Journal of Pharmaceutical Research International

23(4): 1-11, 2018; Article no.JPRI.43471
ISSN: 2456-9119
(Past name: British Journal of Pharmaceutical Research, Past ISSN: 2231-2919, NLM ID: 101631759)

Chimeric Antigen Receptor T Cell (CAR – T) Therapy – A Novel Treatment in Cancer

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Authors' contributions

This work was carried out in collaboration between all authors. Author PB wrote the first draft of the manuscript. Authors VK and AN managed the editing and proof reading of the study. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/JPRI/2018/43471
Editor(s):
(1) Dr. Rahul S. Khupse, Pharmaceutical Sciences, University of Findlay, USA.
Reviewers:
(1) Lívia Garcia Bertolacci-Rocha, Universidade Federal de Goiás, Brasil.
(2) Thompson Akinbolaji, Yuma Regional Medical Center, USA.
(3) Poggi Alessandro, IRCCS AOU San Martino, Italy.
(4) Pratibha Kamble, University of Mumbai, India.
(5) Wagih Mommtaz Ghannam, Mansoura University, Egypt.
Complete Peer review History: http://www.sciencedomain.org/review-history/26188

Review Article

Received 3rd June 2018
Accepted 22nd August 2018
Published 11th September 2018

ABSTRACT

Cancer is one of the most challenging diseases affecting mankind. It continues to defy all the treatment modalities and advancements and claims millions of lives every year. Chimeric Antigen Receptor –T (CAR-T) cell therapy is one of the breakthroughs in cancer treatment. Chimeric antigen receptor is a recombinant immunoreceptor that enables the T cells to recognise and kill tumour surface antigens in a non MHC (Major Histocompatibility Complex) restricted manner. There are four generations of CAR-T cells, of which the second generation CAR-T cells have maximum efficacy clinically. In 2017, two CAR-T cell therapies were approved by FDA for the treatment of ALL (Acute Lymphoblastic Leukaemia) and NHL (Non-Hodgkin's Lymphoma). More than 80% remission is achieved with these tumours which are resistant to all other treatment modalities. Now CAR-T cell therapies are being tested for non – haematological malignancies also. However, the adverse

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effects of CAR-T cell therapies are very severe like Cytokine Release syndrome (CRS) and on - target off - tumour toxicity. This review will attempt to cover the basic concept and the applications of CAR-T cells, their adverse effects, challenges faced and the future prospects of the CAR-T cells in treatment of cancer.

Keywords: CAR-T therapy; chimeric antigen; anticancer agent.

1. INTRODUCTION

Cancer is the world's second leading cause of death. The standard therapies for cancer include surgery, radiotherapy and chemotherapy. In spite of development in all these primary modalities of treatment, the survival rate of cancer patients has not improved significantly [1]. Newer avenues of treatment are being explored for treatment of cancer. One of the breakthroughs in modern cancer treatments is the advent of Immunotherapy. Here the patient's immune system is enlisted and strengthened to attack the tumour. The various types of immunotherapy include Monoclonal Antibodies, Cytokines, Treatment Vaccines and Adoptive Cell Transfer [2].

2. ADOPTIVE CELL TRANSFER [3]

Adoptive Cell Transfer (ACT) attempts to boost the natural ability of the body's T cells to fight cancer. The various types of ACT include:

1. **Tumour Infiltrating Lymphocytes (TILs):** The lymphocytes that have penetrated the environment in and around the tumour are genetically engineered to attack the tumour cells and kill them. It is being tested in melanoma and cervical cancer.
2. **T Cell Receptors (TCRs):** Naturally occurring T Cell Receptors are used to recognise antigens on the tumour cells. These antigens are then presented to the immune system through the Major Histocompatibility Complex (MHC). These are being tested against melanoma and sarcomas.
3. **Chimeric Antigen Receptors (CARs):** Patient's T-Cells are extracted. Using an inactivated virus as a vector, the T-cells are genetically modified so that they produce receptors (CARs) on the cell surface capable of recognising tumour proteins or antigens. The modified T-cells are then multiplied and reinfused into the patient's blood stream. The engineered cells further multiply in the patient's body and recognise and kill cancer cells through the CARs.

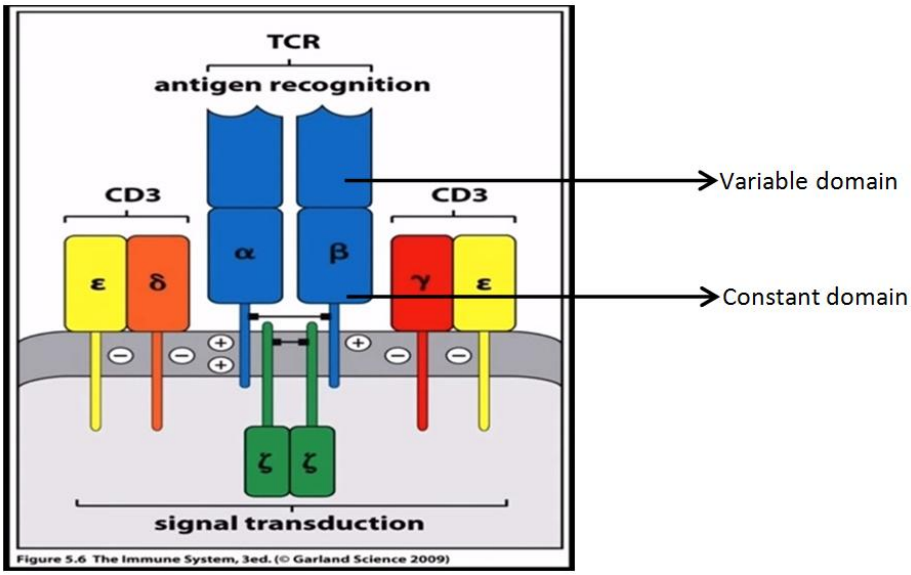

Fig. 1. the structure of a T-Cell receptor [4]

2.1 History of CAR-T Cell Development:

The normal T-cell receptor consists of a heterodimer with either α and β subunits or γ and δ subunits. The variable region of these subunits contains the antigen binding domain (presented by the antigen presenting cells in a MHC restricted manner). The receptor is complexed to the CD3 molecule consisting of the γ, δ and ε subunits with the cytoplasmic tails of ζ. The binding of an antigen causes phosphorylation and activation of the CD 3 molecule. This triggers downstream signalling pathways that activate T-cells releases cytokines to destroy the antigen of interest.

In two studies the variable region of the TCR was replaced with the variable region of immunoglobulin. It was found that this gave antigen specificity to the T cell and enabled non MHC mediated antigen recognition and T cell activation [5,6]. These two studies where the foundation for the development of CARs.

A study constructed chimeric proteins using CD4, CD8 and CD25 with the cytoplasmic tails of ζ [8] The chimeric proteins led to the biochemical events of early T-cell activation namely release of IL-2 (Interleukin –2) and calcium influx showing that the cytoplasmic tails of ζ could replicate the TCR signalling.

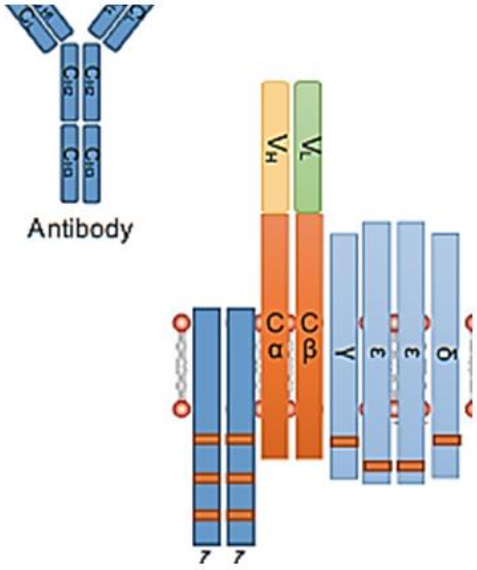

Fig.2. Chimeric receptor construct [7]

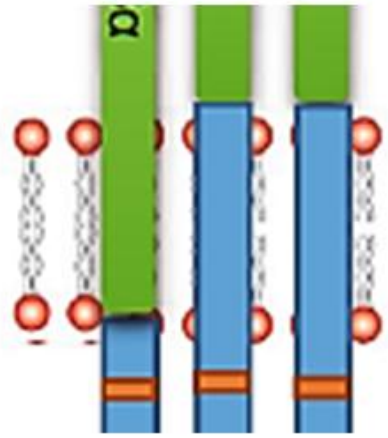

Fig. 3 Chimeric protein construct [7]

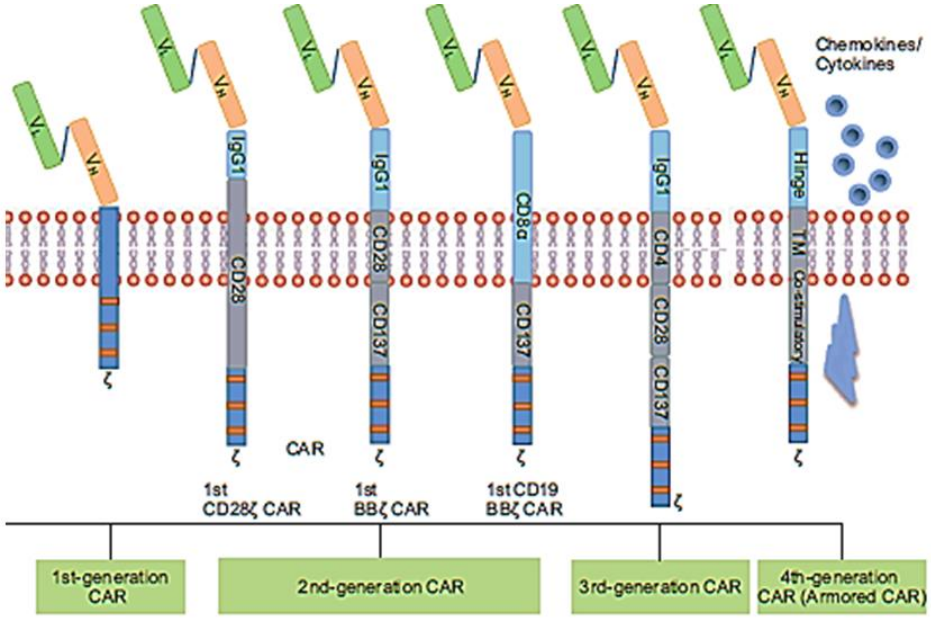

Fig. 4. the generations of CAR-T cell [7]

Taking into account all these advances Eshar et al. [9] designed a gene composed of a single chain variable fragment of an antibody linked with ζ chains. The transfected T-cell hybridoma triggered IL-2 secretion on exposure to antigen, and mediated non – MHC restricted hapten – specific target cell lysis. This was the first generation Chimeric Antigen Receptor. But these receptors can induce limited cytokine production only since they do not cause full activation of T-cells. They are devoid of co-stimulatory molecules like CD 28 that cause activation of naïve or resting lymphocytes. They also undergo apoptosis in the absence of co-stimulatory molecules.

In subsequent models co-stimulatory molecules like CD 28, CD 137 were introduced into the CAR. These are the second generation Chimeric Antigen Receptors. These were superior to the first generation CARs in inducing cytokine production and proliferation of the CAR - T cells.

The third generation CARs contain more than one co-stimulatory domain which results in better persistence of the CAR – T cells *in vivo.* The fourth generation CARs additionally express cytokines to further improve the anti-tumour effect. But these are still in early stages of development. The second generation CARs have shown maximum clinical efficacy in haematological malignancies and certain solid tumours [7].

3. STRUCTURE OF CHIMERIC ANTIGEN RECEPTOR [10]

The Chimeric Antigen Receptor is composed of:

- **Antigen binding moiety –** this is mostly an antibody derived single chain variable fragment (ScFv) and consists of a variable heavy chain region, a flexible linker and a variable light chain region. The ScFvs used are humanized or derived from human monoclonal antibodies.
- **Extracellular spacer –** Links the ScFv to the transmembrane domain. It should be flexible to facilitate antigen recognition. It is derived from IgG subclasses most commonly.
- **Transmembrane domain –** provides stability to the CAR. Commonly derived from CD 28 molecule
- **Co-stimulatory domain –** Augments T-cell activation by increasing cytokine production and by stimulating proliferation, differentiation and persistence of T cells. Initially, CD 28 was used as the co-stimulatory domain. Later CD 137(4-1BB) was also used and found effective. In third generation CARs multiple co-stimulatory molecules are used. In the fourth generation CARs, additional cytokine expression cassette is added.
- **Activation / Signalling domain –** This mediates the T-cell activation signal. The activation domain of choice is CD3ζ

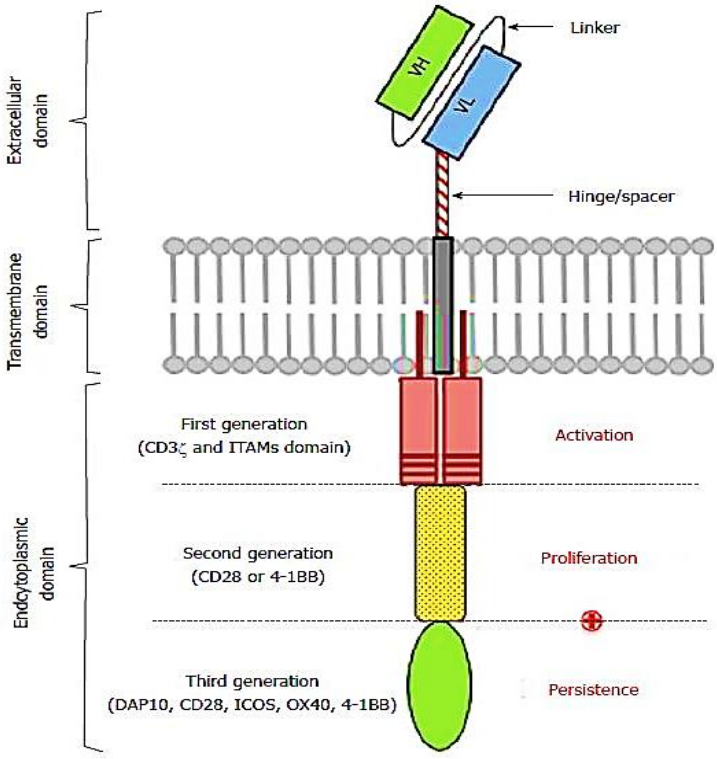

Fig. 5. Structure of CAR [11]

The characteristic of the engineered receptor can be changed by changing the co-stimulatory molecule or the antigen binding domain. The CD 28 domain causes potent cytotoxicity and early tumour killing while the CD 137 domain causes persistence and memory T cell formation.

3.1 Manufacturing CAR-T Cells [12]

To prepare CAR T cell products for the treatment of patients, T cells are obtained from the blood, activated in vitro to facilitate gene insertion, and modified to express the CAR by viral or nonviral gene delivery. CAR T cells are then reinfused into the patient, often after the administration of lymphodepleting chemotherapy to promote engraftment and proliferation of transferred cells.

3.2 Vectors Used [13]

The most commonly used vectors for transfection of T-cells are inactivated γ-retroviral vectors derived from the murine leukaemia virus and lentiviral vectors. Both integrate semi-randomly into the human genome. The other vectors being studied are DNA transposons and transfection through mRNAs.

4. APPLICATIONS OF CAR-T CELL THERAPY

CAR-T cell therapy has become one of the promising treatments for refractory B-cell malignancies with CD-19 as the ideal target antigen since it is expressed in almost all B-cell malignancies, but not in cells of other lineages. Clinical trials using CAR-T cells have been conducted in solid tumours also. But the success rate is very poor in these trials.

4.1 CAR-T Cell Therapy in Haemato-logical Malignancies

The use of second-generation CAR –T cells targeting the pan B-cell marker CD19 has achieved high remission rates. There have been about 16 trials conducted using CD-19 targeted CAR-T cells in acute lymphoblastic leukaemia (ALL). In both adult and paediatric patients, high morphologic complete remission rates (59-95%) were achieved [14]. The remission rates were very high considering the fact that these tumours were refractory to all other treatment modalities. In a study by Maude *et al*, the product CTL019 was found to cause 81% remission in B-cell ALL

patients with refractory disease [15]. This product was then approved as tisagenlecleucel (KYMRIAH) by the FDA on august 30, 2017 [16]. Relapse after CAR therapy remains the major issue with anti CD 19 CAR-T cell therapy and is an important cause of treatment failure.

The anti CD-19 CAR-T cell therapy has produced modest remission rates in Non-Hodgkin's Lymphoma (NHL) of 33-55% in Diffuse Large B cell Lymphoma (DLBL) [14]. The remission rates were higher in the treatment of CD 19 positive follicular lymphoma also [7]. But due to severe adverse effects and lack of persistence of the CAR-T cells the trials were terminated with follicular lymphoma. The ZUMA-1 trial was conducted using the product axicabtagene ciloleucel and caused 54% complete response rate in patients with DLBL [17]. This study led to the approval of the second CAR-T cell therapy by the FDA. Axicabtagene ciloleucel (YESCARTA) was approved on October 18, 2017 for the treatment of DLBL and received orphan drug status [18] On-going trials with CD 20 as the target antigen are being evaluated in DLBL.

In chemotherapy refractory high risk Chronic Lymphocytic Leukaemia (CLL) patients, CD 19 directed CAR-T therapy caused 13-50% remission in various studies [7]. The decreased remission rates may be due to inhibitory tumour microenvironment or disease intrinsic mechanisms like defects in circulating T cells in CLL patients [1].

In Hodgkin's Lymphoma (HL) CD 30 directed CAR-T cells are being tried in refractory cases. There was partial remission in 39% of the cases. This is further to preclinical studies which have shown a good response with CD 30 CAR-T in humanized mice with HL [7].

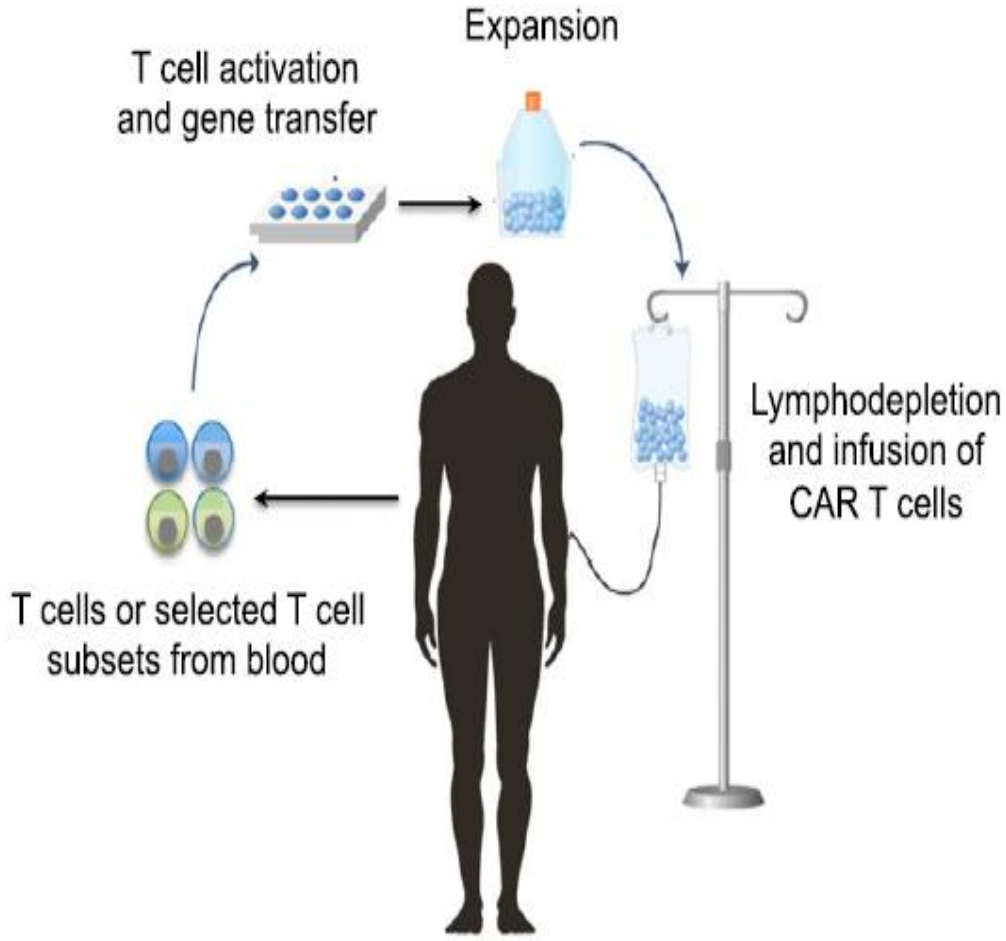

Fig. 6. Adoptive cell therapy with CAR modified T-cells [12]

CAR-T cells have been designed to target CD 123 and Lewis Y molecules on Acute Myeloid Leukaemia (AML). CAR-T cell therapy is also being investigated in the treatment of multiple myeloma. The target antigen is B-Cell Maturation Antigen (BCMA) and CS 1 [12].

5. CAR-T CELL THERAPY IN SOLID TUMOURS

The various studies conducted on solid tumours using CAR-T cell therapy have been summarised in Table 1 [19-25].

In all the above studies, the engineered T-Cells persisted for a very short time in vivo.

5.1 Other Applications of CAR-T Cells [26]

CAR-T cells are being explored in:

- Multiple Sclerosis – against myelin oligodendrocyte glycoprotein (MOG)
- Inflammatory intestinal diseases like Irritable Bowel Syndrome
- Autoimmune diseases like Pemphigus vulgaris where Chimeric Autoantibody receptors (CAART)
- Human Immunodeficiency Virus (HIV) type 1

5.2 Adverse Effects of CAR-T Cell Therapy

- Tumour lysis syndrome & Cytokine Release Syndrome (CRS):
  This occurs due to overproduction of cytokines from activated CAR-T cells resulting in tumour lysis, macrophage release – Macrophage activation Syndrome (MAS) and further cytokine release. CRS is associated with elevated circulating cytokines like IL-6,IFN-γ, TNF-α and can cause high fever, multi-organ failure, hypotension and hypoxia. CRP levels can be a predictive biomarker for CRS [1]
  Treatment: IL-6R inhibitor tocilizumab has been used as an effective treatment in CRS. TNF-α inhibitor infliximab has also been used. Mild adverse effects can be managed with coricosteroids [7]. But the use of corticosteroids decreases the overall efficacy of the CAR-T treatment. Allopurinol can be used to prevent tumour lysis syndrome. Lymphodepletion chemotherapy and other immuno suppressive treatments can be used to prevent the occurrence of adverse effects [1].
- On-target off- tumour toxicity:
  It is due to the recognition of the target antigen expressed in normal tissues by the CAR-T cells. Eg. B-Cell aplasia caused by the anti-CD 19 CAR-T cells. This can be treated by I.V.Immunoglobulin infusion [7] But the adverse effects are more severe in solid tumours. It has even led to death [22]. To avoid this two strategies are being adopted:
  i. To enhance the CAR-T cell selectivity to recognise tumour cells only – by using tumour specific antigens like EGFR vIII, combinatorial antigen targeting
  ii. To control the CAR-T cell activity when severe toxicity occurs – by using suicide genes [7]
- Neurologic toxicity:
  Can range from confusion and delirium to aphasia, myoclonus, obtundation and seizures. It is mostly self limiting. The etiology is unknown but can be due to elevated cytokine levels. It is treated with high dose steroids [1].

Table 1. CAR-T cell therapy in solid tumours

Antigen targeted	Cancer targeted	Outcome
α folate receptor	Ovarian carcinoma	Disease progressed
CD 171, GD 2	Neuroblastoma	CD 171- no persistence of CAR-T cells GD 2 – complete remission in 3/11 patients
HER 2	Colorectal cancer	Died due to cytokine release syndrome (CRS) – 1 patient
IL13R α2	Glioblastoma	1/4 patient had a good response
CEA	Liver metastases from adenocarcinoma of G.I tract	Disease progressed
HER 2	sarcoma	No clinical benefit

- Anaphylaxis:
  Occurs when the ScFv used is of non-human origin. Upon receiving a second infusion there can be fatal anaphylaxis. Human anti- mice antibodies were found in patients who had received murine ScFv derived CAR-T cell therapy [27]. Following this, the ScFvs were derived from human monoclonal antibodies or humanised antibodies.
- Risk of insertional mutagenesis
  If the viral vectors used get inserted close to proto-oncogenes, there can be activation of the proto-oncogenes resulting in malignancy. Primary immunodeficiency disorders may also occur. These have not occurred in any human trials so far [1].

5.3 Challenges for CAR-T Cell Therapy

Challenges with CD 19 CAR-T cell therapy [10]:

Relapse after CD 19 therapy is the major hurdle. The relapse can be of two types:

a) CD 19 positive relapse – this occurs due to loss of persistence of CAR-T cells. This can be managed by
   i. using optimal CAR constructs - CD 137 co-stimulatory domain helps in increasing the persistence
   ii. preconditioning – using a lymphodepleting chemotherapy helps in increasing persistence
   iii. Increasing the ratio of early lineage T-cells
   iv. Multiple CAR-T cell infusions
b) CD 19 negative relapse – this is called tumour antigen loss escape leading to an outgrowth of tumour escape variant cells like CD 20 positive cells

This can be managed by:

i. T-cell expressing CAR which contains two different ScFvs in tandem – called TanCARs
ii. Two different CARs targeting two different antigens – called dual-signalling CAR.

It is practically difficult to design the TanCAR or the dual-signalling CAR because it is difficult to find two targets for a single tumour which is not expressed on normal cells and to find suitable epitopes. But preclinical proof of concept studies has shown promise in these two strategies.

Challenges with solid tumours:

1. Selection of target tumour antigens [1] – It is difficult to find target proteins that are expressed exclusively on the solid tumour cells. So it will be difficult for the T-cells to differentiate between normal cells and tumour cells.

Strategies adopted:

I. Identification of selectively expressed proteins – eg. Mesothelin, GD 2
II. Identification of neo-antigens as targets – neo-antigens are short peptides of 8-12 amino acid sequences that occur as a result of tumour specific mutations. Eg. EGFR vIII

2. Antigen escape due to outgrowth of antigen null cells[10]– avoided by combining CAR-T cells that target separate antigens, use of TanCARs and dual signalling CARs
3. Delivery of CAR-T cells to the tumour site [1] – there are highly restricted areas in solid tumours. It is difficult to deliver CAR-T cells into these areas. In preclinical studies, it has been found that using CAR modified immune cells other than T-cells (like Natural Killer cells) may help in better delivery of CAR-T cells to the restricted areas.
4. Persistence of the CAR-T cells [1]– The persistence and proliferation of CAR-T cells can be improved by the use of the increased ratio of central memory T cells with naïve markers like CD62L. Integration of IL-12 and IL-15 genes also improves persistence. Multiple cell infusion is another approach.
5. Immunosuppression – the solid tumours have a hostile tumour micro-environment (TME) that decreases the efficacy of the CAR-T cells.

The characteristics of the TME are:

a) Hypoxia, low pH and absence of vital nutrients that impair the functioning of T-cells
b) Existence of inhibitory pathways –
   - stromal and immune cells release suppressive factors

- tumour and infiltrating cells may express inhibitory receptor ligands that can directly suppress tumour specific T-cells

These pathways are circumvented by

- The use of immune checkpoint inhibitors targeting CTLA-4 [13]
- Facilitating the expression of survival genes like BCL-X [1]
- Co-expression of CD 25, CD 28 and CD 137 can protect the T-cells from the hostile TME – these are called armoured CAR-T cells [10]
- CAR-T cells that are engineered with inducible IL-12 expression are called TRUCKs – T-cells redirected for universal cytokine mediated killing – they resist the hostile environment and have additional anti-tumour property [1]
- Chemotherapeutic agents like fludarabine and cyclophosphamide can be used before infusion of the CAR-T cells to help them resist the immunosuppressive factors [1]

5.4 Points to Ponder

- There has been no suitable dose range that has been optimised for therapy
- Whether the dose range would be the same for different targets and different diseases has to be addressed
- The optimal ratio of the subsets of T-cells to be used (CD4, CD8, memory T cells) is not known.
- There is no protocol to judge which patients should receive multiple CAR infusions.
- The optimal conditioning regimen has not been determined.
- The patients who have received Hematopoietic Stem Cell Transplants (HSCT) have shown mixed responses to CAR-T cell therapy. Further studies in patients who have undergone HSCT may be required to see whether they are suitable candidates for receiving CAR-T cell therapy.

6. CONCLUSION

CAR-T cell therapy has emerged as a miracle therapeutic option in the treatment of refractory and relapsed haematological malignancies. The cells are manufactured in a personalised manner since autologous T-Cells are used. This reduces the transmission of blood borne diseases. Many barriers exist for its use in solid tumours. These challenges must be addressed in future studies and efforts should be made to decrease the severity of the adverse effects. Transition should be made from proof of concept studies to clinically implementable technologies. The underlying mechanisms by which resistance to therapy occurs should be elucidated and effective combination therapies should be developed. CAR-T cell therapy can become an effective treatment option for diseases without alternative treatment options in the future.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sciencedomain.org/review-history/26188