Apoptosis of CD19+ chimeric antigen receptor T cells after treatment with chemotherapeutic agents

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Abstract. The use of chemotherapeutic agents prior to treatment with infusion of cluster of differentiation (CD)19-chimeric antigen receptor (CAR)-T cells is important for the efficacy of clinical therapies against hematological malignancies. However, the effect of chemotherapeutic agents on CD19-CAR-T cells and the associated underlying mechanisms remain unknown. The first aim of the present study was to determine the effect of chemotherapeutic agents on CAR-T cells using the *in vitro* Cell Counting kit 8 assay. The second aim was to evaluate the abilities of fludarabine (FDR) and mafosfamide (MFA; a metabolite of cyclophosphamide) to induce apoptosis of CD19-CAR-T cells via the use of Annexin V/propidium iodide double staining. In addition, a JC-1 fluorescent probe was used to detect alterations in cell membrane potential, and flow cytometry analysis was used to measure concentrations of caspase-3/7 to identify apoptotic pathways of CD19-CAR-T cells. The data of the present study suggested that FDR and MFA inhibit the activities of CD19-CAR-T cells. Alterations to the mitochondrial membrane potential and an increase in the concentration of caspase-3/7 indicated early apoptosis of FDR- and MFA-treated CD19-CAR-T cells. The present study laid a theoretical foundation for the development of programs for clinical treatment.

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Abbreviations: FDR, fludarabine; MFA, mafosfamide; CAR, chimeric antigen receptor; CCK-8, Cell Counting kit 8; OD, optical density; FITC, fluorescein isothiocyanate; CD19, cluster of differentiation 19

Introduction

Chimeric antigen receptor (CAR) T cells contain an antibody-derived target region and a single chain fragment variable, which is an antibody-derived target region infused to the membrane of T cells to control signaling. They are divided into four types according to their structure and activity. CAR-T cells are cellular immunotherapies that have been studied internationally for nearly 20 years, although the use of these engineered cells only began to be widespread in recently. At the 2017 ASH Meeting on Hematologic Malignancies (1), the exceptional performance of CD19-CAR-T cells in the treatment of hematologic malignancies was recognized. However, as suitable targets for CAR-T solid tumors are often expressed in healthy tissues leading to CAR-T cells attacking normal cells their use is limited and the prospects for CAR-T therapy for solid tumors are not good. The use of the cluster of differentiation (CD)19 antigen has led to the emergence of several associated complications, such as low gamma globulin hematic disease, cytokine storm and off-target effects (2). Hence, there is a need to explore more effective regimens of existing treatments to reduce such complications. Certain studies have focused on the incorporation of suicide genes into fourth generation CAR-T cells to stimulate apoptosis of target cells; however, complications have still been experienced (3,4). Chemotherapeutic agents have many useful clinical applications, although the exact effects of these on CAR-T cells remain unknown. Therefore, the aims of the present study were to determine the effect of chemotherapeutic agents on CAR-T cells using the *in vitro* Cell Counting kit-8 (CCK-8) assay and to evaluate the abilities of fludarabine (FDR) and mafosfamide (MFA) to induce apoptosis of CD19-CAR-T cells through the use of Annexin V/propidium iodide double staining, a JC-1 fluorescent probe for detection of alterations in cell membrane potential and flow cytometric analysis to assess concentrations of caspase-3/7 to identify the apoptotic signaling pathways of CD19-CAR-T cells. Since CD19-CAR-T cells have demonstrated excellent response rates in patients with acute lymphoblastic leukemia, a common hematological disease (5-8), CD19-CAR-T cells were used in the present study.

Materials and methods

Treatment of CD19-CAR-T cells with chemotherapeutic agents. CD19-CAR-T cells were donated by Biothera Pharmaceuticals, Inc. (Eagan, MN, USA) and cultured in serum-free primary cell culture medium (Hangzhou Union Biotechnology Co., Ltd., Guangzhou, China) at 37°C and 5% CO_2. CD19-CAR-T cells were cultured at a concentration of $2x10^5$ cells in 90 μl immune cell serum-free medium (Youkang serum free medium; Union Biotechnology Co., Ltd., Hangzhou, China) supplemented with FDR (Genzyme Europe B.V., Naarden, Netherlands) at concentrations of 6.25, 12.5, 25, 50 or 100 μg/ml, or MFA (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) at concentrations of 1.25, 2.5, 5, 10 or 20 μg/ml for 24, 48, 72 or 96 h at room temperature. Each sample was prepared in triplicate. Serum-free medium (10 μl) and 90 μl $2x10^5$ CD19-CAR-T cells in immune cell serum-free medium served as a negative control.

Inhibition of CD19-CAR-T cell viability by the CCK-8 assay. Inhibition of CD19-CAR-T cells incubated with FDR and MFA for 24, 48, 72 and 96 h were tested using a CCK-8 assay (Biyuntian Biological Engineering Co., Ltd., Shanghai, China), according to the manufacturer's protocol. At each time point, each concentration was distributed among 3 wells; normal, control and blank control wells. The normal well received cells, culture medium and chemotherapeutic agents (A dosing group). The control well received cells and culture medium (A0 dosing group). The blank control well received culture medium (A blank group). After culture for 24, 48, 72 and 96 h, 10 μl CCK-8 solution was removed from each well and incubated at 37°C and 5% CO_2 for 2 h, and the optical density (OD) was measured using a SpectraMax M Series Multi-Mode Microplate reader (Molecular Devices, LLC, Sunnyvale, CA, USA) at 450 nm wavelength. The % cell viability was calculated as (the OD value of the A0 dosing group - the OD value of the A dosing group)/(the OD value of the A0 dosing group - the OD value of the A blank dosing group) x100%.

Annexin V/propidium iodide, caspase-3/7 and mitochondrial membrane potential analysis of CD19-CAR-T cells by flow cytometry. CD19-CAR-T cells were cultured in serum-free medium (Youkang serum free medium; Union Biotechnology Co., Ltd., Hangzhou, China) and stimulated with 2% interleukin-2 (Novoprotein Biotechnology Co., Ltd., Shanghai, China) every 2-3 days until the cell concentration reached $2x10^5$ cells per 90 μl. Then, FDR (12.5 μg/ml) and MFA (10 μg/ml) were added to the cultures, for 12, 24 or 48 h and divided into normal and control groups. CD19-CAR-T cells were cultured in serum-free medium (normal group). Annexin V positive and propidium iodide positive using a fluorescein isothiocyanate (FITC) Annexin V/Dead Cell Apoptosis kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA) were added to CD19-CAR-T cells (control group). Apoptotic cells were induced by cytokine depletion by washing and suspending cells in serum-free medium three times. Following incubation for 24 h, apoptotic cell death was detected using a CKX41 inverted microscope (Olympus Corporation, Tokyo, Japan) to observe the changes in the shape of cells, and then examined by flow cytometry. Following incubation with FDR and MFA for 24 and 48 h, apoptotic cell death was examined using a phycoerythrin (PE) CellEvent™ Caspase-3/7 Green Flow Cytometry Assay kit (Thermo Fisher Scientific, Inc.). Alternatively, after incubation with FDR and MFA for 12 and 24 h, apoptotic cell death was examined using the MitoProbe™ (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-benz-imidazol-carbocyanine iodide; JC-1) assay kit (Thermo Fisher Scientific, Inc.) by flow cytometry and the ApoAlert reagent kit (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocols. A total of 10,000 events were acquired for each sample treated with FDR and MFA, and analyzed using the FACScan™ automated flow cytometer system (BD Biosciences, Franklin Lakes, NJ, USA) with C6 Plus software version 1.0.264.21 (BD Biosciences) and WinMDI software version 2.7 (kindly made available by Dr Joe Trotter; The Scripps Research Institute, La Jolla, CA).

Statistical analysis. Differences in cell inhibition among the various dosage groups were compared using an analysis of variance with a factorial design and one-way analysis of variance (ANOVA). Differences in apoptosis rates among the groups were compared using a paired t-test, depending on data distribution. Two-way ANOVA followed by the Turkey's post hoc test was used. All analyses were performed using SPSS version 15.0 software SPSS, Inc. (Chicago, IL, USA). $P \leq 0.05$ was considered to indicate a statistically significant difference. All values are expressed as the mean ± standard error.

Results

Inhibition of CD19-CAR-T cells by chemotherapeutic agents. Both FDR and MFA were demonstrated to inhibit the viability of CD19-CAR-T cells. The inhibition ratio increased with increasing drug dosage and incubation time (P<0.05; Fig. 1). With MFA the inhibition ratio increased with increasing drug dosage and incubation time within 72 h (Fig. 2). At 96 h, there was ≥90% inhibition of CD19-CAR T cells by FDR at 1.55 μg/ml. The 50% inhibition ratio (IC_{50}) of CD19-CAR-T cells treated with FDR for 24 h was 1.55 μg/ml (data not shown). At a dose of 10 μg/ml MFA, the % inhibition was >90% at 72 h; however, the % inhibition was lowest at 96 h. Based on the % inhibition at 72 h, the IC_{50} was 3.34 μg/ml (Fig. 2).

Apoptosis of CD19-CAR-T cells treated with FDR and MFA. Following treatment with FDR and MFA for 24 h, early apoptotic CD19-CAR-T cells assumed irregular shapes and increased cell debris, due to eversion of phosphatidylserine in the cell membrane. The data demonstrated that the number of early apoptotic cells [Annexin V positive, propidium iodide negative, (R2 segment of the plots)] and dead cells [Annexin V positive (R1+R2 sections of the plots)] increased following treatment with FDR and MFA (P<0.05 vs. control group; Fig. 3). There were significant differences between the FDR or MFA-treated, control and normal groups (P<0.05 vs. control group; Fig. 3).

The expression of active caspase-3/7 (M2 gate; Fig. 4), a downstream effector of the apoptotic signaling cascade, was significantly increased in CD19-CAR-T cells after treatment with FDR and MFA (P<0.05 vs. control group; Fig. 4) compared with the control group. Apoptosis of CD19-CAR-T

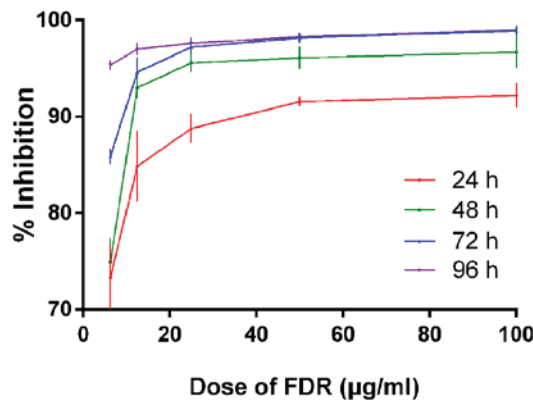Figure 1. Inhibition of CD19-CAR T cell viability by FDR. The doses of FDR were at concentrations of 6.25, 12.5, 25, 50 or 100 µg/ml. The % inhibition was elevated in a dose-dependent manner following treatment with FDR for 24, 48, 72 and 96 h. Data are presented as the mean ± standard error. FDR, fludarabine; CD19, cluster of differentiation; CAR, chimeric antigen receptor.
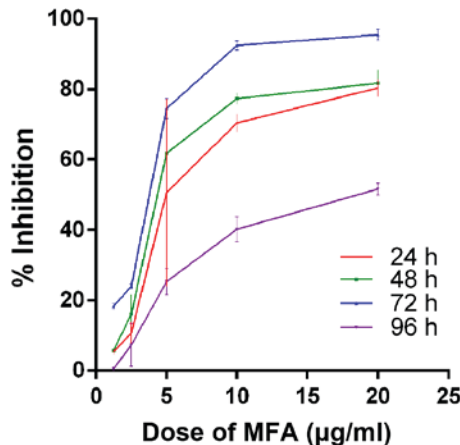

Figure 2. Inhibition of CD19-CAR T cell viability by MFA. The doses of MFA were at concentrations of 1.25, 2.5, 5, 10, or 20 µg/ml. The % inhibition was elevated in a dose-dependent manner after treatment with MFA for 24, 48, 72 and 96 h. Data are presented as the mean ± standard error. MFA, mafosfamide; CD19, cluster of differentiation; CAR, chimeric antigen receptor.
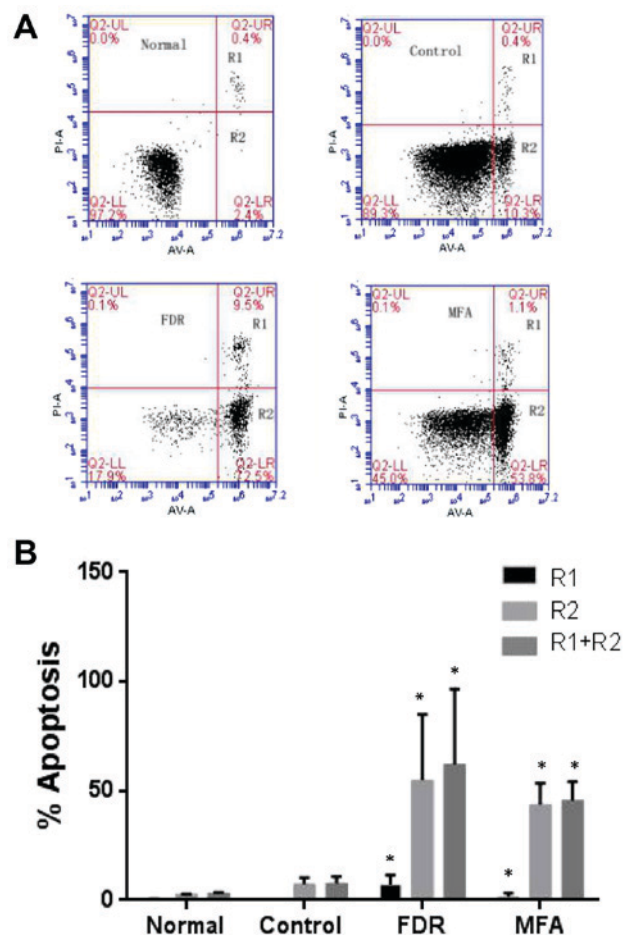

Figure 3. Effect of FDR and MFA on apoptotic CD19-CAR-T cells. (A and B) CD19-CAR-T cells were cultured in serum-free medium (normal group). Apoptosis was induced by cytokine depletion (control group). FDR (12.5 µg/ml) and MFA (10 µg/ml) were added to the cultures for 24 h. The cells were stained with Annexin V-fluorescein isothiocyanate and PI. R2 denotes early apoptotic cells (Annexin V positive and PI negative) and R1+R2 represents total dead cells. The data demonstrated that FDR increased apoptosis and total cell death (*P<0.05 vs. control). MFA significantly induced apoptosis and total cell death (*P<0.05 vs. control). Data are presented as the mean ± standard error (n=3). PI, propidium iodide; MFA, mafosfamide; FDR, fludarabine; CD19, cluster of differentiation; CAR, chimeric antigen receptor.

cells treated with FDR occurred within 48 h, and within 24 h MFA treatment.

Mitochondria with normal membrane potential (Δψm) concentrates JC-1 into aggregates (red fluorescence in FL2), whereas in depolarized mitochondria, JC-1 forms monomers (green fluorescence in FL1). Compared with normal CD19-CAR-T cells, FDR and MFA-treated groups had greater proportions of cells containing JC-1 monomers (R2 gate; green fluorescence), suggesting a drop in Δψm (P<0.05 vs. control group; Fig. 5). Next, CD19-CAR-T cells were incubated with FDR for 24 h, or MFA for 12 h, and the fluorescence of Δψm altered from red to green (P<0.05 vs. control group; Fig. 5). Together, these results suggested that FDR and MFA induced apoptosis of CD19-CAR-T cells.

Discussion

FDR and MFA are chemotherapeutic agents most commonly used as a conditioning regimen prior to acute leukemia therapy. Lukenbill and Kalaycio (9) reported that T cells may

be inhibited by FDR for up to 3 years, with the lowest concentration of T cells observed in the first year, which then recover to a normal level in the third year. Essentially, CD19-CAR-T cells may be inhibited by FDR, as shown in the experiments of the present study where an increase in the inhibition ratio with time was observed. Therefore, when deciding upon a regimen, the time point must be considered both before and after bone marrow transplantation to optimize the effects of CD19-CAR-T cell infusion.

Previous research has indicated that activities of regulatory T cells (Tregs) may be inhibited by administration of cyclophosphamide (10). MFA is an analogue of cyclophosphamide, which is a known inhibitor of T cells (11). The results of the present study revealed that inhibition of cell viability in MFA-treated CD19-CAR-T cells was increased after 72 h. Prior to infusion of CD19-CAR-T cells, the interval time between MFA and CD19-CAR-T cells must be ≥3 days. Therefore, after bone marrow transplantation, MFA may be used to kill CD19-CAR-T cells remaining after 3 days.
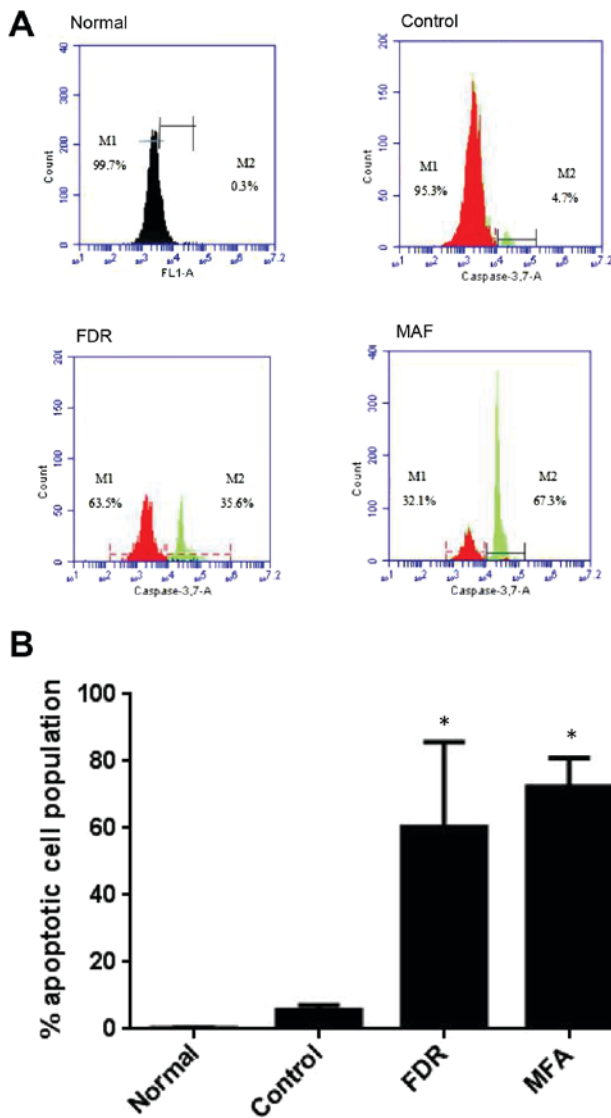
Figure 4. Effect of FDR and MFA on expression of caspase-3/7 in CD19-CAR-T cells. (A) The red fluorescence was produced when the mitochondrial membrane potential was higher, green fluorescence when it was lower. The decrease of cell membrane potential can be detected by the change of caspase-3/7 from red to green fluorescence, which is a marker for early apoptosis. Black means there was no change in mitochondrial membrane potential. In flow cytometry, FL1 and FL2 represent each channel. FL1 corresponds to FITC and FL2 corresponding to PE. CD19-CAR-T cells were stained with caspase-3/7-PE (FL2). (B) The data demonstrated that FDR and MFA significantly increased caspase-3/7 expression (P<0.05 vs. Control; n=3). Data are presented as the mean ± standard error. *P<0.05 vs. Control. MFA, mafosfamide; FDR, fludarabine; CD19, cluster of differentiation; CAR, chimeric antigen receptor; FITC, fluorescein isothiocyanate; PE, phycoerythrin.
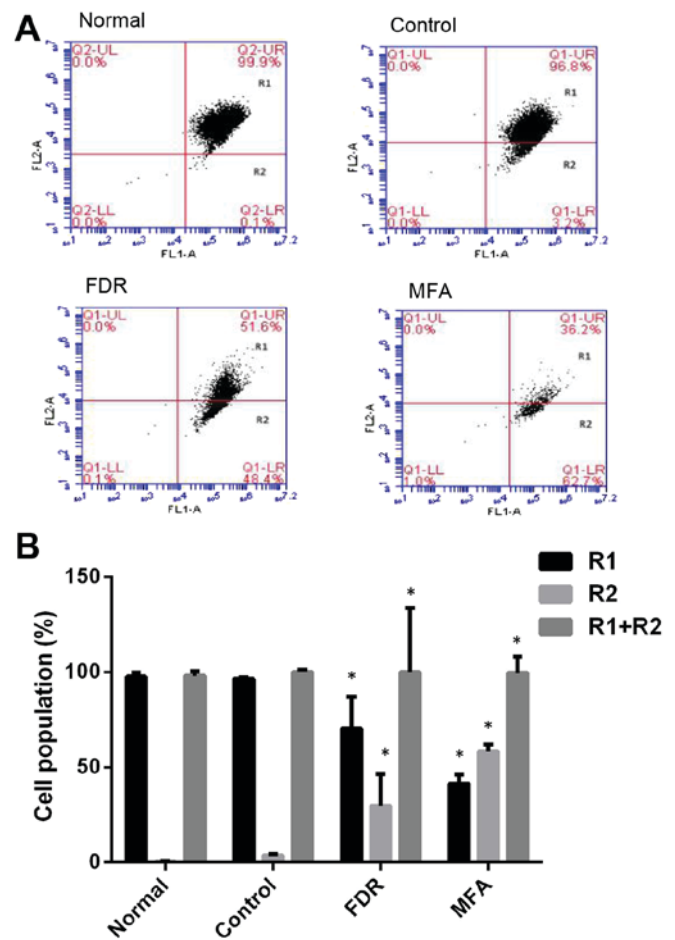

Figure 5. Effects of FDR and MFA on mitochondrial membrane potential of CD19-CAR-T cells. (A) CD19-CAR-T cells were stained with JC-1 reagent. (B) FDR and MFA increased mitochondrial membrane damage (*P<0.05 vs. Control; n=3). Data are presented as the mean ± standard error. MFA, mafosfamide; FDR, fludarabine; CD19, cluster of differentiation; CAR, chimeric antigen receptor.

The pathogenesis of several diseases by detection of apoptosis of peripheral blood leukocytes has been reported in many clinical studies (12-15). At different developmental stages of lymphocytes, the inhibition ratio increases with time (16). The present study is the first to reveal that FDR and MFA induce apoptosis of CD19-CAR-T cells, as confirmed by the eversion of phosphatidylserine in the cell membrane using the Annexin V and propidium iodide apoptosis kit, which is currently a robust method for measuring early apoptosis. In the present study, after the cells were incubated with the two chemotherapeutic agents for 24 h, lipid membrane rollover by phosphatidylserine to the outer membrane was detected by the FITC Annexin V/Dead Cell Apoptosis kit, which suggested apoptosis of CD19-CAR-T cells.

According to the mitochondrial apoptotic pathway theory (17), red fluorescence changing to green fluorescence indicates early apoptosis of CD19-CAR-T cells. The results of the present study revealed that MFA induced apoptosis of CD19-CAR-T cells earlier than FDR. However, these results may have been influenced by the dosage and the different underlying mechanism of actions of the drugs.

A change in caspase-3/7 activity indicates an important role in the mitochondrial apoptotic singling pathway. Activated caspase-3 cleaves the corresponding nuclear cytoplasmic substrates, resulting in apoptosis. Caspase-3 may also activate caspase-7 (18), which further promotes apoptosis (19). The results of the present study showed that caspase-3/7 activity, an indicator of early apoptosis in CD19-CAR-T cells, significantly increased after treatment with FDR and MFA.

In conclusion, this study may be the first to demonstrate that FDR and MFA added separately inhibited the activities of CD19-CAR-T cells, as confirmed by the CCK-8 assay. These findings lay the foundation for future cell immunotherapies and the sequencing of therapeutic regimens. Apoptotic cell death of

CD19-CAR-T cells warrants further research for the advancement of cellular immunotherapies. The present study focused on the cellular level. Future studies should establish animal models with a B-cell malignant cell line to test whether the activities of CD19-CAR-T cells were affected by FDR and MFA treatment.

Availability of data and materials

The datasets generated and/or analyzed during the current study are not publicly available due to ongoing research but are available from the corresponding author on reasonable request.

Authors' contributions

WY was a major contributor in writing the manuscript and the design and performance of the experiments; FP, GL and CZ were responsible for collecting samples; WD was in charge of manufacture and tested the CD19-CART cells; XW, YH, MY and XF designed experiments; HL and ZP wrote the article and analyzed the data; and, CL was responsible for paper guidance, writing of the manuscript and designing the study. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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