CRISPR/Cas9-Engineered Universal CD19/CD22 Dual-Targeted CAR-T Cell Therapy for Relapsed/Refractory B-cell Acute Lymphoblastic Leukemia

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ABSTRACT

Purpose: Autologous chimeric antigen receptor T (CAR-T) cell therapy is an effective treatment for relapsed/refractory acute lymphoblastic leukemia (r/r ALL). However, certain characteristics of autologous CAR-T cells can delay treatment availability. Relapse caused by antigen escape after single-targeted CAR-T therapy is another issue. Therefore, we aim to develop CRISPR-edited universal off-the-shelf CD19/CD22 dual-targeted CAR-T cells as a novel therapy for r/r ALL.

Patients and Methods: In this open-label dose-escalation phase I study, universal CD19/CD22-targeting CAR-T cells (CTA101) with a CRISPR/Cas9-disrupted *TRAC* region and *CD52* gene to avoid host immune-mediated rejection were infused in patients with r/r ALL. Safety, efficacy, and CTA101 cellular kinetics were evaluated.

Results: CRISPR/Cas9 technology mediated highly efficient, high-fidelity gene editing and production of universal CAR-T cells. No gene editing–associated genotoxicity or chromosomal translocation was observed. Six patients received CTA101 infusions at doses of 1 (3 patients) and 3 (3 patients) $\times$ 10^6 CAR^+ T cells/kg body weight. Cytokine release syndrome occurred in all patients. No dose-limiting toxicity, GvHD, neurotoxicity, or genome editing–associated adverse events have occurred to date. The complete remission (CR) rate was 83.3% on day 28 after CTA101 infusion. With a median follow-up of 4.3 months, 3 of the 5 patients who achieved CR or CR with incomplete hematologic recovery (CR/CRi) remained minimal residual disease (MRD) negative.

Conclusions: CRISPR/Cas9-engineered universal CD19/CD22 CAR-T cells exhibited a manageable safety profile and prominent antileukemia activity. Universal dual-targeted CAR-T cell therapy may offer an alternative therapy for patients with r/r ALL.

Introduction

Autologous chimeric antigen receptor T (CAR-T) cell therapy has exhibited remarkable efficacy as a treatment for relapsed/refractory acute lymphoblastic leukemia (r/r ALL). CD19 CAR-T cells have been extensively studied and have shown to result in high complete remission (CR) rates of approximately 76%–93% in patients with r/r ALL (1–8). Currently, three kinds of autologous CD19 CAR-T cell products have been approved by the FDA for the treatment of patients with r/r ALL or non–Hodgkin lymphoma. However, the inherent limitations associated with autologous CAR-T cells, including a lengthy waiting period between leukapheresis and CAR-T cell infusion, possible manufacturing failures, low viability and inferior quality of CAR-T cells due to prior chemotherapy, and high cost, can delay the availability of the treatment.

Universal CAR-T (UCAR-T) cells, which are off-the-shelf products, might be an alternative option. Preliminary data from several UCAR-T clinical trials demonstrated the feasibility of this treatment (2, 9, 10). Trials with transcription activator-like effector nuclease (TALEN)-engineered universal CD19-targeted CAR-T cells have indicated that this treatment has an acceptable safety and efficacy profile in patients with r/r ALL (2, 9). Compared with TALEN gene editing technology, CRISPR/Cas9 technology has a simpler design, higher editing efficiency, and wider versatility (11–13). Clinical data from trials of the product generated using CRISPR/Cas9 gene editing technology have demonstrated its safety and feasibility including through single-antigen targeting (CD7) in r/r T cell ALL (10, 14–17).

Antigen escape-mediated relapse is another limitation of CD19-targeted CAR-T therapy. Several possible mechanisms, including early loss of CAR-T cell persistence, CD19 antigen loss/down-regulation, and the preexistence of splice variants of the CD19 molecule have been proposed (18–20). Evidence from preclinical models of solid tumors has shown that dual or multiantigen-targeting CAR-T cells may exhibit synergistic effects, permitting the optimization of response rates compared with those achieved by targeting a single antigen (21, 22). Some studies, using CD19/CD22 bispecific, cocktail, or sequential autologous CAR-T therapy infusion, have demonstrated promising therapeutic efficacy with low $CD19^-$ relapse rate (5, 23–25). Here, we combined these approaches to develop a universal CD19/CD22 dual-targeting CAR-T cell product (CTA101) whose *TRAC* region and *CD52* gene were

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Translational Relevance

Limitations associated with autologous chimeric antigen receptor T (CAR-T) cells, including an undesirable waiting period between leukapheresis and CAR-T cell infusion, possible manufacturing failures, low viability and inferior quality of CAR-T cells due to prior chemotherapy, and high cost, might delay their availability for use in treatment of acute lymphoblastic leukemia. CD19$^-$ relapse after CD19 CAR-T therapy is another limitation. In this study, we developed CRISPR-edited allogeneic off-the-shelf CD19/CD22 dual-targeted CAR-T cells as a treatment strategy for relapsed/refractory acute lymphoblastic leukemia. Potent antileukemia activities were identified, and no CRISPR gene editing–associated genotoxicity, immunogenicity, or GvHD were observed. Moreover, all patients received infusion within 8 days of enrollment. Our data demonstrate that CRISPR-edited allogeneic CD19/CD22 dual-targeted CAR-T cells may improve the availability and accessibility of CAR-T therapy.

disrupted by using CRISPR/Cas9 technology to avoid host immune-mediated CAR-T cell rejection and support alemtuzumab (anti-CD52 antibody)-mediated targeted depletion of patients' autologous T cells, respectively (26). Preclinical data demonstrated the safety of CRISPR gene editing and the efficacy of CTA101. We also conducted a phase I clinical trial of CTA101 in adult patients with r/r ALL (NCT04227015).

Patients and Methods

Construct design and CTA101 generation

A dual-targeted CAR lentiviral construct was developed by incorporating CD19 and CD22 single-chain variable fragments derived from Clone FMC63 and Clone m971, respectively, followed by 4-1BB costimulatory and CD3ζ signaling domains. Lentivirus was produced in 293T cells, cryopreserved at −80°C, and thawed immediately before transduction. CD3$^+$ T cells were isolated and activated by anti-CD3/CD28 Dynabeads (Gibco, catalog no: 40203D). The cells were then cultured in X-VIVO 15 medium (Lonza, catalog no.: 04-418Q) supplemented with 5% CTS Immune Cell SR (Gibco, catalog no.: A2596101) and 100 U/mL IL2 and transduced with lentivirus at a multiplicity of infection of 2, 1 day after stimulation.

The Dynabeads were removed before electroporation of Cas9 (Thermo Fisher Scientific) and single-guide RNA (sgRNA) ribonuclease complex (RNP), which was performed on day 4. Briefly, T cells were washed twice with OPTI-MEM (Gibco, catalog no.: 11058021) and resuspended in OPTI-MEM at a final concentration of 1–3 × 10^8 cells/mL. Subsequently, 0.4 mL of cells was mixed with Cas9 RNP and electroporated in a 4 mm cuvette. Next, 40 μg of Cas9 protein and 40 μg of total sgRNA targeting *TRAC* and *CD52* at an equal molecular ratio (Genescript) were electroporated into the cells using a BTX Agile Pulse at 500 V and 1 ms. Following electroporation, the cells were immediately cultured at 37°C and 5% CO_2 until negative selection was conducted. Residual T-cell receptor (TCR)/CD3$^+$ cells were depleted using CliniMACS (Miltenyi Biotec) on day 8. The enriched TCR-less CTA101 CAR-T cells were expanded for an extra 3 days before cryopreservation in dose aliquots of 50 million cells per bag.

The genomic sgRNA target sequences, with the protospacer adjacent motif in bold, were:

TRAC: 5′-AGAGTCTCTCAGCTGGTACA**CGG**-3′

and

CD52: 5′-TTACCTGTACCATAACCAGG**AGG**-3′.

In vitro cytotoxicity assay

K562-Luci tumor cells were generated and used in a luciferase-based CTL assay. Briefly, firefly luciferase–encoding lentivirus was transduced into K562 tumor cells to generate a parental cell for target cell line preparation. CD19 and CD22 antigens were introduced into K562-Luci cells using lentiviral transduction to generate CD19/CD22 single- and double-expressing cell lines. The resulting target cells were resuspended at 1 × 10^5 cells/mL in X-VIVO 15 medium and incubated with different ratios of T cells (e.g., 30:1, 15:1) overnight at 37°C. Then, 100 μL of the mixture was transferred to a 96-well white luminometer plate. Next, 100 μL of substrate was added, and the luminescence was immediately determined. The results are reported as the percentage of killing based on the luciferase activity in the wells with tumor cells but no T cells [% killing = 100 − ((relative light units (RLU) from well with effector and target cell coculture)/(RLU from well with target cells) × 100)].

Whole-genome sequencing of CTA101

Genomic DNA was extracted from nontransduced, CAR-transduced but nongene-edited, and CAR-transduced and gene-edited CTA101 products using the Fast Pure Cell/Tissue DNA Isolation Mini Kit (Vazyme, catalog no.: DC102-01). A total amount of 1.5 μg DNA per sample was used as input material for the DNA sample preparations. Sequencing libraries were generated using the Truseq Nano DNA HT Sample Preparation Kit (Illumina, catalog no.: FC-121-4003) following the manufacturer's recommendations, and index codes were added to attribute sequences to each sample.

Briefly, the DNA sample was fragmented by sonication to a size of 350 bp, and then the DNA fragments were end-polished, A-tailed, and ligated with the full-length adapter for Illumina sequencing using further PCR amplification. Finally, the PCR products were purified by AMPure XP system (Beckman, catalog no.: A63881), and the libraries were analyzed for size distribution using the Agilent 2100 Bioanalyzer and quantified using real-time PCR. The constructed libraries were sequenced using the Illumina Nova6000 platform, and 150 bp paired-end reads with an insert size of around 350 bp were generated. Potential off-target sites were estimated using Cas-OFFinder for the whole genome. The top 1,000 sites with less than 8 bp mismatches with the sgRNA target sequence were selected for further analysis.

Detection of CTA101 chromosomal translocation

qPCR assays were used to detect the potential occurrence of a chromosomal translocation of *CD52* and *TRAC*. These two translocations were labeled as *TRAC:CD52* and *CD52:TRAC*, respectively. This template contained tandem sequences for the four forward and four reverse primer flanking sequences corresponding to the four probes (Supplementary Table S1). The concentration of the standard plasmid was determined using stock solution, and the copy number was calculated accordingly. The sequences of the primers and probes are shown in Supplementary Table S1. Genomic DNA was extracted using the FastPure Cell/Tissue DNA Isolation Mini Kit. Quantitative real-time PCR was performed, and the gene copy numbers of genomic DNA were calculated according to the standard curve and C_q value.

Residual Cas9 measurement

ELISA was conducted to detect residual Cas9 protein in CTA101, and the results were validated for accuracy, precision, range, and lower limit of quantification. The CRISPR/Cas9 Protein ELISA Kit (NOVATEINBIO, catalog no.: NB-E1372PR) was used for assay development according to the manufacturer's instructions. The Cas9 proteins in the samples were tightly and stably spotted onto the strip wells. The bound Cas9 proteins were then recognized using a detection antibody followed by a color development reagent. The Cas9 ratio was proportional to the absorbance intensity. The absolute amount of Cas9 was quantitated by comparison with the Cas9 control.

Clinical protocol design

A phase I clinical trial of CTA101 in adult patients with r/r ALL was conducted. This clinical trial was designed to assess the safety and efficacy of universal bispecific CD19/CD22 dual-targeted CAR-T cell therapy in patients with r/r ALL. The inclusion criteria were as follows: (i) age of 3 to 70 years, (ii) diagnosis of r/r B-cell ALL (B-ALL), (iii) HLA donor-specific antibody (DSA)-negative status, (iv) Eastern Cooperative Oncology Group performance status of less than 2, (v) estimated survival time longer than 3 months, (vi) good organ function, and (vii) no GvHD.

The protocol was approved by the Institutional Review Board of the First Affiliated Hospital of Zhejiang University School of Medicine (Hangzhou, China). All enrolled patients provided written informed consent. The enrolled patients received a single dose of CTA101 with traditional 3+3 dose escalation (dose level: 1 and 3 $\times$ 10^6 cells/kg). One week before CTA101 infusion, patients received a preconditioning chemotherapy regimen composed of cyclophosphamide, fludarabine, and alemtuzumab (Supplementary Table S2). The primary endpoint was the frequency of dose-limiting toxicity (DLT) and adverse events (AE). The secondary endpoints included overall response rate (ORR), CR rate, overall survival (OS) rate, and cellular peak kinetics.

Assessment of CTA101 expansion and persistence *in vivo*

Serial bone marrow and peripheral blood samples after CTA101 infusion were collected in K_2EDTA BD vacutainer tubes (BD Biosciences). The persistence of CTA101 from fresh peripheral blood and bone marrow was determined using flow cytometry (FCM) and quantitative PCR. Circulating CTA101 numbers per microliter were calculated on the basis of the measured absolute $CD45^+$ T lymphocyte counts. Simultaneously, CAR DNA copies were evaluated as another method of determining CTA101 expansion and persistence. Genomic DNA was extracted using an AxyPrep Blood Genomic DNA Miniprep Kit (Axygen) from cryopreserved peripheral blood and bone marrow. CAR DNA copies were assessed using quantitative real-time PCR as described in the Supplementary Materials and Methods.

Assessment of serum cytokine levels

The levels of the cytokines including IL6, IL8, IL2RA, monocyte chemoattractant protein (MCP)-1, IFNγ, and IL2 in serum were measured in a multiplex format according to the manufacturer's instructions as described in the Supplementary Materials and Methods.

Statistical analysis

The data cut-off date was August 31, 2020. Variables were tabulated and summarized with descriptive statistics. For the time-to-event analyses, Kaplan–Meier curves were constructed. All the *P* values presented are two-tailed. *P* values less than 0.05 were considered statistically significant. Statistical analysis was performed using GraphPad Prism 7.

Results

CTA101 manufacturing

Non-HLA-matched off-the-shelf CTA101 was manufactured from six nonrelated healthy donor T cells under Good Manufacturing Practice conditions, using the electrotransfer of RNPs comprising

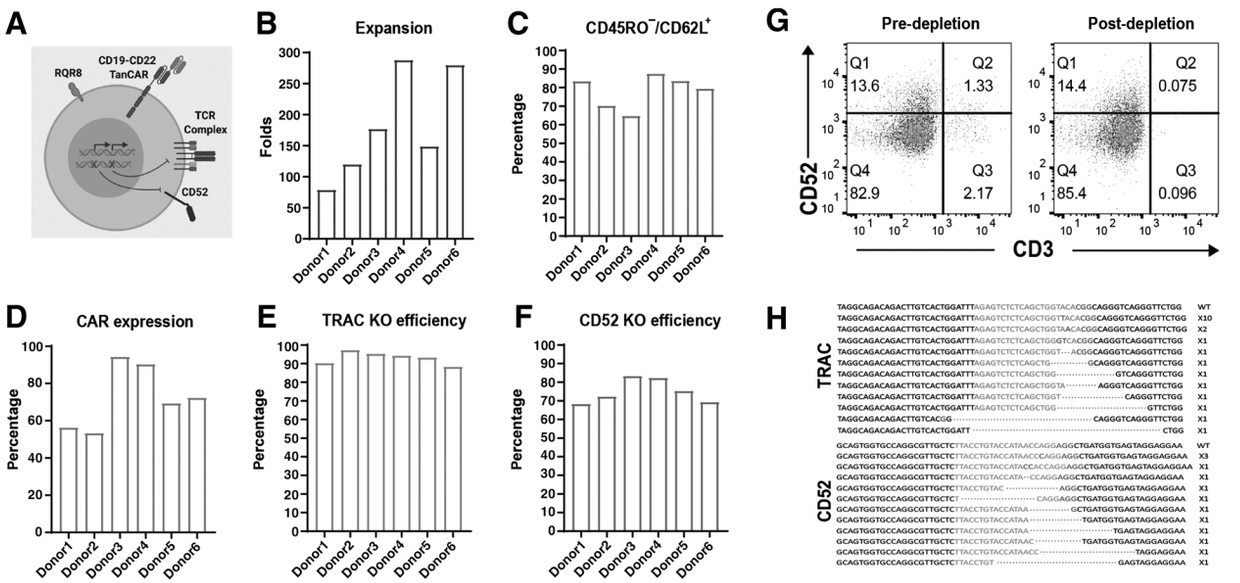

Figure 1.

Characteristic of CTA101. **A,** Schematic illustration of CD19/CD22 dual-targeting CTA101 design. **B–F,** CTA101 was manufactured by electroporation of *TRAC*- and *CD52*-targting Cas9 RNP, followed by lentiviral transduction of the CAR transgene. Expansion, phenotype, CAR expression, and *TRAC* and *CD52* knockout efficiency are shown. **G,** TCR/CD3 and *CD52* expression before and after CliniMACS magnetic bead depletion. **H,** Gene editing events of CTA101 as confirmed by Sanger sequencing.

recombinant Cas9 loaded with equimolar mixtures of *TRAC*- and *CD52*-targeting sgRNA, followed by lentiviral transduction of the CD19-CD22-CAR transgene. A suicide switch, RQR8, composed of a CD34 epitope cell enrichment moiety and CD20 epitope rituximab target moiety, was designed for rituximab-mediated depletion of CTA101 cells in case of adverse effects (**Fig. 1A**; ref. 27).

The CTA101 manufacturing process, including apheresis, T-cell activation, lentiviral transduction, gene knockout, expansion, negative selection, and formulation, was extensively investigated. These steps took around 11 to 13 days, followed by 14 days for release testing. For each healthy donor, one lot of UCAR-T cells were manufactured. All six batches were successfully expanded to above 100-fold by the time of harvest (**Fig. 1B**). Above 60% of the final products exhibited a stem cell memory phenotype as confirmed by $CD45RO^-/CD62L^+$ expression (**Fig. 1C**). CAR expression was assessed using FCM staining for anti-FMC63 antibody, which ranged from approximately 40% to 80% of T cells in the final product (**Fig. 1D**). The frequency of editing determined using FCM was consistently above 85% (88%–97%) for *TRAC* and above 65% (68%–83%) for *CD52* (**Fig. 1E** and **F**). The cells were sorted using CliniMACS magnetic bead–mediated depletion of residual $CD3^+$ cells to diminish the risk of GvHD. Below 1.0% of cells had detectable surface TCR/CD3 expression (**Fig. 1G**). Above 70% of the final products expressed RQR8 as determined by rituximab staining; at the same time, above 50% of the final products exhibited the "ideal" phenotype defined by TCR^-, $CD52^-$, CAR^+, and $RQR8^+$ expression (Supplementary Fig. S1). The results of Sanger sequencing confirmed the presence of on-target edits that were indicative of nonhomologous end joining (insertions, deletions, and indels) at a frequency of > 70% at both the *TRAC* and *CD52* loci (**Fig. 1H**).

CTA101-mediated $CD19^-$ escape mitigation

Thirty samples from adult patients with r/r ALL were analyzed to determine the expression profiles of CD19 and CD22 in leukemia cells. As shown in **Fig. 2A**, CD22 was expressed in 87.0% (32.7%–97.6%) of $CD19^+$ B-ALL and 70.5% (60.0%–98.7%) of $CD19^-$ B-ALL blasts, representing a potential candidate for dual antigen targeted therapy. *In vitro* cytotoxic assays were performed to evaluate the $CD19^-$ tumor relapse prevention capability of CD19/CD22 dual-targeted CTA101 products. CTA101 was cocultured with K562 cells engineered to express CD19, CD22, or both antigens at an effector-to-target ratio of 2:1. Next, the *in vitro* cytotoxicity of the CD19/CD22 mono-specific CAR-T cells and CTA101 was evaluated using a luciferase-based assay. All three CAR-T cells exhibited antigen-specific cytotoxicity, while only CTA101 had an equivalent cytotoxicity among all three target cell lines, indicating that CD19/CD22 dual targeting could mitigate $CD19^-$ escape (**Fig. 2B**; ref. 28).

Gene editing–associated safety assessment of CTA101

Whole-genome sequencing was conducted to assess the on-target and off-target editing events in the UCAR-T cells of the CTA101 final product. The genomic localization of identified DNA cleavage sites occurred as expected, given the chromosomal location of *TRAC* and *CD52* target genes on chromosomes 14 and 1. While most mutations were on target, a few off-target mutations were identified (**Fig. 3A**; Supplementary Fig. S2).

To study the safety and genotoxicity of the multiplex CRISPR/Cas9 genome editing of two chromosomes, qPCR assays were conducted to quantify the potential rearrangements that could occur with the simultaneous editing of *TRAC* and *CD52* loci during the manufacturing process. A positive reference sample from the synthetic template plasmid was evaluated as an assay control. An amplicon flanking the *CD52* genomic target region was used as an internal control. While chromosomal rearrangements were observed in 4% of previously reported TALEN-edited UCART19 cells, no *CD52:TRAC* rearrangements were detected in CTA101. *CD52:TRAC* rearrangement declined in frequency to <0.15% at cell harvest. (**Fig. 3B**). Karyotype abnormalities were not observed in the final products (Supplementary Fig. S3).

To avoid unpredictable immunogenicity of the *Streptococcus pyogenes* Cas9 protein, residual Cas9 proteins were quantified during the manufacturing process. The levels were below 0.195 fg/cell (the detection limit of sensitive ELISA) in the final product (**Fig. 3C**). The products that met the quality standard were transported to the hospital by cold chain in advance and stored in liquid nitrogen prior to patient infusion.

Patient characteristics

From January 2020, to the data cutoff on August 31, 2020, 8 adult patients were screened and 6 were enrolled (**Fig. 4A**). There were two screening failures due to positive DSA and alanine aminotransferase >3.0× upper limit of normal, respectively. All enrolled patients underwent the preconditioning chemotherapy regimen in compliance with the protocol (**Fig. 4B**). All 6 enrolled patients received one infusion of CTA101, with no more than 8 days between enrollment and infusion. The median age of the 6 patients was 49 years old (range, 26–56). The median number of prior lines of therapy was 5 (range, 2–8), and the median marrow blast percentage was 52% (range, 1–82; **Table 1**; Supplementary Table S3). Prior to

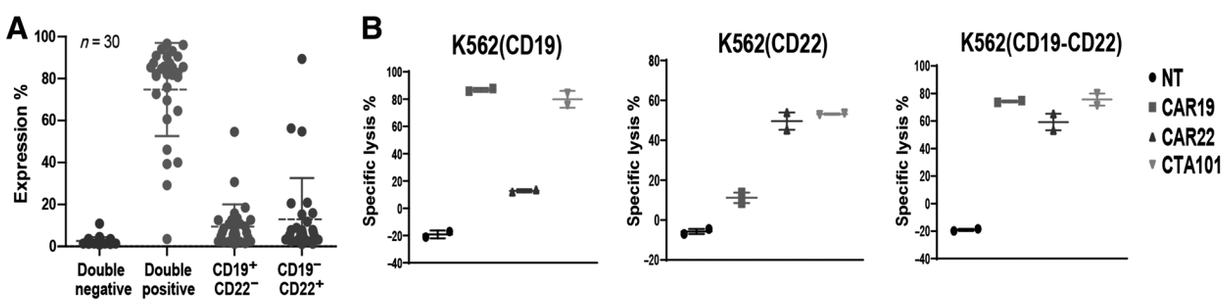

Figure 2.

CTA101-mediated $CD19^-$ escape mitigation. **A,** CD19 and CD22 expression profile in B-ALL patient samples. **B,** Cytotoxicity of CD19/CD22 single-antigen targeting and CTA101 CAR-T cells against CD19/CD22 single- and double-positive tumor cells was measured using a luciferase-based assay. NT, nontransduced T cell.

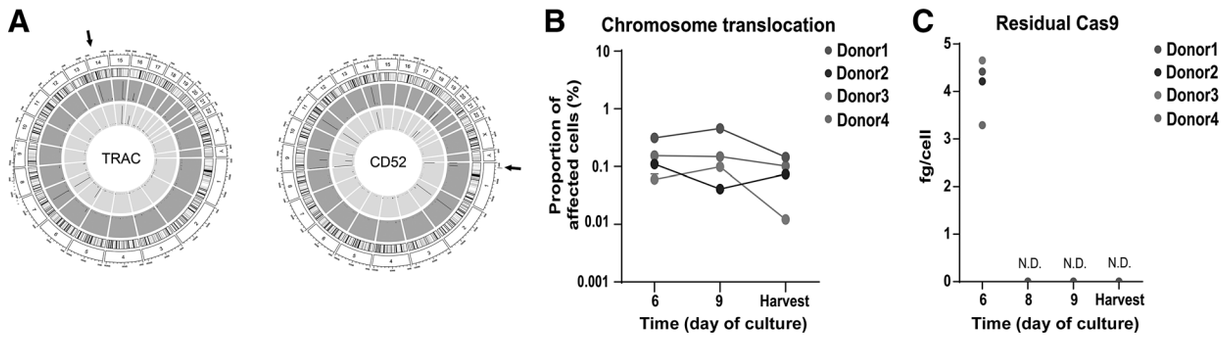

Figure 3.

Gene editing–associated safety assessment of CTA101. **A,** On-target and off-target event analysis of CRISPR-edited CTA101 cells using whole-genome sequencing. Nontransduced T cells were used as a control. Yellow circles represent CAR-transduced T cells, and green circles represent CAR-transduced CRISPR-edited CTA101 T cells. Arrowheads indicate the location of sgRNA. **B,** Chromosomal rearrangements determined using qPCR. *CD52* represents endogenous CD52 genomic sequence control, *TRAC-CD52* represents chromosome translocation between the *TRAC* and *CD52* genomic loci. **C,** Quantification of residual Cas9 protein during the manufacturing process of CTA101 over time is shown. N.D., not detected.

enrollment, 3 patients had hyperleukocytosis and one had $CD3^+$ T lymphocytopenia. One patient relapsed within 3 months of prior autologous CD22-targeted CAR-T therapy, and 3 patients had high-risk genomic lesions including two cases with *BCR-ABL1 T315I* translocation and one case with *BCR-ABL1* translocation. No patients received bridging chemotherapy prior to preconditioning chemotherapy.

CTA101 safety profile

The two earliest batches of UCAR-T products from two independent healthy donors were used for clinical evaluation. Three patients received an infusion of 1×10^6 CAR-T cells/kg (dose level 1), and the other 3 patients received an infusion of 3×10^6 CAR-T cells/kg (dose level 2). All 6 patients were available for the evaluation of safety and efficacy.

No DLTs, GvHD, or immune effector cell–associated neurotoxicity syndrome has occurred to date. Cytokine release syndrome (CRS) occurred in all patients. The median time from infusion to the onset of CRS symptoms was 4 days (range, 1–8), and the median duration was 8 days (range, 5–14). Five of the 6 patients (83.3%) had grade 1–2 CRS. One of the 6 patients (16.7%) had grade 3 CRS with hypoxia and required facemask oxygen supplementation (15 L/minute), and symptoms resolved within 7 days with the administration of one dose of tocilizumab (8 mg/kg) and glucocorticoids.

Three patients (50%) experienced infections with a severity ≥ grade 3, which included cytomegalovirus reactivation/infection (two cases), bacterial pneumonia (one case), and fungal sepsis (one case). Three of the 6 patients (50%) experienced cytopenia lasting beyond day 28 after CTA101 infusion. Patient 6 suffered from cardiac arrest on day 54 after CTA101 infusion and successfully resuscitated but finally died of heart

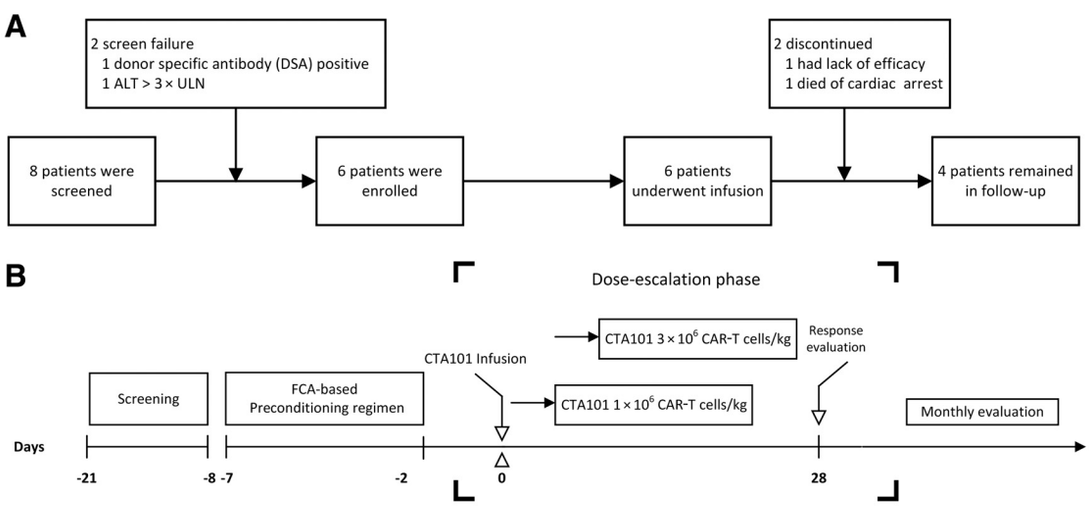

Figure 4.

Clinical trial procedure. **A,** Patient enrollment flow chart. **B,** This phase I open-label dose-escalation study consisted of a 2-week screening period, a 1-week preconditioning regimen phase, a 4-week dose-escalation phase, and a follow-up phase of up to 1 year after CTA101 infusion. During the screening period, before CTA101 infusion, bone marrow examinations were performed. The DLT observation period was 28 days. At the end of the DLT observation period for each cohort of three patients, decisions were made regarding further escalation or deescalation, according to this plan. FCA: fludarabine, cyclophosphamide, alemtuzumab.

Table 1. Patient characteristics and summary of response.

Patient	Age	Previous lines of therapy	Source of PBMC	Phenotype of tumor cell	BM blasts prior to preconditioning therapy, %	Gene fusion	Dose level	CRS grade	Initial response	Follow-up (days)
1[a]	54	6	Donor 1	$CD19^+$ and $CD22^+$	82	BCR-ABL1 T315I	DL1	1	CR, MRD^-	MRD negative, (228)
2	45	2	Donor 1	$CD19^+$ and $CD22^+$	50	—	DL1	1	CR, MRD^-	Underwent haplo-HSCT in remission on D60 (182)
3[a,b]	26	4	Donor 2	$CD19^+$ and $CD22^+$	54	—	DL1	2	CRi, MRD^-	MRD negative, (128)
4[a]	56	3	Donor 2	$CD19^+$ and $CD22^+$	72	—	DL2	3	CRi, MRD^-	Received salvage chemotherapy due to primary disease recurrence (95)
5	40	8	Donor 2	$CD19^+$ and $CD22^+$	4	BCR-ABL1	DL2	1	NR	Received salvage chemotherapy on day 35 (94)
6	53	6	Donor 1	$CD19^+$ and $CD22^+$	1	BCR-ABL1 T315I	DL2	2	CRi, MRD^-	Death (57)

Abbreviations: BM, bone marrow; PBMC, peripheral blood mononuclear cell.
[a]Hyperleukocytosis prior to enrollment.
[b]Relapsed within 3 months following prior autologous CD22-targeted CAR-T.

failure. Details of AEs suspected to be related to CTA101 treatment within 8 weeks are shown in Supplementary Table S4. No replication competent lentivirus was detected, and no CRISPR/Cas9 genome editing–associated AEs occurred.

Marked elevations in several serum cytokines, especially IL6, IL8, IL2RA, MCP-1, and IFNγ in patients with CR or CR with incomplete hematologic recovery (CR/CRi) were observed. Most of T_{max} of these cytokines occurred on day 7. The maximum level of IL6 was approximately 3.8-fold higher in patients (3,931 pg/mL) with grade 3 CRS compared with patients (1,009 pg/mL) with grade 1–2 CRS. Details of the kinetics of cytokines within 28 days after CTA101 infusion are shown in Supplementary Fig. S4.

CTA101 efficacy

On day 28 after CTA101 infusion, 5 of the 6 patients (83.3%) achieved CR/CRi, and 5/5 patients (100%) achieved a minimal residual disease (MRD)-negative (sensitivity for negative value < 0.01%) status according to the results of FCM (**Fig. 5A**). As for 3 patients with high-risk genomic lesions, 2 patients achieved MRD-negative CR/CRi, while the other one had no response. With a median follow-up of 4.3 months (range, 2–8 months), 3 of the 5 patients remained MRD negative. Patient 4 was identified MRD-positive CR on day 47 and confirmed $CD19^+/CD22^{dim}$ relapse on day 47 after CTA101 infusion. This patient developed grade 3 CRS and received one dose of tocilizumab (8 mg/kg) and methylprednisolone infusion from day 8 to day 15 (total dose, 1,380 mg). Temperature and IL6 increased significantly on day 7 but dropped rapidly after the use of tocilizumab and methylprednisolone (Supplementary Fig. S5). Patient 2 received myeloablative allogeneic hematopoietic stem cell transplantation (allo-HSCT) in remission on day 60.

CTA101 cellular pharmacokinetics

CTA101 expansion measured using qPCR was observed in all 6 patients and peaked between day 10 and day 14 (**Fig. 5B**). The T_{max} occurred on day 10 in the majority of CR/CRi patients, and on day 14 in the nonresponding patient. The mean peaked expansion was 629,541 copies/μg genomic DNA in CR/CRi patients and 27,347 copies/μg genomic DNA in the nonresponding patient. Expansion measured using FCM was only observed in 5 patients who achieved CR/CRi on day 28 (**Fig. 5C**). The median peaked absolute CAR^+ CTA101 cells count was 166.21 (range, 28.56–2072.37) cells/μL in CR/CRi patients and 0.70 cells/μL in the nonresponding patient. The mean absolute count of $CD3^+$ T cells within 7 days of infusion was 0.04 cells/μL in CR/CRi patients and 2 cells/μL in the nonresponding patient. The median duration of CTA101 persistence measured using qPCR was 42 days after infusion (range, 21–114). The phenomenon, that CAR copy numbers increased significantly on day 7 but dropped rapidly on day 14, was observed in patient 4 who received tocilizumab and high-dose methylprednisolone for CRS.

Discussion

UCAR-T cells modified with gene editing have attracted much attention because of their widespread and prompt availability for patients. Dual-targeted CAR-T cells may reduce the likelihood of single-target loss-induced relapse, similar to the well-established paradigm of multiagent combination chemotherapy regimens. To our knowledge, this is the first CRISPR-edited allogeneic off-the-shelf CD19/CD22 dual-targeted CAR-T cell product to achieve CR in patients with B-ALL. No CRISPR gene editing–associated genotoxicity, immunogenicity, or GvHD were observed, demonstrating the

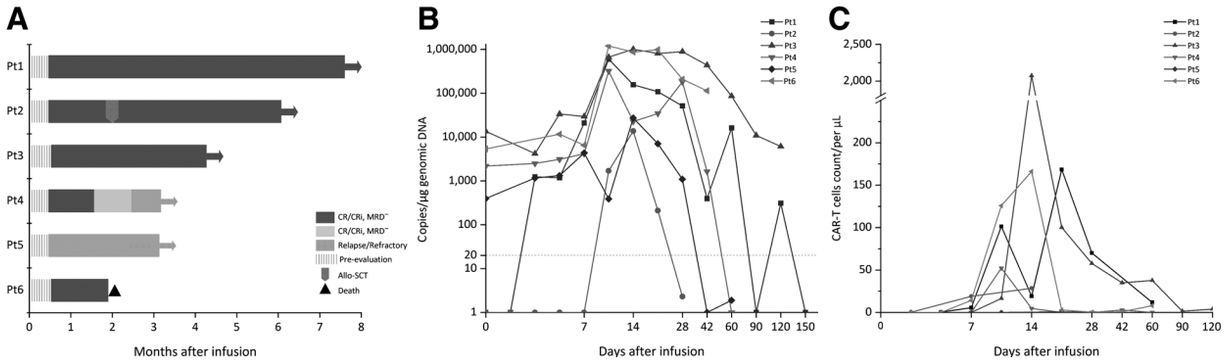

Figure 5.

Clinical response and cellular pharmacokinetics of 6 patients with r/r ALL. **A,** Clinical outcomes of the 6 patients. **B,** Peak levels and persistence of CTA101 in peripheral blood measured using qPCR. The horizontal line at 20 copies/μg genomic DNA represents the lower limit of quantification of the assay. **C,** Absolute count of CTA101 cells in peripheral blood measured using flow cytometry. Expansion of CTA101 was not observed in patient 5, who did not achieve CR.

safety and feasibility of CRISPR/Cas9 multiplex genome-edited allogeneic T cells for the treatment of r/r hematologic malignancies.

Although substantial chromosomal translocations had been reported in TALEN-edited UCART19 cells (29), no chromosomal translocations were detected in our CRISPR gene-edited CTA101 products. Although no clear conclusion can be drawn, the distal positions of the sgRNA *TRAC* and *CD52* targeting sites may disfavor rearrangements. Little genotoxicity was observed in Cas9 RNP-edited CTA101 cells; this might account for the reduction in the culture time required for sufficient product yield. The longer culture time required for TALEN mRNA-mediated gene editing usually leads to senescence and the halting of T-cell proliferation. As a shorter culture time correlated with a younger phenotype, gene editing with Cas9 RNP may surpass TALEN mRNA as the optimal strategy for multiplexed genome-edited T-cell manufacturing.

GvHD is one of the potential risks associated with UCAR-T therapy. Several strategies, such as TCR knockout in UCAR-T cells mediated by different gene editing techniques (2, 10, 29, 30) or the use of CAR-engineered natural killer cells or macrophages that do not cause GvHD (31, 32), have been applied to prevent GvHD. Because of the limited efficiency of current gene-editing technology, TCR cannot be knocked out thoroughly. Consequently, the final UCAR-T products contain a small number of TCRαβ-T cells, usually less than 1% of the total UCAR-T cells (29). One study, which enrolled 20 patients with r/r ALL, showed that 2 patients experienced acute skin GvHD (both grade 1; ref. 2). No GvHD was observed in two other studies that enrolled 5 patients with r/r T cell ALL and 12 patients with r/r large cell or follicular lymphoma, respectively (9, 10). In the present study, no GvHD has occurred to date. Owing to the small sample size and short follow-up duration of these studies, it is currently difficult to evaluate the risks of GvHD caused by residual UCAR-TCRαβ T cells.

Because of the apheresis procedure, manufacturing, and logistics after enrollment, the median waiting period for infusion is usually 2 to 5 weeks for autologous CAR-T therapy (3, 33–36). Up to 20% of enrolled patients might fail to receive infusion due to disease progression, apheresis or manufacturing failures, or other reasons. Patients who have a high tumor burden prior to apheresis, such as in leukocytosis, are at risk of disease progression or severe morbidity and mortality caused by leukostasis and tumor lysis syndrome during the time-consuming process of manufacturing autologous CAR-T cells (1). In light of the possible negative impact of high tumor burden on CAR-T cell manufacturing and clinical efficacy (37), patients with high tumor burden prior to apheresis may fail to receive or benefit from autologous CAR-T therapy. In this study, CTA101 was transported to the site and cryopreserved in liquid nitrogen after being authorized by an ethics committee. All patients enrolled successfully received infusions within 8 days. UCAR-T therapy with a much shorter waiting period for infusion and without the risks of manufacturing failures might decrease the drop-out rate prior to infusion. Three patients with leukocytosis and 1 patient with lymphopenia prior to enrollment, for whom autologous CAR-T therapy was not suitable, received CTA101 successfully, demonstrating that CTA101 could be available to more patients with leukemia.

CAR-T cells targeting CD19 achieved remarkable success in B-ALL, yet relapse remains an enormous challenge in CAR-T therapy. The long-term persistence of CAR-T and prolonged B-cell aplasia were associated with long-term remission after CD19-targeted autologous CAR-T therapy (3, 7), and high antileukemia potency of CAR-T cell with short-term persistence might also achieve long-term remission (8). However, the challenge of $CD19^-$ relapse needs to be resolved, accounting for up to 39% of patients after CD19-targeted CAR-T therapy (38). To overcome this challenge, other B-lineage markers have been proposed as candidate targets. CD22 expression in B-ALL is high, ranging from 50% to 100% (39). Fry and colleagues reported that CD22-targeted CAR-T therapy could induce remission in patients with r/r ALL with $CD19^-$ or CD19-dim relapse after CD19-targeted CAR-T therapy (28).

The majority of patients enrolled in this study had heavily pretreated disease, and one experienced relapse within 3 months of prior autologous CD22-targeted CAR-T cell therapy. The ORR was 83.3% on day 28 after infusion, and all patients who had response achieved MRD-negative remission. Preliminary data demonstrated that CTA101 had potent antileukemia properties. The tumor cell phenotypes of the patient receiving CD22-targeted CAR-T therapy priorly was $CD19^+$ and $CD22^+$, and the patient achieved MRD-negative CR/CRi on day 28 after CTA101 infusion. This indicates that CTA101 can be effectively applied to patients relapsed with negative or low levels of CD19 or CD22 expression. Because of the short follow-up time, the risk of recurrence needs further evaluation.

To decrease the risk of relapse, bridging to HSCT after CAR-T cell therapy was also applied in ALL therapy. The necessity of transplantation after CAR-T therapy is still controversial (1, 8, 33). Previous

autologous CAR-T therapy in our site demonstrated that bridging to HSCT promoted long-term remission (1). Considering the relative short duration of CTA101 persistence, and patients enrolled who had high tumor burden and were more refractory, HSCT after CTA101 infusion was adopted in this study. HSCT was also suggested for patients with delayed hematopoietic recovery after CTA101 infusion. Patient 2 received haplo-HSCT in remission on day 60 and was MRD negative to date. Patient 3 is supposed to receive allo-HSCT in the following days. The influence of CTA101 infusion on the occurrence and severity of AEs associated with the subsequent HSCT needs to be verified.

The causes of cardiac arrest in patient 6 remains unknown. The patient experienced grade 2 CRS and recovered within 21 days of infusion. Fungal sepsis was observed on day 25. After 2 weeks of antimicrobial therapy, the patient's condition improved significantly. Other causes such as electrolyte disturbances and drug-induced cardiac arrest were not identified.

It is also worth noting that patient 5, without achieving CR/CRi, had a rather low peak expansion of CTA101 as measured using qPCR, while not detected using FCM. A similar paradoxical phenomenon has also been observed in the nonresponding patient who received autologous CAR-T therapy. A lack of expansion of functional CAR-T cells was attributed to this phenomenon and to poor disease control in these patients. We observed that many more $CD3^+$ T cells appeared in the nonresponding patient than in CR/CRi patients within 7 days of infusion. Rejection mediated by those cells was also considered to be a factor of expansion and treatment failure. The reason for the premature appearance of excessive $CD3^+$ T cells has yet to be identified. Therefore, there is a need for further investigation of the preconditioning regimen and tumor burden, and further analysis of the phenotype of residual $CD3^+$ T cells.

In conclusion, CTA101 exhibited a manageable safety profile and prominent antileukemia activity, especially for patients that were ineligible for autologous CAR-T cell therapy. Moreover, ready-to-use CTA101 therapeutic CAR-T cells may be obtained off the shelf at a low cost. In addition to their potentially widespread applicability, these universal bispecific CAR-T cells have the potential to target CD19 or CD22 antigens without the need to produce reedited T cells. Further multicenter phase II clinical trials are required to establish universal CD19/CD22 dual-targeted CAR-T cell immunotherapy as a standard treatment strategy for patients with r/r ALL.

Authors' Disclosures

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Authors' Contributions

Y. Hu: Conceptualization, resources, methodology, writing–original draft, project administration, writing–review and editing. **Y. Zhou:** Resources, data curation, methodology. **M. Zhang:** Resources, data curation, validation. **W. Ge:** Methodology, writing–original draft, writing–review and editing. **Y. Li:** Resources, methodology. **L. Yang:** Resources, validation, methodology. **G. Wei:** Software. **L. Han:** Validation, writing–original draft. **H. Wang:** Resources. **S. Yu:** Resources, software. **Y. Chen:** Resources, software. **Y. Wang:** Resources. **X. He:** Resources. **X. Zhang:** Resources. **M. Gao:** Resources. **J. Yang:** Data curation, validation. **X. Li:** Validation. **J. Ren:** Conceptualization. **H. Huang:** Conceptualization, supervision, project administration.

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