Comprehensive Overview of CAR-T Cell Therapy, Engineering Process and Future Prospects

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Abstract. Chimeric antigen receptor (CAR)-T cell therapy is a revolutionary treatment method which applies the technology of modifying patients' immune T cells to eliminate cancer cells. The immune system recognizes invading cells by noticing antigens on the foreign cells. The receptors of T cells bind to the antigens which notifies the rest of the immune system to eradicate the foreign invaders. CAR-T cell therapy has gained achievement in the treatment of hematologic malignancies such as B-ALL. CAR-T cell engineering process contains four steps including leukapheresis and the expression of the CAR on the T cells. Among the process, the Sleeping Beauty transposon system shortens the time between genetic modification and infusion so that patients can receive the modified T cells on site. GMP (Good Manufacture Practice) also ensures quality and safety of the CAR-T cells before infusing into the patients. CAR-T cells damage tumor cells by three major pathways. T cells utilize perforin and granzyme to lyse open antigen-positive tumor cells and use Fas and Fas ligand to target antigen-negative tumor cells. The derivation of cytokines from CAR-T cells sensitizes the tumor stroma and enhances tumor killing ability. The development in CAR-T cell designs has made a huge contribution to the success of the treatment where five generations of CAR-T cells have already been investigated. However, there are still some challenges associated with the treatment such as antigen escape relapse and on-target off-tumor toxicities observed in solid tumors. The technology can be further innovated by overcoming antigen escape loss, enhancing safety of CAR-T cells, and improving the persistence of CAR-T cells using the combination of oncolytic viruses with CAR-T cells. This review mainly focuses on the CAR-T cell engineering process and killing mechanisms as well as some obstacles and potential improvement for the technology.

Keywords: CAR-T cells, Cancer immunotherapy.

1. Introduction

The important relationship between the immune system and cancer was first emphasized by Rudolf Virchow about 150 years ago, as he observed the elevated numbers of leukocytes in tumors [1]. The first immunotherapy, performed by William Coley and colleagues who treated patients with sarcoma, showed that patients with a bacterial infection had greater tumor regression compared to uninfected patients [2]. Through the advancement of cancer research, studies have now indicated the correlation between the increased incidence of cancer and a defective immune system and shown that the infusion of autologous tumor-infiltrating lymphocytes (TILs) can lead to regression of metastatic melanoma [3]. The first adoptive immunotherapy that targets specific tumor grafts by infusing immunocompetent lymphocytes into murine models was reported more than 60 years ago by Mitchison and colleagues. This technique of using the immune system to treat cancer has a high potential to overcome several challenges on vaccine-based approaches for cancer treatment, including the de novo activation and expansion of tumor-specific T cells in vivo, especially in immune compromised patients [4]. However, in cancer immunotherapy, breaking tolerance to "self" antigens is a significant challenge. Tumor cells can develop "foreign" antigens brought on by viruses or as a

result of genetic mutations specific to a given tumor that can result in epitopes that T cells and B cells can identify. This type of immune response against foreign substances is comparable to those induced by infections, so they are less affected by the regular mechanisms that regulate self-tolerance [4]. On the contrary, several tolerance mechanisms can limit the immune reactions against self-antigens on tumors, such as negative selection in the thymus and T cell exhaustion by the immunosuppressive tumor microenvironment (TME) [3]. Therefore, although T cells play an important role in eliminating the tumor, the amount of endogenous T cells with high-affinity to tumor antigens is insufficient for controlling human cancer.

One solution to this problem is the development of genetically engineered receptors called chimeric antigen receptors (CARs), which combines the specificity of monoclonal antibodies and the tumor killing efficacy of T cells. The extracellular domain is composed of a single-chain variable fragment (scFv) derived from the variable region of heavy and light chains of an antibody fused via a flexible linker, which allows for the specific recognition of antigens with high affinity. The transmembrane domain is a hydrophobic alpha helix that spans the membrane that provides stability to the receptor. The intracellular domain contains the CD3ζ T cell receptor (TCR) intracellular signaling domain, which will be activated upon antigen recognition [5]. This approach redirects and boosts patients' T cells so that their anti-tumor activity is tumor-specific, high-affinity and major histocompatibility complex (MHC)-independent.

Nevertheless, this strategy has its limitations that require improvements. First, it is shown that some cancer patients relapsed with tumor cells lacking antigen expression after a complete response after CAR-T cell therapy [6], which makes CAR-T cell therapy no longer effective against tumors. Second, CAR-T cell therapy elicits toxicities, such as cytokine release syndrome (CRS) and CAR-T cell-related encephalopathy syndrome (CRES), limiting the use of CAR-T cell therapy widely [7]. Third, it has been a challenge to improve the persistence of CAR-T cells. There are currently five generations of CAR-T cell design, in which the fifth generation improved the persistence, as well as vaccination and combination therapy of CAR-T cell with oncolytic viruses [8].

Even though there are many challenges in widely implementing CAR-T cells on both blood and solid tumors, CAR-T cell therapy remains to be an essential strategy in the field of cancer immunotherapy. This review discussed the engineering process of CAR-T cells that involves blood removal from patients, T cell activation, gene delivery, CAR-T cell expansion, quality assessment and infusion, and the killing mechanism of CAR-T cells via three pathways, including the perforin and granzyme axis, the Fas and Fas ligand axis, and cytokine secretion. This review also highlighted possible improvements to overcome the limitations of CAR-T cell therapy.

2. CAR-T cell designs

The first-generation CAR-T cells include an extracellular scFv from a monoclonal antibody that is linked by a spacer to the transmembrane domain connected to the intracellular domain CD3ζ only (Figure 1a) [3]. However, the first-generation CAR-T cells fail to be clinically effective due to limited expansion and short persistence, especially when the tumor cells do not express costimulatory molecules that are essential for T cell activity [9]. Therefore, similar to native T cells, CAR-T cells with only the CD3ζ sequence cannot carry out their normal function in the absence of the costimulatory signal [5]. The second-generation CAR-T cells contain an additional costimulatory intracellular domain, such as CD28 or 4-1BB, in CAR molecules (Figure 1b). This modification of the CAR design leads to an improved activation, survival, and expansion of modified cells. However, it is still unknown which costimulatory domain is the best. Hence, more studies on the safety and efficacy of the second-generation CAR-T cells are needed. The third-generation CAR-T cells are engineered in a fashion that multiple costimulatory signals are incorporated into the receptor (Figure 1c), attempting to improve tumor killing ability. Third-generation CAR-T molecules, such as CD3ζ-CD28-OX40 or CD3ζ-CD28-41BB, have a higher potency with better persistence, cytokine production, and anti-tumor activity. Nevertheless, no improved outcomes were observed compared

to the second-generation CAR-T cells, and two costimulatory molecules may lead to uncontrolled T cell activation and severe toxicity [3], [5]. The fourth-generation CAR-T cell, which is also known as armored CAR-T cell, is engineered to express a constitutive or inducible cytokine and receptor production cassette together with the second-generation CAR (Figure 1d). T cells redirected towards universal cytokine killing (TRUCK) CAR-T cells are designed to secrete cytokines to disrupt the TME abundant of immunosuppressive cytokines in solid tumors, which could potentially promote an immune-activating TME and improve anti-tumor activity of CAR-T cells as well as resident immune cells. Cytokine modulating CAR-T cells are another type of armored CAR-T cells designed to modify cytokine function by changing how CAR-T cells react to cytokines. Antibody-secreting CAR-T cells are the last type of CAR-T cells that are created to secrete antibody-like proteins. This approach employs a single therapy to doubly target antigens on cancer cells using both the CAR and the secreted antibodies, strengthening tumor recognition. Moreover, this type of CAR-T cells also boosts cancer cytotoxicity because antibodies could mediate several killing mechanisms, such as antibody-dependent cell-mediated cytotoxicity (ADCC). Since the combination of both CAR-T cells and checkpoint inhibitors has shown to be clinically beneficial relative to CAR-T cells alone, armored CAR-T cells that secrete checkpoint inhibitors are being developed to interfere immune suppression in solid tumors [10].

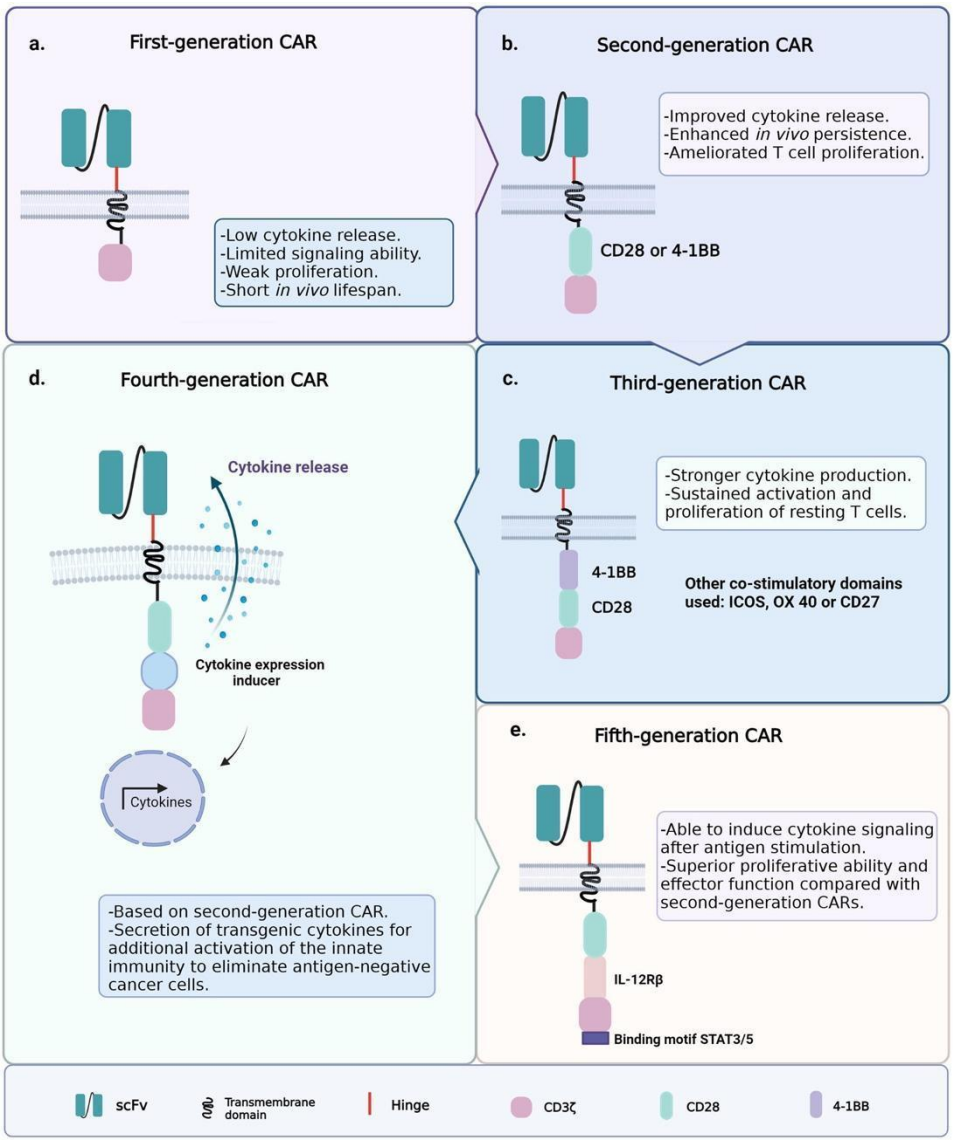

Figure 1. Five generations of CAR-T cells construct models [11].

3. CAR-T cells Engineering Process

The underline mechanism of CAR-T cells production is mainly divided into four major steps. It starts by removing the blood from the patients and undergoes purification and leukapheresis which separates the T cells. Secondly, using a viral or non-viral vector enables the T cells to produce CAR on their surface. Afterward, the engineered CAR-T cells will be incubated and expanded to a large population, which will then be under quality assessment and transfused into the patient. Finally, the killing process of CAR-T cell begins

Leukapheresis is a process of separating lymphocytes in the blood and returning the rest of the blood to the patient. The collected leukocytes undergo anticoagulant buffer wash and counterflow centrifugal elutriation based on different sizes and densities [12]. At the level of T cells, the CD4 or CD8 subtype could be further separated using antibody bead conjugation [13]. As activated T cells are needed for the next steps which could be usually done through two processes: by either the activation of MHC on the antigen-presenting cells or with costimulatory molecules like CD28 or 4-1BB. Experimentally, the CD28/CD3 monoclonal antibodies are usually involved since activity through antigen-presenting cells is not normally feasible [13]. A strong proliferative signal is provided by anti-CD3 beads, and a further costimulatory signal is accomplished by anti-CD28 beads [14]. The combination of anti-CD3 beads and anti-CD28 beads allows exponential growth of the T cells as well as a significant amount of cytokines produced [15].

Viral and non-viral methods are two ways that direct the CAR into the T cells. For the viral method, the retrovirus is commonly used for effective gene delivery as the helper gene of the virus could be substituted by a therapeutics gene and further integrated into the host genome after reversely transcribing its RNA to DNA [16]. Other types of viral vectors such as lentiviral vectors are also adopted. The third generation of lentiviral vector consists of three plasmids, two separate packaging plasmids one encoding for *gag* and *pol* and another for *rev*. The third plasmid derived from the VSV-G contains the gene of interest [17]. Besides viral factors, non-viral factors such as CRISPR or Sleeping Beauty transposon systems provide another path for inserting the gene. Comparatively, the non-viral way is cheaper and not size-restricted since there are no packaging steps.

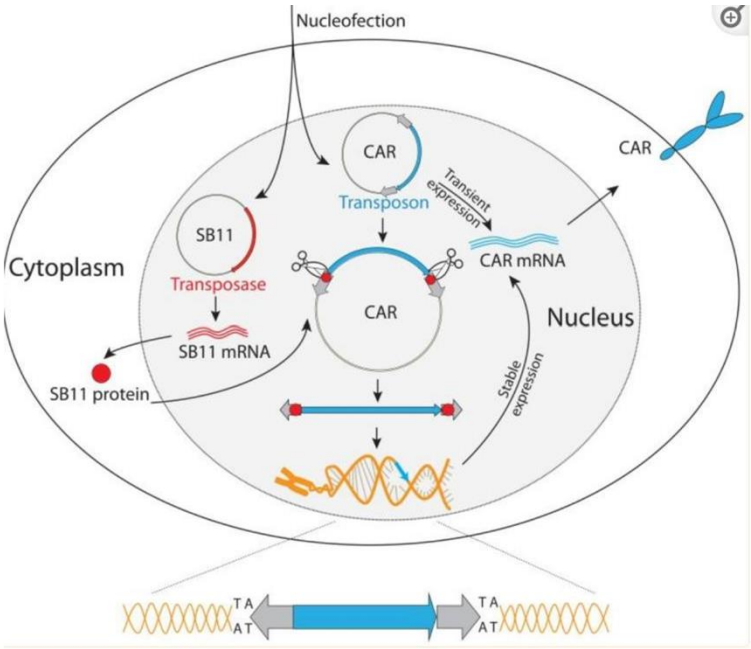

Figure 2. Schematic of Sleeping Beauty (SB) system [18, p. 19].

As shown in the figure the SB transposon system consists of two kinds of transposon systems. SB11 transposase and CAR transposon. Firstly, the SB11 will be transcribed as SB11 mRNA and will be further translated into SB11 protein which will flank the CAR transposon. Through a cut-and-

paste mechanism, the CAR will be inserted into TA sites in the host T-cell genome so that the CAR can be expressed on the surface [18, p. 19].

Further cultivation and expansion are needed for growing the engineered CAR-T cell into a large population. Two systems namely the WAVE Bioreactor and the G-Rex Bioreactor are used. The WAVE Bioreactor adopts a rocking platform to expand the CTL019 with a special perfusion function allowing for automatic feeding and waste removal. Another culture system, G-Rex (Wilson Wolf), can expand cells from low densities [14]. By using these bioreactors for 9-11 days the cells can rapidly expand to 5-10 liters. The concentration by Haemonetics CellSaver is required which provides a closed and sterile system for the washing and concentration steps [19]. The final concentrated form of CAR-T cells will be assessed for several criteria before being infused into the patient.

Quality assessments are conducted by CGMPs (Good Manufacture Practice) to inspect the production materials used in the engineered process such as cells and vectors, and to monitor the key production parameters for consistent products. Further tests for the purity, safety, and efficacy of the final CAR-T cells are carried out. Qualified and validified CAR-T cells with specific criteria will be released. CAR-T cells are then transported and finally reached the patient's body after intravenous injection, which travels to the tumor site to perform their killing process [20].

4. Killing Mechanism

One of the major pathways that T cells utilize to mediate their cytolytic effector functions is exocytosis of cytotoxic granules that contain perforin and granzymes, targeting antigen-positive tumor cells. Cytotoxic granules that are linked to the microtubules of the effector cell migrate towards the interface and fuse to the plasma membrane in the region of cSMAC after IS formation [21]. The granules containing cytolytic cargo are released into the synaptic cleft, and perforin causes the membrane of the target cell to develop pores that allow pro-apoptotic granzymes access. After entering the cytoplasm of the target cell, granzymes can cause caspase-dependent and independent apoptotic cell death to cleave their substrates [22], [23]. The perforin and granzyme axis are essential for quick, efficient, and specific CAR-T cell lysis of target cells.

Along with the exocytosis of cytotoxic granules, the Fas and Fas ligand (FasL) is another important axis that mediates the lysis of target tumor cells by CAR-T cells. Trimerization of the Fas receptor upon Fas ligand binding initiates the Fas and FasL pathway [24]. As a result, pro-caspase 8 and the adapter protein Fas-associated death domain (FADD), which together make up the death-inducing signaling complex (DISC), activate caspase 8. After that, caspase 8 then processes pro-caspase 3 to produce mature caspase 3, which subsequently mediates cell death by cleaving key cellular substrates, carrying out apoptosis [25]. It is also reported that CAR-T cells can mediate tumor lysis against antigen negative fractions in a tumor environment with both antigen-positive and -negative tumor cells. This antigen independent and cell-cell contact-mediated tumor lysis is only observed when activated CAR-T cell interact with the antigen positive fraction [17]. Thus, the Fas and FasL axis represents a different molecular approach through which CAR-T cells can cause tumor cell lysis. The antigen-independent mechanism could be beneficial in overcoming antigen-loss associated diseases.

Although direct interactions between T cells and tumor cells are the foundation of CAR-T cell design, the release of cytokines by activated CAR-T cells may improve their tumor killing ability. It is demonstrated that cytokines derived from CAR-T cells sensitize the tumor stroma and promote immune cell re-education that includes the macrophage polarization to the anti-tumoral M1 phenotype [27].

5. Limitations and Improvement

CAR-T cell therapy has a significant effect in certain cancer types, but there are still some limitations reducing the durability of CAR-T cells in hematological malignancies and solid tumors. One of the most common challenges is antigen escape relapse. Patients who were treated with single

antigen targeting CAR cells would lose the target antigen expression partially or completely [28]. According to Majzner et al., approximately 70% to 90% of relapsed pediatric B-ALL patients had enduring responses to CD19 targeting CAR-T cell therapy, while the persistence of the responses was based on the outgrowing CD19-negative leukemia [29]. A phase I trial conducted by Children's Hospital of Philadelphia reported that 36% of the patients would have a relapse after the CD19 CAR T-cell therapy whereas 13 out of 55 patients (24%) would experience a CD19-negative relapse [29]. It demonstrated that CD19-negative antigen escape was the main cause of antigen loss relapse after CD19 CAR-T cell therapy. Another challenge is on-target off-tumor toxicity, which is associated with cytokine release syndrome (CRS). When CAR-T cells bind to targeting antigens in solid tumors, it would trigger the activation of infused CAR-T cells and release many inflammatory cytokines subsequently [30]. The CRS reactions can be categorized into 5 grades from mild (grade 1) to death (grade 5). IL-6 is the main mediator of the CRS. The toxicity is also associated with the antigen expression in normal healthy living cells. When CAR-T cells bind to targeting antigens in solid tumors, the antigens are also found in healthy tissues. The toxicity released from binding of CAR-T cells would damage healthy cells. For example, neurotoxicity was detected in high affinity GD2-specific CAR-T cell therapy during neuroblastoma treatment [30]. Thus, incorrect selection of antigens may cause on-target off-tumor toxicities.

As mentioned before, some issues and limitations have been found in CAR-T cell therapy. Scientists have developed several novel strategies to improve the technology. First, researchers are developing a technology for generating T cells, which can identify multiple antigens to overcome antigen loss relapse in B-ALL patients. They studied on the combination of antigen targeting CD19 and CD123 CAR-T cells. It was found that the T cells have 2 patterns, either modifying 2 various CAR molecules with 2 distinct binding domains or modifying 1 CAR molecule with 2 distinct binding domains in tandem, which is also called Tan-CAR [31]. The dual-targeted CAR-T cells have antigen input that would induce vigorous activities to fight against tumor cells, and the dual-targeted CAR always has alternative antigen input which could prevent CD19 escape relapses under the condition of one antigen loss. However, there are still some burdens in this strategy such as limited selections of clinically proven antigens and epitope selections based on the presence of Tan-CAR [31].

Scientists are also developing several strategies to prevent toxicities produced from on-target/off-tumor effects in solid tumors. The first strategy was to enhance selectivity of CARs. Since CAR-T cells only attack antigen-targeting cells, it is better to target truly tumor-specific antigens such as EGFRvIII to eliminate off-tumor toxicities [31]. The results showed that the infusion of EGFRvIII-specific antigen to patients having EGFRvIII positive glioblastoma was successful in the absence of off-tumor toxicities. According to Posey et al., glycosylated antigen-Tn-MUC1 could be used as a targeting antigen to identify Tn- and STn-positive tumor cells by a newly developed technology of CAR with scFv from antibody 5E5 [32]. Combinatorial antigen targeting method could also be applied to enhance the specificity of CARs. Dual-targeting antigens could be administered using 2 receptors with distinct binding domains, which is like the concept of the application of overcoming the antigen escape relapse. One receptor used was a CAR with CD3z signaling domain targeting one specific antigen that helped to turn on the T cell activation function, while the other receptor was a CCR targeting a second antigen which helped to initiate the CD28 and CD137 costimulation signaling [31]. The engineered T cells would only be initiated when both antigens were present. Researchers also introduced another method of synthetic Notch receptors (syn-Notch). The receptors could identify extracellular molecules and trigger intracellular responses. The scientists designed 2 combinatorial antigen targeting circuits including CD19 syn-Notch, GFP syn-Notch, and CD19 CAR, and they found that the receptors would successfully express the second specific antigen when syn-Notch targeted the first specific antigen [33]. Syn-Notch CAR expression was also studied in Jurkat T cells where CD19 and mesothelin were used. It was found that GFP mesothelin CAR had an expression with a half-time of approximately 6 hours ($t_{1/2}$ ~ 6 hrs), whereas CAR T cell activation had an expression with $t_{1/2}$ ~ 13 hrs, meaning that tumor cells with one single targeting antigen would not lead to complete activation [33]. Syn-Notch CAR expression was further studied in human

primary T cells. They tested α-GFP syn-Notch receptor to derive α-CD19 4-1BBζ CAR expression. It was clinically found that Gal4-VP64 activation domain would lead to a good syn-Notch expression. In addition, tuning the sensitivity of CAR to epidermal growth factor receptor could differentiate between tumor cells and normal tissue cells while sustaining the potency of anti-tumor activities [34].

The 5th generation of CAR-T cells was improved based on the 2nd generation CAR-T cells by including an additional IL-12 receptor β-chain and STAT3 (Figure 1e). The 5th generation of CAR-T cells appeared to have the superior activation and proliferative activity. The generations of CAR-T cells proved that each component of the CAR construct had an impact on the persistence of CAR-T cells. Furthermore, the combination of CAR-T cell therapy with oncolytic viruses improved CAR-T cell trafficking to the tumor sites and anti-tumor activity. The treatment of solid tumor using CAR-T cell therapy alone would cause endogenous immune cell death, whereas the addition of oncolytic virus treatment expressing cytokines and immune checkpoint inhibitors would enhance CAR function and effector T cells (Figure 3). For example, in the neuroblastoma model, CAR-T cells were combined with Ad5Δ24 virus, RANTES chemokine, and IL-15 cytokine. The results demonstrated that Ad5Δ24 virus made a robust cytotoxic effect on the tumor cells and enhanced apoptosis, whereas antigen-targeting anti-GD2 CAR-T cells were not affected [35]. The preclinical research proved that the combined treatment of oncolytic viruses and CAR-T cell therapy would not only advance the persistence of CAR-T cells, but also improve anti-tumor activities.

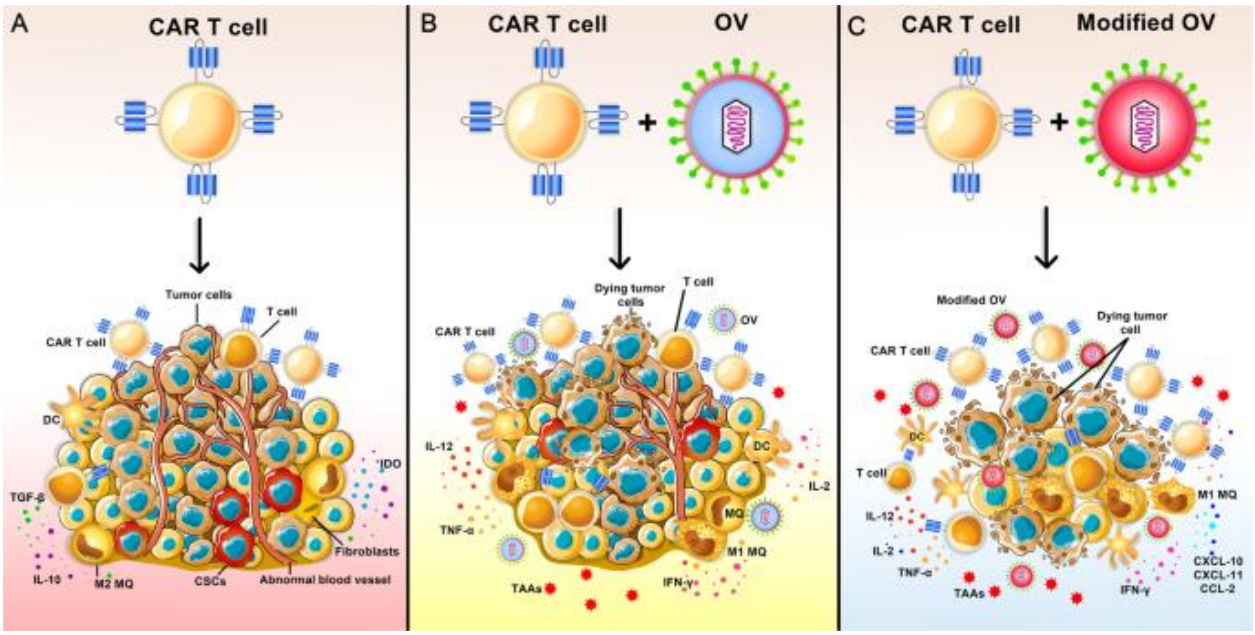

Figure 3. The combination of CAR-T cell therapy and oncolytic viruses. A) CAR-T cell therapy treatment alone in solid tumors failed because of low penetration rate and immunosuppressive TME. B) Treatment of oncolytic viruses before inserting CAR-T cells would cause innate immune cell death. C) The combination of CAR-T cell therapy and modified oncolytic viruses enhances CAR- T cell function and anti-tumor activities [36].

6. Conclusion

The insufficiency of T cell in recognizing cancer cell due to immunosuppressive tumor environment led to the development of CAR (chimeric antigen receptors). It is used mainly for enhancing the anti-tumor ability of the patients' T cells in an MHC-independent way, and simply constructed of extracellular scfv domain, transmembrane domain and intracellular domain. After three evolutionary CAR-T cells development, the fourth one with inducible cytokine production with higher cytotoxicity was finally generated. The production of such CAR-T cells usually undergoes four steps, including leukapheresis, T cells activation and vectors transduction, cultivation and quality assessment. Through intravenous injection of the final CAR-T cells, the killing process of tumor cells

starts by mainly by cytotoxic granules with perforin and granzymes or through Fas and Fas ligands. Several challenges such as antigen escape or on-target off-tumor toxicity remain. Overcoming those challenge has been a goal for the scientists by developing new innovative insights. By using dual targeted CAR-T cells can prevent CD19 targeted relapse. Further enhancement in the selectivity of the CARs and using combinatorial antigen targeting method prevents the toxicity of on-target/off-tumor effects in solid tumors. The development of the fifth generation of the CAR-T cells, as well as more advanced clinical research deepens the CAR-T cells' therapeutic potential as a effective cancer immunotherapy treatment.

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