Review Article
J Immunol Clin Microbiol. 2019;4(2):34-51

J Immunol Clin Microbiol

Immunotherapeutic CAR T-Cell Engineering

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Abstract

Cancer is a significant problem in our age and sought alternative therapies. Immunotherapeutic Car T-cell is a different kind of modality treatment under adoptive immunotherapy and has many promising clinical trials in recent years. CARs are synthetic receptors with a modular design. CAR T-cells recognize the antigen by antigen-sensitive antibody header (scFv). In this way, by activating the intracellular signaling pathway, it begins to proliferate and secrete various cytokines to kill the cell they recognize. Research on treating solid tumors with Car T-cell still is a controversial and uncertain issue compared to it frequently uses in cancers of the blood-producing cells (hematologic cancer) such as leukemia and lymphoma. Although engineering immune cells to treat cancer has various side effects such as off-target recognition, neurotoxicity, cytokine release syndrome (CRS) and toxicity, many clinical trial results are optimistic.

Key words: *Cancer, Chimeric antigen receptors, CAR T-cell, Immunotherapy, Adoptive cell transfer therapy, Genetic engineering, Toxicity*

Introduction

Cancer is a multi-stage process involving mutations in the cells or for various reasons, changes in the invasion of uncontrolled proliferating cells and apoptosis due to DNA damage. Various treatment methods found for this disease which causes the death of many people today. According to the data in 2016, the death of many people in the world is caused by cancer after cardiovascular disease (1).

As known, the current treatment methods are as in the table. Immunotherapy, which is one of these treatment methods, attracts attention in recent years (Fig.1).

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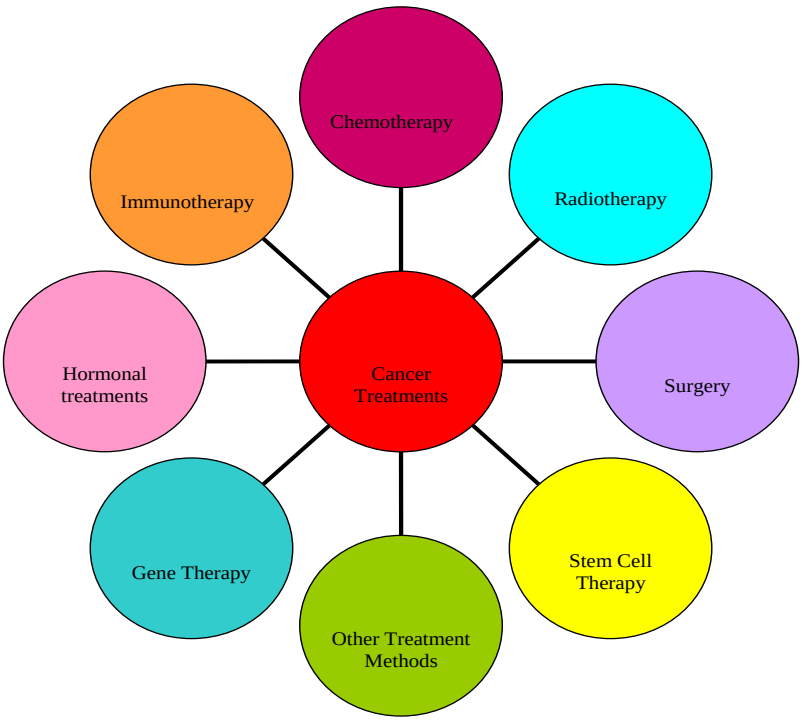

Figure 1. The most common cancer treatment methods.

Immunotherapy

Immunotherapy (immuno-oncology or biologic therapy) is a kind of cancer treatment that the body's activated immune system can directly attack tumor cells but leaves healthy cells unharmed. It is a treatment that aims to strengthen the immune system. There are two main types, specific immunotherapy and non-specific immunotherapy. Specific immunotherapy responds to a specific cell or antigen, including adaptive cell therapy. In non-specific immunotherapy; it is intended to stimulate the entire immune system (2, 3).

In 1866, Wilhelm Busch reported that they developed tumor suppression after the disease caused by Streptococcus species. The first known tumor immunotherapy was attempted to see tumor suppression in 1868 by a sarcoma patient infected with the same infection after the operation. Here, the patient's immune system to produce tumor necrosis (TNF) factor is provided (4).

Immunotherapy is used to treat many cancers, commonly used varieties; cytokines, antibodies, cells, cancer vaccines, adoptive cell therapy. In recent years, chimeric antigen receptor (CAR) T-cell therapy and therapeutic blockade of immune control points (CTLA-4 (cytotoxic T lymphocyte-related protein 4) and PD-1 / PD (programmed cell death protein 1 pathway)) have been promising for cancer patients (5- 7).

Adoptive Immunotherapy

Adoptive immunotherapy is the event of immunologically active cells being administered to the patient to prevent the treatment and formation of the disease. Cells used in this method;

T cells. These cells are infiltrative tumor lymphocytes (TIL), cancer-specific TCR (T cell receptor), and chimeric antigen receptor (CAR). Adoptive immunotherapy is a kind of treatment that can use in solid metastatic tumors, bone marrow and peripheral stem cell transplantation after relapses, latent viral infections and in AIDS. The worst disadvantage of this treatment is toxicities. Successful results have been reported in pretreatments. It is a method with increasing efforts and its disadvantages are tried to be eliminated (8 -10).

The Emergence of Immunotherapeutic CAR T-Cell

Zelig Eshharand and Friends proposed the first CAR theory at the Weizmann Institute in 1989 with the idea of equipping the T cell with CAR to direct it against a specific tumor antigen. Later in 2008, Malcolm Brenner and Houston had their first success at the clinic related to CAR (11).

This method is aimed to activate the body's immune system through genetically modified T cells. The target antigen, such as the CD19 receptor of B-cells, is a genetically engineered T cell expression chimeric-antigen receptor (CAR) that recognizes and destroys the target (12) (Fig.2).

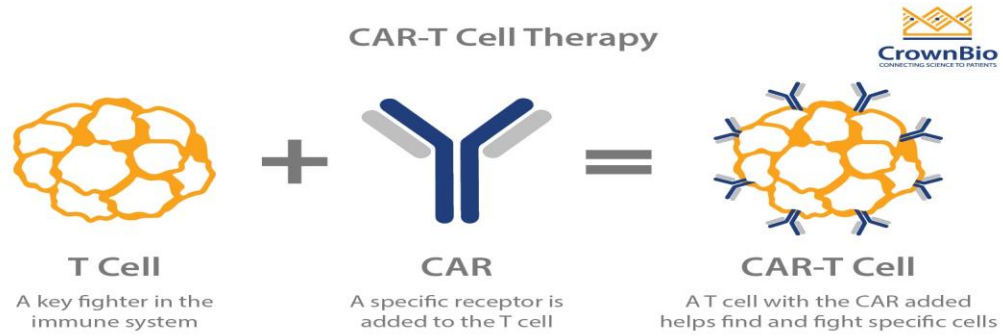

Figure 2. Crown Bio Connecting Science To Patients (13).

CAR Structure

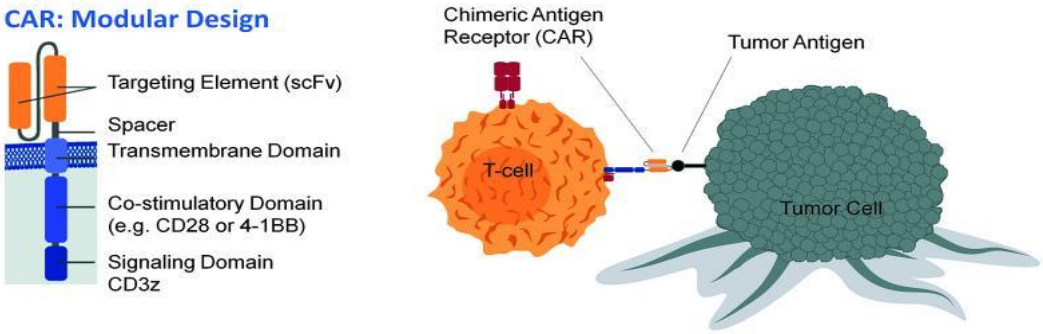

Figure 3. CARs are synthetic receptors with a modular design. The CAR T- cell starts the cytotoxic molecular secretion in order to proliferate, produce cytokines and kill the known cell by activating the intracellular signaling pathway by recognizing the antigen with the antigen-sensitive antibody header (scFv) (14).

Targeting Element (scFv): It is made from the Single-chain Variable Fragment (ScFV) portion of a specific antibody directed against the target antigen that will be activated when it recognizes the cancer-specific antigen.

Spacer: Generally made from IgG 1, it affects the flexibility of the extracellular domain and activation of CAR T-cell.

The Transmembrane Domain: TM field is mostly derived from type I membrane proteins such as CD3, CD4, CD8 or CD28. It acts as a bridge and signal transducer and affects the expression of CAR on the T-cell membrane.

Signaling Domain: Consists of a CD3 signaling pathway that activates the T cell after binding to the target cell. This structure is the first-generation CAR T-cells, which do not require antigen presentation by human leukocyte antigen (HLA), allowing it to bypass the HLA-I restriction.

For first-generation CAR T-cells, even when the CAR T-cell mechanism was active, T cells did not proliferate in vivo, and also a robust cytokine response does not observe after recognition of a tumor cell. This finding led to the production of second and third-generation CAR T-cells.

Tumor-associated antigens (TAAs) membrane proteins in tumor cells indicate higher expression level than normal tissues (Fig.3). The non-cellular scFv domain recognizes the TAA, the transmembrane domain which transmits extracellular signals, then the intracellular signaling area, external stimulus T converts to cell signals. This formation of CAR allows T cells to react individually to tumor cells with specific antigens. Secondary and third version CAR T-cells are produced by combining with the signal fragments of CD28 or CD137 proteins together with the CD3zeta activation signal fragment in order to recognize and kill cancer cells more effectively (15–21).

CAR Types

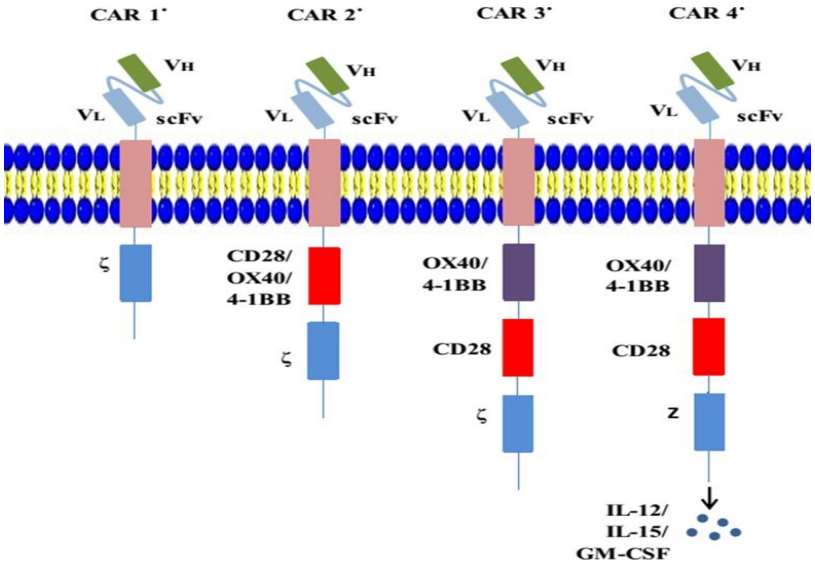

Figure 4. Structure of the four-generation chimeric antigen receptor (22).

Four kinds of CAR T-cells were produced after various studies. The first generation includes ScFV with antigen identification and the CD3ζ signal chain as the intracellular domain. As in the first generation, it contains the second generation ScFV and CD3ζ signal chain, but it has a common stimulus area such as 4-1BB (CD137) or CD28. In addition to the first generation features, the third generation includes both an auxiliary stimulator field, including 4-1BB and CD28 (23-25) (Fig.4).

Fourth-generation CAR T-cells, also known as "TRUCKs" or "armored CARs", have a constitutive or inducible expression domain for a protein that needs to secrete. Fourth generation CAR T-cells are capable of Interleukin-15 (IL-15) and Interleukin-12 (IL-12) secreting cytokines additionally to their second generation. (26-28).

Biomarkers

Biomarkers, which are an essential part of cancer treatments, are used in analyzing potential risk, screening, the probability of one disease versus that of other diseases possibly (differential diagnosis), prognosis, prediction of treatment response and disease progression. These biomarkers are related to the surface of cancer cells and serve as targets for directing and regulating the response of the immune system (direction of cytotoxic T cells).

With the emergence of CAR T cell therapy, a new therapeutic biomarker was searched. These markers aim to direct CAR T-cells to cancer cells. CAR T-cells can kill them after distinguishing the specific target antigen in tumor cells. The first biomarker target for CAR T-cell therapy was CD19 which is a B cell marker expressed in highly malignant B cells (Fig.5).

After the CD19 molecule for the success of Acute Lymphoblastic Leukemia (ALL), Non-Hodgkin's Lymphoma (NHL) and chronic lymphocytic leukemia (CLL) the search was in quest of a new biomarker. From 2018, several clinical studies are targeting 25 different surface biomarkers in most human tissues. (29 – 32).

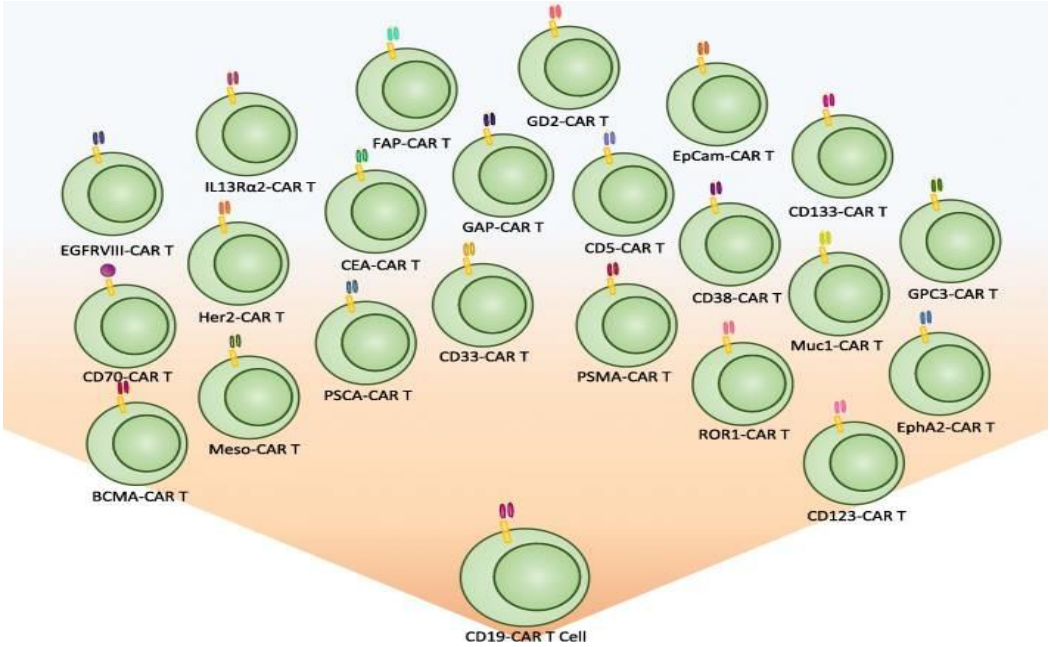

Figure 5. CAR T-cells with various biomarkers (30).

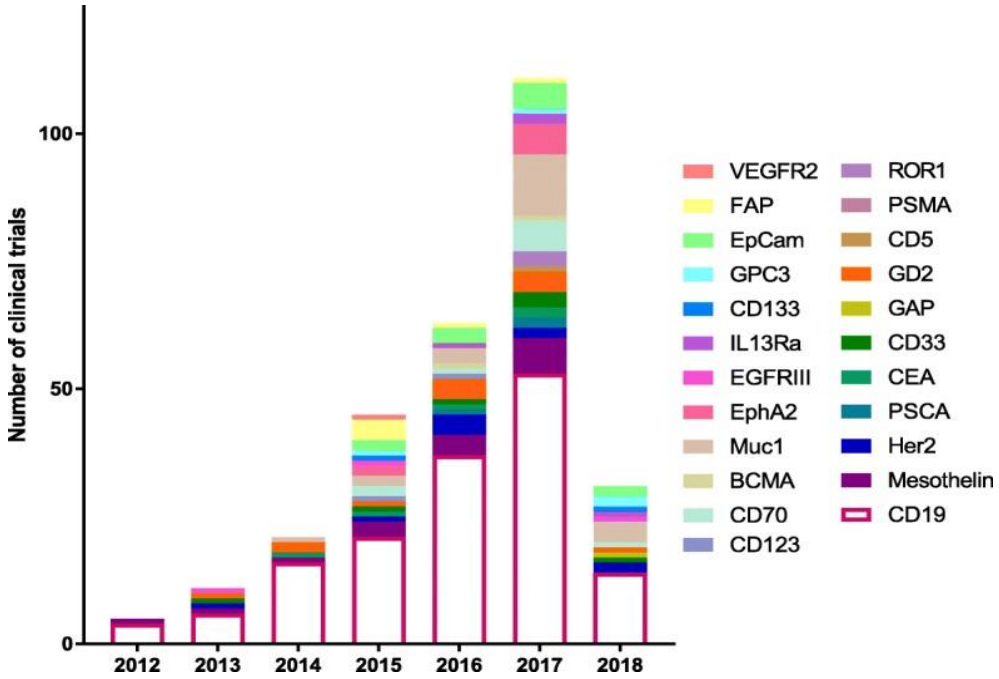

Figure 6. Biomarkers used in clinical studies between 2012 and 2018 (30).

With the advancement of immunotherapeutic CAR T-cell, CD19 biomarker was initially considered the targeted therapy. Subsequently, clinical studies were performed targeting Mesothelin. The number of biomarkers tested also increased to 25 (Fig.6). Immunotherapeutic CAR T-cell has been successful against hematologic cancers, but in solid tumors, it is not the same. There are several difficulties in solid tumors. There are currently 17 biomarkers in clinical trials for solid tumors to overcome difficulties. As immunotherapeutic CAR T-cell progresses, new biomarker targets for hematological and solid malignancies are being investigated (30).

CAR T-Cell Production and Application

CAR T-cells manufacturing for a clinical application involves five steps, autologous T cell collection, T cell activation, genetic modification of T cells, CAR T-cell expansion, CAR T-cell formulation and freezing storage. Following these five steps, generated CAR T-cells are administered to a patient for clinical treatment. First, autologous T cells are isolated from the peripheral blood of the patient by leukapheresis, followed by apheresis. Then, induction of T cell activation by monoclonal antibodies (e.g., anti-CD28 and anti-CD3, or cytokines (such as IL-2, IL-15 and IL-17) performed. The transgene encoding the CAR transfected into the T cell via viral or non-viral approaches, such as retroviral and lentiviral vectors, transposon and plasmid. Clinical studies have generally used retroviral vectors (33).

Genetically engineered T cells are amplified to capture the desired activity. This process takes about two weeks. The last step is to vaccinate genetically engineered T cells to the patient (34-36). Besides treatment and replication processes of T cells include procedures that require specific awareness and different recommended applications. The fundamental

advantage of CAR T-cells is that they are active drugs that can carry on and proliferate in the patient's body (28, 37).

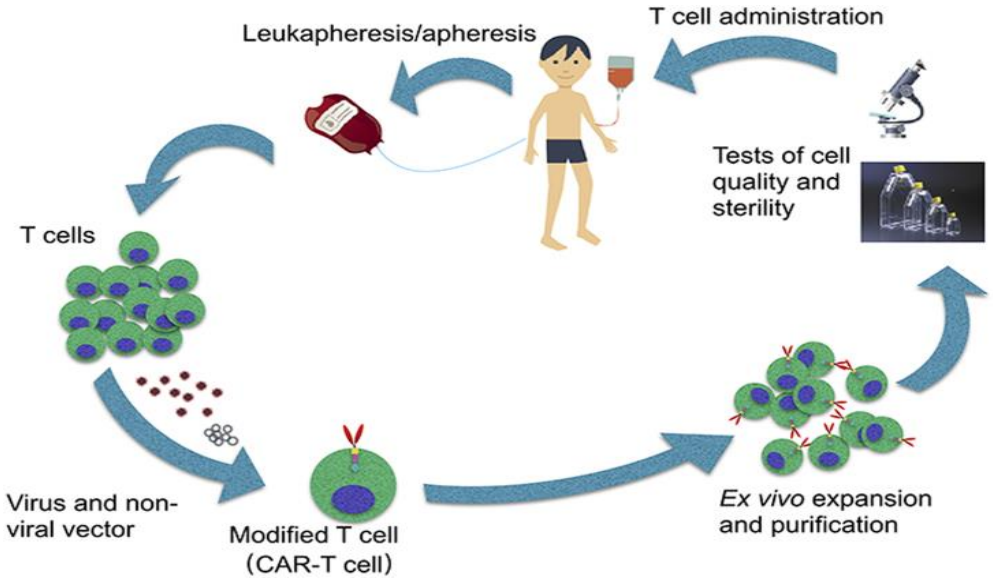

Figure 7. Chimeric antigen receptor T cell (CAR T-cell) production scheme (38).

Car T-Cell Production Techniques

Viral or non-viral vectors carrying the CAR structure can be transfected into T cells (39). Viral vectors have high gene transfer efficiency (40). Among viral vectors, retroviral or lentiviral vectors are most commonly used, but there are some health risks such as an immune response potential, toxicity, additional mutagenesis, or another tumorigenicity inducer (41, 42).

Non-viral vectors that are more interested in researchers; they have features such as non-infectiousness, easy access to large-scale preparation and relatively unlimited vector capacity and controllable chemical structure. Transposon-based systems constitute the main class of non-viral vectors and include Sleeping Beauty, PiggyBac, and Tol2 transposon systems (43- 45).

RNA-based electroporation of lymphocytes, which is a less effective method than the lentiviral method, is noteworthy because it is safer and more economical. Once the advantages and disadvantages of these methods have been assessed, it may be possible to select the ideal method for CAR-modified T-cell production (37) (Table.1).

Table 1. Advantages and disadvantages of vectors used in immunotherapeutic CAR T-cell engineering (28).

Vector	Special Features	Restrictions
Gammaretroviral	Integration into the cell genome (15) Permanent expression of the gene (16) Availability of multiple packaging systems (15)	Additive oncogenesis (15) High costs (16) Influencing active dividing cells (15) Decrease in CAR expression after a while (16)
Lentiviral	Affecting non-dividing cells Improved cargo capacity (17) A possibility of reduction of placement oncogenesis (18)	Lack of comprehensive, accessible vector packaging systems (18) Multi-party to party specifications (17)
Transposon	Stable integration into the cell genome (19)	Low efficiency (19)
DNA plasmid	Low cost (20) Low immunogenicity (21) Low risk of placement oncogenesis (21)	Reduced activity (22) Reduced genome integration (22) Early depletion of T cells (21) Limited permanence and expansion of engineered cells (20)
Messenger RNA	Temporary expression of the transgene (1 week) (23)	No integration into the cell genome (23)

FDA-approved CAR T-Cell Therapies

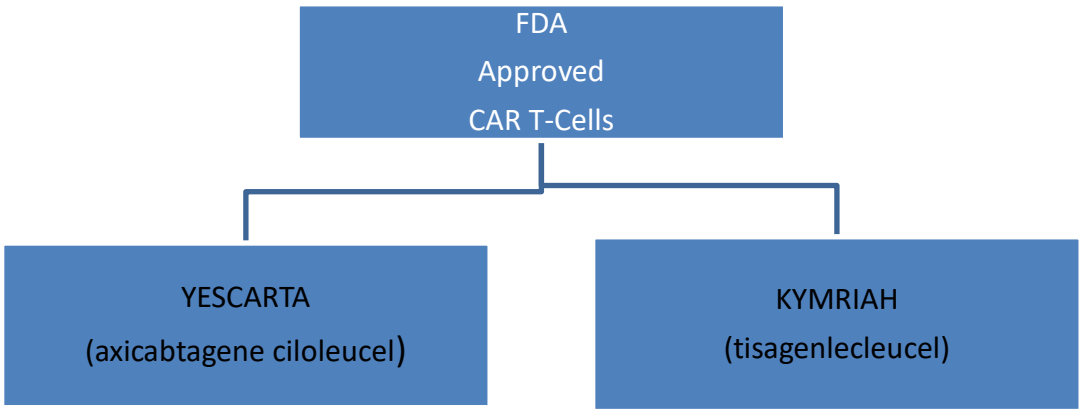

Figure 8. Car T-Cell products with FDA approval (46).

Immunotherapeutic CAR T-cell engineering showed successful results in hematologic cancers (32,46,47). Among the most successful clinical trials are the FDA-approved second-generation CD19-targeted CAR T products manufactured in Novartis and Kite Pharma. Axicabtagene ciloleucel (Yescarta ™ ovKite) was approved for leukemia (August 2017) and lymphoma (May 2018) and Tisagenlecleucel (Kymriah ™ –Novartis) lymphoma (October 2017) (48) (Fig.8).

Promising preliminary data were produced with Lisocabtagene Maraleucel JCAR017 (liso-cel, Celgene), a third generation CAR T-cell product used in current studies (7).

FDA approved Yescarta and Kymriah are second-generation CAR T products (49, 50).

Third-generation CAR T-cells have also been investigated and the desired success in clinical trials has not been achieved (51).

The 4th generation CAR T-cells, later known as TRUCKs, was developed but its initial pre-clinical success resulted in unexpected toxicities and could not reproduce in clinical trials (NCT01236573, NCT01457131). Toxicities led to the end of work (52- 57).

Which types of cancer are suitable for CAR T-cell ?

Car T-cell therapy frequently uses in hematological malignancies such as acute lymphoblastic leukemia, chronic lymphocytic leukemia, lymphoma and multiple myeloma (58) (Fig.9). CD19-targeted CAR T-cells have achieved the most successful results with CAR T-cell technology to date. Other targets such as CD20, CD30, and CD138 have also achieved some success with CAR T-cell therapy (59,60,61). CAR T-cells have not yet been successful in the clinical applications of solid tumors. In solid tumors, the majority of treatment results in CAR T-cells attacking and inadequate for the microenvironment of the tumor (62 -65). In this case, the applicability of CAR T-cell technology in the treatment of solid tumors has been the subject of debate (38, 66).

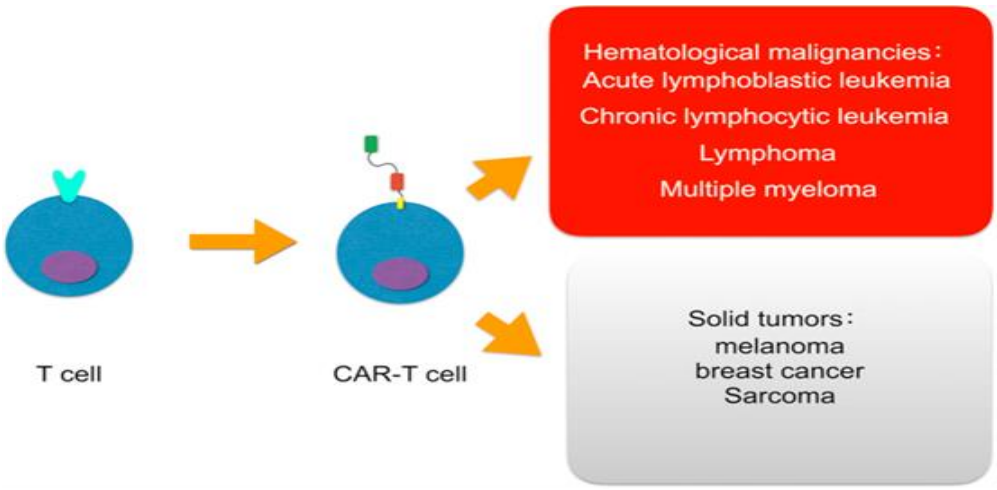

Figure 9. CAR T-cell technology is shown to be applied to what type of hematological tumors and solid tumor (38).

CAR T-Cell Application in Solid Tumors with Metalloproteinases

CAR T-cell technology, which has succeeded in hematological malignancies, has some obstacles in solid tumors. The reason for this is thought to be related to the particular

properties of the solid tumor microenvironment. For example, the lack of particular antigens may limit the formation of CAR T-cells and the low-efficacy CAR T-cell may also cause severe off-target effects. On the other hand, the immunosuppressive microenvironment of solid tumors disrupts the function of CAR T-cells (67). Collagen fibers produced by cancerous cells and cancer-related fibroblasts prevent immune cells from settling into tumors. This facilitates the spread of the tumor. Failure of T cells to pass through collagen barriers has also been demonstrated in breast, lung, and pancreatic carcinomas (68- 70). Matrix metalloproteinases (MMPs) are endopeptidase proteases capable of proteolysis of the majority of ECM components (71). MMPs play a significant role in the development of organs, inflammatory disorders and tissue remodeling as well as in the pathogenic role in cancer (72, 73). MMP2, MMP3 and MMP9 were confirmed to be involved in metastatic niche formation (74-76). MMP8 is a protease with pro-metastatic activity, also known as collagenase-2. In studies on melanoma and breast adenocarcinoma, MMP8 expression, anti-cancer and anti-metastasis activities have been reported to be associated with good prognosis and survival (77). Recent studies have found that although the anti-PD-1 blocking antibody in the microenvironment of some solid tumors has overcome the barriers of CAR T-cell therapy and the desired success has not yet been achieved (78, 79). In a written study, hypothetically, MMP8 allows CAR T-cells to overcome collagen barriers from solid ECM and solid tumors. If CAR T-cells overexpress MMP8, the solid tumor may increase the effectiveness of CAR T-cell technology and successful results can be achieved in solid tumors (Fig.10).

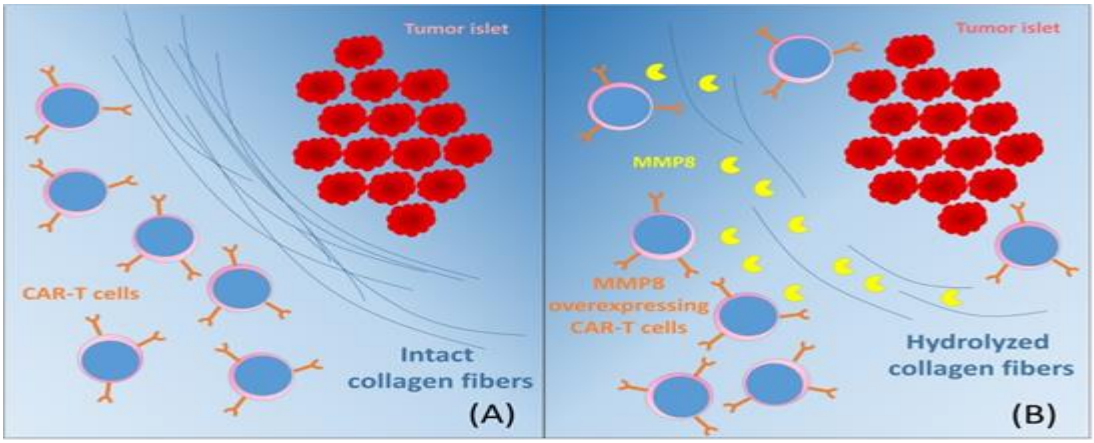

Figure 10. As a comment, MMP8 can destroy collagen fibers covering the tumor and may increase collection of CAR T-cells (80).

Natural Killer Cells and CAR T-Cell

Side effects such as cytokine release syndrome (CRS), severe neurotoxicity, non-target tumor toxicities and graft versus host disease (GVHD), which are disadvantages of CAR T-cell technology, have fatal consequences. New strategies should be developed to ameliorate CAR T-cell technology for solid tumors and to eliminate fatal side effects. Therefore, natural killer T (NKT) cells transfected with CAR attract attention (81). CSPG4-specific CAR-NKT cells using RNA electroporation generated for possible use in cancer immunotherapy. In a phase 1 study with patients with melanoma, it was shown that NKT cells could be used. Natural killer T (NKT) cells carrying a combination of natural killer (NK) cells and T cells

are known to migrate to non-lymphoid tissues. This suggests a beneficial effect for use in solid tumors. Thus, equipping NKT cells with CARs may represent a safer and equally valid approach for use in solid tumors than conventional CAR-T cell therapy (82). CAR-NKT cells can simultaneously attack two different pathways by recognizing the tumor-specific surface antigen. It performs its attack specifically through its CARs or through its internal anti-tumor activities via endogenous TCRs. Therefore, CAR-NKT cells can still be activated by endogenous TCRs when tumor cells try to escape without immune recognition, and their anti-tumor activity can be increased. Compared with conventional CAR-T cells, cytokine secretion of CAR-NKT cells was generally lower. CAR-NKT cells have been shown to produce antigen cytokines upon stimulation with melanoma cells specifically. CAR-NKT cells are specific cytotoxicity to that of conventional CAR T-cells. Therefore, CAR-NKT cells may be an alternative to conventional CAR T-cells for cancer immunotherapy (81,82).

New Modified Chimeric Antigen Receptor (CAR) Designs

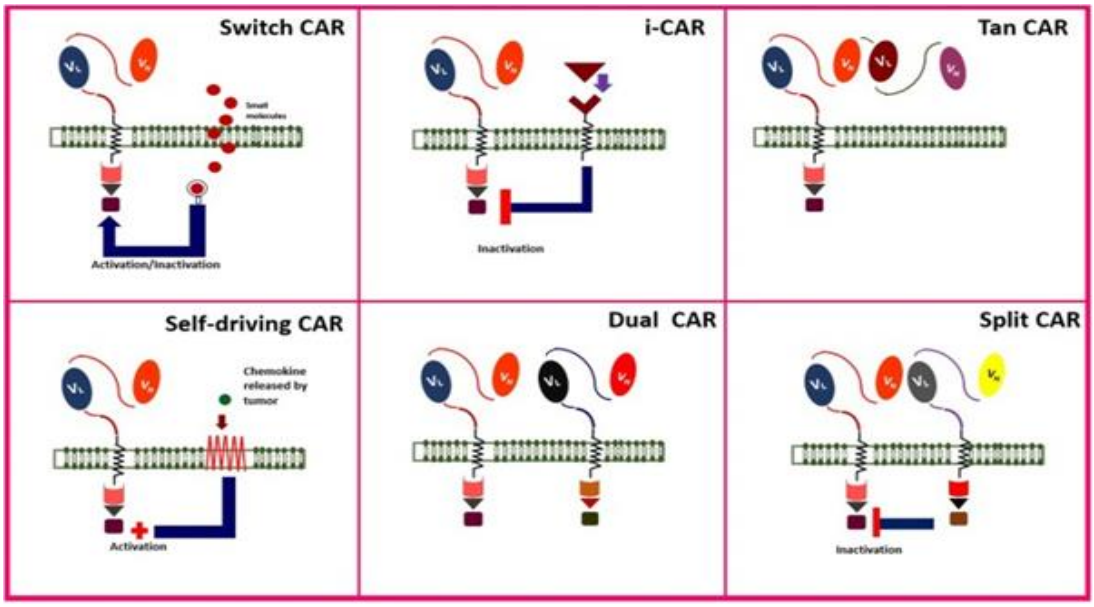

Figure 11. Especially in solid tumors, creating smarter CAR T-cells to improve therapeutic efficacy and reduce adverse effects (28).

CAR T-cell therapy is an adaptive cell therapy and consists of a mixture of immune, gene, and cell therapy (83, 84). More intelligent and advanced CAR T-cells technology improve the potential, quality, safety, efficacy, and anti-tumor function of cells. The application of CAR T-cells for anti-tumor benefits, particularly in solid tumors, is accompanied by some adverse effects. Several CAR T-cells have been designed to prevent this. There are models of these cells designed in figure 11.

Toxicity of CAR T-Cell Therapy

CAR T-cells can produce cytokines, kill targeted tumor cells, and even cause T cells to proliferate, causing an excessive response. A rapid immune response can be a double-edged weapon. Although the future is a brilliant treatment, in clinical trials, toxicities have emerged intensively due to the activity of T cells equipped with CAR. According to published reports,

fatal complications observed in some patients treated. (see Data Clusters EV3 and EV4 for published adverse events) (85, 86). Especially in 2016, many deaths reported. These are deaths caused by neurotoxicity caused by cerebral edema in the CD19 targeted CAR studies funded by Juno Therapeutics. Following the first reported deaths, the trial was interrupted. After some changes, it resumed, but two more news was published (87).

Cytokine swing syndrome (CRS)

To date, the most common side effect after infusion of CAR T-cells is the onset of immune activation, which is known as CRS, resulting in high inflammatory cytokines (88, 89). In most patients, CRS occurs 1-14 days after CAR-T cell infusion. CRS management requires appropriate grading to define ratings and criteria. According to the damage it creates, it is called as mild CRS (fever), moderate CRS and severe CRS (sCRS; 3rd-degree organ toxicity, potentially life-threatening) (46, 88).

Currently, there is three CRS classification scale. The National Cancer Institute created the first scale used to define CRS, but this system is not specific for cellular therapeutic approaches. Later, Lee et al. proposed a specific scale for a new rating system. Currently, it is the most widely used scale (88, 90). Fever is often the first and most necessary sign of CRS. Following CAR T-cell infusion, fatigue, fatigue, myalgia, nausea, anorexia, hypotension, capillary leakage, cardiac dysfunction, renal failure, liver failure, spreading intravascular coagulation and cytokine elevations such as interferon-gamma, granulocyte macrophage colony stimulating factor, IL-10 and IL-6, have been reported most frequently symptoms in trials with CD19-CARs. (88 – 93). Systemic corticosteroid has been shown to rapidly reverse sCRS symptoms without compromising its antitumor response (91,92). The drug Tocilizumab was successful in almost reversing CRS as a blockade of IL-6 receptor (IL-6R) with approval from the Food and Drug Administration (FDA) (46, 47).

Neurotoxicity

Another severe toxicity observed in patients receiving CD19-specific CAR T-cells. Neurological toxicities such as confusion, delirium, expressive aphasia, occlusion, myoclonus and seizures have been reported (47, 92). The causative pathophysiology of these neurological side effects is unknown. Although the cause of toxicity is not known, it is thought to be caused by the attack of CAR T-cells or general cytokine-induced inflammation (94).

Although not a clear expression of CD19 in the affected brain regions, some groups have documented that CAR T-cells have infiltrated cerebrospinal fluid (CSF) in most patients with neurotoxicity (47, 91, 92). To date, neurological toxicity has been reversible in the majority of fatal cases. Nevertheless, the CD19 17 CAR T study included patients who died in neurotoxicity (NCT01865617). It is not clear whether neurological toxicities are limited or not only in CD19-specific CAR T-cells (91, 92).

Target/non-tumor recognition

It is the toxicity of genetically targeted T cells directly attacking normal tissues containing the common expression of the targeted antigen. Given the effect of directed T cells, toxicity on non-pathogenic tissues expressing low antigen levels can be quite harmful. In a recent study at Erasmus University, cholestasis formation has been described in renal cell carcinoma patients vaccinated with CAR-modified T-cells that are physiologically expressed

on bile duct epithelial cells, specific for carbonic anhydrase IX (95). The most severe target toxicity reported in a study targeting ErbB2 in patients with lung carcinoma. One patient died of rapid respiratory failure and multiple organ dysfunction, as ErbB2 was recognized in normal lung cells (96). Because of the toxicities of this kind, the selection of specific antigen specific to the tumor is the most critical determinant.

Non-Target Toxicity

Most genetically modified T cells contain antigen recognition sites derived from monoclonal antibodies (mAb). This is the toxicity that occurs when cells unexpectedly attack another antigen than intended. The safety of some mAb profiles is unclear. In vitro data suggest that artificial synthetic structures may carry risks of recognition outside the target (97). For example, as seen in a patient treated with mesothelin-specific CAR T-cells, the presence of infusible external components resulting in acute anaphylaxis was recognized as a host. In one of four patients treated with multiple infusions of mesothelin-specific CAR T-cells, cardiorespiratory insufficiency reported at the end of the third infusion. Therefore, this possibility should be kept in mind for future developments when CAR T-cells target the new tumor-associated antigen (98).

Other Toxicities

In addition to the toxicities mentioned above, there are many other types of toxicity. For example, immunosuppressive delivery to recipients before T cell infusion is associated with much more antitumor activity. Unfortunately this situation; lymphoma and non-myeloablative regimen, anemia, coagulopathy and neutropenic sepsis with well-known toxicities. The mortality of this toxicity is approximately 1% and is the main fatal risk of T-cell therapy in the experience of the National Cancer Institute Surgical Branch (99). The integration of the viral vectors used to facilitate stable expression in primary T cells in the presence of genotoxicity may pose a potential risk of oncogenic placement mutagenesis, including deterioration of normal gene expression as observed in uncontrolled SCID-X1 therapy.

In most cases, the addition of a retroviral vector to LMO-2 oncogene is involved (100). The insertion of a transgene into differentiated T cells also carries the risk of induced malignant transformation. Although there are no reports of toxicity, such as vector-borne causes of death, clonal expansion, or enrichment for integration sites, it well is known that this is an issue to be considered. In light of the different toxicity spectra associated with the application of T cells, it is reasonable to find a balance between tumor elimination and unexpected toxicities. In order to establish this balance, more studies are needed on the suicide gene, targeted activation and other innovative gene therapy strategies. Some scientists believe that lowering the T cell dose to prevent toxicity and using second generation CARs instead of third generation CARs will prevent such toxicity.

Recent Results and Future Perspectives

Car T-cell technology is a rapidly growing innovative cure for cancer and can survive for a long time on the body. The success of CAR T-cell technology in cancer such as leukemia and lymphoma is painting a promising picture. In solid tumors, besides some difficulties have been encountered that prevents the optimized function. There is a need to increase the efficiency of CAR T-cells, especially against solid tumor cells. Changes in the construction

of CARs affect the level of expression, recognition power, specificity, and even T cell reaction rate. It is, therefore, necessary to systematically compare functionally optimized CARs. More attention should be paid to CRS what is the most critical problem of CAR T-cell therapy and other side effects. Toxicity management should become the focus of practice. It hopes that CAR T-cells will be able to predict and manage improved quality and toxicity in all types of cancer treatment. It will also enable each patient to reach treatment from a socio-economic point of view by commercializing the immunotherapeutic technology at an acceptable cost. In conclusion, more studies are needed to optimize the treatment by considering the positive and negative aspects of all probabilities during the anti-cancer immunotherapeutic treatment of CAR T cells.

Ethics Committee Approval: NA
Informed Consent: NA
Peer-review: Externally peer-reviewed.
Conflict of Interest: No conflict of interest was declared by the author.
Financial Disclosure: The author declared that this study has received no financial support.

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