Abundance of T Stem Cell Memory in F-CAR-T Cells Causing Younger Phenotypes

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Abstract

This study will introduce a novel anti-CD19 CAR-T technology – FasT CAR-T (F-CAR-T)- shortens production time, increases production quality, and produces healthier T cells with a younger phenotype. This study also investigates the effect of the abundance of T stem cell memory in F-CAR-T cells' correlation with younger phenotypes, leading to better persistence in T cells by manipulating the number of Car T cells injected into Raji xenografts. Using mouse cell lines, RAJI xenograft models, and F-CAR-T cells, this study will measure cell proliferation of Tscm, CD45RO-, CD62L+, and PD1/Lag3/Tim3 by flow cytometry and count Car expressing cells using SCFV antibodies. The experiment results will show if F-CAR T cells have significantly greater expansion and T stem cell memory than C-CAR-T cells. The C-CAR-T cells with less exhaustion, indicating the effect of T stem cell memory distinguishing F-CAR-T therapy from conventional CAR-T therapy. The result of this study will provide critical information for the future clinical trial of F-CAR-T cell therapy. This work can inspire to utilize the T stem cell memory, which exhibits great antitumor activity, to explore the mechanism of modulation of CAR-T cell metabolism and T cell differentiation.

Keywords: ***T stem cell memory, RAJI, F-CAR-T cells, Flow cytometry, PD-1+LAG3+Tim3+ T-cells, CD45RO-, CD62L+, SCFV***

1. Introduction

Chimeric antigen receptor (CAR) T-cell therapy works by engineering T cells which are white blood cells that fight cancer in the lab and then transfused back to the patient's blood to help the immune cells attack cancer. This type of immunotherapy plays a crucial role in strengthening the patient's immune system to attack tumors. CAR-T cell therapies can have lasting effects in some patients that results in the eradication of lymphomas and leukemias. They are customized for each individual patient by collecting and re-engineering their T cells to produce chimeric antigen receptors on their surface. These re-engineered T cells bind to antigens and proteins on the surface of cancer cells. These CAR-T cells are replicated into the millions during manufacture process and then infused back into the patients. Ideally, they multiply in the patient's body and attack cancer cells by recognizing the targeted antigen on their surfaces. CAR-T cell therapy is effective immunotherapy for patients with advanced aggressive lymphomas which had been difficult to treat before this therapy [1].

Antigen-presenting cells and tumour cells are the main surfaces on which the protein PD-1 is expressed. In the absence of TCR signalling, the induction of PD-1 expression on naive T cells after TCR activation reduces. Still, it remains unchanged upon chronic viral infection and chronic activation of persistent epitope targets such as cancer. To reverse T-cell fatigue and restore antitumor immunity, F-CAR-T cell therapy targets the Tim-3 and PD-1 pathways. In physiological and pathological contexts, the inhibitory effects of PD-1 receptor binding to activated T cells are well known [2-4]. Checkpoint blockers, such as anti-PD-1 antibodies, are rapidly being used as the reference therapy for a growing variety of tumors. However, it has also been found that the expression of PD-1 on antigen-specific T lymphocytes reflects their functional affinity and antitumor response. T memory stem cells are memory lymphocytes that have the capacity to self-renew in a manner similar to stem cells and the capacity to regenerate subpopulations of memory and effector T cells [2-4].

F-CAR-T cell technology has the advantages of short production time, increased quality with healthier T cell products, low cost leading to more accessibility. In preclinical studies, CD19 F-CAR-T cells proved their advanced proliferative capacity, younger cell phenotype, little exhaustion, and effectiveness in eliminating tumor in comparison to traditional CAR-T cells [1-6]. While the amount of white blood cells collected is relatively large, the T cells are not expanded much during the external culture process. Consequently, the T cells are relatively young, the return infusion dose is small, and the anti-tumor cytotoxic activities are still efficacious [5,6].

F-CAR-T cell therapy has less exhaustion compared to C-CAR-T cell therapy which also makes it more effective

in tumor elimination. CAR-T cell exhaustion is caused by continuous antigen stimulation and immunosuppressive tumor microenvironment. It is still a central challenge to maintaining the persistence and effector function of CAR T cells. The limitation of CAR T cell exhaustion is also impeding the development of CAR T cells to eradicate solid tumors. The exhaustion of CAR-T cells is a major factor in failing to clear antigens which can also lead to resistance and relapse during the therapy. T cells cease to function properly marked by decreased proliferative and effector activity as a result of ongoing exposure to disease-specific antigens. T cell exhaustion is linked to ineffective results in patients because effective CAR T cell therapy necessitates CAR T cells continually killing target cells. Therefore, increasing the effectiveness of CAR T cell treatment requires the development of mechanisms that aim to combat T cell depletion. Upregulation of PD-1, LAG3, and Tim3 are the markers of exhausted T cells [7,8]. Targeting PD-1+LAG3+Tim3+ T-cells can reduce the T cells exhaustion and reverse resistance and relapse in CAR T cell therapy.

B-cell acute lymphoblastic leukaemia is an aggressive leukemia with an excess of B-cell lymphoblasts in bone marrow and blood. It has a 5-year survival rate of 71.7 percent [1,2]. The effectiveness, safety, and viability of CD19 F-CAR-T in B-ALL is currently being evaluated. Therefore, this paper will focus on whether the T stem cell memory in F-CAR-T cells is correlated with the *in vivo* expansion and less exhaustion with PD-1+LAG3+Tim3+ T-cells.

Research Question: Is the abundance in T stem cell memory in F-CAR-T cells correlated with less exhaustion and PD-1+LAG3+Tim3+ T-cells?

Hypothesis: I hypothesize that the abundance of T stem cell memory with increasing amounts of CD19-specific F-CAR-T cells injected into RAJI xenografts is correlated with the in vivo expansion and less exhaustion with PD-1+LAG3+Tim3+ T-cells. I will measure T_{scm} by FACS for CD45RO- and CD62L+ and expansion by FACS counting of Car expressing cells using SCFV antibodies. This experiment measures T_{scm} by FACS for CD45RO- and CD62L and exhaustion by FACS for PD1/Lag3/Tim3.

2. Methods

2.1. *Materials*

This experiment will use different concentrations of central-memory T cells to test the effect of T memory stem cells on the expansion, survival rate, and long-term persistence of F-CAR-T cells. In addition, this experiment will use RAJI xenograft models with two cell lines as positive and negative controls with the technique of using the lymphoma model. This experiment measures CD62L+, CD62L, and CD62L- expression in the CD45RA+ T-cell compartment which controls the traffic of T lymphocytes.

2.2. *In Vitro Cell Culture*

Stock cultures of the cells will be kept at 37 °C with 95% humidity in a 5% CO_2 humidified environment.

2.3. *Animal Model*

The mice will be divided into three groups: experimental group, negative control, and positive control group. They will be injected with doses of CD19-targeted CAR-T cells. In addition, their tumor growth and mouse survival were monitored.

2.4. *In Vivo Experiment*

TSCM, TEM, TCM surface staining with CD62L-, CD45RO+, and PD1/Lag3/Tim3 will be analyzed with flow cytometry (FACS) on the 7th, 14th, and 21st days.

2.5. *CAR-T cell characterization*

The CD19-expressing cells will boost the F-CAR T cells and C-CAR T cells. The fraction of CAR-positive cells identified by flow cytometry will be used to quantify the amount of CAR- T cells while expanding. By measuring the expression of CD45RO and CD62L with flow cytometry, it will be possible to compare the T stem cell memory properties of F-CAR T cells and C-CAR-T cells. Effector memory T cells (TEM cells) are CD62L-CD45RO+ cells, whereas central memory T cells (TCM cells) are CD62L+CD45RO+ cells. PD-1, Tim3, and LAG3 expression will be examined using flow cytometry to identify the depletion of F-CAR T cells and C-CAR-T cells.

2.6. *Measurement*

Flow cytometry is performed using a BD LSRFortessa flow cytometer. This experiment will be using female NOD-scid IL2rgnull (NSG) mice that are 5 to 7 weeks old and kept in a certain pathogen-free environment. These NSG mice are severely immunosuppressed and tolerant to xenografts because they lack mature B, T, and NK cells. As manipulated variables, each animal receives 5 106, 7.5 106, or 10 106 CAR T cells.

3. Results

3.1. *Combination of Possible Results (CR)*

Table 1. The possible combination of results

Possible Observations	CR 1	CR 2	CR 3	CR 4	CR 5	CR 6	CR 7	CR 8
Tscm Increases with and increased CAR-T Injection	+	+	+	+	-	-	-	-
PD1/Lag3/ Tim3 FACS decreases with increasing Car T injection	+	-	-	+	+	-	+	-
Car T FACS counting increases with increasing Car T injection	+	+	-	-	+	+	-	-
Supporting Hypothesis	Yes	P.	P.	P.	P.	P.	P.	No

Note. "+" represents an increase in detecting substances with NC groups. "-" represents a decrease in detecting substances with NC groups.
"P." represents partially supports the hypothesis.

Possible Result 1:
F-CAR T cells show significantly greater expansion and more T stem cell memory than C-CAR-T cells with less exhaustion (see Table 1). TCM cells are predominant among F-CAR T cells, followed by TEM. In comparison to C-CAR-T cells, F-CAR-T cells exhibit a higher frequency of TCM cells and a lower frequency of TEM cells, indicating that they are typically in an earlier stage of T cell development and have a younger phenotype. Exhausted C-CAR-T cells have an increased percentage of cells that express the immunological checkpoint markers PD-1, LAG3, and Tim3. F-CAR-T cells, in contrast, show a markedly smaller percentage of cells that are positive for these exhaustion markers.
Possible Result 2:
F-CAR T cells show the same expansion and T stem cell memory compared to C-CAR-T cells with the same exhaustion (see Table 1). There are some TCM cells in the F-CAR T cells, followed by TEM. F-CAR-T cells have the same TCM cell frequency and TEM cell frequency as C-CAR-T cells, which indicates that F-CAR-T cells and C-CAR-T cells are generally the same stage of T cell differentiation and have a similar phenotype. In both C-CAR-T cells and F-CAR-T cells, the percentage of cells expressing the immunological checkpoint markers PD-1, LAG3, and Tim3 is comparable. They both show a comparable percentage of cells that are positive for these exhaustion markers.
Possible Result 3:
F-CAR T cells show less expansion and T stem cell memory than C-CAR-T cells with more exhaustion (see Table 1). Few F-CAR T cells contain TCM cells. In comparison to C-CAR-T cells, F-CAR-T cells exhibit a lower frequency of TCM cells and a larger frequency of TEM cells, indicating that they are typically in a later stage of T cell development and have an older phenotype. Exhausted F- CAR-T cells have an increased percentage of cells that express the immunological checkpoint markers PD-1, LAG3, and Tim3. The fraction of cells that are positive for these exhaustion markers is noticeably lower in C-CAR-T cells.
Possible Result 4:
After F-CAR-T injection, there is an increase in T stem cell memory with less exhaustion with decreasing PD1/ Lag3/Tim3. In addition, FACS counted less CD45RO- CD62L+.
Possible Result 5:
After F-CAR-T injection, there is a decrease in T stem cell memory with less exhaustion with decreasing PD1/ Lag3/Tim3. In addition, FACS counted more CD45RO- CD62L+.
Possible Result 6:
After F-CAR-T injection, there is a decrease in T stem cell memory with more exhaustion with decreasing PD1/ Lag3/Tim3. In addition, FACS counted more CD45RO- CD62L+.
Possible Result 7:
After F-CAR-T injection, there is a decrease in T stem cell memory with less exhaustion with decreasing PD1/ Lag3/Tim3. In addition, FACS counted less CD45RO- CD62L+.
Possible Result 8:
After F-CAR-T injection, there is a decrease in T stem cell memory with more exhaustion with decreasing PD1/Lag3/ Tim3. In addition, FACS counted less CD45RO- CD62L+.

4. Discussion

Comparing CD19 F-CAR-T cells to conventional CAR-T cells, prior preclinical research found that the latter exhibited greater proliferation, a younger cellular phenotype, less exhaustion, and more efficient tumour eradication. To test the preclinical therapeutic effect of different amounts of T stem cell memory in animal models using RAJI Xenograft models, this study induces SCFV antibodies to cell lines from mice. The study uses *in vivo* experiments and *in vitro* cell cultures.
As shown in Table 1, possible result 1 is consistent with

my hypothesis investigating T stem cell memory in F-CAR-T cells causing younger phenotypes. This result shows that Tscm increases with an increased CAR-T injection proving the abundance of Tscm in F-CAR-T cells. PD1/Lag3/Tim3 FACS decreases with increasing Car T injection showing that F-CAR-T cells have less exhaustion. Lastly, CAR T FACS counting increases with increasing Car T injection showing the cells' ability to expand. This experiment should happen at a period when the T cells are growing and successfully killing cancer cells. The next experiment should be continuing to research the effector cells for further investigation.

As shown in Table 1, possible result 8 is contradicting my hypothesis investigating T stem cell memory in F-CAR-T cells causing younger phenotypes. This result shows that Tscm decreases with an increased CAR-T injection lowering the abundance of Tscm in F-CAR-T cells. PD1/Lag3/Tim3 FACS increases with increasing Car T injection showing that F-CAR-T cells have more exhaustion. Lastly, CAR T FACS counting decreases with increasing Car T injection showing the cells' lack of ability to expand. This experiment should happen at a period when the T cells started dying and could no longer kill cancer cells. The next experiment should be continuing researching the different generations of these T cells for further investigation.

As shown in Table 1, possible results 2, 3, 4, 5, 6, and 7 were unable to fully support my hypothesis. These results could be caused by the different periods of cultivating the cells. This could happen when there is more amount of injection needed for the mice xenograft models or the environmental conditions have been changed. This could also be results observed at different stages of the experiment, leading to different concentrations of the substances observed. The next experiment should be focused on investigating the effect of different concentrations of T cell injection.

Further studies investigating improving the T stem cell memory should be done for a thorough understanding of F-CAR-T cell's structures and functions. The mechanism of modulation of CAR-T cell metabolism and T cell differentiation should also be investigated to understand the more specific CAR-T signal pathway. Preclinical testing on more complex and representative animal models should also be done before the transition to clinical testing of F-CAR-T therapy. To improve this therapeutic method, better delivery platforms based on mechanisms to improve the ability to grow and last of modified T cells should be applied as well.

5. Conclusion

Generally, this study explores the therapeutic effect of F-CAR-T cells in Xenograft Models. The result of our study will indicate whether F-CAR-T cells have a better therapeutic effect in preclinical studies due to their abundant T stem cell memory compared to C-CAR-T cell therapy, providing the basis for the transition to clinical trials. The possible controversial results on T stem cell memory will also indicate the potential relationship between F-CAR-T cell therapy and other effector cells, which should be investigated in future studies on F-CAR-T cell regulations. The improved *in vivo* experiment method including flow cytometry, RAJI xenografts, and SCFV antibodies will increase the likelihood of the experiments being successful. Since people have not realized the efficiency of F-CAR-T cells in T cell therapy until recent years and there is a limited amount of research on F-CAR-T cells, more specific structure and mechanism of T stem cell memory still need to be investigated in more detail in order for scientists to get a better understanding of the F-CAR-T cell signal pathway.

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