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Original Article

PiggyBac-modified CD19-expressing 4T1 cell line for the evaluation of CAR construct

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Abstract: Reliable and stable target cell lines are required for evaluating the efficiency and studying the mechanism of chimeric antigen receptor T (CAR-T) immunotherapy both *in vitro* and *in vivo*. Jurkat cells can be used as an alternative for human primary lymphocytes to evaluate the constructs and function of the "CAR". This study established a murine 4T1-CD19 cell line that stably expressed a *cd19* gene. The 4T1-CD19 cells had similar growth kinetics to its parent cell 4T1. The protein CD19 expression of the 4T1-CD19 was detected by reverse transcription-polymerase chain reaction (RT-PCR) and western blot. The second-generation CAR was constructed and transfected into Jurkat cells. The expression of CAR protein was analyzed by flow cytometry and western blot. Finally, the interaction between the CAR and CD19 was confirmed by the upregulation of the *IL-2* mRNA level of Jurkat-CAR stimulated by 4T1-CD19. Therefore, the 4T1-CD19 cell line and Jurkat-CAR have been successfully established, and may be used to access the function of various CAR constructs both *in vitro* and *in vivo*.

Keywords: 4T1, Jurkat, IL-2, murine, CAR-T

Introduction

Autologous T cells are usually modified to express chimeric antigen receptors (CARs), and these T cells are named CAR-T. These cells are redirected to killing specific tumor cells. This is a novel and revolutionary anti-cancer strategy for tumor immunotherapy and it is specifically efficient against CD19-positive B cell malignancies [1-3]. CARs usually consist of an extracellular part responsible for the recognition of specific tumor antigen, and an intracellular segment that can improve cytotoxic and proliferation activity of CAR-T. The binding properties of CARs to the chosen specific tumor antigen are normally determined by a single-chain variable fragment (scFv), which is derived from a monoclonal antibody. These scFv-derived fragments acquire different binding affinity, and major histocompatibility complex (MHC)-independent binding with its ligands are usually with specific tumor antigens. The clinical trials of the first-generation CARs were disappointing. Then, second-generation CARs were developed, with the scFv part combining with a co-stimulatory part (often 4-1BB or CD28) and a T cell pro-activator cytotoxic fragment (CD3z) [4, 5]. Recent clinical trials based on anti-CD19 CAR-T cells of second-generation exhibited astonishing results in patients with acute lymphoid leukemia (ALL), non-Hodgkin's lymphoma, and chronic lymphocytic leukemia. These clinical results have been summarized by Lorentzen and Straten [6] and Wang et al. [3]. The efficiency of these CARs is now being tested in an international multi-center clinical trial. Two leading products, tisagenlecleucel (Kymriah, Novartis) and axicabtageneciloleucel (Yescarta, Kyte-Gilead), are already authorized by the Food and Drug Administration (FDA) of North America (U. S. and Canada) and Europe for clinical use. According to the published results, response rates vary between 50% and 85% with impressive disease-free and overall survival [6].

However, despite these positive data, this T cell-based treatment can cause severe toxicities. These engineered CAR-T cells harm non-tumor cells because of on-target/off-tumor effect. If this effect occurs in the important organs like heart, lung or liver, then the CAR-T cells with strong cytotoxicity could threaten the life of the patient [7]. Besides, the killing efficiency of CAR-T cell remains disappointing in the settings of solid tumors [8, 9]. Some conditions exist that may mitigate the killing capacity of CAR-T against solid tumors; for example, differently expressed tumor-associated antigens could cause tumor escape during treatment [10]. Thus, the CAR construct should be optimized to increase its efficiency while simultaneously decreasing the toxicity. Wu et al. designed the "ON-switch" CARs that enable small molecules to control T cell functions without affecting the antigen specificity [11]. This method would help physicians to precisely control the timing, distribution, and dosage of CAR-T, which could improve the efficiency with mitigated toxicity. In this kind of research, corresponding cancer cells expressing a specific ligand of the CARs should be established. Usually, the efficiency of the CAR-T cells is evaluated in an NSG mice model which lacks autologous immunity. This kind of *in vivo* model cannot study the involvement of the host immune system during the CAR-T treatment process. So, murine-origin cancer cells expressing corresponding ligand should be developed as a tool for the evaluation of the CAR construct.

We therefore built a strategy to generate a CD19 expressing murine 4T1 cell line, which is a highly metastatic murine adenocarcinoma with rapid growth, derived from a BALB/c mouse with spontaneous mammary tumor [12] and the interaction between CD19 and CAR was confirmed by the 4T1-CD19 induced activation of Jurkat-CAR. This 4T1-CD19 cell line and Jurkat-CAR reported here will be further used in our lab as a tool to evaluate the CAR construct in a pre-clinical study.

Materials and methods

Construction of anti-CD19 CAR lentiviral vector and PBDP-CD19 vector

The anti-CD19 scFv derived from FMC63 was synthesized (GenScript Biotech, China) and inserted into a lentiviral vector plvx-acgfp-N1. The human CD19 gene was synthesized and sub-cloned into PiggyBac Dual promoter. The ultima vectors were confirmed by gene sequencing (Tsingke Biotech, China).

Cell lines

4T1 cells and Human embryonic kidney (HEK) 293T cells were purchased from the Cell Bank of Chinese Academy of Sciences (SGST, China) and maintained in DME/F12 (Hyclone, USA) containing 10% fetal bovine serum (FBS) (Zhejiang Tianhang Biotechnology, China). Jurkat cells were obtained from the Cell Bank of Chinese Academy of Sciences (SGST, China) and maintained in RPMI-1640 medium (Hyclone, USA) containing 10% FBS. The different kinds of cells were cultured in a cell incubator at 37°C and 5% CO_2.

Generation of 4T1-CD19 cell line and CAR expressing Jurkat cells

4T1 cells were transfected with the plasmid PBDP-CD19 and plasmid Super PiggyBac transposase using Lipofectamine 3000 (Invitrogen, USA) following the protocol introduction. CD19-expressing cells were selected with 6 μg/ml of puromycin (BioFroxx, Germany) diluted in DME/F12 containing 10% FBS and the selected culture medium was replaced every 2-3 days until most of the 4T1 cells were GFP-positive. Following this stage, the cells were digested and seeded in 96-well culture plate at a density 1-2 cells/well to obtain monoclonal 4T1-CD19 cells. The lightest cells were selected for further culture. For the generation of Jurkat-CAR cells, lentivirus was added to the cultures at a multiplicity of infection (MOI) of 5, and 6 μg/ml transduction enhancer polybrene (Solar Bio, China) was added. The culture medium was replaced every 2-3 days and the cell density was maintained at 1-3 × 10^6 cells/ml.

Production of lentivirus

In the stage of lentivirus' packaging, 293T cells were transfected with plvx-acgfp-h1928z, psPAX2, and pMD2.G. The packaging plasmids were added at a ratio of 2:1:2. The day before transfection, 293T cells were seeded into a T75 cell culture flask and maintained in DME/F12 (Hyclone, USA) containing 10% FBS (Zhejiang Tianhang Biotechnology, China). When the cell confluence reached 60%~80%, the transfection was conducted using Lipofectamine 3000 (Invitrogen, USA). Then, the cells were

cultured in a cell incubator at 37°C, and 5% CO_2. The supernatants containing viral particles were harvested after 48 hours and mixed with Lenti-X™ concentrator (Takara Bio, Japan) overnight at a temperature of 4°C. The next day, the supernatants containing viruses were centrifuged at 1,500 × g for 45 minutes at 4°C, suspended in phosphate buffered saline (PBS) (Jet Bio-Filtration, China), and stored at -80°C for a later experiment.

Flow cytometry

Monoclonal 4T1-CD19 cells were incubated with fluorescence labeled antibody directed against human CD19 (BD Pharmingen, USA) for 20 minutes at 4°C and washed once with PBS. The sample analysis was conducted on a BD C6 flow cytometer and analyzed with FlowJo VX software. Expression of CD19-CAR was verified according to the GFP positive rate. The untreated Jurkat cells were used as a control group.

Jurkat-CAR cells function assay

An in vitro assay was conducted to evaluate the biologic function of Jurkat-CAR cells. Briefly, 4T1-CD19 cells were seeded in a 96-well plate at a density of 10^4 cells per well. Effector cells and medium were added at an equal volume at an E/T ratio of 8:1 and 16:1. After 24 hour incubation, cells were obtained, the mRNA was extracted and reverse transcribed to cDNA, and finally stored at -20°C.

Quantitative real-time PCR

To further investigate the expression level of CD19 and IL-2, qPCR was conducted through a StepOne™ Real-Time PCR system (Applied Biosystems). RNA was extracted with RNAsimple Total RNA Kit (TianGen, China) and reverse transcribed with HiScript Q RT SuperMix (Vazyme, China). The human CD19 gene was detected through the following primers: 5'-TACCTGATCTTCTGCCTG-3' and 5'-TCATCCTCTTCCTCTTCC-3'. The human IL-2 gene was detected through the following primers: 5'-GGACTTAATCAGCAATATCAA-3' and 5'-AAGGTAATCCATCTGTTCA-3'.

Statistical analysis

All results are presented as the mean ± standard deviation (SD). Statistical analysis was performed with GraphPad Prism software version 5.0. For comparison of the two groups, two-tailed unpaired t tests were used and *P*-values <0.05 were considered significant.

Results

Generation of the CD19 expression 4T1-CD19 cell line

The eukaryotic expression plasmid PBDP-CD19, containing puromycin expression cassette, was constructed and the amino acid sequence was confirmed to have 100% identity with CD19 sequence (NCBI Reference Sequence: NP_001761.3). In order to screen the CD19 expressing 4T1-CD19 cells, the killing curves of 4T1 cell under different concentrations of puromycin ranging from 1 to 10 μg/ml were determined (**Figure 1A**). Following 8 days of puromycin disposal, complete cell death was observed at 6 μg/ml on the 4th day. The plasmid PBDP-CD19 and PiggyBac transposase were both transfected into 4T1 cells, and 6 μg/ml of puromycin was used for the recombinant cell selection. After 7 days of selection, only 6-9% of 4T1 cells had survived and most of them were GFP positive (**Figure 1B**). Then, the cells were digested and seeded in 96-wells culture plate to obtain monoclonal 4T1-CD19 cells. After about 4 rounds of passage, the highly CD19 expression of 4T1-CD19 cell line was selected with the positive rate being 99.7% (**Figure 1C**).

Characterization of the 4T1-CD19 cell line

Cell numbers of 4T1 and 4T1-CD19 were determined at different time points. The growth curves of the 4T1-CD19 and 4T1 were depicted according to the results. The curves indicated that CD19 insertion did not affect the proliferation activity of 4T1 (**Figure 2A**). The expression of CD19 was further confirmed through RT-PCR and western blotting. CD19 specific primer was applied to detect the exogenous CD19 mRNA level. The relative expression level of CD19 in 4T1-CD19 was ~4,200 (4,210.86±938.65) fold, which was significantly higher than that of the control 4T1 (**Figure 2B**). Immunoblotting was performed to confirm the protein expression of CD19. The 4T1-CD19 lane showed a 95 kilo-Dalton (kD) exogenous band, whereas the control 4T1 cell lane did not show any band (**Figure 2C**). Collectively, these results showed that the *cd19* gene was successfully inserted into the genome of 4T1, and that the CD19 protein can also be detected.

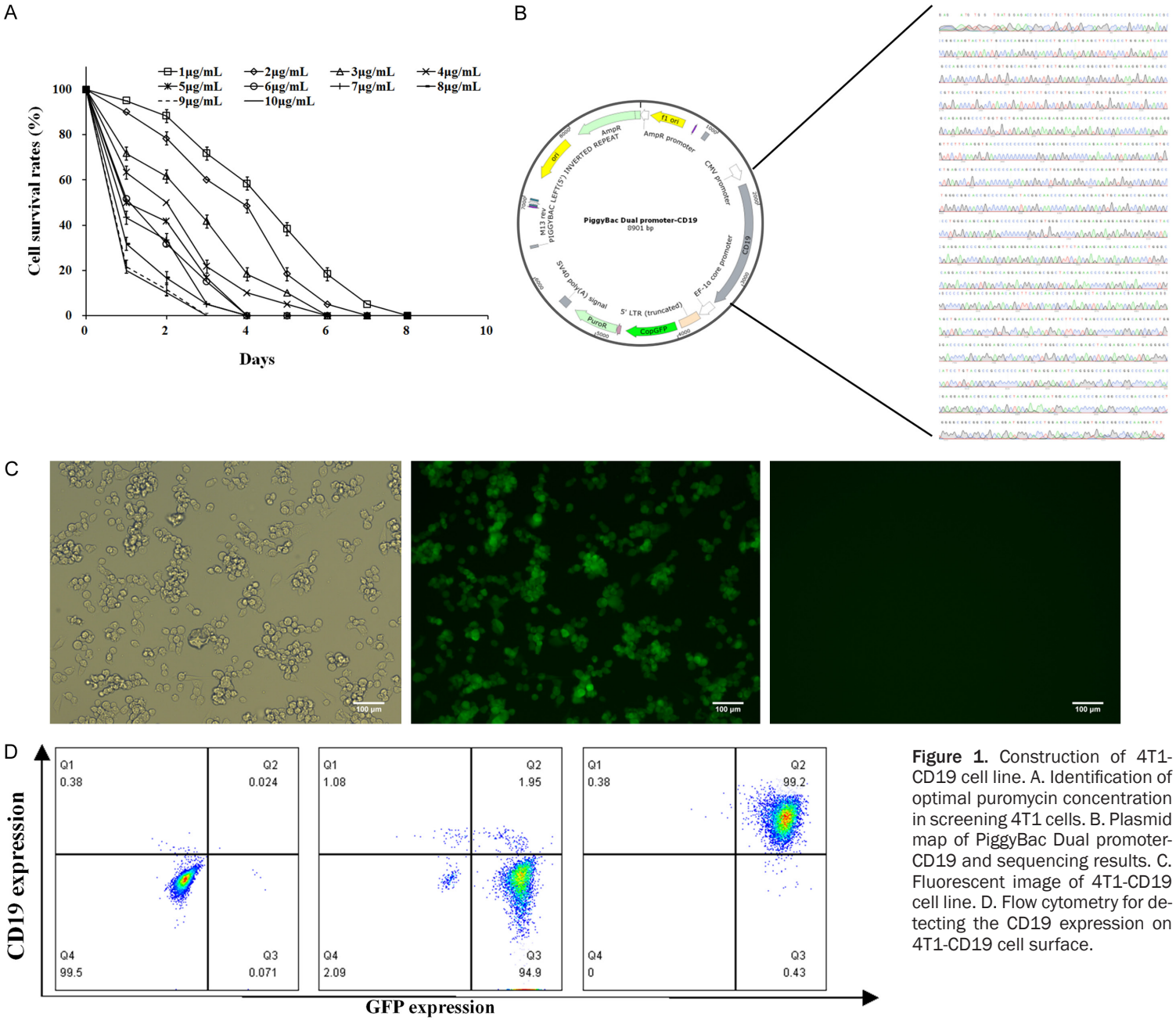

Figure 1. Construction of 4T1-CD19 cell line. A. Identification of optimal puromycin concentration in screening 4T1 cells. B. Plasmid map of PiggyBac Dual promoter-CD19 and sequencing results. C. Fluorescent image of 4T1-CD19 cell line. D. Flow cytometry for detecting the CD19 expression on 4T1-CD19 cell surface.

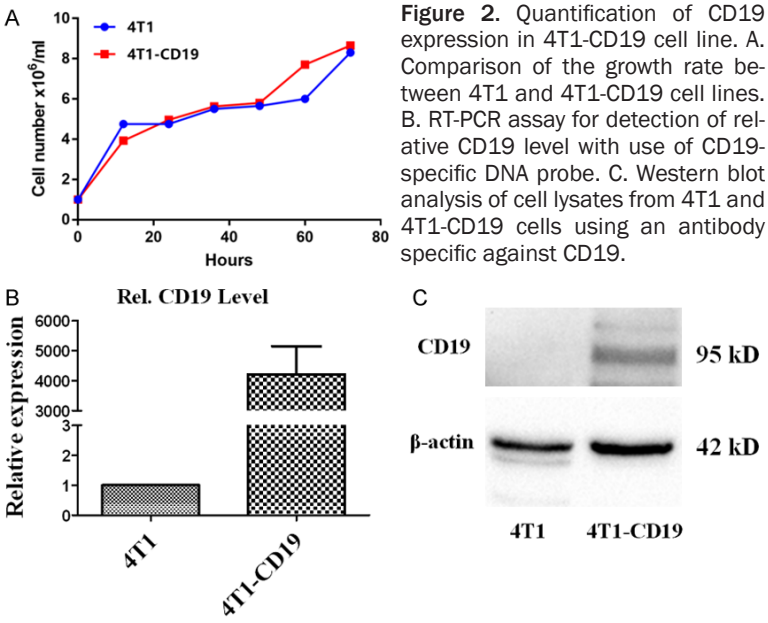

Figure 2. Quantification of CD19 expression in 4T1-CD19 cell line. A. Comparison of the growth rate between 4T1 and 4T1-CD19 cell lines. B. RT-PCR assay for detection of relative CD19 level with use of CD19-specific DNA probe. C. Western blot analysis of cell lysates from 4T1 and 4T1-CD19 cells using an antibody specific against CD19.

Construction and identification of Jurkat-CAR cells

Compared with the first-generation of chimeric antigen receptor T cells, the second-generation cells additionally possess an anti-CD19 specific scFv linked to a hinge domain, a part of the CD28 costimulatory molecule and cytoplasmic portion of the TCR-ζ molecule (**Figure 3A**). The fragment was inserted into a lentiviral vector system, plvx-acgfp-N1. A green fluorescence protein (GFP) was added after the CAR to verify the transfection efficiency. The expression of CD19-CAR was characterized by flow cytometry and western blot. The flow cytometry showed expression of CD19-CAR on transfected Jurkat cells, but not on the control Jurkat (**Figure 3C**). The transfection efficiency of Jurkat-CAR was over 60% (**Figure 3C**). Immunoblotting of the Jurkat-CAR lane showed a band between 55-70 kD (calculated molecular weight of CAR), whereas the control Jurkat lane exhibited no band (**Figure 3B**).

Jurkat-CAR cells produced IL-2 after stimulation by 4T1-CD19 cells

To verify the biologic function of CAR and CD-19 construct, Jurkat cells were incubated with 4T1-CD19 cells at different effector-to-target (E/T) ratio of 8:1 and 16:1 for 12 h, separately. After incubation, the Jurkat cells were collected to conduct RNA extraction. The relative expression level of cytokine IL-2 was detected. Compared with the control group, the expression of IL-2 mRNA was upregulated in Jurkat-CAR co-treated with CD19-4T1, with corresponding 17.48 and 5.37-fold increase, respectively under different E/T ratios (**Figure 4**).

Discussion

In this study, we established a monoclonal murine cancer cell line, 4T1-CD19. Furthermore, the membrane localization of CD19 was observed by immunofluorescence and flow cytometry. Then, the interaction of CD19 and CAR was confirmed by the activation of Jurkat-CAR. 4T1-CD19 could be further used *in vitro* and *in vivo* to study both the functional and structural properties of a specific construct of CARs and its ligands.

CD19 (95 kDa), the B lymphocyte antigen receptor, is an important co-receptor for membrane immunoglobulin (mIg). It belongs to the immunoglobulin super family that is expressed in almost all growth stages of B-cells and also could be identified on most acute B-lymphoblastic leukemia (ALL) cells, chronic B-lymphocytic leukemia (CLL), and non-Hodgkin lymphoma [13]. These properties determined CD19 as the best target for CAR-T therapy against B-lymphoid malignancies. According to the published results, CART-CD19 cells showed specific, effective, and persistent killing activity against $CD19^+$ tumor cells [14]. Thus, we tried to develop a solid murine tumor 4T1 expressing CD19, which could offer an ideal cell line to study the killing mechanism of CAR-T cells against solid tumors both *in vitro* and in immunocompetent mice model. PiggyBac (PB), an efficiently transposon system, has always been used to generate induced pluripotent stem cells from somatic cells, without gene alterations [15]. It is a highly active transposon derived from the *cabbagelooper moth* that can provide sustained transgene expression of human T lymphocytes [16]. Thus, we used PiggyBac transposon systems as a methodology for stable genetic modification. The plasmid

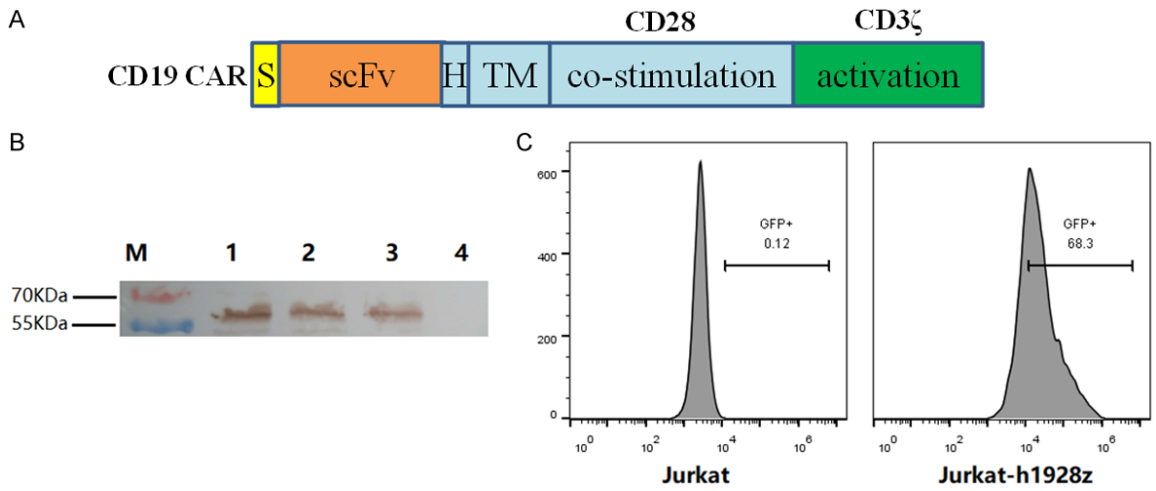

Figure 3. h1928z CAR gene structure and characterization of Jurkat-CAR cells. A. Schematic illustration of CAR containing gene. B. Detection of CAR by immunoblotting assay using an antibody specific to CD3z. CAR presented at ~59 kD. C. Flow cytometry assay to determine positive ratio of Jurkat-CAR cells.

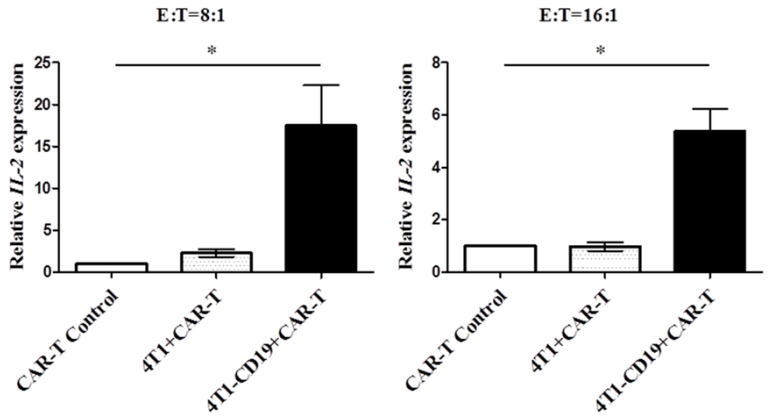

Figure 4. Study of Jurkat-CAR cell function. Jurkat-CAR cell incubation with the 4T1-CD19 cell line in different E:T ratio; IL-2 expression level determined with IL-2 specific probe.

PBDP-CD19 was introduced into 4T1 cells and positive cells were selected based on the resistance to puromycin; the highest level of CD19-expressing cell clone was selected and the 4T1-CD19 cell line was established. The integration of CD19 into 4T1 was confirmed by the RT-PCR on mRNA level and western blot on protein level. The passaged 4T1-CD19 cells exhibited similar proliferation characteristics when compared with the parent 4T1 cells according to the growth curves.

The 4T1 mammary carcinoma, originally obtained by Fred Miller and colleagues, is a metastatic tumor cell line [17]. Unlike other tumor models, the 4T1 tumor is very tumorigenic, invasive, and can initially metastasize from the primary sites of mammary gland to different distant sites including blood, liver, lymph nodes, brain, lung, and bone [18]. The 4T1 tumor has several features which makes it an appropriate cell line for the study of human mammary cancer. To the best of our knowledge, there are limited reports about the expression of specific protein from gene modification of murine cancer cells using a PB system.

Jurkat was obtained from a patient with acute lymphoblastic leukemia [19]. The Jurkat cell line could be used to study the effects of various natural or synthetic compounds on $CD4^+$ T-cell activation, proliferation, and apoptosis. Wu et al. also reported that Jurkat T cell could be used to evaluate the construction of CAR and its specific ligand [11]. Jurkat-CAR secretes IL-2 upon its ligand-specific stimulation. In this study, lentivirus was used to introduce a second-generation CAR gene into Jurkat cells. Compared with the first-generation CAR, the second-generation CAR incorporates classical costimulatory cytoplasmic domains, like CD28, 4-1BB (CD137), OX40 and ICOS, either separately or in combination, which can boost the

biologic function and proliferation of CAR-T [20]. In this study, transfection efficiency of CAR gene into Jurkat cells was analyzed by flow cytometry. After 20 day culture of Jurkat cells, the GFP could still be detected, which indicates stable expression of CAR gene in Jurkat cells. Then, the Jurkat-CAR was incubated with 4T1-CD19 and 4T1, separately. The real time PCR results of IL2 proved that the CD19 cognate ligand and the CAR had been successfully constructed and it was sufficient to trigger IL-2 expression of Jurkat-CAR.

In summary, the monoclonal murine 4T1-CD19 cell line established here not only provided platforms for evaluation of a CAR construct *in vitro* but in the future could also be used to study the activity of CAR-T against solid tumor in an immunocompetent murine model.

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Disclosure of conflict of interest

None.

Abbreviations

CD19, Cluster of Differentiation 19.

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