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Review Article

Concomitant Use of CD138- and CD19-directed Chimeric Antigen Receptor-modified T Cells Enhances Cytotoxicity Towards Multiple Myeloma

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Abstract: Multiple myeloma (MM) is a malignancy characterized by abnormal proliferation of clonal plasma cells, and it is the second most common hematologic malignancy in the world after non-Hodgkin lymphoma. In recent years, significant progress has been made in the clinical treatment of MM. In particular, certain novel drugs, such as bortezomib, lenalidomide, and carfilzomib, have greatly improved the survival rate of patients with MM. However, because of drug resistance, most MM patients eventually suffer a relapse and die of the disease. In this study, the chimeric antigen receptor-modified T cell (CAR-T cell) technology, which has achieved success in recent clinical trials for B-cell acute lymphoblastic leukemia (B-ALL), was used. In view of the high CD138 expression in MM cells and the presence of the CD138–/CD19+ phenotype in a small subset of MM cells, and based on preliminary findings of effective killing of MM cells by CD19-CAR-T cells in clinical studies, CD138- and CD19-directed CAR-T cells were constructed. Through in vitro experiments and the use of a mouse model, we proved that these two types of CAR-T cells possess strong biological activity in the specific killing of target cells, and that the concomitant use of these cells significantly enhances the killing effect in an MM mouse model.

Keywords: Multiple Myeloma, CAR-T, CD138 (syndecan-1), CD19

1. Introduction

Multiple myeloma (MM) is an extremely severe plasma cell malignancy, in which the tumor cells originate from plasma cells in the bone marrow. It is characterized by abnormal proliferation of bone marrow plasma cells, accompanied by excessive production of monoclonal immunoglobulins or M-proteins. MM is usually accompanied by multiple lytic bone lesions, hypercalcemia, anemia or renal failure. MM affects 5 in every 100,000 people, accounts for approximately 10% of hematologic malignancies, and is the second most common hematologic malignancy worldwide. Conventional treatment methods include autologous stem-cell transplantation; however, this treatment method has severe drawbacks, as high dosages result in high morbidity and mortality rates. In recent years, immunomodulators, such as lenalidomide, and pomalidomide, as well as proteasome inhibitors, such as bortezomib, have yielded clinical successes. Clinical application of these novel drugs has significantly improved the survival rate of patients with MM. However, because of drug resistance, the vast majority of MM patients eventually suffer a relapse and die of the disease. Therefore, there is an urgent need for the development and application of new treatment strategies.

The chimeric antigen receptor (CAR) T cell technology involves the modification of T cells to recognize tumor-associated antigens (TAA) on the tumor surface. Through the use of such modified T cells, tumor cells can be effectively and specifically killed [1]. A CAR-T cell typically consists of an extracellular antigen-recognition and -binding domain (usually a single-chain variable fragment, scFv), a costimulatory domain, and CD3ζ signaling molecules. The scFv enables the selective recognition of tumor cells by T cells [2], and the immunoreceptor tyrosine-based activation motifs of CD3ζ, effectively activate T cells and enhance their killing ability [3-4]. First-generation CAR-T cells harbored only a CD3ζ domain; therefore, the cells had limited persistence and killing ability [5], resulting in a lack of clinical efficacy. The second-generation CAR expanded upon the first-generation CAR with the incorporation of a costimulatory domain, such as OX40 (CD134), CD28, 4-1BB (CD137), or CD27 [2, 6-8], which enhances the proliferation and survival of CAR-T cells in the body. In a clinical study on the treatment of B-cell acute lymphoblastic lymphoma, success was achieved with CD19-directed second-generation CARs, which yielded a complete response rate of 88% [9]. The third-generation CAR consists of two costimulatory domains, typically CD28 and CD137, which are linked [10]. Third-generation CARs produced more beneficial cytokines *in vitro* than second-generation CARs; therefore, an enhanced effect may be achieved for certain target cells [11].

High expression of CD138 (syndecan-1) has been found in MM cell lines and plasma samples of patients with MM [12-15]. For example, in the study conducted by Lin et al. in 2004 on 306 MM patients, CD138 expression was observed in 100% of case. However, CD138 is not expressed on T cells and B cells [16]. In contrast, CD138 promotes the growth and proliferation of myeloma cells, as well as tumor-associated angiogenesis [17-19]. These findings have made CD138 an extremely promising target for MM treatment, and the activity of CD138-directed first-generation CARs has been proven *in vitor* [20]. A clinical study on the use of CD138-directed 4-1BBζ second-generation CARs in five MM patients was conducted at the General Hospital of the People's Liberation Army [21]. Although their clinical efficacy was limited, the treatment regimen was proven to be safe.

Incurability, high relapse rate, and drug resistance in MM are mainly attributed to the presence of a small amount of MM cancer stem cells (MM-CSCs). Interestingly, many studies reported CD138 negativity in stem cells of MM cell lines and patient samples [22-24], and CD138 negativity in MM is closely linked with the proliferation of and drug resistance in MM [25]. CD19 expression is nearly absent in myeloma plasma cells; however, poorly differentiated populations of CD19+ cells are associated with drug tolerance and disease progression [36]. Matsui et al. [23] recently reported a small subpopulation of MM-CSCs (<5%) in MM cell lines and patient samples. Currently, controversies regarding the MM-CSC phenotype still exist owing to the lack of sensitive and feasible identification methods [27]. Garfall et al. [28] recently reported the use of CAR-T cells (CTL019) derived from autologous T-cells in the treatment of a patient with refractory MM. Although CD19 expression was absent in 99.95% of the patient's neoplastic plasma cells, the patient showed a complete response to the therapy. Although positive results have been achieved in preliminary studies on the sole use of CD138- or CD19-directed CAR-T cells for treatment, these treatment methods still have major drawbacks. With CD138-CAR-T cells, the small portion of CD138–cells that are capable of differentiation cannot be killed, and there is a high likelihood of relapse, and treatment success with CD19-CAR-T cells was only achieved in a single case. Based on the facts that MM cells are of the CD138+ phenotype and that a small amount of MM-CSCs with a CD138–/CD19+ immunophenotype exist in MM patients, in the current study, CD138- and CD19-directed CAR-T cells were separately constructed, and the *in vivo* effects of single or concomitant administration of the CAR-T cells were teste.

2. Materials and Methods

2.1. Cell Lines and cell Culture

The human myeloma-derived cell lines RPMI8226 and U266, the human erythroleukemic cell line K562, and the mantle cell lymphoma cell lines MAVER-1 and JeKo-1 were obtained from the American Type Culture Collection (Manassas, VA). All cells were cultured in complete medium consisting of RPMI 1640 (Hyclone,), 10% fetal bovine serum (Biowest), 2 mM L-glutamine, 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin at 37°C in a humidified 5% CO_2-containing atmosphere. HEK293T cells were cultured in DMEM (Hyclone) containing 10% fetal bovine serum. Peripheral blood samples were obtained from several healthy donors. Mononuclear cells were isolated by Ficoll-Paque density gradient centrifugation (Ficoll-Paque, Piscataway, NJ) and T cells were cultured in TexMACS^{TM} GMP medium (Miltenyi, Bergisch Gladbach, Germany).

2.2. CD138-CAR and hCD19-CAR Construction

A second-generation CAR construct was used for CD138-CAR construction, and a third-generation CAR construct was used for hCD19-CAR construction. The constructs were generated as previously described [29].

2.3. Lentivirus Production and T Cell Transduction

Lentiviruses were prepared as previously described [30]. To generate CAR-T cells, The mononuclear cells from peripheral blood was collected and cultured in a 24-well culture plate. Approximately 1×10^6 T cells were seeded in 1 mL of culture medium in each well. Next, 10 μL of anti-CD3/CD28 beads (Miltenyi, Bergisch Gladbach, Germany) was added. After 48 h of activation, the T cells were transduced with viral supernatant (MOI=5). The cells obtained after transduction were CD138-CAR-T cells and hCD19-CAR-T cells, respectively. The activation,

transduction, and subsequent culturing of T cells were performed in TexMACS^{TM} GMP medium. supplemented with 40 IU mL^{-1} IL-2. Transduction efficiency was determined using by a flow cytometry (FACSCalibur; BD Biosciences).

2.4. Flow Cytometry and Western Blotting

RPMI8226, U266, JeKo-1, MAVER-1, and K562 cells were collected and stained with the indicated antibodies. RPMI8226 and U266 were stained with FITC-conjugated CD138 antibody (BD Biosciences), while JeKo-1 and MAVER-1 were stained with the PE/Cy5-conjugated CD19 antibody. T cells were stained with APC-conjugated Fc antibody (BD Biosciences), and the positive rate was determined. T cells were also respectively stained with FITC-conjugated CD4 antibody (BD Biosciences) and FITC-conjugated CD8 antibody (BD Biosciences), and the phenotypic profiles were determined. For K562 cells, background expression levels of CD138 and CD19 were determined. After construction, CD138-expressing K562 cells (K562-CD138) and CD19-expressing K562 cells (K562-CD19) were stained with the CD138 antibody and CD19 antibody, respectively. After staining, all cells were incubated at 37°C for 20–25 min, washed thrice with PBS, then analyzed on a FACSCalibur flow cytometer.

Total protein was extracted from appropriate quantities of the transduced CAR-T cells and untreated T (UNT) cells for western blotting. The primary antibody used was mouse anti-CD3ζ (BD Biosciences), and the secondary antibody used was HRP-conjugated goat anti-mouse (Solarbio).

2.5. Construction of K562-CD138 and K562-CD19 cells

To validate the killing specificity of the constructed CAR-T cells, CD138 and CD19 expression constructs were transformed into K562 cells to be used as positive control cells in subsequent experiments, as described previously [30]. CD138 (NM_002997) and CD19 (AH005421.2) sequences were retrieved from NCBI, processed using the SignaIP 4.1 Server and UniProt, respectively inserted on pCDH-CMV-MCS-EF1-CopGFP vectors, and packaged into lentiviruses. K562 cells were then transduced for 6 h and subsequently cultured. After staining with the corresponding antibodies, sorting was performed using a FACSAria^{TM} III cell sorter (BD Biosciences) to obtain a positive rate of 100%.

2.6. In Vitro Killing Experiment

In the *in vitro* killing experiment, the target cells used for testing CD138 CAR T cells were the MM cell lines RPMI8226 and U226, as well as the constructed K562-CD138 cells, while the target cells used for hCD19 CAR T cells were the mantle cell lymphoma cell lines MAVER-1 and JeKo-1, as well as the constructed K562-CD19 cells. Untransduced T cells (UNT) cells were used as control effector T cells. Two methods were used to assess the killing of target cells, the first being the carboxyfluorescein succinimidyl ester (CFSE)/ 7-aminoactinomycin D (7-AAD) flow-cytometric assay [31], as described previousely [30].

The second method used to assess the killing of target cells was the measurement of lactate dehydrogenase (LDH) release from tumor cells using the CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega, USA) in strict adherence to the manufacturer's instructions [20]. Fifty microliters of killer cell supernatant of each group was placed in 96-well plates, and 50 μL of CytoTox 96® reagent was added to each well. After reacting for 30 min, 50 μL of stop solution was added to each well. Absorbance at 490 nm for each group was recorded with a microplate reader after 1 h of reaction, and apoptotic target cell death, apoptotic effector cell death, and total target cell death were measured. The killing rates for the respective groups were calculated based on the following formula:

$$\text{Killing rate (\%)} = (\text{experimental group cell number-apoptotic effector cell death-apoptotic target cell death})/(\text{total target cell death-apoptotic target cell death}) \times 100\%$$

2.7. Measurement of IFN-γ and Granzyme B release

To prove the effective activation and target cell killing activity of T cells, IFN-γ and granzyme B release by UNT cells and CD138-CAR-T cells were measured. Two groups with effector: target (E:T) ratios of 0.25:1 and 0.5:1 were established. For each group, 4×10^5 target cells in a 1.5 mL culture system were incubated for 18 h, and then analyzed by the cytometric bead array (CBA) method for IFN-γ and granzyme B release using an IFN-γ Kit (Human IFN-γ Flex Set; BD) and a granzyme B Kit (Human granzyme B Flex Set; BD), respectively. Specifically, for each group, 50 μL of supernatant was obtained, cell factor capture beads were added, and the cells were incubated at room temperature for 1 h. Next, the corresponding cell factor antibody was added, cells were incubated at room temperature for 2 h, washed twice with PBS, and analyzed with a benchtop flow cytometer (NovoCyte; ACEA). Data were processed using FCAP Array v3, and three parallel experiments were carried out for each group.

2.8. In Vivo Experiments

To determine the effect of treatment with the CAR-T cells in vivo, a mouse model was used in this study. Twenty-four NODPrkdc^{em26Cd52} Il2rg^{em26Cd22}Nju mice (NCG, Model Animal Research Center of Nanjing University) between 5–6 weeks of age were procured. RPMI8226 cells were cultured in a 100-μL culture system consisting of 70 μL of PBS and 30 μL of matrigel (BD Biosciences). The mice were subcutaneously injected with 5×10^6 RPMI8226 cells into the right shoulder. Treatment was commenced when tumor sizes reached 50 mm^3. The mice were randomly allocated to four groups, with six mice in each group: (i) injection with 100 μL of PBS during each treatment; (ii) injection with 1×10^7 UNT cells (100 μL) during each treatment; (iii) injection with 1×10^7 CD138-CAR-T cells during each treatment (100 μL); (iv) injection with 1×10^7 CD138-CAR-T cells (100 μL) and 2×10^6 hCD19-CAR-T cells (50 μL) during each treatment. The

CAR-T cells were administered via tail vein injections, and three treatments were respectively provided on the 7th, 9th, and 13th day after tumor cell injection. Body weight and tumor size of each mouse were measured 2–3 times per week. The following formula was used to calculate tumor size: $4\pi/3\times(\text{tumor length}/2)\times(\text{tumor width}/2)^2$ [20].

The animal experiment was performed in strict adherence to the experimental animal management regulations of the Institutional Animal Care and Use Committee of Soochow University. The mice were euthanized in a humane manner when death was imminent or when tumor size reached 15 mm.

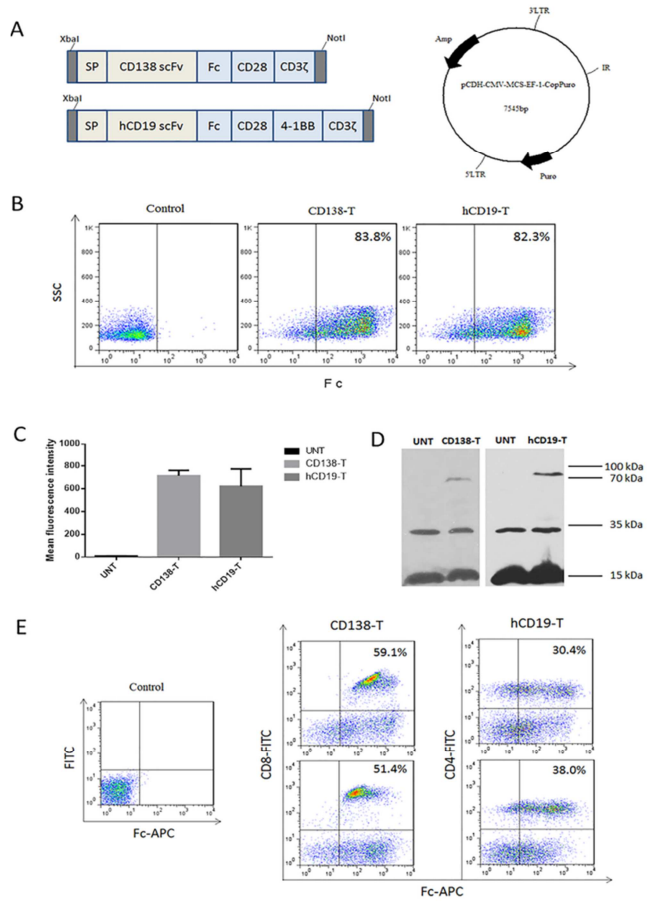

Figure 1. *Design of CAR and characterization of CAR-T (A) The structure of CD138-CAR and hCD19-CAR and lentiviral vector. A CD138-CAR was generated by a tandem sequence contained a signal peptide (SP), CD138-scFv consisting of V_H-linker-V_L, Fc extracellular domain, CD28 transmembrane domain (TM), co-stimulatory domain CD28 and CD3ζ chain, that defined the second generation CAR. And hCD19-CAR consisting two co-stimulatory domains (CD28 and 4-1BB) is a third generation CAR. (B) The detection of positive of CD138-T and hCD19-T. Transfected CAR-T cells were harvested respectively, stained with anti-Fc Ab, and analyzed using flow cytometry. The transfection efficiency and mean fluorescence intensity (MFI) were presented respectively in (B) and (C). Data are presented as the mean±SD of the three separate experiments. (D) Immunoblot analysis of CD3ζ fusion protein expression in CD138-T and hCD19-T cells. After lysates CAR-T cells, protein was harvested and separated by SDS-PAGE under reducing conditions. Immunoblot analysis was performed with a mouse anti-CD3ζ chain-specific mAb followed by exposure to an HRP-conjugated goat anti-mouse antibody and chemiluminescent detection. (E) The detection of CD8 and CD4 to CAR-T cells. The transfected T cells was stained with anti-Fc mAb and anti-CD8 or anti-CD4 mAb respectively, and analyzed using flow cytometry.*

2.9. Immunohistochemistry Tests

Immunohistochemistry was assessed in tumors removed from the mice, using the following procedure. After receiving three CAR-T treatments, the mice were fed for four days and then euthanized. Subcutaneous tumor tissues were removed and immediately fixed in formalin overnight. Fixed tissues were dehydrated, embedded in paraffin, and sectioned, and the sections were dewaxed. Sections subjected to antigen retrieval were sealed in TBST buffer with 10% serum at room temperature for 1 h, stained with purified mouse anti-human CD19 antibody (NB100-65672, Novus) and rabbit anti-human CD138 antibody (DF6367, Affinity) at 4°C for 10 h, and then with HRP-conjugated goat anti-mouse secondary antibody and HRP-conjugated goat anti-rabbit secondary antibody at room temperature for 1 h. Subsequently, the sections were stained with diaminobenzidine. Alternatively, dewaxed sections were directly subjected to H&E staining and observed under an upright fluorescence microscope.

2.10. Data Analysis

For the *in vitro* experiments, data were processed using GraphPad Prism 5. The experiments were repeated at least three times for each group, and statistical analysis was performed using Student's *t*-test, with p-values < 0.05 indicating significance. For the *in vivo* experiments, survival curves of mice were processed using the Kaplan–Meier method, and survival data were analyzed using the log-rank (Mantel–Cox) test, with p-values < 0.05 indicating significance.

3. Results

3.1. Detection of CD138 on the Cell Surface of MM Cell Lines and CD19 on the Cell Surface of Lymphoma Cell Lines

CD138 and CD19 expression on the surfaces of MM and lymphoma cells, respectively, was first investigated. Both MM cell lines RPMI8226 and U266 highly expressed CD138 on their surfaces, with the positive CD138 expression rates for RPMI8226 and U266 being 98.0% and 99.1% respectively (Figure 7A). For the CD19+ tumor cells, the high CD19-expressing MAVER-1 and JeKo-1 cell lines were selected; the CD19 expression rates were 92.9% and 75.0% respectively (Figure 7B).

3.2. Construction and Expression on CD138- and hCD19-specific CAR-T Cells

A second- and third-generation CAR construct was used in the construction of CD138-CAR and hCD19-CAR, respectively (Figure 1A). The cDNAs of CD138 and hCD19 consisted of heavy and light chains, with a signal peptide sequence (MLLLVTSLLLCELPHPAFLLIP) followed by the sequences of the Fc domain, CD28 costimulatory domain, and CD3ζ region. The sequences were inserted in a

pCDH-CMV-MCS-EF1-CopPuro vector containing a puromycin resistance gene (Figure 1A). Activated T cells from healthy human donors were transduced with the CD138-CAR and hCD19-CAR constructs. The antigen expression rates on CD138-CAR-T and hCD19-CAR-T cells were 65–85% and 70–85%, respectively (Figure 1B). Furthermore, the mean fluorescence intensities for CD138-CAR-T cells and hCD19-CAR-T cells staining with the APC-conjugated Fc antibody were extremely high (Figure 1C). The CD4+- and CD8+-positive rates of transduced T cells are shown in Figure 1E.

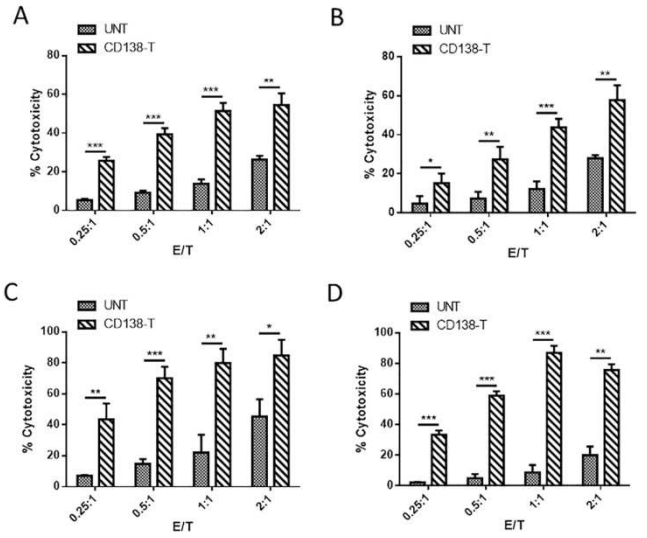

Figure 2. *Enhanced cytotoxicity of CD138-T cells against CD138 positive MM cells. The Killing detection of transfected CD138-T against MM cell lines RPMI8226 (A) and U266 (B) was performed. Effector and target (4×10^5) were co-culture for a period of 18 hours in the presence of CSFE/7-AAD at E:T ratios of 0.25:1, 0.5:1, 1:1 and 2:1. Meanwhile, we analyze the cytotoxicity of CD138-T to RPMI8226 (C) and U266 (D) by LDH release assay. Data presented are the mean±SD of the three separate experiments. *p<0.05, **p<0.01, ***p<0.001 compared with UNT at the same E:T ratio.*

To confirm the insertion and expression of CARs in the T cells, western blotting was performed to detect the expression of the CD3ζ fusion protein (Figure 1D). The molecular mass of the ζ chain was approximately 15 kDa, and the molecular mass of the dimer was 30 kDa. The CD138-CAR-T and hCD19-CAR-T CD3ζ fusion proteins were clearly detected on the blots, indicating they were effectively expressed by the T cells.

3.3. CD138-CAR-T Cells Show Enhanced Ability to Kill MM Cells

The ability of CD138-CAR-T cells to kill MM cells was compared to that of UNT cells, first by using the CFSE/7-AAD staining method. E:T ratios of 0.25:1, 0.5:1, 1:1, and 2:1 were used, and the assay time was 18 h.

CD138-CAR-T cells had a significantly enhanced killing ability towards RPMI8226 cells as compared to UNT cells (e.g., 13.9±2.3% vs. 51.5±4.1%, p=0.0001 for E:T=1:1; 26.3±2.0% vs. 54.5±6.1%, p=0.001 for E:T=2:1) (Figure 2A). Similarly, CD138-CAR-T cells had a significantly stronger killing ability towards U266 cells than did UNT cells (e.g., 12.3±1.5% vs. 43.7±3.2%, p=0.0007 for E:T=1:1; 28.0±1.5% vs. 57.9±7.5%, p=0.002 for E:T=2:1) (Figure 2B). UNT cells showed nearly no killing activity towards U266 cells when an E:T ratio of 0.25:1 was used, and the killing rate was only 28% for an E:T ratio of 2:1.

To confirm the high killing ability of CD138-CAR-T cells towards MM cells, and LDH release assay was used, with the same assay time and E:T ratios as for the CFSE/7-AAD test. The results confirmed that the killing ability towards RPMI8226 and U266 cells was greatly enhanced in CD138-CAR-T cells. (Figure 2C and Figure 2D). The target-cell death rates for the experimental group obtained using the LDH release assay were approximately 20% higher than those obtained by flow cytometry, while differences in target-cell death rates for the control group were insignificant. This may be due to differences in accuracy between two detection methods.

Taken together, these results indicated that the insertion of the CD138-CAR construct in T cells significantly enhances their cytotoxicity towards MM cells.

3.4. hCD19-CAR-T Cells Show Enhanced Ability to Kill CD19-expressing Cells

As MM cells have extremely low CD19 expression, MM cells could not be used as target cells to directly determine the killing ability of the constructed hCD19-CAR-T cells towards MM cells; therefore, we used the mantle cell lymphoma cell lines MAVER-1 and JeKo-1. Again, we first used the CFSE/7-AAD staining method to assess the killing ability during an assay time of 18 h and with E:T ratios of 0.25:1, 0.5:1, 1:1 and 2:1.

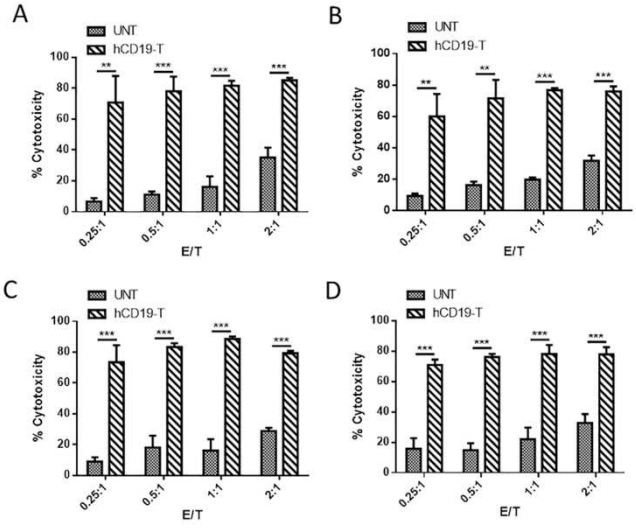

Figure 3. *Enhanced cytotoxicity of hCD19-T cells against CD19 positive cells. The Killing detection of transfected hCD19-T against mantle cell lymphoma MAVER-1 (A) and JeKo-1 (B) was performed. Effector and target (4×10^5) were co-culture for 18 hours in the presence of CSFE/7-AAD at E:T ratios of 0.25:1, 0.5:1, 1:1 and 2:1. Meanwhile, we analyze the cytotoxicity of CD19-T to MAVER-1 (C) and JeKo-1 (D) by LDH release assay. Data presented are the mean±SD of the three separate experiments. *p<0.05, **p<0.01, ***p<0.001 compared with UNT at the same E:T ratio.*

The killing rates towards both types of target cells by hCD19-CAR-T cells were much higher than those by UNT cells. For MAVER-1 target cells, the killing rate by hCD19-CAR-T cells was 70–85%. Even at the lowest E:T ratio, a 70% killing rate was achieved in the experimental group. In contrast, the killing rate by UNT cells was less than 10% (e.g., 16.2±3.8% vs. 81.6±3.2%, p=0.0001 for E:T=1:1; 35.2±6.4% vs. 82.5±1.5%, p=0.0002 for E:T=2:1) (Figure 3A). For JeKo-1 target cells, the killing effect by hCD19-CAR-T cells significantly stronger than that of UNT cells as well (e.g., 19.8±1.3% vs. 76.9±1.2%, p=0.0001 for E:T=1:1; 31.8±3.4% vs. 76.0±3.1%, p=0.0001 for E:T=2:1) (Figure 3B).

An LDH release assay with the same assay time and E:T ratios confirmed the findings of the flow cytometric assay (Figure 3C and Figure 3D). Thus, both assay methods provided strong proof of the high killing ability of hCD19-CAR-T cells towards CD19-expressing cells.

3.5. CD138-CAR and hCD19-CAR Confer High Specificity Towards CD138+ and CD19+ Cells, Respectively

To assay the specificity of the killing activities of CD138-CAR-T and hCD19-CAR-T cells towards CD138+ and CD19+ cells respectively, K562 cells, which are CD138– and CD19– (Figure 8B) were transformed to stably express CD138 (K562-CD138) or CD19 (K562-CD19). CD138-CAR-T and hCD19-CAR-T cells were then evaluated for their killing activity towards these two types of constructed cells and unmodified K562 cells. Effector and target cells were co-cultured at E:T ratios of 0.25:1, 0.5:1, 1:1, and 2:1 for 18 h. The LDH release assay method was used for detection, and the positive rate for both the constructed K562-CD138 cells and K562-CD19 cells was 96% (Figure 8A).

The results indicated that the killing ability of CD138-CAR-T cells towards K562-CD138 cells was substantially higher than that of UNT cells. For an E:T ratio of 0.25:1, the killing effect of CD138-CAR-T cells was 5 times that of UNT cells, and for an E:T ratio of 2:1, the killing effect of CD138-CAR-T cells was 40% higher than that of UNT cells (Figure 4A). The killing effects of CD138-CAR-T and UNT cells towards the parent K562 cells were comparable at all four E:T ratios used. (Figure 4C). As for K562-CD19 cells, the killing ability of hCD19-CAR-T cells was substantially higher than that of the control group. For the lowest E:T ratio, the killing rate of K562-CD19 cells by hCD19-CAR-T cells was more than 4 times that of the killing rate by UNT cells. (Figure 4B). For K562 parent cells, hCD19-CAR-T and UNT showed no differential effect at all E:T ratios (Figure 4D).

From the above results, it can be concluded that the constructed CD138-CAR-T cells are highly specific towards their respective target cells.

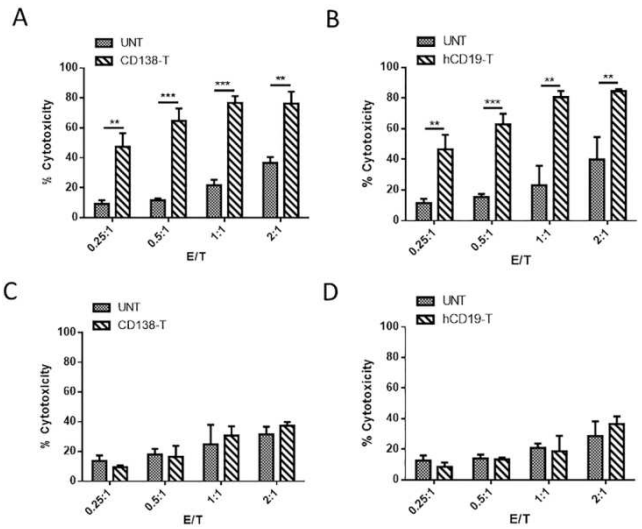

*Figure 4. Sensational specificity of CD138-T and hCD19-T cells to CD138 and CD19 antigen respectively. The K562 cell expressing CD138 (K562-CD138) (A) and K562 cell expressing CD19 (K562-CD19) (B) stayed with CD138-T and hCD19-T respectively for 18 hours followed with analysis by LDH release assay. The cytolytic activity of CD138-T (C) and hCD19-T (D) against untreated K562 cell was also detected by LDH release assay. Data presented are the mean±SD of the three separate experiments. *p<0.05, **p<0.01, ***p<0.001 compared with UNT at the same E:T ratio.*

3.6. High levels of Granzyme B and IFN-γ Are Released After Reaction of CAR-T Cells with Target Cells

T cells confer cytotoxicity towards tumors through two ways: (1) release of perforin and granzymes, and (2) activation of death receptors through the Fas/FasL or TNF/TNFL signaling pathway. For CAR-T cells, besides the killing effect through TAA recognition, the interaction of IFN-γ released by CAR-T cells and IFN-γR on tumor surfaces causes a cytotoxic effect towards tumor cells as well [32]. In addition, CAR-T cells show higher IFN-γ release than do T cells [33]. This indicates that granzyme B and IFN-γ are cytotoxic towards tumor cells and also provides convincing proof of the activity of CAR-T cells. The experiment was conducted using different E:T ratios, and for each ratio, CAR-T and tumor cells were co-cultured for 18 h, and the supernatant was collected for testing.

The amounts of granzyme B released after CD138-CAR-T cells were respectively co-cultured with target cells RPMI8226 and U266 were significantly higher than those in the UNT cell control group (e.g., 6097.8±212.6 pg mL^{-1} vs. 95.6±6.4 pg mL^{-1}, p=0.008 for E:T=0.5:1 towards RPMI8226; 3284.6±325.8 pg mL^{-1} vs. 22.9±13.6 pg mL^{-1}, p=0.0001 for E:T=0.5:1 towards U266) (Figure 5A). Similarly, the amounts of IFN-γ released were substantially higher for the CD138-CAR-T than for UNT cells (e.g., 1633.9±67.5 pg mL^{-1} vs. 15.8±9.0 pg mL^{-1}, p=0.0001 for E:T=0.5:1 towards RPMI8226; 1104.8±69.7 pg mL^{-1} vs. 8.69±5.5 pg mL^{-1}, p=0.0001 for E:T=0.5:1 towards U266) (Figure 5B).

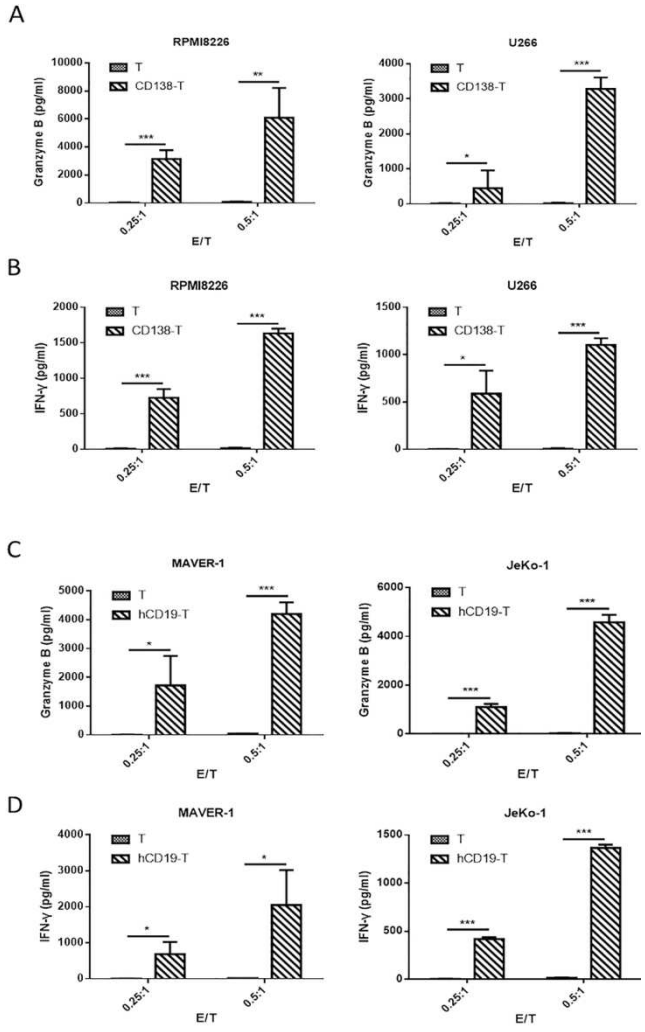

Figure 5. *Enhanced release of Granzyme B and IFN-γ as CAR-T cells in response to tumors. The effector cell CD138-T was co-cultured with a constant of target cells (4×10^5) for 18 hours at a various E:T ratios in 24-well microplates within a final volume of 1.5 ml RPMI1640 complete medium. A part of supernatant was collected and used for detection of Granzyme B (A) and IFN-γ(B) using Cytometric Bead Array (CBA) by flow cytometry. In addition, the assay that Granzyme B (C) and IFN-γ(D) released by hCD19-T cells co-culturing with MAVER-1 and JeKo-1 was performed. Data presented are the mean±SD of the three separate experiments. *p<0.05, **p<0.01, ***p<0.001 compared with UNT at the same E:T ratio.*

This was in accordance with the higher killing ability of hCD19-CAR-T cells than that of UNT cells. Results of granzyme B detection indicated that the amount released by hCD19-CAR-T cells was much higher than that released by UNT cells (e.g., 4202.5±403.3 pg mL^{-1} vs. 41.7±3.4 pg mL^{-1}, p=0.0001 for E:T=0.5:1 towards MAVER-1; 4578.4±309.1 pg mL^{-1} vs. 33.2±3.8 pg mL^{-1}, p=0.0001 for E:T=0.5:1 towards JeKo-1) (Figure 5C). The killing of target cells by hCD19-CAR-T cells also promoted IFN-γ release (e.g., 562.8±120.6 pg mL^{-1} vs. 5.1±4.4 pg mL^{-1}, p=0.025 for E:T=0.25:1 towards MAVER-1; 419.3±17.9 pg mL^{-1} vs. 5.27±0.8 pg mL^{-1}, p=0.0001 for E:T=0.25:1 towards JeKo-1) (Figure 5D). These results sufficiently showed that the co-culture of CAR-T and tumor cells resulted in the release of high levels of granzyme B and IFN-γ, which likely contributed to the death observed in tumor cells after treatment with the CAR-T cells.

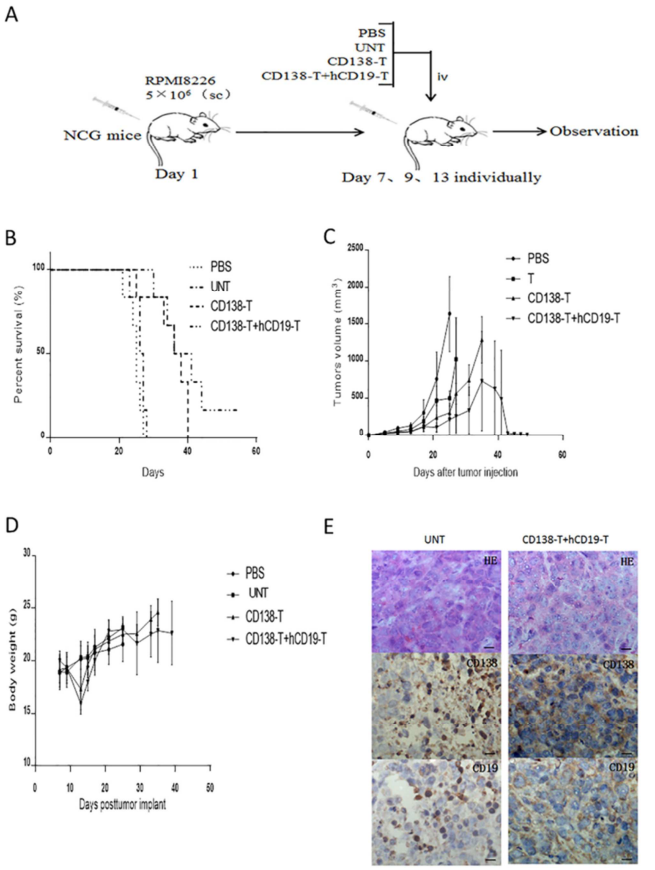

Figure 6. *Combination treatment with CD138-T and hCD19-T against CD138-positive MM cell can work in xenograft mouse model. (A) 5 to 6-week-old female NCG mouse were injected subcutaneously in the right dorsa with RPMI8226 (5×10^6), and after one week, mice were randomly divided into four groups (6 per group) receiving treatment intravenously on day 7, 9 and 13: group 1 received PBS, group 2 received UNT cells, group 3 received CD138-T cells and group 4 received CD138-T combined with hCD19-T cells. (B) Mice were humanely sacrificed when tumors reached 15 mm in diameter. Survival was evaluated from the first day of tumor injection until death. Statistical analysis was performed using the log-rank (Mantel-Cox) text. *p<0.05, **p<0.01, ***<0.001 compared with UNT. (C) Tumor volume of the mice was measured every 2-3 times one week using the following formula: $4\pi/3\times(\text{tumor length}/2)\times(\text{tumor width}/2)^2$. (D) Body weights of the mice were measured every 2-3 times one week. The values are presented as the mean±standard error of the mean.(E) Immunohistochemistry slides of subcutaneous xenograft sections from UNT group and double-CAR group were presented. The CD138 and CD19 antigen were detected respectively. And the sections were stained with hematoxylin and eosin to present its structure. Magnification: ×400. (Scale bar 50μm).*

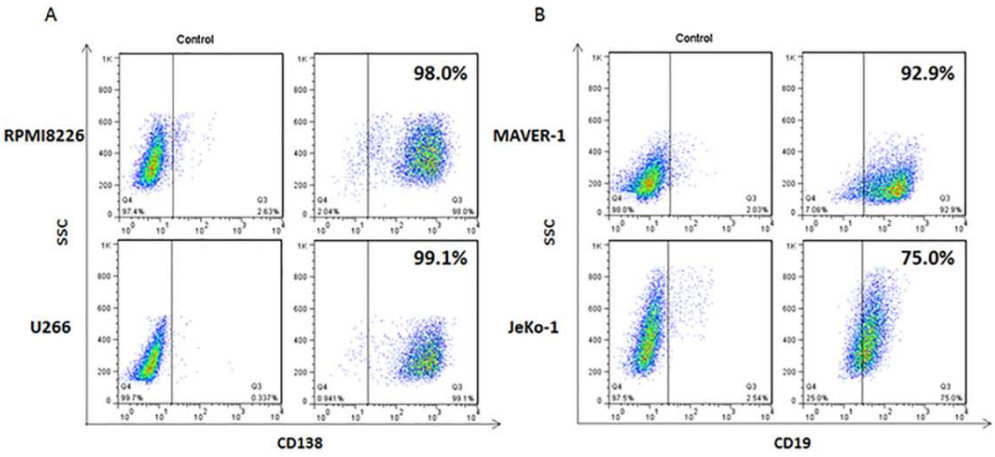

Figure 7. *High expression of corresponding antigen in multiple myeloma and mantle cell lymphoma. (A) To test the expression rate of CD138 antigen in MM cell lines RPMI8226 and U266, both cell lines were stained FITC-conjugated anti-CD138 mAb, and analyzed using flow cytometry. (B) Mantle cell lymphoma MAVER-1 and JeKo-1 exposed to PE-cy5-conjugated anti-CD19 mAb, and analyzed using flow cytometry.*

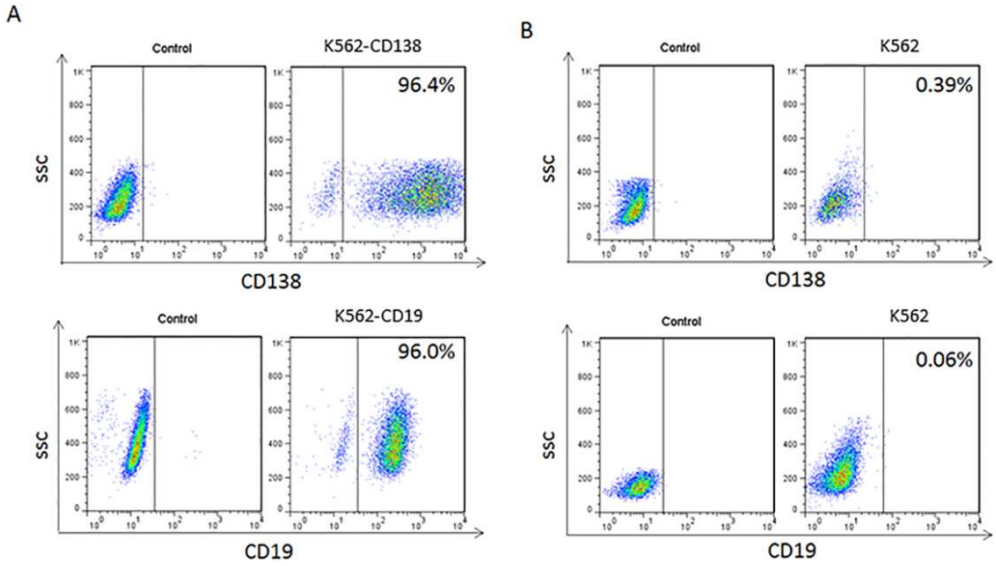

Figure 8. *The structure of K562 expressing CD138 or CD19 antigen. Untreated K562 cell is negatively express CD138 and CD19 antigen, as shown in (B). K562 cell was stained FITC-conjugated anti-CD138 mAb or PE-cy5-conjugated anti-CD19 mAb and placed for 20 minutes at rt followed by washing three times with PBS, and analyzed using flow cytometry. (A) K562 cell was transfected by lentivirus containing the cDNA sequence of CD138 or CD19 antigen for 6 hours and sorted by FACSAria™III Cell Sorter. Established K562-CD138 and K562-CD19 cells were incubated with anti-CD138 mAb and anti-CD19 mAb individually.*

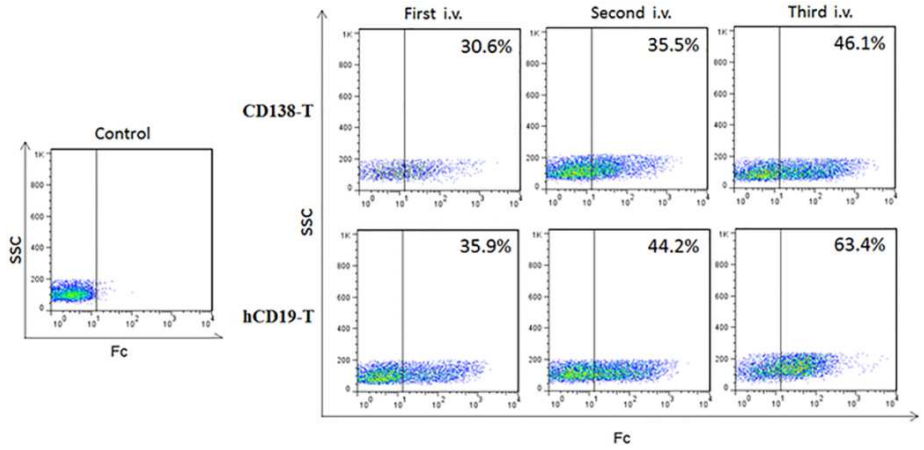

Figure 9. *Detection of transfected efficiency of CAR-T cells used to injection. Activated T cells was infected by lentivirus containing the cDNA sequence of CD138 or CD19 mAb and cultured for about one week, and was stained APC-conjugated anti-Fc mAb before analyzed.*

3.7. Treatment of MM in the Mouse Model

To determine whether CD138-CAR-T and hCD19-CAR-T cells exert effects *in vivo*, the NCG mouse model was used. RPMI8226 MM cells were subcutaneously injected in the mice, and 1 week later, the control groups received tail vein injections of PBS or UNT cells, while the experimental groups received injections of CAR-T cells. Three treatments on the 7^{th}, 9^{th}, and 13^{th} day after tumor cell injection were administered, after which the survival times of the mice were observed (Figure 6A). The CAR-positive rates of CD138-CAR-T cells administered during the three treatments were 30.6%, 35.5%, and 46.1% respectively, and the CAR-positive rates of hCD19-CAR-T cells were 35.9%, 44.2%, and 63.4% (Figure 9). The results indicated that the treatment effect was significantly pronounced in the experimental groups as compared to the PBS and UNT cell control groups. Survival times for the CD138-CAR-T cell group (single-CAR group) and the CD138-CAR-T plus hCD19-CAR-T group (double-CAR group) were significantly higher than those in the PBS and UNT cell groups. Complete remission and ultimate tumor disappearance was achieved in one particular mouse of the double-CAR group, which indicates that the treatment effect of the double-CAR group was significantly superior to that of the single-CAR group. Data analysis using the log-rank (Mantel–Cox) test indicated that the treatment effect of single-CAR vs. UNT cells was significantly different ($p=0.0102$). When the double-CAR group was compared with the UNT cell group, the p-value was less than 0.0006, which indicates that the differences were extremely significant. The median survival times of mice in the PBS, UNT cell, and single-CAR groups were 24.6, 26.2, and 35.4 days, respectively, and they were even longer for the double-CAR group (Figure 6B).

Tumor growth was significantly suppressed in the single- and double-CAR groups after the first treatment, while rapid tumor growth was observed in the PBS and UNT cell groups. Differences in tumor growth remained significant during subsequent treatments, e.g. tumor volume in the single-CAR group was lower than that in the UNT cell group on day 25 (306.8 ± 84.7 mm^3 vs. 503.0 ± 97.8 mm^3, $p=0.0095$), and tumor size in the double-CAR group was significantly lower than that in the UNT cell group on day 21 (111.4 ± 59.0 mm^3 vs. 473.7 ± 195.0 mm^3, $p=0.0014$) (Figure 6C). In general, tumor growth was slower in the double-CAR group than in single-CAR group, which indicates that double-CAR treatment has a stronger effect than single-CAR treatment. Changes in body weight of the mice were also closely monitored during the experiment, and the results indicated that there were no major differences among the various groups (Figure 6D). Immunohistochemistry tests on tumor tissues obtained from the mice revealed that fewer CD138 and CD19 antigens were present on tumor surfaces in the double-CAR group than on those in the UNT cell group (Figure 6E).

4. Discussion

CAR-T cell therapy is a novel form of immunotherapy, and its development is the most promising among the various tumor treatment strategies. The TAA targeted by the scFv in a CAR is major histocompatibility complex-independent, and targets can be selected from a diverse range of molecules [34-35]. In particular, the CAR-T cell therapeutic product CTL019, developed by Novartis, targets CD19 on the surfaces of hematologic tumors. Significant results have been obtained in clinical applications, and CTL019 has been approved as the first gene-modified cell therapy product available in the market. In this preclinical study, the effects of CAR-T technology in MM treatment were investigated, and good results were achieved. The strategy adopted for this study was the concomitant use of CD138- and CD19-directed CAR-T cells. It is widely known that MM cell lines and bone marrow plasma cells of patients highly express CD138 [12-14, 16]. MM cells also express high levels of CD38, another common target for MM treatment; however, CD38 is also expressed by T and B cells, which poses difficulties for CAR-T cell therapy in distinguishing MM cells from T and B cells [36]. Successful applications of radioimmunotherapy drugs, such as iodine-131-labeled anti-CD138 mAb, in clinical trials have indicated to some extent that CD138 is a high-potential target, especially in the treatment of refractory and relapsed MM [37]. A phase II clinical trial by Jagannath et al. using BT062, an antibody-drug conjugate directed against CD138, also indicated the potential of CD138 as a target in MM treatment [38].

Recent studies have identified the B cell maturation antigen (BCMA) as another extremely promising target for CAR-T cell therapy. Significant MM treatment effects of BCMA-directed second-generation CAR constructs in mice have been previously reported, with 100% of the mice being cured of the disease [39]. MM cells express high levels of BCMA, while plasma cells and mature B cells express low levels or do not express BCMA [40]. In addition, BCMA is not expressed in normal tissues and on the surface of hematopoietic stem cells [39]. BCMA is a member of the tumor necrosis factor receptor superfamily and provides anti-apoptotic signals for MM cells [41]. To date, at least four cases of clinical application of immunotherapy using BCMA-directed CAR-T cells have been reported in the U.S., and fairly good results were obtained in all cases, which also proves the safety of *in vivo* application of BCMA-directed CAR-T cells [42-45]. As CD138 is expressed on dermal tissues, the use of anti-CD138 antibody drugs often leads to skin and mucosal damage; therefore, toxicity issues may arise with CD138-CAR-T cell therapy [46]. Currently, BCMA is the most promising target for MM treatment [46]; however, this does not imply that CD138 is an unsuitable target for MM treatment. For instance, in a clinical trial of BCMA CAR-T cells, BCMA-positive MM cells in some patients gradually decreased with repeated doses of

BCMA-CAR-T cells, and the patients eventually suffered a relapse [42]. In addition, BCMA is not expressed in all MM patients. Ali et al. analyzed bone tumor sections of 85 patients and found that in 33 patients, MM cells did not express BCMA. On the other hand, a study conducted by Lin et al. (2004) on 306 MM patients indicated that abnormal plasma cells of all patients expressed CD138, which shows that CD138 has a wider application range as a target for MM treatment than does BCMA. Thus, these two types of target cells have their respective advantages and disadvantages.

Guo et al. [21] reported the construction of a second-generation CD138-directed CAR with a 4-1BB costimulatory domain for the treatment of chemotherapy-tolerant patients with MM. Out of five patients who received 3–6 infusions of CD138-CAR-T cells, four patients developed fever and their condition remained stable for 3–7 months, while the other patient died because of disease progression. The results achieved in the study were very limited, and the data obtained lacked comprehensiveness. Although the clinical efficacy of CD138-CAR-T cells in the aforementioned study was limited, the study has offered substantial proof of the safety of CD138-CAR-T cells in clinical applications. In contrast to the construct used in the aforementioned study, the antibody sequence used in this study, a patented sequence developed by our research team, harbored CD28 as a costimulatory domain for the CAR constructs. Furthermore, the methods of preparation of the CAR-T cells used in both studies are fundamentally different. Therefore, a number of factors could have caused the results of our *in vitro* experiment to be significantly superior to those obtained by Guo et al.

With regard to the construction strategy, a second-generation CAR construct with CD28 as the costimulatory domain was selected for the construction of CD138-CAR, while a third-generation CAR construct with two costimulatory domains, CD28 and 4-1BB, was selected for the construction of hCD19-CAR. The current literature on CAR-T cell construction strategies have not shown sufficient proof that the third-generation construct is significantly superior to the second-generation construct. In some studies, experiments in mice showed that the third-generation CAR had no significant advantages over the second-generation CAR, and was even found to be slightly less effective [47]. While the precise underlying reasons are still unclear, it has been proposed that the dual stimulation increased activation-induced cell death and decreased the antitumor efficacy of the third-generation CAR [48]. In spite of these findings, during the construction of CD19-CAR in this study, it was found that the third-generation CD19-CAR constructed in our laboratory had a higher cytokine production capacity than the second-generation CD19-CAR (data not shown); therefore, the third-generation CAR construct was used in this study.

In this study, CD19 was selected as a second target for the dual treatment of MM. A major experimental hurdle in this study was the inability to obtain sufficient primary CD138–/CD19+ MM cells. The fraction of CD19+ in primary MM cells is extremely low [24], and these cells are difficult to detect by routine methods. Lin et al. [15] analyzed bone marrow aspirates of several hundred MM patients by flow cytometry, and the results indicated that CD19+ MM cells were found in only 1% of cases. Gupta et al. [49] analyzed bone marrow aspirates of 103 MM patients and found that CD19+ MM cells were seen in only 3.7% of cases. Therefore, to effectively validate the activity of the CD19-CAR-T cells developed in this study, CD19+ tumor cells, specifically, the mantle cell lymphoma cell lines MAVER-1 and JeKo-1, were selected as the experimental model. For *in vitro* cytotoxicity testing, two approaches were adopted: the CFSE/7-AAD assay and the measurement of LDH released by dying target cells. The results showed that high killing rates could be achieved at low E:T ratios. The rate of tumor cell death was not increased when the E:T ratio was increased to 2:1, which might be explained by the methods of operation and calculation for the LDH release assay. For instance, when the E:T ratio was increased, the increase in OD value was greater for groups with only effector cells than for those with both effector and target cells, which led to calculation errors. However, the presence of such errors does not affect the proof of high toxicity of CAR-T cells towards tumor cells. The results obtained by the LDH assay method and the CFSE/7-AAD assay method were similar.

CAR-T cell therapy has achieved remarkable results in recent years, especially in B-ALL and B-NHL indications. However, MM-directed CAR-T cell therapies are still in a preliminary stage of development in terms of the number and scale of clinical studies. The CD138-CAR-T cells constructed in this study demonstrated good activity *in vitro*, and relatively good results were also obtained in the *in vivo* killing experiments using a mouse model. In particular, complete remission was achieved in one particular mouse of the double-CAR group, and the median survival time of the double-CAR group was significantly higher than that of the single-CAR group, which indicates that the concomitant use of CD138-CAR-T and hCD19-CAR-T cells resultes in a significant enhancement of treatment efficacy, thereby offering greater potential for clinical applications.

5. Conclusion

This preclinical study tested the applicability of the novel CAR-T cell technology for the treatment multiple myeloma. CD138- and CD19-directed CAR-T cells were developed and their killing activities were assessed *in vitro* and *in vivo*. Both cell types showed significant and specific killing activity in vitro, and concomitant application resulted in enhanced effect. Further, our dual approach showed substantially stronger treatment efficacy than a previously reported CD138-directed CAR.

Conflicts of Interest

L. Yang is a co-founder of PsesonGen Biotherapeutics Co., Ltd. which focuses on R&D of CAR-T and CAR-NK technologies and translating them to clinics. There is no potential conflict of interest to disclose.

Authors' Contributions

S. B. Z. performed the experiments, acquired and interpreted the data, and wrote the manuscript. C. W. was involved in the construction of plasmid and lentiviral vectors. J. L. L. and Z. X. L. contributed to the animal studies. Y. F. L. performed the cell killing assays. H. M. M. and G. L. A. cultured some cells. L. Y. designed the experiments, analyzed and interpreted the data, and gave an elaborate guidance for this project.

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