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Effects of CAR structure and culture conditions on memory CAR-T cells

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Abstract. – Adoptive CAR T cell therapy (chimeric antigen receptor T-Cell) has received increasing attention in recent years; however, its efficacy is undesirable and differs from person to person. Understanding how to overcome this obstacle is important to improve therapy. Infusion of poorly differentiated CAR-CD62L+ T cells, such as T memory stem cell populations, leads to enhanced T cell implantation, expansion, and persistence, which ultimately leads to more stable tumour regression. Here, we reviewed emerging findings demonstrating that CAR structure and cell culture conditions can influence CAR T cell differentiation and antitumour efficacy.

Key Words:
CAR T, Memory T cells, CAR structure, Cell culture condition.

Introduction

T memory stem cell (T_{SCM}) have extremely long lifespan, potentially proliferative ability to reconstitute to other subsets, is ideal for adoptive cell transfer immunotherapy[1,2]. Adoptive cell transfer immunotherapy is becoming a powerful treatment strategy for cancer patients, evidenced by increasing success in clinical trials of naturally existing or genetically engineered lymphocytes reactive to tumour cells[2]. Although these regimens can induce complete and long-lasting tumour regression in advanced cancer patients, response rates are still mostly inadequate; therefore, much work needs to be done. There is now extensive evidence that objective responses are closely related to early T cell implantation levels and early amplification peaks. The persistence of T cells is also associated with the possibility of objective responses in many trials and may require sustained remission. These factors are greatly influenced by the composition of the infused T cell product, as T cell subsets differ greatly in their ability to proliferate, reconstitute immune subsets and survive term. In fact, infusion of cells with longer telomeres or cell products containing a higher proportion of CD62L+, CD28+ or CD27+ T cells was associated with objective tumour response in patients, suggesting that T cells with lower degrees of differentiation are superior to TTE cells. Notably, the implantation and expansion of T cells expressing a CD19-specific CAR or suicide groups correlated with the frequency of infused CD8+CD45RA+CCR7+ TSCM cells.

Consistent with developmental grade, the least differentiated TSCM cells mediate a more potent anti-tumour response than TCM cells, similarly, TCM cells are more potent than terminal differentiated TEM cells[3] (Figure 1).

Tumour-infiltrating lymphocytes are usually in a state of terminal differentiation and normal exhaustion, which makes the separation of early memory T cell subsets impractical. Therefore, the selection of a less differentiated subset of T cells in the context of immunotherapy has become a necessary requirement. Actually, isolation of a less differentiated T cell population also has the advantage of reproducibly producing more defined T cell products. In fact, PBMC composition varies widely between individuals due to age, pathogen exposure, and previous systemic treatment. In addition, unselected populations containing high proportions of TEM and effector

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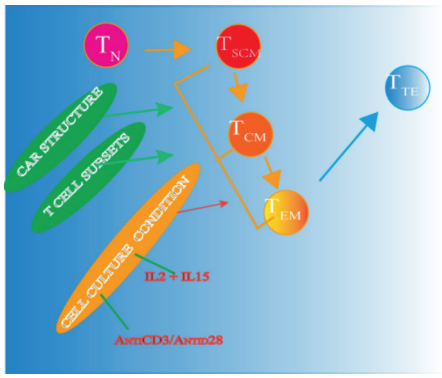

Figure 1. Effects on CAR-T cells differentiation[1]. After antigen initiation, naive T (TN) cells gradually differentiate into a variety of memory T cell subsets and eventually differentiate into terminally differentiated effector T (TTE) cells.

cells may not produce viable clinical products due to poor cell expansion *in vitro*. Several clinical trials of CD19-specific CAR T cells from isolated TCM cells are currently underway[4].

CAR Structure Affects Memory T Cell Differentiation

Early T cell failure is a major factor limiting the antitumour efficacy of CAR-expressing T cells, and the CAR structure plays an important role in making CAR T cells susceptible to chronic activation and failure.

The chimeric antigen receptor (CAR) links the antigen recognition domain to the intracellular signalling domain to redirect T cell specificity and function[5-7]. CAR T cells with expression of the CD28/CD3 or 4-1BB/CD3 signalling domain are effective in the treatment of refractory B cell malignancies but exhibit differences in effector function, clinical efficacy and toxicity, and these differences are caused by the activation of different signalling cascades. Mass spectrometry analysis of stimulation-induced phosphorylation events in primary human CD8+ CD28/CD3+ and 4-1BB/CD3+ CAR T cells revealed that both CAR structures activated similar signalling intermediates. CD28/CD3 CAR stimulation activates protein phosphorylation with faster and more dramatic changes associated with effector T cell-like phenotypes and functions. In contrast, 4-1BB/CD3+ CAR T cells preferentially express T cell memory-associated genes and exhibit sustained antitumour activity against established tumours *in vivo*. Mutagenesis of the CAR CD28 signalling domain indicates that a certain increase in CD28/CD3 CAR signal intensity is related to the constitutive association of Lck with this domain in the CAR complex. The CAR signalling pathway cannot be predicted only by the domain used to construct the receptor, but signal intensity is a key determinant of T cell fate. Therefore, CAR design based on signal intensity can improve clinical efficacy and reduce toxicity.

Destruction of the TET2 gene enhances the efficacy of CAR T cells. Ninety-four percent of CAR T cells are derived from a single clone in which a lentiviral vector-mediated CAR transgene insertion disrupts the methylcytosine dioxygenase TET2 gene. One analysis revealed a suballelic mutation in the patient's second TET2 allele. The CAR T cell with the TET2 gene mutation exhibited an epigenetic profile consistent with altered T cell differentiation at the peak of amplification, with a central memory cell gene phenotype. The experimental knockdown of TET2 reproduced the potential effect of TET2 dysfunction in CAR T cells of this patient. These findings indicate that progeny of individual TET2 modified CAR T cells induce leukaemia remission and can be used to improve immunotherapy[8].

IL-15 enhances CAR T cell efficacy. Successful outcomes are associated with engraftment and long-term persistence of CAR T cells. Long-term immunosurveillance by persisting CAR T cells is likely key to achieving durable responses in adoptive cell therapy[9]. T memory stem cells (TSCMs) have excellent potential for long-lasting persistence but making such T cell subsets is challenging because they are rare in circulating lymphocytes.

A clinically relevant method for generating CAR T cells with the potential to retain the TSCM phenotype was generated using the Sleeping Beauty platform. Since IL-15 is essential for T memory cells, its co-stimulatory properties are integrated by co-expression of the CAR with membrane-bound chimeric IL-15 (mbIL15). mbIL15-CAR T cells do not rely on CAR signalling to signal T cell persistence through signal transduction and transcriptional activator 5 production, avoiding significant autonomic growth or transformation, and achieving effective rejection of CD19+ leukaemia. One long-lived T cell line is CD45ROnegCCR7+CD95+, which is most similar in phenotype to the TSCM phenotype, and the cells have a memory-like transcriptional profile. In general, CAR T cells can maintain a memory stem cell phenotype through mbIL15 signalling, resulting in long-term persistence. This observation needs to be evaluated in clinical trials.

Furthermore, CD19-specific CAR-engineered T cells with a lack of an IgG1 Fc spacer have a stronger therapeutic effect against B-ALL than those with the spacer[10].

Memory T Cell Subsets and CAR T Cell Efficacy

Human CD4+ and CD8+ T cells are composed of different subpopulations that have different proliferation and sustained antitumour effects after *in vitro* expansion and adoptive transfer[11,12]. Compared with elderly patients, young patients with acute myeloid leukaemia (AML) have a significantly different distribution of memory T cell subsets, while patients with complete remission of AML have distinct T cell subsets, and T cell subsets, especially memory stem cells, change significantly during treatment of these patients. The heterogeneity of the infused CAR T cell subset makes it difficult to distinguish the elements of amplification and persistence, antitumour response, and toxicity of CAR T cells in these early trials. Selecting a clear subset of T cells for genetic modification and its identification via a defined ratio of CD4+/CD8+ markers will provide a more consistent CAR T cell product for clinical applications, resulting in reproducible *in vivo* activity and contributing to the identification of factors associated with efficacy or toxicity. CAR T (CD4+/CD8+) cell treatment of glioblastoma did not achieve better results than standard of care[13], and CD4+ CAR T cells attenuated the CD8+ CAR T cell effect, indicating that different types of tumours respond differently to different CAR T cell subgroups. Patients with a large number of CD8+ TCM cells in the blood were subjected to CAR T cell production using CD8+ TCM cells. In patients with a small number of CD8+ TCM cells, those with severe lymphopenia or high lymphoma burden, a subpopulation with a greater proportion of CD8+ T cells, were selected for CAR transduction.

Cell Culture Conditions Affect Memory T Cell Differentiation *Short-Term Anti-CD3/Anti-CD28 Stimulation Is Conducive to Tscm Formation*

Alvarez-Fernandez et al[14] showed that short-term stimulation with anti-CD3/anti-CD28 magnetic beads and low concentrations of IL-7 and IL-15 increased the frequency of CD4+ and CD8+ TSCM cells in primary T cells and enhanced their proliferative capacity. In addition, IL-21 was added to the culture conditions to further enrich and amplify CD4+ and CD8+ TSCM cells. Importantly, the conditions described in the study allow efficient transfection of CD4+ and CD8+ TSCM cells with lentiviruses. This study reveals a new method for the enrichment and expansion of TSCM cells *in vitro*, which may have clinical relevance for adoptive cell therapy in cancer patients.

Ghassemi et al[15] found that CART19 cells harvested on day 3 had enhanced proliferative capacity after restimulation with their cognate ligands relative to day 9 CART19 cells. On day 9, CAR T cells show a potential decrease in proliferative capacity due to prolonged exposure to agonistic anti-CD3/anti-CD28 magnetic beads during culture, leading to T cell depletion. On day 9, CAR T cells show a potential decrease in proliferative capacity due to prolonged exposure to agonistic anti-CD3/anti-CD28 magnetic beads, leading to earlier memory T cell depletion.

The number of adoptively transferred Tscm cells in the product on day 9 will be proportionally reduced compared to that in the day 3 product. In summary, after shortening the duration of T cell stimulation *in vitro*, T cells have the ability to enhance proliferation and secrete effector cytokines and have excellent antitumour activity.

The Influence of IL-2 on Memory T Cells

Currently, the most commonly used cytokine treatment for culturing T cells is IL-7/IL-15 or IL-2[16-20]. Other cytokines and combinations, such as IL-2/IL-15 or IL-21, have also been used[21-24].

The *in vivo* persistence of CAR T cells is associated with clinical response, and specific cell subpopulations in CAR T cell products affect their subsequent expansion and persistence *in vivo*. Xu et al[20] analysed 14 patients with B cell malignancies infused with autologous CAR T cells. CD19-CAR T cells expanded *in vitro* with IL-2, and the researchers found that *in vivo* amplification was related to the frequency of the CD8+CD45RA+CCR7+ subpopulation in the infusion product. Its phenotype is closest to that of T memory stem cells. Preclinical models have shown that increasing the frequency of CD8+CD45RA+CCR7+ CAR T cells in infused cell subsets via culture with IL-7 and IL-15 enhances CAR T cell resistance to death and produces stronger antitumour activity. Removal of CD45RA+CCR7+ cells from IL-7- and IL-15-cultured CAR T cell products abolished this advantage, even though all other T cell subsets remained intact.

Effect of Short-term IL-2 Culture on Memory CAR T Cells

Interleukin-2 (IL-2) is widely used to promote immune cells in the body, especially T cells. IL-2 is a cytokine that has been used to activate and expand T cells for many years. Due to its potent T cell growth-inducing function, IL-2 has been used for many years in the culture and expansion of various T cells, including tumour-infiltrating lymphocytes (TILs), T cell receptor T (TCR-T) cells and genetically engineered T cells with chimeric antigen receptors (CAR T cells). Long-term culture with IL-2 enhances T cell activation and proliferation at the expense of terminal differentiation of effector T cells, which in turn impairs the efficacy of adoptive T cells. However, using IL-2 short-term culture, a sufficient number of effective central memory CAR T cells can be produced[25].

Summary Outlook

The cell surface molecules CD45RA, CD45RO, CD62L and CCR7 can divide memory T cells into different subpopulations, which is of great significance for the functional study of memory T cells and antitumour immunity. The formation and maintenance of memory T cells after antigen stimulation are extremely complex processes involving the combined action of antigen stimulation intensity and type, cytokines and cell surface molecules. In addition to considering the role of cytokines and small-molecule drugs in T cell therapy, the sorting and maintenance of cell subsets are also important, such as Tcm cell sorting (Figure 1). At present, mainly lentiviral transfections and plasmid transfections of T cells by the piggyBac transposon system are used. Both have advantages and disadvantages, including high transfection efficiency and high preparation cost with lentiviral transfection, while plasmid electro transfection improves transfection efficiency and the transfected cell product. Survival is critical, and recently developed polymer-loaded plasmids have been used to improve transfection efficiency. Memory T cell differentiation is a complex multifactor process that plays an important role in the progression from isolating the gene from the carrier itself to cell isolation and culture.

Conclusions

CAR structure itself affects memory T cell differentiation and its antitumour efficacy. In addition, cell culture conditions such as short-term anti-CD3/anti-CD28 stimulation or low levels of IL-2 affect memory T cell differentiation. In addition, a defined ratio of different subpopulations may provide a more consistent CAR T cell product for clinical applications.

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Conflict of Interest

The Authors declare that they have no conflict of interests.

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