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 \textit{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.

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 recent years. 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 hematologic 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 \textit{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% CO₂. CD19-CAR-T cells were cultured at a concentration of 2x10⁵ 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 2x10⁵ 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, culture medium and chemotherapeutic agents (A blank 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% CO₂ 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 2x10⁵ 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 CCKX41 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 phycoerythin (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-benzimidazol-carboxyanine 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 FACScalibur™ 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<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 (IC₅₀) 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 IC₅₀ 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. 4).

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
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.
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.

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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.

References
1. Lee SY, Olsen P, Lee DH, Kenoyer AL, Budde LE, O’Steen S, Green DJ, Heimfeld S, Jensen MC, Riddell SR, et al: Preclinical optimization of a CD20-specific chimeric antigen receptor vector and culture conditions. J Immunother 41: 19-31, 2018.
2. Davila ML and Brentjens R: Chimeric antigen receptor therapy for chronic lymphocytic leukemia: What are the challenges? Hematol Oncol Clin North Am 27: 341-353, 2013.
3. Deniger DC, Switzer K, Mi T, Maiti S, Hurton L, Singh H, Huels H, Olivas S, Lee DA, Chaplin RE and Cooper LJ: Bispecific T-cells expressing polyclonal repertoire of endogenous γδ T-cell receptors and introduced CD19-specific chimeric antigen receptor. Mol Ther 21: 638-647, 2013.
4. Zhou X, Di Stasi A, Tey SK, Krance RA, Martinez C, Leung KS, Durett AG, Wu MF, Liu H, Leen AM, et al: Long-term outcome after haploidentical stem cell transplant and infusion of T cells expressing the inducible caspase 9 safety transgene. Blood 123: 3893-3905, 2014.
5. Kochenderfer JN, Dudley ME, Feldman SA, Wilson WH, Spaner DE, Marcic I, Stetler-Stevenson M, Phan GQ, Hughes MS, Sherry RM, et al: B-cell depletion and remissions of malignancy along with cytokine-associated toxicity in a clinical trial of anti-CD19 chimeric-antigen-receptor-transduced T cells. Blood 119: 2709-2720, 2012.
6. Hao W, Lee DW, Shah NN, Stetler-Stevenson M, Yuan CM, Pastan IH, Dimitrov DS, Morgan RA, Fitzgerald DJ, Barrett DM, et al: Anti-CD22-chimeric antigen receptors targeting B-cell precursor acute lymphoblastic leukemia. Blood 121: 1165-1174, 2013.
7. Porter DL, Levine BL, Kalos M, Bagg A and June CH: Chimeric antigen receptor-modified T cells in chronic lymphoid leukemia. N Engl J Med 365: 725-733, 2011.
8. Grupp SA, Kalos M, Barrett D, Aplenc R, Porter DL, Rheingold SR, Teachey DT, Chew A, Hauck B, Wright JF, et al: Chimeric antigen receptor-modified T cells for acute lymphoid leukemia. N Engl J Med 368: 1509-1518, 2013.
9. Lukenbill J and Kalaycio M: Fludarabine: A review of the clear benefits and potential harms. Leuk Res 37: 986-994, 2013.
10. Kanakry CG, Ganguly S, Zahurak M, Bolaños-Mirande J, Thoburn C, Perkins B, Fuchs EJ, Jones RJ, Hess AD and Luznik L: Aldehyde dehydrogenase expression drives human regulatory T cell resistance to posttransplantation cyclophosphamide. Sci Transl Med 5: 211ra157, 2013.
11. Goldstein M, Roos WP and Kaina B: Apoptotic death induced by the cyclophosphamide analogue mafosfamide inhuman lymphoblastoid cells: Contribution of DNA replication, transcription inhibition and Chk5 signaling. Toxicol Appl Pharmacol 229: 20-32, 2008.
12. Aleman M, Beigier-Bompadre M, Borghetti C, de la Barrera S, Abbate E, Isturiz M and Sasiani MC: Activation of peripheral blood neutrophils from patients with advanced ductal carcinoma in situ. J Exp Med 190: 87-95, 2001.
13. Smith JA and Weidemann MJ: Further characterization of the neutrophil oxidative burst by flow cytometry. J Immunol Methods 162: 261-268, 1993.
14. Jung YJ, Kim YJ, Kim LH, Lee SO, Park BL, Shin HD and Lee HS: Putative association of Fas and FasL gene polymorphisms with clinical outcomes of hepatitis B virus infection. Interlovirology 50: 369-376, 2007.
15. Maloy KJ, Salaun L, CabiI R, Dougan G, Saunders NJ and Powrie F: CD4+CD25+ T(R) cells suppress innate immune pathology through cytokine-dependent mechanisms. J Exp Med 197: 111-119, 2003.
16. Hua YY, Wang XS, Zhang Y, Yao CG, Zhang XM and Xiong ZA: Intense picosecond pulsed electric fields induce apoptosis through a mitochondrial-mediated pathway in HeLa cells. Mol Med Rep 5: 981-987, 2012.
17. El Kebir D and Filer JG: Role of neutrophil apoptosis in the resolution of inflammation. ScientificWorldJournal 10: 1731-1748, 2010.
18. Stanczyk J, Ospelt C, Gay RE and Gay S: Synovial cell activation. Curr Opin Rheumatol 18: 262-267, 2006.
19. Zhang H, Gao G, Clayburne G and Schumacher HR: Elimination of rheumatoid synovium in situ using a Fas ligand ‘gene scalpel’. Arthritis Res Ther 7: R1235-R1243, 2005.