IL-6/IFN-γ double knockdown CAR-T cells reduce the release of multiple cytokines from PBMCs in vitro

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1. Introduction

In 1989, the concept of chimeric antigen receptor T (CAR-T) was first proposed to establish adoptive cell immunotherapy with tumor-specific recognition capabilities. The first-generation CAR was designed by simply replacing the extracellular portion of the T cell receptor with a single-chain antibody variable region fragment (scFv) segment of a specific monoclonal antibody. However, the clinical trial results of the first-generation CAR were very disappointing, and the best-performing had only two out of 11 patients with long-term remission. With the deepening of the understanding of immunology, the CD3ζ signal, while inducing the activation and short proliferation of T cells, was found to later induce the energy of T cells. In 1998, two laboratories reported that the signal domain of CD28 could provide auxiliary co-stimulation for the CD3ζ signal. Thus, a second-generation CAR was designed on the basis of the first-generation CAR by introducing the components of the co-stimulating molecules, such as the intracellular signal domain of CD28 or CD137 (4–1BB). For the third-generation CAR, a functional signal domain of two co-stimulating molecules selected from CD27, CD28, 4–1BB, ICOS, and OX40 was introduced. Among them, the combination of CD28 and 4–1BB is the most common. At present, the main clinical trial of CAR involves the second-generation CAR. The efficacy of CAR-T cells has been recognized by the scientific community and regulatory agencies. At present, 3 anti-CD19 CAR-T cell products have been approved by the Food and Drug Administration (FDA). In addition, numerous CD19-targeted CAR-T clinical trials are under way worldwide and have achieved good clinical results.

The side effects and cytotoxicity of anti-CD19 CAR-T therapy coexist with their effectiveness. Since CD19 is chosen as the target, the input anti-CD19 CAR-T cells kill autologous normal B cells together with the tumor cells. On the other hand, in the early stages of CAR-T cell input, due to the massive amplification of T cells and the secretion of large amounts of cytokines when tumors are killed by T cells, CRS occurs, which is mainly characterized by fever, low blood pressure, hypoxia, and significantly upregulated serum expression levels of certain cytokines, including interferon γ (IFN-γ), fractalkine, granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin-5 (IL-5), interleukin-6 (IL-6), Feline McDonough Sarcoma-like tyrosine kinase 3 ligand (Flt-3 L), and interleukin-10 (IL-10). Novartis’ CD19-targeted product, CLT019, alleviated 80% patients, but it also caused CRS in 64% patients. Similarly, KTE-C19, a product used by Kite Pharma in California to treat non-Hodgkin’s lymphoma, caused CRS in 94% patients and two deaths in tests.

In general, high-dose steroid hormones, such as prednisone, can quickly reverse the clinical symptoms of CRS. However, steroids have the risk of reducing the efficacy of CAR-T therapy and increasing the recurrence rate. At present, the best
treatment for CRS is tocilizumab, a blocked monoclonal antibody drug of the interleukin-6 receptor (IL-6 R). The FDA has now officially approved Roche’s tocilizumab (anti-IL-6 R) for the treatment of CRS risks that may arise in CAR-T therapy. However, tocilizumab is expensive and thus a financial burden for patients. If the release of cytokines can be inhibited from the source, the risk of CRS can be greatly reduced and the safety of CAR-T treatment can be enhanced. At the same time, the financial burden of patients can also be reduced.

RNA interference (RNAi) is a gene silencing mechanism at the post-transcriptional level that is common in organisms with strong sequence specificity. Currently, the most commonly used interference in animals is small RNA molecules such as small interfering RNA (siRNA), shairpin RNA (shRNA), and artificial microRNA. Nowadays, oligonucleotide annealing is the main method for constructing shRNA interference expression vectors.

Because IL-6 and IFN-γ are both cytokines that play crucial roles in CRS, we propose a new paradigm in the management of CRS-associated with CAR-T cell therapy via the inhibition of IL-6 and IFN-γ as a preventive strategy. In this study, we disrupted IL-6 and IFN-γ by shRNA interference during the preparation of anti-CD19 CAR-T cells to obtain double knockdown (KD) anti-CD19 CAR-T cells. To verify whether double knockdown CAR-T cells can reduce the CRS response caused by CAR-T cells, modeling CRS in vitro is necessary. Peripheral blood mononuclear cell (PBMC)-culture was used as an in vitro model related to the in vivo features of CAR-T therapy. The IL-6/IFN-γ double KD anti-CD19 CAR-T cells can inhibit the release of multiple cytokines from PBMCs, while maintaining the effectiveness of CAR-T, thereby reducing toxic reactions during anti-CD19 CAR-T cell therapy.

2. Materials and methods

2.1. Structure of CAR

The rat anti-CD19 scfv with the clone number FMC63 was adopted. The sequences of shRNAs targeting IL-6 and IFN-γ were checked on the Sigma-Aldrich China website, and three sequences of each shRNA were selected. Using the traditional cloning-to-vector method, we inserted two IL-6/IFN-γ siRNA sequence-encoding DNA oligonucleotides into the vector. The DNA oligonucleotide contained 21 nucleotide-long forward siRNA sequences that have been successfully connected to its reverse complementary sequences by Ambion scientists using 9-nucleotide spacing sequences TTTACAGAG. Five thymines were added to the 3’ end of the oligonucleotide as a termination signal, and the restriction enzyme cutting site as required was also added while designing. Four primers were designed for each shRNA sequence using the four-primer anneal-synthesis method. The primer sequences were shown in Table 1.

The four designed primers of each shRNA were synthesized by Genewiz Co., Ltd. (Suzhou). The four primers were annealed to form a short segment of DNA.

The anti-CD19 CAR, shIL-6, and shIFN-γ were cloned into the lentivirus vector pH to obtain clones co expressing shIL-6 and/or shIFN-γ (Figure 1a–c).

2.2. Screening and establishment of shIL-6 and shIFN-γ clones

IL-6 and GFP genes were cloned in sequence into the lentivirus vector pH to obtain the pH-IL-6-GFP plasmid (Figure 1d). IFN-γ and GFP genes were cloned in sequence into the lentivirus vector pH to obtain the pH-IFN-γ-GFP plasmid (Figure 1e). GFP is a reporting gene used to detect the efficiency of gene silencing.

| Table 1. The primer sequences of shRNA. |
|----------------------------------------|
| shRNA | Primer sequence |
|-------|----------------|
| shIL-6-1 | shIL-6-1-1st ATGCTTCTCATGAATGCAGTTTTGC |
| shIL-6-1-2 | shIL-6-1-2nd ATAAGCTTCTCATGAATGCAGTTTTGC |
| shIL-6-1-3 | shIL-6-1-3rd GTTCTTCTCATGAATGCAGTTTTGC |
| shIL-6-1-4 | shIL-6-1-4th AAGGAGACAATTTGGCTCTGCGGC |
| shIL-6-2 | shIL-6-2-1st ATTATCCGCTACATCTGAATGTTTTTGC |
| shIL-6-2-2 | shIL-6-2-2nd ATATCCGCTACATCTGAATGGGC |
| shIL-6-2-3 | shIL-6-2-3rd ATTATCCGCTACATCTGAATGGGC |
| shIL-6-2-4 | shIL-6-2-4th ATTATCCGCTACATCTGAATGGGC |
| shIL-6-3 | shIL-6-3-1st ATTATCCGCTACATCTGAATGGGC |
| shIL-6-3-2 | shIL-6-3-2nd ATTATCCGCTACATCTGAATGGGC |
| shIL-6-3-3 | shIL-6-3-3rd ATTATCCGCTACATCTGAATGGGC |
| shIL-6-4 | shIL-6-4-1st ATTATCCGCTACATCTGAATGGGC |
| shIL-6-4-2 | shIL-6-4-2nd ATTATCCGCTACATCTGAATGGGC |
| shIL-6-4-3 | shIL-6-4-3rd ATTATCCGCTACATCTGAATGGGC |
| shIL-6-4-4 | shIL-6-4-4th ATTATCCGCTACATCTGAATGGGC |
| shIFN-1 | shIFN-1-1st ATTATCCGCTACATCTGAATGGGC |
| shIFN-1-2 | shIFN-1-2nd ATTATCCGCTACATCTGAATGGGC |
| shIFN-1-3 | shIFN-1-3rd ATTATCCGCTACATCTGAATGGGC |
| shIFN-1-4 | shIFN-1-4th ATTATCCGCTACATCTGAATGGGC |
| shIFN-2 | shIFN-2-1st ATTATCCGCTACATCTGAATGGGC |
| shIFN-2-2 | shIFN-2-2nd ATTATCCGCTACATCTGAATGGGC |
| shIFN-2-3 | shIFN-2-3rd ATTATCCGCTACATCTGAATGGGC |
| shIFN-2-4 | shIFN-2-4th ATTATCCGCTACATCTGAATGGGC |
| shIFN-3 | shIFN-3-1st ATTATCCGCTACATCTGAATGGGC |
| shIFN-3-2 | shIFN-3-2nd ATTATCCGCTACATCTGAATGGGC |
| shIFN-3-3 | shIFN-3-3rd ATTATCCGCTACATCTGAATGGGC |
| shIFN-3-4 | shIFN-3-4th ATTATCCGCTACATCTGAATGGGC |
Figure 1. Establishment and screening of shIL-6 and shIFN-γ clones. (a-b). Establishment of IL-6 and IFN-γ knockdown (KD) clones. (c). Establishment of the IL-6/IFN-γ double KD clone. d. IL-6 and GFP cloned into the lentivirus vector pHRI in sequence. IL-6 and GFP are fusion proteins. e. IFN-γ and GFP cloned into the lentivirus vector pHRI in sequence. IFN-γ and GFP are fusion proteins. f. The IL-6 KD clones and IL-6/IFN-γ double KD clones effectively induced IL-6 mRNA expression in 293 FT cells, as determined by quantitative polymerase chain reaction (Q-PCR). g. The IFN-γ KD clones and IL-6/IFN-γ double KD clones effectively inhibited IFN-γ mRNA expression in 293 FT cells, as determined by Q-PCR. h. The IL-6 KD clones and IL-6/IFN-γ double KD clones effectively inhibited IL-6 protein expression in 293 FT cells, as determined by flow cytometry. i. The IL-6 KD clones and IL-6/IFN-γ double KD clones effectively inhibited the average fluorescence intensity of IL-6 at the protein level, as detected by flow cytometry. j. The IFN-γ KD clones and IL-6/IFN-γ double KD clones effectively inhibited IFN-γ protein expression in 293 FT cells, as determined by flow cytometry. k. The IFN-γ KD clones and IL-6/IFN-γ double KD clones effectively inhibited the average fluorescence intensity of IFN-γ at the protein level, as detected by flow cytometry. **P < .01, ***P < .001, mean ± SEM.
Two IL-6 KD clones as well as one IL-6/IFN-γ double KD (shIL-6-1 and shIFN-γ-1) clone were co-transfected with pHR-IL-6-GFP through calcium phosphate transfection. CaCl$_2$ and two plasmids were mixed with water in a certain proportion and dripped slowly into the culture supernatants of 293 FT cells (purchased from ATCC). The 293 FT cells transfected with pHR-IL-6-GFP alone named pHR-IL-6-GFP-293 FT. The 293 FT cells co-transfected with pHR-anti-CD19 CAR and pHR-IL-6-GFP named pHR-anti-CD19 CAR-293 FT. The 293 FT cells co-transfected with shIL-6-1 and pHR-IL-6-GFP named shIL-6-1-293 FT. The 293 FT cells co-transfected with shIL-6-2 and pHR-IL-6-GFP named shIL-6-2-293 FT. The 293 FT cells co-transfected with IL-6/IFN-γ double KD and pHR-IL-6-GFP named double KD-293 FT. After 24 h, the 293 FT cells were obtained, and total RNA was then extracted using MiniBEST Universal RNA Extraction Kit and reverse transcribed into cDNA by using the FastKing RT Kit. Quantitative real-time PCR (Q-PCR) was performed to determine IL-6 mRNA expression using gene specific primers. After the PCR program, the data were analyzed with ABI 7500 Fast system (Applied Biosystems), and quantified with the comparative Ct method (2$^{-\Delta\Delta Ct}$) based on Ct values for IL-6 and β-actin genes in order to calculate the relative mRNA expression level.

In the same manner, two IFN-γ KD clones as well as one IL-6/IFN-γ double KD (shIL-6-1 and shIFN-γ-1) clone were co-transfected with pHR-IFN-γ-GFP through calcium phosphate transfection. The 293 FT cells co-transfected with shIFN-γ-1 and pHR-IFN-γ-GFP named shIFN-γ-1-293 FT. The 293 FT cells co-transfected with shIFN-γ-3 and pHR-IFN-γ-GFP named shIFN-γ-3-293 FT. Q-PCR was performed to determine IFN-γ mRNA expression. The primer sequences were shown in Table 2. The 293 FT cells were loaded onto the Guava easyCyte HT machine (Millipore). The shIL-6, shIFN-γ, and CD19 CAR co-expression vector was constructed and packaged as a lentivirus through a 3-guan system.

### 2.3. IL-6/IFN-γ double KD CAR-T cell culture

The PBMCs (Allcells, Cat: PB005F) were resuspended in CTS$^{TM}$ AIM V$^{TM}$ SFM medium (GIBCO, Cat: A3021002) at a density of 1 × 10$^6$ cells/ml, while 50 ng/ml anti-human CD3 functional grade purified (eBioscience, Cat: 160288–85) and 50 ng/ml anti-human CD28 functional grade purified (eBioscience, Cat: 160288–85) were added to activate T lymphocytes. The cells were then incubated at 37°C with 5% CO$_2$ for 48 h. Concentrated lentiviruses were added to the cultured cells at a multiplicity of infection of 5, while 200 unit/ml of IL-2 (Shandong Quangang Pharmaceutical, Cat: S20020004) and 4 μg/ml polybrene (Sigma, Cat: h9268-5 g) were added and mixed well. The mixture was then incubated at 37°C with 5% CO$_2$ for 6–8 h, followed by centrifugation at 300 g for 5 min and replacement of the medium with IL-2-containing fresh CTS$^{TM}$ AIM V$^{TM}$ SFM medium. Fresh CTS$^{TM}$ AIM V$^{TM}$ SFM medium (with IL-2) was added every 2–3 days. Cell density was maintained at approximately 1 × 10$^6$ cells/ml and amplified for 14 days.

### 2.4. Analysis of cell subsets using flow cytometry

A total of 1 × 10$^6$ virus-infected T cells were collected and centrifuged after 48 h culture. The collected T cells were resuspended using pre-cooled PBS. Then, 10 μl of 0.1 μg/μl FITC-labeled human CD19 (20–291) protein was added to each sample. The samples were then stained in a dark place at 4°C for 1 h. Meanwhile, 5 μl each of anti-CD3, anti-CD4, and anti-CD8 antibodies were added to each sample. The samples were then stained in a dark place at 4°C for 15 min. After being washed three times with pre-cooled PBS, the samples were loaded onto the Guava easyCyte HT machine (Millipore).

### 2.5. In vitro killing test of CAR-T cells

K562 cells were purchased from ATCC (CAT: bio-105947), and K562-C19 was built by our laboratory. The K562 cells and constructed K562-C19 cells were mixed at a ratio of 1:1, and the mixed cells were then inoculated in a 96-well plate. The double KD anti-CAR CAR-T cells, single KD anti-CAR CAR-T cells, anti-CAR CAR-T cells, or T cells were cocultured with the 1:1 mixture of K562 and K562-C19 at both effector cells: target cells (E:T) = 1:1 and E:T = 3:1.

The cells were then cultured at 37°C with 5% CO$_2$ for 24 h. The supernatant (100 μl) was collected from each well and stored at −80°C for further use. Then, 5 μl each of PE anti-human CD19 antibody (Biolegend) and FITC anti-human CD3 antibody (Biolegend) were added to the remaining cells in each well. The cells were then incubated at room temperature for 15 min, centrifuged, resuspended with 200 μl PBS, and finally tested using the Guava easyCyte HT machine. The percentage of specific cytolyis was calculated as follows: Percent specific cytolyis = [1−(experimental Proportion of K562-C19/Proportion of K562)/(target control Proportion of K562-C19/Proportion of K562)] × 100.

### 2.6. Detection of cytokine expression in CAR-T cells using reverse-transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted from the 24-h in vitro-killed double KD anti-CAR CAR-T cells, single KD anti-CAR CAR-T cells, and anti-CAR CAR-T cells, described in Section 2.4, using the MiniBEST Universal RNA Extraction Kit (TAKARA, Cat: 9767), and then reverse transcribed into cDNA by using the FastKing RT Kit (Tiangen, Cat: KR11601). Quantitative real-
time PCR (Q-PCR) was used to examine the expression profiles of some cytokine genes, including IL-6, IFN-γ, IL-17, IL-2, TNF-α, IL-10, and GM-CSF in the different groups using gene specific primers. After the PCR program, the data were analyzed with ABI 7500 Fast system (Applied Biosystems), and quantified with the comparative Ct method \(2^{-\Delta \Delta Ct}\) based on Ct values for cytokine genes and β-actin genes in order to calculate the relative mRNA expression level. The primer sequences of PT-PCR were shown in Table 2.

### 2.7. Detection of cytokine content in *in vitro* killing conditioned medium using ELISA

The E:T = 3:1 *in vitro* killing conditioned medium (as described in Section 2.4) was collected and divided into the T cell group, anti-CID19 CAR-T cell group, single KD anti-CID19 CAR-T cell group, double KD anti-CID19 CAR-T cell group, and tumor cell negative control group (1:1 mixture of K562 and K562-CID19 only). The secretion of IL-6, IFN-γ, IL-17, IL-2 and IL-10 was detected using ELISA MAX™ Deluxe Set Human IL-6 (Biogenecd, Cat: 430504), ELISA MAX™ Deluxe Set Human IFN-γ (Biogenecd, Cat: 430104), ELISA MAX™ Deluxe Set Human IL-17A set (Biogenecd, Cat: 433914), ELISA MAX™ Deluxe Set Human IL-2 (Biogenecd, Cat: 431804), and ELISA MAX™ Deluxe Set Human IL-10 (Biogenecd, Cat: 430604), respectively, following the manufacturer’s instructions.

### 2.8. Detection of the amount of cytokines released from the killing conditioned medium-stimulated PBMCs using RT-PCR

The PBMCs (Allcells) were resuspended in AIM-V medium (GIBCO) at a density of 1 x 10^6 cells/ml. The E:T = 3:1 killing conditioned medium was collected and divided into the T cell group, anti-CID19 CAR-T cell group, single KD anti-CID19 CAR-T cell group, double KD anti-CID19 CAR-T cell group, and negative control group (1:1 mixture of K562 and K562-CID19 only). The conditioned medium (100 μl) of each group was added to 3 x 10^6 PBMCs for stimulation. After stimulation for 24 h, the PBMCs were collected and total RNA was extracted using the MiniBEST Universal RNA Extraction Kit. The RNAs were then reverse transcribed into cDNAs by using the FastKing RT Kit. Q-PCR was performed to detect IL-6, IFN-γ, IL-2, TNF-α, and IL-10 mRNA expression. After the PCR program, the data were analyzed with ABI 7500 Fast system (Applied Biosystems), and quantified with the comparative Ct method \(2^{-\Delta \Delta Ct}\) based on Ct values for cytokine genes and β-actin genes in order to calculate the relative mRNA expression level.

### 2.9. Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics 24 (IBM, CA). Data are expressed as mean ± standard error of mean (mean ±SEM). Statistical analysis was performed by one-way ANOVA to evaluate the difference among all groups, followed by Least Significant Difference, Dunnet’s, Newman-Keuls multiple comparisons post hoc test to compare designated pairs of groups. Statistical significance was assumed at \(p < .05\).

### 3. Results

#### 3.1. Screening of shIL-6 and shIFN-γ clones

The quantitative PCR results showed that there was no significant difference in the expression level of IL-6 between the pHR-IL-6-GFP-293 FT cells and the pHR-anti-CID19 CAR-293 FT cells \((P > .05)\). However, the expression level of IL-6 was significantly decreased in the shIL-6-1-293 FT cells, the shIL-6-2-293 FT cells, and the double KD-293 FT cells, compared with the pHR-anti-CID19 CAR-293 FT cells \((P < .001)\) (Figure 1f).

The quantitative PCR results showed that there was no significant difference in the expression level of IFN-γ between the pHR-IFN-γ-GFP-293 FT cells and the pHR-anti-CID19 CAR-293 FT cells \((P > .05)\). However, the expression level of IFN-γ was significantly decreased in the shIFN-γ-1-293 FT cells, the shIFN-γ-3-293 FT cells, and the double KD-293 FT cells, compared with the pHR-anti-CID19 CAR-293 FT cells \((P < .001)\) (Figure 1g).

The 293 FT cells were obtained after 24 h co-transfection of the pHR-IL-6-GFP and the two IL-6 KD clones and IL-6/IFN-γ double KD clone.

The flow cytometry results revealed that IL-6 protein expression of the pHR-anti-CID19 CAR-293 FT cells was not significantly different compared with the pHR-IL-6-GFP-293 FT cells \((P > .05)\). However, IL-6 protein expression of the shIL-6-1-293 FT cells, the shIL-6-2-293 FT cells, and the double KD-293 FT cells was significantly decreased compared with the pHR-anti-CID19 CAR-293 FT cells \((P < .01)\) (Figure 1h). We then calculated the average fluorescence intensity of each group and found significant decreases in the shIL-6-1-293 FT cells, the shIL-6-2-293 FT cells, and the double KD-293 FT cells compared with the pHR-anti-CID19 CAR-293 FT cells \((P < .001)\), Figure 1i).

The 293 FT cells were obtained after 24 h co-transfection of the pHR-IFN-γ-GFP and the two IFN-γ KD clones and IL-6/IFN-γ double KD clone.

The flow cytometry results revealed that IFN-γ protein expression of the pHR-anti-CID19 CAR-293 FT cells was not significantly different compared with the pHR-IFN-γ-GFP-293 FT cells \((P > .05)\). IFN-γ protein expression of the shIFN-γ-1-293 FT cells, the shIFN-γ-3-293 FT cells, and the double KD-293 FT cells, was significantly decreased compared with the pHR-anti-CID19 CAR-293 FT cells \((P < .01)\), Figure 1j). We then calculated the average fluorescence intensity of each group and found significant decreases in the shIFN-γ-1-293 FT cells, the shIFN-γ-3-293 FT cells, and the double KD-293 FT cells, compared with the pHR-anti-CID19 CAR-293 FT cells \((P < .001)\), Figure 1k).

#### 3.2. Preparation and flow cytometry detection of CAR-T cells

The double KD clones, pCMV vector, and pMD.2 G vector were mixed and co-transfected into the 293 FT cells. After 48 h transfection, the supernatants were collected and the double KD lentivirus was obtained. T cells were then infected with the lentivirus to obtain double KD CAR-T cells. To test whether gene silencing can affect the proliferation of CAR-T cells, we counted the number of cells every two days by trypan blue staining. As shown in Figure 2a, after 14 days of culture, the proliferation of the T cells, anti-CID19 CAR-T cells, single KD anti-CID19 CAR-T cells, and
double KD anti-CD19 CAR-T cells was 219, 204, 222, 227, and 230 fold. \( P > .05, \text{ Figure 2a} \). The phenotypic analysis results revealed that the CD4:CD8 ratio in each group of cells was approximately 1:1, and no significant differences in the ratios were observed among the groups (Figure 2b). As shown in Figure 2c, T cell group was set as negative control group, CAR expression in anti-CD19 CAR-T cells, single KD anti-CD19 CAR-T cells, and double KD anti-CD19 CAR-T cells was 33.9%, 31.4%, 29.7%, and 30.3%. CAR expression was about 30% in each experimental group, with no statistical difference (Figure 2c).

### 3.3. Killing effect of CAR-T and the secretion of cytokines from CAR-T cells

#### 3.3.1. Killing test of CAR-T cells

K562, a human myeloid leukemia cell line, does not express CD19 molecules on the surface, whereas CD19 molecules are expressed on the surface of the K562-CD19 cells constructed in our laboratory. A 1:1 mixture of K562 and K562-CD19 cells was used as target cells. The flow cytometry showed that K562 accounted for 100% of K562 and K562-CD19 cell mixture in the CD19 CAR-T cell group, the single KD CD19 CAR-T cell group and the double

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**Figure 2.** The proliferation rate, cell phenotype, and infection efficiency of double knockdown (KD) anti-CD19 CAR-T cells did not change compared with those of original anti-CD19 CAR-T cells. a. The proliferation rate of anti-CD19 CAR-T cells, single KD anti-CD19 CAR-T cells, and double KD anti-CD19 CAR-T cells. NS \( P > .05 \), mean ± SEM. b. Phenotype of anti-CD19 CAR-T cells, single KD anti-CD19 CAR-T cells, and double KD anti-CD19 CAR-T cells, as detected by flow cytometry. c. CAR expression in anti-CD19 CAR-T cells, single KD anti-CD19 CAR-T cells, and double KD anti-CD19 CAR-T cells, as detected by flow cytometry.
KD anti-CD19 CAR-T cell group at E:T = 3:1 (Figure 3b). It showed that the anti-CD19 CAR-T cells, single KD anti-CD19 CAR-T cells, and double KD anti-CD19 CAR-T cells did not kill the K562 cells, whereas the K562-CD19 cells were killed specifically. The mean killing efficiency of the T cells, anti-CD19 CAR-T cells, single KD anti-CD19 CAR-T cells, and double KD anti-CD19 CAR-T cells to the K562 and K562-CD19 cell mixture at E:T = 1:1 was 17%, 54.3%, 59%, 56.7%, and 63.3%, whereas the killing efficiency of all groups at E:T = 3:1 was 16%, 100%, 100%, 100%, 100%. Statistical analysis revealed no significant difference in the specific killing efficiency among the single KD anti-CD19 CAR-T cell, double KD anti-CD19 CAR-T, and anti-CD19 CAR-T cell groups (P > .05, Figure 3a,b).

3.3.2. mRNA expression of cytokines decreased significantly in double KD anti-CD19 CAR-T cells

Detection at mRNA level of the expression of factors causing cytokine storms, including IL-6, IFN-γ, IL-17, IL-2, TNF-α, IL-10, and GM-CSF, is important for evaluating the occurrence of CRS. The RT-PCR results of the killing test of CAR-T cells revealed that compared with the T cells, the anti-CD19 CAR-T cells increased the mRNA levels of IL-6, IFN-γ, IL-17, IL-2, TNF-α, IL-10, and GM-CSF significantly, thereby inducing the release of multiple cytokines (P < .001, Figure 4a–g). Compared with the anti-CD19 CAR-T cells, the single KD anti-CD19 CAR-T cells displayed a significant reduction in the mRNA levels of IL-6, IFN-γ, IL-17, IL-2, TNF-α, IL-10, and GM-CSF, thereby inducing reduction in the levels of multiple cytokines (P < .001, Figure 4a–g). Compared with the single KD anti-CD19 CAR-T cells, the double KD anti-CD19 CAR-T cells showed a significant reduction in the mRNA levels of IL-6, IFN-γ, IL-17, IL-2, TNF-α, IL-10, and GM-CSF, thereby inducing reduction in the levels of multiple cytokines (P < .01, Figure 4a–g).

3.3.3. Cytokines released by double KD anti-CD19 CAR-T cells decreased significantly at the protein level

Detection of the expression of cytokines at the protein level can more intuitively reflect the situation of cytokine-induced CRS. ELISA was performed to detect the secretion of IL-6, IFN-γ, IL-17, IL-2, and IL-10 by CAR-T cells. The results of ELISA revealed that compared with the T cells, the amount of IL-6, IFN-γ, IL-17, IL-2, and IL-10 released in the anti-CD19 CAR-T cells was significantly increased (P < .001, Figure 5a–e). Compared with the anti-CD19 CAR-T cells, the amount of IL-6, IFN-γ, IL-17, IL-2, and IL-10 released in the single KD anti-CD19 CAR-T cells was significantly reduced (P < .01, Figure 5a–e). Compared with the single KD anti-CD19 CAR-T cells, the double KD anti-CD19 CAR-T cells showed a significant reduction in the amount of IL-6, IFN-γ, IL-17, IL-2, and IL-10 released (P < .05, Figure 5a–e). This result indicates that after the knockdown of both IL-6 and IFN-γ genes, the amount of multiple cytokines released can be reduced.

3.4. Expression of cytokines released from the killing conditioned medium-stimulated PBMCs decreased significantly

In addition to the large amount of cytokines released by CAR-T cells during the killing process, the release of cytokines from other immune cells, including monocyte macrophages, dendritic cells, possible stroma cells, and endothelial cells, is a major cause of cytokine storms. A large number of cytokines released by activated T cells can stimulate the activation of macrophages, induce high levels of proinflammatory cytokines, and further promote the subsequent immune response. Therefore, we used the conditioned medium after CAR-T cell killing as a stimulator to evaluate the effect of cytokines released after CAR-T cell activation on other immune cells in vitro. To verify the effects of the double KD anti-CD19 CAR-T cells on other immune cells, PBMCs were extracted from the blood and stimulated by the killing conditioned medium. The effect of the killing conditioned medium on cytokine production in PBMCs was detected through RT-PCR. The RT-PCR results showed that compared with the PBMCs stimulated by the T cell killing conditioned medium, the amount of IL-6, IFN-γ, IL-2, TNF-α, and IL-10 released by the PBMCs stimulated with the anti-CD19 CAR-T cell killing conditioned medium was significantly increased (P < .001, Figure 6a–e). Compared with the PBMCs stimulated by the anti-CD19 CAR-T cell killing conditioned medium, the amount of IL-6, IFN-γ, IL-2, TNF-α, and IL-10 released by the PBMCs stimulated with the single KD anti-CD19 CAR-T cell killing conditioned medium was significantly reduced (P < .01, Figure 6a–e). These results indicate that double KD anti-CD19 CAR-T cells can induce more significant reduction of cytokines released from other immune cells.

4. Discussion

The clinical manifestations of CRS include fever, nausea, fatigue, myalgia, depression, low blood pressure, hypoxia, clotting disorders, capillaries leakage, and/or toxic multiorgan dysfunction. CRS was observed in 30%–94% of patients, including 1%–48% of patients who experienced CRS at level 3 and above. CRS often occurs 1–22 days after infusion of CAR-T cells, with a median seizure time of 2–3 days. Severe CRS usually occurs earlier than the mild cases. High fever is the first clinical manifestation of CRS in most patients, and the body temperature of some patients can even reach more than 40°C. After CAR-T cell therapy, the cytokines involved in CRS include IFN-γ, IL-6, IL-2, TNF-α, and IL-10.

Two types of strategies are used to reduce CRS: prevention strategies are used to reduce the occurrence of severe toxicity and remedial strategies are used to minimize CAR-T cell-associated fatal toxicity. The best example of remedial strategies is adding ‘suicide’ or ‘elimination’ genes to CAR-T cells, which allows patients to selectively induce CAR-T cells apoptosis in the event of a severely toxic CRS reaction or off-target effect while receiving CAR-T treatment. Studies have reported that the activation of induced caspase-9 (iCasp9) enzymes can lead to rapid death of iCasp9-expressing T cells when they are exposed to the synthetic dimeric drug.
Figure 3. The specific cytotoxicity of double knockdown (KD) anti-CD19 CAR-T cells did not change compared with that of original anti-CD19 CAR-T cells. a. Expression of CD19 protein in K562 and K562-CD19 cells detected by flow cytometry. WT: wild type. The specific cytotoxicity of anti-CD19 CAR-T cells, single KD anti-CD19 CAR-T cells, and double KD anti-CD19 CAR-T cells, as detected by flow cytometry. NS P > .05, mean ± SEM. b. Specific cytotoxicity of anti-CD19 CAR-T cells, single KD anti-CD19 CAR-T cells, and double KD anti-CD19 CAR-T cells, as detected by flow cytometry. NS P > .05, mean ± SEM.
Figure 4. The mRNA expression of IL-6 (a), IFN-γ (b), IL-17 (c), IL-2 (d), TNF-α (e), IL-10 (f), and GM-CSF (g) in double knockdown (KD) anti-CD19 CAR-T cells decreased significantly, as detected by Q-PCR. **P < .01, ***P < .001, mean ± SEM.
Currently, several clinical trials evaluating iCasp9-modified CAR-T cells are in progress (NCT02274584 and NCT02414269). However, the high cost and poor availability of AP1903 may limit the use of this suicide system in remedial strategies.

We adopted a preventive strategy of using gene silencing technology to knockdown the most important CRS-inducing cytokines, IL-6 and IFN-γ. The results showed that the amount of IL-6, IFN-γ, IL-2, TNF-α, and IL-10 released by the PBMCs stimulated with the double KD anti-CD19 CAR-T cell killing conditioned medium was significantly reduced (Figure 6a–e). We suppressed the release of cytokines from the source, thereby reducing the impact of CRS, improving the safety of CAR-T, and reducing the toxic and side effects of CAR-T.

To validate this hypothesis, we constructed plasmids that co-expressed shIL-6, shIFN-γ, and CD19 CAR. To screen the effective silent shRNA, we screened the shRNA targeting IL-6 through co-transfection of plasmids of IL-6 KD clones and shRNA targeting IL-6. The results showed that the amount of IL-6, IFN-γ, IL-2, TNF-α, and IL-10 released by the PBMCs stimulated with the double KD anti-CD19 CAR-T cell killing conditioned medium was significantly reduced (Figure 6a–e).
The finding indicated that the two IL-6 KD clones can effectively inhibit the expression of IL-6 in the 293 FT cells by inducing IL-6 gene silencing. Meanwhile, we screened the shRNA targeting IFN-γ through co-transfection of plasmids of IFN-γ KD clones and pHR-IFN-γ-GFP. The finding indicated that the two IFN-γ KD clones can effectively inhibit the expression of IFN-γ in the 293 FT cells by inducing IFN-γ gene silencing. Thus, we successfully screened two shRNAs targeting IL-6 and two shRNAs targeting IFN-γ. In the same manner, we also verified the IL-6/IFN-γ double KD clone.

We successfully created IL-6/IFN-γ double KD anti-CD19 CAR-T cells and performed a series of validations on the function of the improved CAR-T cells. Our results also showed that the transduction efficiency, proliferation rate, cell phenotype, tumor affinity, and killing function of the double KD anti-

![Figure 6](image_url)
CD19 CAR-T cells did not change compared with those of the original anti-CD19 CAR-T cells. These observations indicate that this improvement does not affect the function and effectiveness of CAR-T cells. The transduction efficiency of CAR is approximately 30%. The results showed that the mean killing efficiency to the K562 and K562-CD19 cell mixture at E:T = 1:1 was approximately 60%, whereas the killing efficiency at E: T = 3:1 was 100%, and the amount of cytokines released by T cells at mRNA and protein levels is not much. This indicates that CAR-negative T cells do not affect the safety and effectiveness of CAR-T treatment. First, CAR-negative T cells will not expand and release cytokines in patients, thus producing side effect. Secondly, the effectiveness of CAR-T treatment can guarantee the CAR-positive cell quantity by increasing the total number of cells, so as to ensure the effect of CAR-T treatment.

We further experimentally verified the effects of the double KD anti-CD19 CAR-T cells on the safety of cell therapy, including detection of cytokine release at mRNA and protein levels. The results indicate that while the IL-6 KD anti-CD19 CAR-T cells maintained the original advantages of CAR-T cells, IL-6 expression decreased at both mRNA and protein levels. Surprisingly, the decrease in the expression of IFN-γ, IL-17, IL-2, TNF-α, IL-10, and GM-CSF cytokines occurred at the same time as the decrease in the expression of IL-6 at the mRNA level. The results of ELISA showed that the release of IFN-γ, IL-17, IL-2, and IL-10 was also significantly reduced at the protein level. The IFN-γ KD anti-CD19 CAR-T cells showed characteristics similar to those of the IL-6 KD anti-CD19 CAR-T cells. After the knockout of both IL-6 and IFN-γ genes, the amount of multiple cytokines released decreased significantly compared with the single KD anti-CD19 CAR-T cells. These results indicate that the double KD anti-CD19 CAR-T cells can effectively reduce the release of many important cytokines that cause cytokine storms and reduce the risk of CRS.

However, simply reducing the amount of cytokines released from the CAR-T cells is not sufficient to mitigate cytokine storms, as cytokine release from other immune cells is also a very critical factor in CRS occurrence. Therefore, after IL-6 and IFN-γ knockdown, determining the effect of the double KD anti-CD19 CAR-T cells on the release of cytokines from the immune system is also an important assessment for understanding whether the double KD cells can reduce the risk of CRS.

Many types of cells comprise PBMCs, such as lymphocytes, monocytes, and dendritic cells, and the proportion of these cells in PBMCs is the most similar to that of immune cells in human peripheral blood. In this study, we used PBMCs to build an in vitro model of immune cells in human peripheral blood, and the results showed that the double KD anti-CD19 CAR-T cells not only reduced the amount of cytokines released from the CAR-T cells but also significantly reduced the amount of IL-6, IFN-γ, IL-2, TNF-α, and IL-10 released from other immune cells contained in PBMCs. The amount of multiple cytokines released was more significantly reduced by the IL-6/IFN-γ double KD anti-CD19 CAR-T cells than the single KD anti-CD19 CAR-T cells (P < .001), indicating that compared with the single KD anti-CD19 CAR-T cells, the double KD anti-CD19 CAR-T cells can more effectively inhibit the release of cytokines from other immune cells, which has a more positive effect on reducing the risk of CRS. It is worth noting that we also detected the expression of IL-1 released from PBMCs stimulated by anti-CD19CAR-T cell killing conditioned medium, but the expression level was lower than the detection line. We speculate that IL-1 is not a key cytokine leading to CRS production in our model.

Rosalie M. Sterner prepared GM-CSF CRISPR knockout CAR-T cells. The results show that the CRISPR Cas9 GM-CSF k/o CART19 exhibit reduced GM-CSF production compared to wild type CART19, but IL-2 and IFN-γ production are not inhibited by the GM-CSF gene disruption. In this study, the results showed that IL-6/IFN-γ double knockout CAR-T cells reduce the release of IL-6 and IFN-γ while reducing the release of other key cytokines. This may indicate that IL-6 and IFN-γ gene disruption is a better choice. We can't explain this appearance yet, so more experiments are needed to reveal the possible mechanism.

Shiv D Kale stated the view that IFN-γ-JAK-STAT pathway leads to T cell driven CRS. In this study, as shown in Figs 4–5, a large amount of IFN-γ was released by the CAR-T cells, and it indicated that IFN-γ plays an important role in T cell driven CRS. Delong Liu stated the view that myeloid cells including macrophages and monocytes, not the CAR T cells, were found to be the major cells mediating CRS by releasing IL-6 among other cytokines. In this study, as shown in Figure 6, a large amount of IL-6 was released by the stimulated PBMCs, and this view was supported again.

In conclusion, both single and double KD anti-CD19 CAR-T cells maintained the advantages of the original anti-CD19 CAR-T cells in terms of the infection efficiency, proliferation rate, cell phenotype, tumor affinity, and killing function, and additionally reduced the release of cytokines from CAR-T cells and other immune cells. Therefore, we suggest that this gene knockdown strategy can improve the safety of CAR-T without affecting the effectiveness of CAR-T cell therapy.

We clearly realize that our results are preliminary, and these results help us put forward a possible hypothesis. It should be pointed out that our study was not adequately designed regarding power to infer associations of any magnitude. After IL-6 and IFN-γ knockdown, the molecular mechanism that leads to the reduction in the amount of cytokines released by the CAR-T cells and other immune cells needs to be further explored.

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Disclosure statement

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