Chimeric Antigen Receptor T Cells Engineered to Secrete CD40 Agonist Antibodies Enhance Antitumor Efficacy

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Research

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Abstract

Background

Although chimeric antigen receptor (CAR) T-cell therapy has made remarkable achievements against hematological malignancies, the efficacy of it against solid tumors has been limited. By being combined with immune checkpoint inhibitors, such as PD-1, PD-L1, or CTLA-4 antibodies, this therapy has been shown to be a promising strategy to enhance the antitumor efficacy of CAR-T cells. However, due to the fact that acquired resistance to checkpoint inhibitors will occur in most patients, it is vital to investigate other strategies to further improve the antitumor efficacy of CAR-T cell therapy in solid tumors. Recently, CD40 agonist antibodies have been shown to possess potential antitumor efficacy by activating the CD40 pathway.

Results

Based on the piggyBac transposon system, rather than the widely used viral vector, we constructed a meso3-CD40 CAR-T targeting region III of mesothelin (MSLN) that possesses the ability to secrete anti-CD40 antibodies. The results show that compared with meso3 CAR-T, which does not secrete the anti-CD40 antibody, meso3-CD40 CAR-T secreted more cytokines and had a relatively higher proportion of central memory T (T_{CM}) cells after being stimulated by the target antigen. In addition, compared with meso3 CAR-T, we found that meso3-CD40 CAR-T had a more powerful cytotoxicity effect on target cells at a relatively low effector to target ratio. More importantly, we demonstrated that meso3-CD40 CAR-T also had enhanced antitumor activity in a human ovarian cancer xenograft *in vivo*.

Conclusions

In conclusion, these results showed that anti-CD40-secreting CAR-T cells generated by non-viral vectors could be a potential clinical strategy for improving the efficacy of CAR-T cell therapies.

1. Background

Recent developments have demonstrated that chimeric antigen receptor (CAR)-T cell therapy can achieve a durable antitumor response in patients with refractory or relapsed B-cell malignancies [1,2]. However, CAR-T cell treatment has been largely ineffective in patients with advanced solid tumors [3]. A critical factor that contributes to the limitation of CAR-T cell therapy to treat patients with advanced solid malignancies may be the immunosuppressive tumor microenvironment. Recently, strategies that utilize an inhibitory checkpoint blockade to modulate the microenvironment can enhance the efficacy of CAR-T cells in some patients with hematologic malignancies[4]. However, no significant benefit of the PD-1 blockade was found in an early-phase trial of a GD2-CAR for treating neuroblastoma patients[5]. This result illustrates the need to consider approaches that employ other immune mechanisms to improve the efficacy of CAR-T cell immunotherapy for solid cancers.

In addition, inhibitory immune regulators and stimulatory checkpoint pathways may also serve as promising targets for cancer immunotherapy. CD40, a member of the TNF receptor superfamily, is expressed primarily by antigen presenting cells (APCs) and B cells. However, other studies have shown that T cells also express CD40 and can directly receive the signal of CD40L or anti-CD40 antibody so as to promote the secretion of cytokines and the generation of memory cells, thus making T cells have stronger antitumor activity[6,7]. As a result, various strategies to activate CD40 in patients with tumors have been investigated, and both CD40L and anti-CD40 antibodies have shown some encouraging results [8-10].

Our previous study demonstrated that modified CAR-T cells targeting membrane proximal (region III) epitope of mesothelin exhibited strong antitumor activity against various solid tumors [11,12]. Given this, CAR-T cells were generated with the ability to secrete the anti-CD40 antibody that target region III of the mesothelin to potentially achieve a more powerful efficacy. For viral vectors, the expensive cost and potential presence of replication-competent virus in the final cell products have impeded their wider implementation in the industry[13]. In this study, we manufactured CAR-T cells using the piggyBac transposon system to overcome some of these obstacles. More importantly, compared with the treatment with meso3 CAR-T, the approach of the manufactured meso3-CD40 CAR-T cells secreting anti-CD40 antibody used in this study seems to be a more effective strategy for treating tumors. Overall, in terms of the cost and efficacy, the strategy of the generation CAR-T cells that secrete anti-CD40 antibodies by exploiting the piggyBac transposon method seems to have potential for cancer treatment.

2. Materials And Methods

2.1. Cells

The SKOV-3 cell line was obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China), and SKOV-3-luc was established in our laboratory as described previously[11]. Human peripheral blood mononuclear cells (PBMC) from healthy donors were purchased from AllCells (Shanghai, China) and cryopreserved in our laboratory.

2.2. Plasmid construction

The plasmid encoding anti-CD40 antibody, pS338B-CD40, was constructed based on the pS338B vector that was kept in our laboratory. The pS338B-CD40 vector consisted the following components: an EcoRI site, a signal peptide derived from the human immunoglobulin kappa chain, the CD40 scFv heavy chain variable region, a (G4S)3 linker, the CD40 scFv light chain variable region, the human IgG4 Fc (EQ) region, and a SalI site[11].

2.3. CAR-T cell manufacturing and expansion

The manufacture and expansion of meso3 CAR-T cells has been described previously[11]. To generate the meso3-CD40 CAR-T cells, T cells were coelectroporation with a pNB338B-meso3 CAR plasmid and a

pS338B-CD40 plasmid using an electroporator (Lonza) and Amaxa® Human T Cell Nucleofector® Kit (Lonza). After transfection, the meso3-CD40 CAR-T cells were cultured using the same method as used for culturing the meso3 CAR-T cells.

2.4. ELISA

ELISA was used to determine the concentration of anti-CD40 antibodies in the supernatant of the CAR-T cells. A total of 1×10^6 CAR-T cells were seeded in six-well plates, and the supernatant of CAR-T cells were then collected after 24 h or 72 h.

The 96-well ELISA microplate was coated with 1 μg/ml of rhCD40 (ACROBiosystems) at 4°C overnight. After the microplate was sealed with 1% BSA for 2 h at 37°C, the collected supernatant was added to the well and incubated for 1 h at 37°C. After washing the wells three times, a mouse anti-human IgG4(HRP) (Abcam) was added to the well at a dilution of 1:30,000 and incubated for 30 min at 37°C. After washing the wells three times, TMB (3,3',5,5'-tetramethylbenzidine) was added to develop the color. All of the experiments were performed in triplicate.

2.5. Cytokine release assays

Cytokine-release assays were performed by coculturing 1×10^6 T cells with immobilized rhMSLN antigen (ACROBiosystems) in six-well plates. After 24 hours, the supernatants were assayed for the presence of all cytokines using the Cytometric Bead Array according to manufacturer's instructions (BD Biosciences). The values represent the mean of the triplicate wells.

2.6. Flow cytometry

To detect the CAR expression on the surface of the cells, the cells were stained with biotin-conjugated rhMSLN antigen (ACROBiosystems) and PE-conjugated streptavidin (BD Biosciences). The following monoclonal antibodies were used for the phenotypic analysis: PE-Cy5 anti-human CD45RO (BioLegend), PE anti-human CD62L (BioLegend), FITC anti-human CD197 (BioLegend), PE-Cy5 anti-human CD25 (BioLegend), PC5 anti-human CD69 (BioLegend), and PE-CY5 anti-human CD107α (BD Biosciences). The acquisition and analysis were performed using the NaviosTM flow cytometer (Beckman Coulter, USA) and using Kaluza Analyse software.

2.7. Real-time cytotoxicity assay (RTCA)

The method to conduct the real-time cytotoxicity assay was described previously [11]. Briefly, targeted cells were seeded in 16-well E-plates at a density of 10,000 cells per well. After 20–24 hours, the CAR-T cells were added at different effector to target ratios (E:T) of 1:2 and 1:4, while the control T cells were set at an E: T ratio of 2:1.

2.8. *In vivo* experiments

All of the animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of the Shanghai Runnuo Biological Technology Co. Ltd. (Shanghai, China). Six to eight-week-old female NOD/SCID/IL2Rγ−/−(NSG) mice were purchased from the Shanghai Runnuo Biological Technology Co. Ltd. Xenograft tumors were established by subcutaneous injection of 1×10^7 SKOV-3-luc in the presence of a 50% solution of Matrigel (BD Biosciences) in a phosphate buffer solution (PBS). Eight days post inoculation, mice bearing established tumors were treated with intravenous injections of 5×10^6 meso3 CAR-T or meso3-CD40 CAR-T cells. The bioluminescence was measured using serial imaging on a Xenogen IVIS Spectrum System (Life Technologies).

2.9. Statistical analysis

The data are presented as means ± standard error for the means (SEM) or medians with ranges, as stated in the figure legends. To compare two selected groups, the Student's t test was used. Statistical significance was defined at $P < 0.05$. All statistical analyses were performed using Prism software version 6.0 (GraphPad).

3. Results And Discussion

The schematic representation of the plasmid vector encoding the anti-CD40 antibody is shown in Figure 1A. The scFv sequence of the anti-CD40 antibody was derived from a fully human IgG2 anti-human anti-CD40 antibody clone 21.4.1[14]. To prevent the potential target cell, namely, CAR-T cells that express CD40, death caused by the immune effector functions of the anti-CD40 antibody, such as ADCC (Antibody-dependent cell-mediated cytotoxicity) or CDC (Complement dependent cytotoxicity), the scFv was fused to a human IgG4 Fc, which has a very limited ability to elicit those effector functions. In addition, to further reduce the effector function of the human IgG4 Fc, a human IgG4 variant with two points mutations at L235E and N297Q was generated, which has been demonstrated to further reduce the effector function of the antibody[15].

In our previous studies, it was shown that the piggyBac transposon system is an efficient vector to generate CAR-T cell products [12,16]. In this study, we demonstrated that CD40-secreting meso3-CD40 CAR-T cells can be successfully manufactured using coelectroporation of CAR-T cells with the two plasmids, pS338B-CD40 and pNB338B-meso3 CAR. Moreover, the introduction of additional anti-CD40 antibody genes did not affect the level of CAR expression on the surface of the T cell (Fig. 1B and 1C). Other similar studies have also demonstrated that the introduction of an additional antibody gene in the generation of CAR-T cells does not severely affect the expression of CAR[17,18].

After generating the meso3-CD40 CAR-T cells, the ability of meso3-CD40 CAR-T cells to secrete anti-CD40 antibody was determined using ELISA. After 24 h of culture of 1×10^6 meso3-CD40 CAR-T cells, the concentration of anti-CD40 antibodies in the supernatant was approximately 0.17 μg/ml, and the concentration rose to approximately 0.28 μg/ml after 72 h of culture, while no detectable antibody secretion level was present in the cell culture supernatant of the meso3 CAR-T (Fig. 1D). The results

showed that the meso3-CD40 CAR-T cells secreted antibodies continuously and stably. This was consistent with the levels of antibodies secreted by CAR-T cells found in similar studies, although those studies used viral vectors to generate the antibody-secreting CAR-T cells[17,18].

It has been previously reported that CD40-CD40L signaling is essential for the development of memory T cells that depend on fatty acid metabolism for long-term survival [19] , and the expression of CD40 on T cells is fundamental for $CD8^+$ T cell memory generation [6], Therefore, the memory phenotypes of T cells were then detected. The results suggested that there was no significant difference on the effector memory (T_{EM}, $CD45RO^+CD197^-$) cells, while the meso3-CD40 CAR-T cells exhibited a higher proportion of central memory (T_{CM}, $CD45RO^+CD197^+CD62L^+$) cells than the meso3 CAR-T cells (Fig. 2A and 2B). T_{EM} potentially home to peripheral lymphoid tissues and possess a rapid effector function. In comparison, T_{CM} tend to home to secondary lymphoid tissues and maintain a long-lived T-cell memory. Given the close correlation between T_{CM} and the prevention of long-term tumor recurrence, a higher proportion of T_{CM} may lead to enhanced antitumor effects *in vivo*[20,21].

CD25, CD69, and CD107a have been known as activation markers of lymphocytes, and they play pivotal roles in immune responses. To characterize the CAR-T cells, the activation markers on CAR-T cell surfaces were detected upon stimulation with MSLN antigen-coated plates for 24 h. The data revealed that after stimulation, the activation makers (CD25, CD69, and CD107α) on the meso3 CAR-T cells and meso3-CD40 CAR-T cells were all at a high expression level (Fig. 2C–F). This demonstrated that meso3-CD40 CAR-T cells and meso3 CAR-T cells were sensitized to the MSLN antigen and could be effectively activated by the MSLN antigen so that they could have a positive role in killing tumor cells.

In addition, cytokine secretion levels after CAR-T stimulated by the target antigen were determined. The results showed that, compared with the meso3 CAR-T cells, the meso3-CD40 CAR-T cells secreted higher IFN-γ and IL-2 after receiving antigen stimulation (Fig. 3A). A previous study showed that CD40 expression on T cells is essential for the high levels of cytokine secretion[6], while in our study, it was demonstrated that the presence of anti-CD40 antibody can further enhance the secretion of cytokines. Given that IFN-γ is closely correlated with efficacy, it is reasonable to anticipate that meso3-CD40 CAR-T may have an improved antitumor efficacy when compared with meso3 CAR-T. Indeed, as shown in Figure 3B, although the CAR-T cells of both groups showed potent cytotoxic activity against SKOV-3, we observed that the meso3-CD40 CAR-T cells had a stronger cytotoxic activity compared with the meso3 CAR-T cells *in vitro*.

In our previous studies, the cytotoxic activity of meso3 CAR-T cells were analyzed at the E:T ratios of 4:1 to 1:1. Considering their potent cytotoxic activity, if may be difficult to observe the difference in the cytotoxic activity of meso3 CAR-T and meso3-CD40 CAR-T cells because the CAR-T cells from both groups can lyse target cells in a short time. In this study, we adopted a relatively low E:T ratio so that the CAR-T cells could lyse the target cell more gently and, in this case, we observed the benefit of the anti-CD40 antibody.

The results of this study showed that the meso3-CD40 CAR-T had more potent antitumor activity against MSLN-positive cells than meso3 CAR-T cells *in vitro*, even at a low effector to target ratio. Next, we investigated whether there was a similar effect *in vivo*. Xenograft tumor models were established by subcutaneously inoculating with 5×10^6 SKOV-3-luc cells. Eight days post inoculation, mice were treated with intravenous injections of 5×10^6 meso3 CAR-T or meso3-CD40 CAR-T cells. *In vivo* cancer imaging intuitively reflected that both meso3 CAR-T and meso3-CD40 CAR-T effectively inhibited tumor growth *in vivo* (Fig. 4A). Encouragingly, meso3-CD40 CAR-T cells exhibited faster and stronger effects than meso3 CAR-T cells. Consistent with the *in vivo* cancer imaging, the fluorescence intensity of the group treatment with meso3-CD40 CAR-T attenuated more quickly than the meso3 CAR-T group (Fig. 4B and 4C). In addition, the weights of the mice were measured continuously, and the results showed that there were no obvious changes in mouse weights after treatment (Fig. 4D). Overall, these results validated that meso3-CD40 CAR-T cells exhibited better antitumor activity than meso3 CAR-T in a xenograft mouse model.

CD40 is also expressed in APC cells and is indispensable to empower APC cells to cross-prime naïve T cells [7]. Therefore, the CD40-CD40L signaling pathway not only regulates $CD4^+$T- $CD8^+$T cell cross-talk to promote an effective response [22], but also possesses an important effect on DC-T cell cross-talk to activate adaptive immunity [7]. In view of this, it can reasonably be inferred that CAR-T cells that secret the CD40 antibody would seem to possess potential effectiveness to activate the immune response against malignant tumors in humans. This conclusion can be made because the mouse model used in this study was NSG mice, which are severely immune deficient mice.

4. Conclusions

Meso3-CD40 CAR-T was found to secrete more cytokines and had a higher proportion of central memory T cells than meso3 CAR-T after being stimulated by the target antigen. Notably, meso3-CD40 CAR-T also had a more potent antitumor response *in vitro* and *in vivo*. It is worth mentioning that the use of the non-viral piggyBac transposon in this study provides strong competitiveness for industrialization development due to its considerable cost savings. Therefore, CAR-T cells piggyBac-engineered to secrete anti-CD40 antibody could be an effective and low-cost therapeutic strategy to further improve the efficacy of CAR-T cell therapy for solid tumors.

Abbreviations

chimeric antigen receptor T-cell : CAR-T

mesothelin : MSLN

Institutional Animal Care and Use Committee : IACUC

Declarations

Ethics approval and consent to participate

Not applicable

Availability of data and materials

All the authors confirm that the data supporting the findings of this study are available within the article and its supplementary materials.

Competing interests

No potential conflicts of interest were disclosed.

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Authors' Contributions

Conceptualization, Q.Q. and Y.Z.; Investigation, Y.Z., P.W. and T.W.; Data curation, P.W. and T.W.; Funding acquisition, Q.Q. ,T.W; Resources, Y.F.; Supervision, Y.D.; Writing—original draft, Y.Z. and P.W.; Writing—review and editing, T.W. All authors have read and agreed to the published version of the manuscript.

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Consent for publication

Not applicable

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