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Comparison of shearing efficiency of three 2A peptides in CAR-T cells

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Abstract

Chimeric antigen receptor-T (CAR-T) cell therapy is to introduce an artificially designed CAR molecule into T cells, endowed T cells with targeted function, and then transfuse the modified CAR-T cells back into the patients. Great success has been achieved in the treatment of a variety of hematopoietic system malignant tumors. But due to T cell depletion and eventually dysfunction, the curative effect in solid tumors is unsatisfactory. The fourth-generation CAR-T technology can enhance the function of CAR-T cells by co-expressing some secretory peptides. In order to make the expression of secretory peptides in CAR-T cells at an ideal level, we compared the shearing efficiency of three 2A peptides in CAR-T cells for selecting the 2A peptide with the highest shearing efficiency. In our research, mCherry and CAR molecule was co-expressed through different 2A peptide in CAR-T cells. And then CAR-T cells was collected on the third and seventh day after being infected by lentivirus for flow staining, the shearing efficiency of 2A peptides was reflected by detecting the expression intensity of CAR and mCherry molecule. Our results showed that F2A and T2A respectively have the highest shearing efficiency in CAR-T cells under the specific combination mode.

Keywords

CAR-T, Tumor, 2A peptide, CLDN18.2, CAR, mCherry, Shearing efficiency.

1. Introduction

CAR-T cell therapy, known as chimeric antigen receptor T cell immunotherapy, is to collect T cells from the peripheral blood of patients or donors, and introduce an artificially designed CAR molecule into T cells in vitro, endowed T cells with targeted function. After extensive proliferation in vitro, the modified and expanded CAR-T cells was transfused back into patients who have undergone lymphocyte depletion chemotherapy [1-3]. Cause CAR molecules can confer specificity on immune cells to be activated by a specific target, it can enhance the function of T cells to recognize antigen signals and activation. In terms of mechanism, the modified CAR-T cells do not have MHC restriction as the binding signals are transmitted to CAR-T cells when recognized the targeted antigens, and then CAR-T cells are activated by the activation signals of CD3ζ domain and costimulatory domain, resulting in the release of cytokines and the expression of transcription factors, thus promoting T cell survival and function, and finally inducing cytotoxicity to tumor cells [3-5].

The CAR structure was comprised of extracellular antigen recognition domain, transmembrane domain and intracellular signal transduction domain. The extracellular domain is a single-stranded variable fragment (scFv) that can specifically recognize tumor surface antigens. Tumor antigens are usually divided into tumor-associated antigen (TAA) and tumor-specific antigen (TSA), most of which are TAA. The first- generation CAR molecule consists of extracellular antigen binding domain, transmembrane domain and intracellular signal transduction domain of CD3ζ chain to instantly drive T cell proliferation and induce cytokine

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secretion after binding to target cells. But it shows limited proliferation and anti-tumor effects for lack of costimulatory signals. The second generation of CAR molecule was added a costimulatory domain, such as CD28, 4-1BB, OX40 or ICOS, in that having better proliferation ability and releasing more cytokines. And then the third-generation CAR molecule contains two different costimulatory domain, such as CD28 and 4-1BB [5-8]. But up to now, all the CAR-T cell products on the market adopt the second-generation CAR molecule structure. Since the first CAR-T cell product for the treatment of CD19 antigen positive recurrent or refractory B cell acute lymphoblastic leukemia in children and young people has been approved in 2018 by FDA, there are six CAR-T cell therapy products on the market, all of which are of autogenous origin, including Kymriah developed by Novartis, Yescarta and Tecartus by Gilead's Kite company, also Breyanzi by Juno company of BMS, all of which are targeting CD19 for curing the patients suffered from B cell associated malignant tumor [9-11]. By the way, Relma-Cel, another anti-CD19 CAR-T cell product, is also under pre-market review. The other two CAR-T cell products are, respectively, Abecma developed by Juno Company of BMS, and Carvykti, independently developed by Legend Biology. Both of which are targeting B cell mature antigen BCMA and curing the patients with multiple myeloma [5, 12].

Although CAR-T cell therapy has achieved remarkable success in the treatment of a variety of hematopoietic system malignant tumors, its therapeutic effect in the treatment of solid tumors is not satisfactory, partly due to the low efficiency of T cell transport, poor antigen recognition specificity and lack of safety control, but more due to the immunosuppression of tumor microenvironment and T cell dysfunction [13, 14]. In this case, regulating the immune environment of tumor tissue is considered to be the key to improve the efficacy of CAR-T cells. CAR-T cells with transgenic "payload", so-called TRUCKT cells or "fourth-generation" CAR-T cells, can combat T cell depletion related dysfunction and induce effective and long-term anti-tumor immunity by inducing the release of transgenic immune modifiers. When these CAR-T cells are activated, the fourth generation of CAR molecular sequence is transcribed, in which the shearing peptide separates the immune modifier from the CAR molecule through the mechanism of self-cutting, so that the secretory peptide can be secreted into the extracellular fluid, where it not only continuously stimulates CAR-T cells to remain active and induces the formation of memory T cells, but also reactivates the host immune system to respond to restimulation. Li Tang et.al achieved complete remission in a variety of allogeneic and xenograft mouse solid tumor models by developing CAR-T cells with secreting IL-10 in metabolic armor [13]. In addition, the concept of TRUCK is currently being explored using a set of cytokines, including IL-7, IL-12, IL-15, IL-18, IL-23 and their combinations, and has been entered the early experimental stage. And future developments will extend the application to a wider range of proteome release, transforming CAR-T cells into "living factories" of local deposition products with therapeutic activity, which may eliminate some clinical defects of currently used CAR-T cells in the field of solid tumors [15].

After the CAR molecular sequence is transcribed, the self-cutting ability of the shearing peptide is one of the key factors for the secretory protein to maintain a certain concentration. And the 2A peptide is a kind of short peptide derived from virus, often called "self-cutting" peptide and generally 18-25 amino acids in size, which can make a transcriptional product produce a variety of proteins. But the 2A peptide is not completely self-cutting. It works by making ribosomes skip the synthesis of glycine and proline peptide bonds at the C-terminal of the 2A element, resulting in the separation of the terminal and downstream products of the 2A sequence. Compared with the IRES element, 2A peptide has a shorter structure and a good balance of upstream and downstream gene expression [16].

To explore the shearing efficiency of 2A peptides in CAR-T cells and provide theoretical basis for the selection of 2A peptide in the fourth generation of CAR technology, we co-expressed CAR and mCherry molecule through the connection of 2A peptide in CAR-T cells. Finally, the positive

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rate of CAR molecule and the expression intensity of mCherry in CAR-T cells were detected by flow cytometry, and then the shearing efficiency of 2A peptides-T2A, F2A and P2A in CAR-T cells could be compared.

2. Materials and methods

2.1. T cell sorting and culture

Peripheral blood was obtained from healthy volunteers. All blood samples were handled following the required ethical and safety procedures. T cells were purified using Easysep human T cell isolation kit (Stemcell) and cultured in T cell expansion medium (OpTmizer CTS T cell expansion basal medium, Gibco) supplemented with IL-7 (20 ng/ml, Peprotech) and IL-15 (20 ng/ml, Peprotech). And some cells were collected for flow cytometry analysis by staining flow antibody, including anti-human CD3 (BD Pharmingen), anti-human CD4 (BD Pharmingen) and anti-human CD8 (BD Pharmingen), to detect the proportion of $CD4^+$ and $CD8^+$ T cells.

2.2. Construction of human CARs

As described, we generated the third-generation CAR molecule targeting CLDN18.2. In short, the complete CAR sequence was composed of a human CD8 signal peptide, antigen-specific scFv, CD8a hinge and transmembrane domain, ICOS and 4-1BB costimulatory domain, CD3ζ intracellular domain. And on this basis, some of which was composed of 2A peptide and mCherry.

2.3. Production and concentration of lentivirus

Lentivirus production is carried out according to the plan of the manufacturer (TAKARA). In short, 293T cells were inoculated in 15 cm petri dishes the day before transfection. During each transfection, the 57.6 μg plasmid was added to the OPTI-MEM solution (Gibco), and then the 3 ml OPTI-MEM solution containing PEI was added. After incubation at room temperature for 15 minutes, the transfection was gently added to the 293T cells. After 6 hours, the liquid was changed, and the supernatant containing the virus was collected after 48 hours of incubation. After the lentivirus solution was centrifuged to remove the cell fragments, 1/3 volume of Lenti-Xconcentrator (TAKARA) was added, mixed and placed at 4 ℃ for at least 45 minutes, then the supernatant was centrifuged and the lentivirus concentrate was prepared by T cell culture medium re-suspension precipitation.

2.4. Flow cytometry analysis

About 10 ^ 6 cells were collected on the 3rd and 7th day after lentivirus infection. After washed twice with DPBS buffer (Gibco), the cells were stained for 30 minutes with a buffer containing dead or alive dye-BD Horizon Fixable Viability Stain 660 (BD Pharmingen), and CLDN18.2 antibody. After centrifugation, cell precipitation was resuspended by eBioscience™ Flow Cytometry Staining Buffer (Invitrogen) for further flow cytometry analysis.

3. Results

3.1. Generation of the expression vector of human CARs constructure

In our research, to explore the shearing efficiency of F2A, T2A and P2A in CAR-T cells, and select the 2A peptide with the highest shearing efficiency in CAR-T cells, we downloaded and synthesized their respective nucleotide sequences on NCBI (https://www.ncbi.nlm.nih.gov/). In addition, in order to successfully prepare CAR-T cells, we designed the CAR molecular expression vector, and introduced mCherry to the CAR structure through the connection of 2A peptide, see Fig. 1.

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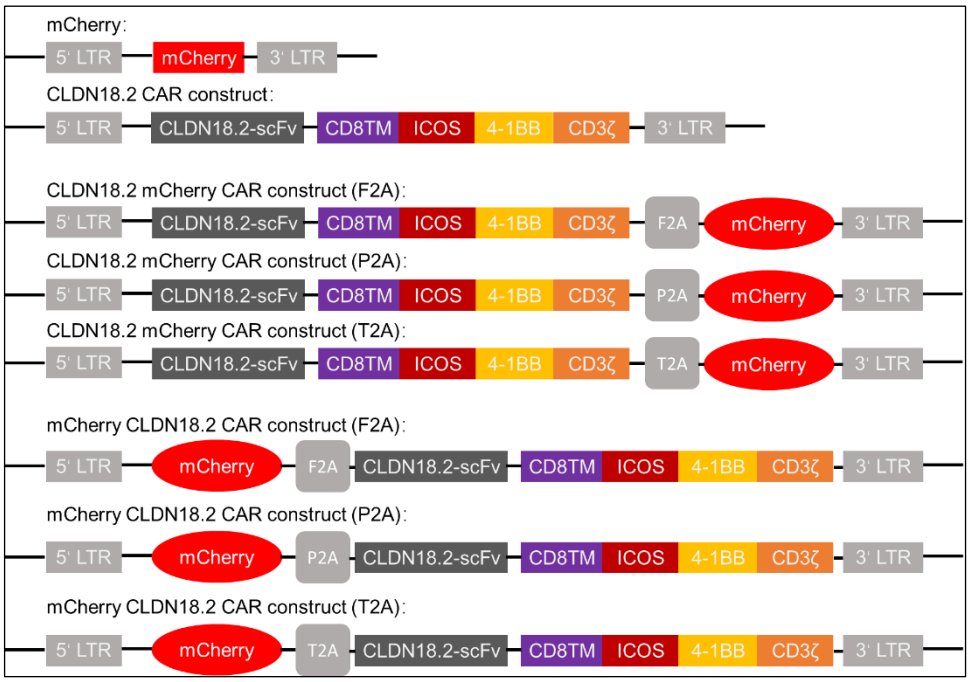

Figure 1. Schematic depicting the CLDN18.2 CAR, mCherry CLDN18.2 CAR and CLDN18.2 mCherry CAR constructs. scFv, single-chain variable fragment; TM, transmembrane domain; LTR, long terminal repeat.

3.2. Preparation of T cells

In order to prepare CAR-T cells, we finally obtained CD3⁺ T cells from human peripheral blood by sorting magnetic beads. Flow cytometry analysis showed that the sample contains more than 98% CD3⁺ T cells, of which CD4 single positive T cell population accounted for 59.1%, CD8 single positive T cell accounted for 36.9%, see Fig. 2.

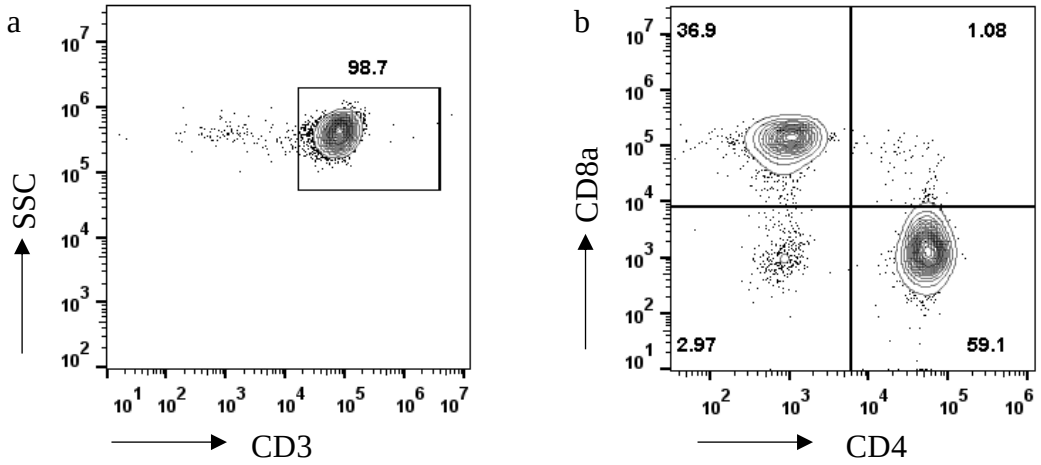

Figure 2. Flow cytometry analysis for prepared T cells. a, Proportion of CD3⁺ cells in the sample; b, The respective proportions of CD4⁺ and CD8⁺ cells in CD3⁺ T cells.

3.3. F2A and T2A respectively have the highest shearing efficiency in CAR-T cells under the specific combination mode

The prepared CD3⁺ T cells were mixed with the corresponding CAR expression vector lentivirus to prepare CAR-T. Generally, the expression of CAR molecule in T cells reached the highest level at 72 hours after lentivirus infection, so we collected cells on the 3rd day after lentivirus infection for flow cytometry. The following flow cytometry analysis shows that on the 3rd day after lentivirus infection, the proportion of CAR-T cells expressing CLDN18.2 alone was up to

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33.09%, and that of mCherry expression group was 77.4%. Clearly, the co-expression of mCherry will reduce the proportion of CAR expression (Appendix Fig. S1). It is worth pointing out that in our experiment, the lower the *CAR⁺/mCherry⁺* ratio, the higher the shearing efficiency of the corresponding 2A peptide in CAR-T cells. Thus, it can be seen from the results that in the combination of CLDN18.2-(2A peptide)-mCherry, F2A had the highest shearing efficiency in CAR-T cells while T2A had the highest in the combination of mCherry-(2A peptide)-CLDN18.2, see Table 1 and Appendix Fig. S1.

In addition, in order to observe the persistence of CAR molecule and mCherry expression, we also collected cells for flow cytometry on the 7th day after lentivirus infection. The results showed that the proportion of CAR⁺ cells decreased on the 7th day, see Table 2 and Appendix Fig. S1.

Table 1. Flow cytometry analysis on the third day

Group	CAR+ (%)	mCherry+ (%)	CAR+/ mCherry+
CLDN18.2 mCherry CAR-T (F2A)	16.35	5.27	3.10
CLDN18.2 mCherry CAR-T (P2A)	13.37	3.14	4.26
CLDN18.2 mCherry CAR-T (T2A)	15.04	3.50	4.30
mCherry CLDN18.2 CAR-T (F2A)	18.88	4.64	4.07
mCherry CLDN18.2 CAR-T (P2A)	14.23	6.11	2.33
mCherry CLDN18.2 CAR-T (T2A)	16.36	7.53	2.17

Table 2. Flow cytometry analysis on the seventh day

Group	CAR+ (%)	mCherry+ (%)	CAR+/mCherry+
CLDN18.2 mCherry CAR-T (F2A)	11.39	2.58	4.41
CLDN18.2 mCherry CAR-T (P2A)	10.58	2.22	4.77
CLDN18.2 mCherry CAR-T (T2A)	11.64	2.00	5.82
mCherry CLDN18.2 CAR-T (F2A)	16.52	3.59	4.60
mCherry CLDN18.2 CAR-T (P2A)	12.61	6.38	1.98
mCherry CLDN18.2 CAR-T (T2A)	15.67	6.74	2.32

4. Conclusion

To compare the shearing efficiency of F2A, P2A and T2A in CAR-T cells, we introduced mCherry into the third-generation CAR structure through the connection of 2A peptide, tried various combinations of mCherry and CAR molecules, and prepared the corresponding lentiviruses. And then, we mixed the pre-prepared T cells with lentivirus concentrate to prepare CAR-T cells. For detecting the proportion of CAR⁺ and mCherry⁺ cells, the cells were collected on the third and seventh day after lentivirus infection. The results of flow cytometry showed that in the combination of CLDN18.2-(2A peptide)-mCherry, F2A had the highest shearing efficiency in CAR-T cells while T2A had the highest in the combination of mCherry-(2A peptide)-CLDN18.2. The significance of this research is to provide data support for the selection of 2A peptide on the structure of the fourth generation CAR-T technology, which is of great significance.

Acknowledgments

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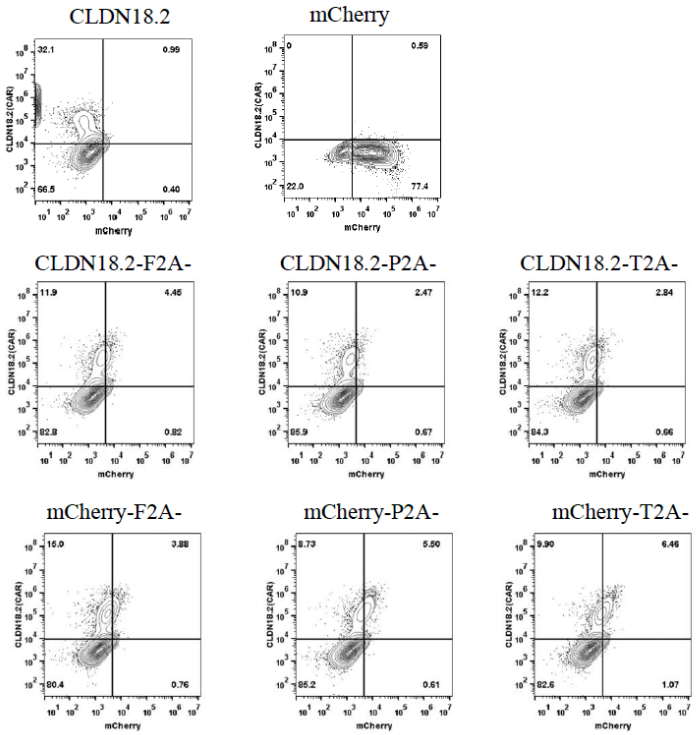
Appendix Figure S1

a



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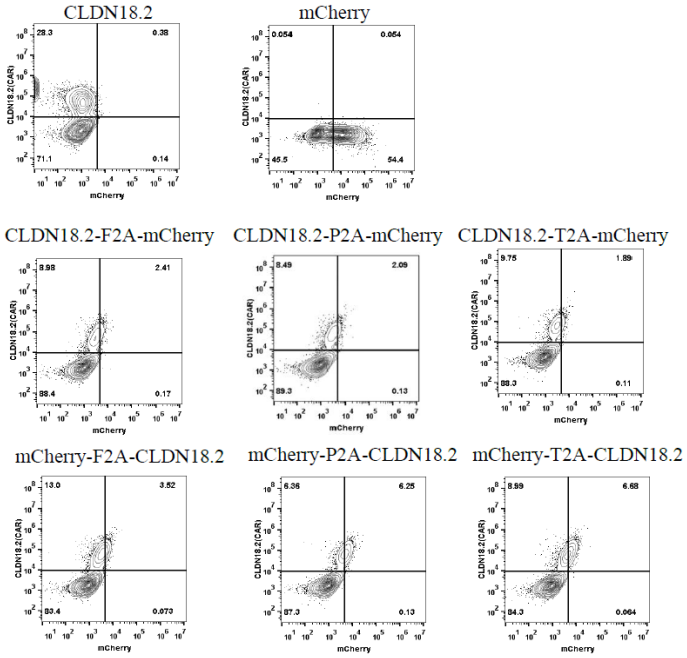

Appendix Figure S1. Flow cytometry results of the expression of CAR and mCherry molecule in CAR-T cells after T cells were infected by corresponding lentivirus. a, Flow cytometry analysis of CAR-T cells on the third day after T cells were infected; b, Flow cytometry analysis of CAR-T cells on the seventh day after T cells were infected.