 CAR-T cells co-cultured with Raji-CLL1, or HL60 cells, but not with the parental Raji cells which do not express CLL1 (Supplementary Fig. 3), these results demonstrating the targeting capacity of the anti-CLL1 CAR-T cells. Next, we investigated the tumor-killing efficacy using human AML-bearing B-NDG mouse model. An increasing dose of anti-CLL1 CAR-T cells ($3 \times 10^6$ and $1 \times 10^7$) were infused into HL60-luc AML-bearing mice and the leukemic burden was evaluated by in vivo imaging methods. As shown in Fig. 1A, B, the AML burden was significantly reduced in anti-CLL1 CAR-T cell-treated group, when compared to their control groups (vehicle and T-mock). Moreover, infusion of anti-CLL1 CAR-T cells, but not the mock T cells, significantly prolonged the survival of the AML-bearing mice, confirming the tumor-killing potential of the anti-CLL1 CAR-T cells in vivo (Fig. 1C).

To initiate the Phase 1/2 clinical trial, 8 children with R/R-AML were enrolled; 6 were male; median age was 12 (range 8–16) years (Table 1). According to the clinical trial protocol, patients (except patient 2) received lymphodepletion conditioning for 3–5 days prior to anti-CLL1 based CAR-T cell infusion on day 0 (Table 1). The general time schedule of anti-CLL1 CAR-T clinical trial was shown in Fig. 2A. The therapeutic responses at 1, 2, 3, 6, 9, and 12 months after infusion were assessed by bone marrow morphology and multiple flow cytometry analysis. Detailed patient characteristics and disease status prior to CAR-T therapy were summarized in Table 1. The median AML tumor burden prior to pre-conditioning was 46.2% (range 14.2% to 92.4%). The CLL1 expression on AML blasts were determined by flow cytometry using anti-CLL1 antibody. The median CLL1 positivity in AML blasts was 88.5% (range 65.0% to 96.0%) among these patients (Table 1). As shown in Table 2, patients 1, 3, 5–8 received 1 million anti-CLL1 CAR-T cells per kilogram, patient 4 received 0.8 million anti-CLL1 CAR-T cells per kilogram. However, due to in vitro inefficient CAR-T cell expansion, patient 2 only received 0.35 million anti-CLL1 CAR-T cells per kilogram. To identify the kinetics of CAR-T cells in patients, we performed flow cytometry analysis to monitor the CAR$^+$ T cell expansion. The CAR-T cells were efficiently expanded in the first month after infusion, with the average peak time varied from day 10 to 12.
CAR-T cell infusion according to the Common Terminology Criteria (Supplementary Fig. 4). and sustained in vivo for varied time in different individuals (Supplementary Fig. 4).

Adverse events (AEs) and clinical outcomes were monitored from the starting point of lymphodepletion until 60 days after CAR-T cell infusion according to the Common Terminology Criteria for Adverse Events (CTCAE), version 5.0. As summarized in Table 3, all anti-CLL1-based CAR-T cells treated patients experienced grade 1–2 CRS in the first month. Satisfyingly, no immune effector cell-associated neurotoxicity syndrome (ICANS) was recorded among these patients. To further confirm the CRS/ICANS, we had continuously monitored the pro-inflammatory cytokine IL-6 release. As shown in Supplementary Fig. 5, the serum IL-6 levels significantly increased in the first month post CAR-T infusion, which was consistent with the occurrence of CRS. Next, the toxicity profiles of the CAR-T cell infusion were evaluated according to the criteria of CTCAE 5.0. Notably, treatment of anti-CLL1 CAR-T cells had no observable toxicity on all the organs according to the criteria of CTCAE 5.0. Notably, treatment of anti-CLL1 CAR-T cells [24, 26].

In addition, patients’ hematologic responses before and after CAR-T cell treatments were tightly monitored. Of note, significant cytopenia was observed in these pediatric AML patients before anti-CLL1 CAR-T treatment (Table 4). Among these patients, patient 2, 3, 6, and 8 did not receive allo-HSCT. Notably, patients 5 and 6 showed recovery of neutrophil and platelet counts on day 60 post CAR-T treatment. Patient 6 showed increased neutrophil count on day 20. The median time from CAR-T treatment to allo-HSCT of these patients was 44 days (range 28–82 days). All together, these observations indicated the myelosuppression effect of anti-CLL1 CAR-T cells on these patients, and suggested that bridging to allo-HSCT after anti-CLL1 CAR-T treatment might be beneficial to the R/R AML patients.

We next assessed the efficacy profiles in these 8 patients with R/R-AML one month after anti-CLL1 CAR-T treatment. As shown in Fig. 2B and summarized in Table 2, morphologic leukemia-free state (MLFS) and MRD negativity were achieved in 4 patients (patient 1, 2, 3, and 8), CRi and MRD positivity were achieved in patient 3, MLFS with MRD positivity were achieved in patient 7, partial remission (PR) was achieved in patient 4, and patient 6 remained at stable disease (SD) status until day 21 after anti-CLL1 CAR-T therapy. To define the long-term effect of anti-CLL1-based CAR-T therapy, we performed a long-term follow-up study among these patients. Among these R/R AML patients, 6 patients (patient 1, 2, 3, 4, 7 and 8) completed allo-HSCT (Fig. 2B and Table 2) after CAR-T treatment. The patient 2 relapsed two months post HSCT and died of GVHD. The patient 4 relapsed six months post HSCT and died of PD. The remaining 4 patients (patient 1, 3, 7 and 8) were still alive and remained complete remission, with the longest 26 months’ follow-up visit. The patient 5 remained at CR status for twelve months without receiving allo-HSCT before relapse. In patient 6, who was at SD status after CAR-T infusion, the percentage of AML blasts decreased from 91.3% to 84.6% two weeks after CAR-T cell therapy, while the remaining AML blasts were CLL1 negative and CD33 positive (Supplementary Fig. 6), then the patient started new chemotherapy on day 21. The patient 6 succumbed to disease progression and lung infection 2 months after CAR-T therapy.

To summarize the interim analysis of the phase 1/2 clinical trial, the anti-CLL1 CAR-T cells displayed an ideal targeting capacity for treating the pediatric R/R AML patients, and should be considered as an alternative strategy for the treatment of AML in the future.

**DISCUSSION**

The application of CD19-directed CAR-T cell therapy has achieved great success for the cure of relapsed or refractory B-cell malignancies (i.e., B-cell acute lymphoblastic leukemia, B-cell lymphoma), also ignited the hope of the treatment of other hematological malignancies. However, the translation of CAR-T cells into AML treatment remains lagged behind, which resulting in immediate reliance on intensified chemotherapy and allo-HSCT. Here, we reported the safety and efficacy profiles of anti-CLL1-based CAR-T cell therapy in eight children with R/R-AML from two independent medical centers.

Up to now, CAR-T cells targeting CLL1, CD13, CD33, TIM3, NK2D, CD123, CD7, NPM1, and FLT3 have been extensively studied and shown to effectively eradicate AML cells in vitro and
**Table 2. Patients’ responses to anti-CLL1 CAR-T cells.**

| Patient # | Sex/Age | Product | Product CAR% | CAR-T Dosage | Best response within 1 month | Bridged HSCT post CAR-T | Current status |
|-----------|---------|---------|--------------|--------------|----------------------------|------------------------|---------------|
| 1         | Male/16Y| CLL1    | 43.2%        | $1 \times 10^6$/kg | MLFS/MRD- | on Day 42 | CR/MRD- |
| 2         | Male/11Y| CLL1    | 12%          | $0.35 \times 10^6$/kg | * | MLFS/MRD- | on Day 46 | Death (GVHD) |
| 3         | Male/13Y| CLL1    | 31.3%        | $1 \times 10^6$/kg | CRI/MRD+ | on Day 75 | CR/MRD- |
| 4         | Female/12Y| CLL1 | 42.9%       | $0.8 \times 10^6$/kg | PR | on Day 38 | Death (PD) |
| 5         | Male/8Y | CLL1    | 40.8%        | $1 \times 10^6$/kg | MLFS/MRD- | No HSCT | Relapsed |
| 6         | Female/13Y| CLL1 | 39.5%       | $1 \times 10^6$/kg | SD | No HSCT | Death (PD) |
| 7         | Male/12Y| CLL1    | 67.6%        | $1 \times 10^6$/kg | MLFS/MRD+ | on Day 82 | CR/MRD- |
| 8         | Male/9Y | CLL1    | 60.7%        | $1 \times 10^6$/kg | MLFS/MRD- | on Day 28 | CR/MRD- |

*CAR-T cells from patient 2 did not expand efficiently as those from other patients. A reduced dosage of CAR-T cells was injected.

Fig. 2 Anti-CLL1 CAR-T induced complete remission in AML patients. A Schematic of the Phase I/II clinical trial design. B Duration of the response and overall survival after the infusion of anti-CLL1-based CAR-T cells. 6 out of 8 patients received allo-HSCT after CAR-T cell infusion. Patient 2 died from GVHD after second HSCT. Patient 4 relapsed and died of PD post HSCT. For the other 2 patients who did not receive allo-HSCT, patient 5 relapsed after twelve months of complete remission, patient 6 died from disease progression and uncontrolled lung infection. CRI/MLFS complete remission/complete remission with incomplete hematologic recovery/morphologic leukemia-free state, MRD minimal residual disease, PR partial remission, SD stable disease.
in vivo [30]. However, the number of successful case reports using CAR-T cells in treating AML patients is limited [31]. For example, NKG2D-targeted CAR-T has been reported to induce CR in one AML patient [32]. Nevertheless, this successful case cannot be replicated later in a large cohort of AML patients. In a dose-escalation Phase 1 clinical trial, five AML patients treated with high-dose of anti-CD123 CAR-T cells from Mustang Bio Inc, two patients achieved CR and the other three remained SD [33]. It has been reported that CLL1-CD33 compound CAR-T cells treated 9 AML patients, 7 of them reached CR [34]. In addition, two successful case reports have utilized CLL1 as single target of CAR-T therapy, suggesting the promising potential of targeting CLL1 in AML treatment [24, 26]. Here, our data further support the efficacy of anti-CLL1-based CAR-T cell therapy in children with R/R-AML.

Satisfyingly, there was no neurotoxicity being observed among these patients. With respect to neurotoxicity of CAR-T therapy, the mechanisms underlying remain poorly understood and the clinical presentations of the neurological side effects are variable between patients. Previous clinical reports [35, 36] have shown that neurotoxicity occurs more frequently in patients who have developed severe CRS, and patients with earlier peak of IL-6 might develop more severe neurotoxicity. In the current study, 3 patients developed grade 2 CRS and have been treated with IL-6 antagonist Tocilizumab or Dexamethasone, while other 5 patients developed grade 1 CRS (Table 3). The timely treatment of CRS might reduce the risk of neurotoxicity in these patients. Moreover, as shown in Supplementary Fig. 5, the average peak time of IL-6 serum levels varied from day 4 to 15 post CAR-T treatment. The lagged peaks of serum IL-6 might also contribute to the low occurrence of neurotoxicity in these patients. It is likely that pre-existing neurological issues, tumor burden, or CAR-T cell dosage could affect the neurotoxicity occurrence. Our clinical study is still recruiting patients, and we will report more evidence regarding to the neurotoxicity issues in the near future.

Several studies have shown that the un-biased tumor associated antigens (TAAs) targeting by CAR-T cells also damaged antigen-expressed healthy tissues (albeit at low levels). For example, treatment with anti-CD123, CD33 CAR-T cells generated a potent anti-AML efficacy while causes severe/prolonged myelosuppression [37]. It has been reported that CLL1 is preferentially highly expressed on AML-stem cells but not on normal HSC, which make it a promising target for AML immunotherapy, although it is also expressed on granulocytes and monocytes [38]. In this study, severe myelosuppression was observed in all responding cases. Given that the myelosuppression effect of anti-CLL1 CAR-T therapy might increase the risk of infection, patients were bridged to allo-HSCT post CAR-T treatment to restore their hematologic system once they reached CR/CRi/MLFS status. Moreover, CAR-T induced loss of tumor antigen and expression of immune inhibitory molecules on tumor cells were frequently observed [39, 40], which could attenuate CAR-T cell in vivo expansion and induce CAR-T cell exhaustion, eventually, resulted in high relapse rate. Previous clinical studies on B-ALL patients with CD19 CAR-T therapy have demonstrated that subsequent allo-HSCT could improve the event-free survival (EFS) and reduce relapse rate [41–43]. However, the evidence to show the benefit of allo-HSCT post CAR-T treatment in AML patient is limited. In the current study, we reported that 4 out of 6 patients who received allo-HSCT after anti-CLL1 CAR-T treatment achieved CR. Among all 8 patients, patient 5 and 6 did not receive
Blood cell count of R/R AML patients.

Table 4.

| Patient | Baseline | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|---------|----------|---|---|---|---|---|---|---|---|
| Lymphocytes (× 10⁹/L) | 3.5–9.5 | 1.1–3.2 | 1.8–6.3 | 125–350 | 110–175 | 110–175 | 110–175 | 110–175 | 110–175 |
| Neutrophils (× 10⁹/L) | 23 | 15 | 10 | 62 | 23 | 23 | 23 | 23 | 23 |
| Platelets (× 10⁹/L) | 34 | 13 | 23 | 34 | 13 | 23 | 23 | 23 | 23 |
| Hemoglobin (g/L) | 71.2 | 74 | 82 | 71.2 | 74 | 82 | 71.2 | 74 | 82 |
| Eosinophils (× 10⁹/L) | 0.09 | 0.06 | 0.07 | 0.46 | 0.16 | 0.06 | 0.04 | 0.06 | 0.46 |
| Basophils (× 10⁹/L) | 0.03 | 0.02 | 0.02 | 0.06 | 0.05 | 0.02 | 0.02 | 0.01 | 0.05 |
| Monocytes (× 10⁹/L) | 0.17 | 0.15 | 0.18 | 0.83 | 0.99 | 0.06 | 0.10 | 0.14 | 0.81 |
| Neutrophils (× 10⁹/L) | 0.02 | 0.04 | 0.04 | 4.91 | 0.26 | 0.02 | 0.03 | 0.03 | 4.88 |
| Lymphocytes (× 10⁹/L) | 1.40 | 0.20 | 0.20 | 0.80 | NA | 0.08 | 0.01 | 0.09 | 0.18 |
| Eosinophils (× 10⁹/L) | 0.10 | 0.10 | 0.10 | 1.20 | NA | 0.00 | 0.00 | 0.00 | 0.59 |
| Basophils (× 10⁹/L) | 0.22 | 0.28 | 0.30 | 0.49 | 0.07 | 0.15 | 0.23 | 0.25 | 0.48 |
| Monocytes (× 10⁹/L) | 0.23 | 0.38 | 0.38 | 0.48 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |

*Normal range: white blood cells (3.5–9.5 × 10⁹/L); lymphocytes (1.1–3.2 × 10⁹/L); neutrophils (1.8–6.3 × 10⁹/L); platelets (125–350 × 10⁹/L); Hemoglobin (110–175 g/L).

Notably, the AML blast burden from the current study was relatively high with median of 46.2% (range 14.2% to 92.4%) (Table 1). However, the high AML blast burden did not impact the response of CAR-T therapy. 7 out of 8 patients responded to the anti-CLL1 CAR-T therapy with CRi/MLFS rate of 75%. A recent clinical study [44] of anti-CLL1 CAR-T treatment in adult R/R AML patients has shown that 7 out of 10 patients responded to the study drug, with a CR/Cri rate of 70%. The median disease burden was 15.5% (range 3.1% to 83.6%) in this study, which is much lower than those from the current study. It seemed like that the lower tumor burden did not guarantee with a higher response rate in these patients. In addition, the tumor antigen expression rate and CAR positivity on T cells may also affect the clinical outcomes of CAR-T therapy. However, we did not observe significant differences of the CLL1 expression rate on tumor cells and the CAR transduction efficiency between these two studies [44].

In addition, we observed that patient 6 poorly responded to anti-CLL1 based CAR-T cells therapy, however, the CLL1 positive AML cells were completely abolished while the remaining AML blasts were CLL1 negative (Supplementary Fig. 6). For the patients with similar outcomes as patient 6, combination of a second CAR-T target depending on the AML immunophenotypic characteristics, such as CD33, CD123, Lewis Y, or CD38, might be helpful in the future to improve the CAR-T cell efficacy. How to balance the risk and benefit when using the CAR-T therapy for R/R-AML treatment remains an important issue in the future. To this end, our results suggested that patients with more than 80% of CLL1 positivity on AML blasts may benefit from single anti-CLL1 CAR-T treatment. However, for patients with less than 80% of CLL1 positivity on AML blasts, an additional CAR to target a different tumor antigen may be beneficial to achieve complete deletion of AML cells.

In summary, our findings demonstrate a very encouraging outcome with a safe and manageable profile and high targeting efficacy for the use of anti-CLL1-based CAR-T cells in the treatment of children with R/R-AML. This is the first report of multi-center based clinical trial for application of anti-CLL1 based CAR-T cells in pediatric R/R-AML.

**DATA AVAILABILITY**

All data generated or analyzed during this study are included in this published article and its supplementary information files. Further inquiries can be directed to the corresponding authors.

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AUTHOR CONTRIBUTIONS
The study was conceived by HZ and GL, designed by HZ, CL, and ML supervised by HZ, CL, and ML, performed the research. HZ, CB, ZP, GL and CL performed the research. HZ, CB, ZP, YH, ZH, and ML performed the research. This work was partially funded by research funds from St. Baldrick’s Medical Center Internal Program (IP-2018-001), and Pearl River S&T Nova Program of Guangzhou (201906010056). This work was also partially supported by grant from National Natural Science Foundation of China (82171025).

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COMPETING INTERESTS
GL, ZZ, WD, YZ and ML are employees of Guangzhou Bio-Gene Technology Co., Ltd., who have potential interest, while other authors have nothing to disclose.

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