CAR T cell therapy: A new era for cancer treatment (Review)

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Abstract. Cancer has recently been identified as the leading cause of mortality worldwide. Several conventional treatments and cytotoxic immunotherapies have been developed and made available to the market. Considering the complex behavior of tumors and the involvement of numerous genetic and cellular factors involved in tumorigenesis and metastasis, there is a need to develop a promising immunotherapy that targets tumors at both the cellular and genetic levels. Chimeric antigen receptor (CAR) T cell therapy has emerged as a novel therapeutic T cell engineering practice, in which T cells derived from patient blood are engineered *in vitro* to express artificial receptors targeted to a specific tumor antigen. These directly identify the tumor antigen without the involvement of the major histocompatibility complex. The use of this therapy in the last few years has been successful, with a reduction in remission rates of up to 80% for hematologic cancer, particularly for acute lymphoblastic leukemia (ALL) and non-Hodgkin lymphomas, such as large B cell lymphoma. Recently, anti-CD19 CAR therapy, or UCART19, has been shown to be efficacious in treating relapsed/refractory hematologic cancer. Several other cell surface tumor antigens, such as CD20 and CD22, found in the majority of leukemias and lymphomas are considered potential targets by pharmaceutical companies and research organizations, and trials have been ongoing in this direction. Although this therapeutic regimen is currently confined to treating hematologic cancer, the increasing involvement of several auxiliary techniques, such as bispecific CAR, Tan-CAR, inhibitory-CAR, combined antigens, the clustered regularly interspaced short palindromic repeats gene-editing tool and nanoparticle delivery, may substantially improve its overall anticancer effects. CAR therapy has the potential to offer a rapid and safer treatment regime to treat non-solid and solid tumors. The present review presents an insight into the advantages and the advances of CAR immunotherapy and presents the emerging discrepancy of CAR therapy over usual forms of therapy, such as chemotherapy and radiotherapy.

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1. Introduction

Chimeric antigen receptors (CARs) are unique receptors that are designed to target a specific tumor antigen to functionally reprogram T lymphocytes. As T lymphocytes are genetically engineered to express these artificial receptors to target cancer cells, the type of therapy may be termed immunotherapy, gene therapy or cancer therapy (1). The human defense system can efficiently identify self and non-self molecules, including bacteria, viruses and abnormal cancer cells. The identification of tumor cells is based on their acquired antigenicity and immunogenicity via the expression of foreign antigens (2). However, cancer cells have the potential to subvert the immune system to their advantage, resulting in inadequate antitumor immunity,

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Abbreviations: ALL, acute lymphocytic leukemia, CLL, chronic lymphocytic leukemia; CAR, chimeric antigen receptor; TCR, T cell receptor; mAb, monoclonal antibody; NK, natural killer; FDA, The United States Food and Drug Administration; scFv, single-chain variable fragment, CRISPR, clustered regularly interspaced short palindromic repeats; PAM, protospacer adjacent motif; Treg, regulatory T cell; PD-1, programmed death 1; PD-L1, programmed death-ligand 1; CTLA-4, cytotoxic T lymphocyte-associated protein 4; *ICOS*, inducible T-cell costimulator; CR rate, complete response rate to the treatment or disappearance of cancer

Key words: chimeric antigen receptor T cell therapy, NK-CAR therapy, CRISPR-CAR, bispecific CAR, tandem CAR, inhibitory CAR

and tumor survival and progression (3). Immunotherapy is also termed biotherapy as the immune system in the body is naturally capable of detecting pathogens and cancerous cells. In recent years, immunotherapy has emerged as an important branch of treatment for similar types of disease; however, its protective mechanism may differ (4). Certain immunotherapies boost the immune system, whereas others directly target the cancer cells. Each treatment type has its advantages and disadvantages depending on the disease type (5). Tisagenlecleucel (Kymriah) is a medication used to treat patients with acute lymphoblastic leukemia (ALL) up to the age of 25 years. Similarly, axicabtagene ciloleucel (Yescarta) is approved for patients with large B-cell lymphoma, such as non-Hodgkin lymphoma (NHL), and those with cancer in a refractory and recurrent state or whose cancer is non-responsive to other treatments (6). With increasing awareness of the immune system, a number of innovative immunotherapies are being developed using methods which include inducing the immune system to function in an impertinent manner to target malignant cells. Another approach involves the administration of immune components, such as synthetic, modified immune proteins that are genetically engineered to target tumor antigens (7).

CAR T cell treatment has achieved success in treating hematopoietic malignancies; however, its effectiveness against solid tumors remains to be determined. In the following sections, the rapid progression in implementing the adoptive transfer of T cells and their mechanism of tumor cell eradication are discussed.

2. CAR T cell therapy

CAR is an emerging immunotherapy for several malignancies. This therapeutic approach is an experimental form of gene therapy that redirects T lymphocytes to eradicate cancerous cells. The initial step in this therapy is leukapheresis or the isolation of a patient's peripheral blood (8,9). Apheresis is widely used to isolate blood from patients and separate it into its components, which are then genetically altered before re-injecting them into the patient's body. Currently, apheresis is used by blood banks to collect platelets and other blood components for the treatment of several diseases, including hematologic and renal disorders. Therefore, it is regarded as a safe practice for healthy individuals and patients (10) (Fig. 1).

3. Architectural ideology of T cell engineering and CAR design

The uniqueness of chimeric receptors exists in their ability to fuse or split discrete vital functions, such as recognition, co-stimulation and activation, in different chains of a receptor molecule by imitating the complexity of the native T cell receptor (TCR) structure (11). T cells do not usually require costimulation for activation and to initiate proliferation, but in the process of establishing CAR T cells, the activation and proliferation of T cells require the presence of costimulatory molecules, which also assist in CAR T cell cytokine production. The strategy involves constructing an engineered chimeric receptor for T cells based on the integration of scFv fragments in the hinge area that separates scFv from the cell membrane. The exposure of scFv on the cell surface, in addition to other small functional molecules, enhances induction of the cytolytic function of the engineered T cell. Together, this coordination of a 'living drug' in the immune system fights against cancer (12). In addition, CAR T cells can remain stable for several years in the body as long-term memory cells. This feature allows them to recognize and kill cancer cells encountered in the circulation in the case of relapse. Another advantage of CAR T cells is that they specifically target only tumor cells and not auto-antigens. Therefore, it is safe and nonlethal to host cells (13). Once the synthetic immunoreceptor is expressed on the surface of an engineered T cell, its scFv specifically binds to target antigens expressed on a cancer cell. This binding subsequently results in the transduction of an activating signal into the genetically edited immune cell. The T cell then elicits its effector anticancer function (14). CAR T cell therapy is considered to be the first approach to reconfigure T lymphocytes with an antibody-specific scFv fragment obtained from monoclonal antibodies (mAbs) by replacing different parts of the TCRα and β chains. A recent report stated the potential of CAR $\gamma\delta$ T cells in curing mucosal-derived malignant tumors as a novel strategy for CAR T cell therapy (15). The hybrid TCR functionally expresses and recognizes the analogous target antigen molecules in a non-MHC-restricted manner. Depending on the diverse biomarker selection and structural complexity, different generations of CAR model have been developed (16).

First-generation CARs. The first-generation CAR T model comprises a CD3ζ chain as a key transmitter of signals from endogenous TCRs. Following a successful outcome in pre-clinical trials, this type of drug entered into phase I clinical trials for leukemia, lymphoma and various other types of cancer, including ovarian cancer and neuroblastoma (17). Despite the inadequate antitumor action owing to the lack of activation, persistent exposure to the tumor environment has resulted in continued therapeutic effects in patients with B-cell lymphoma infused with α-CD20-CD3ζ CAR T cells and a number of patients with neuroblastoma treated with scFv-CD3ζ CAR T cells (18). The heavy and light chains constitute structural parts of the B cell receptor or antibodies, called scFv, which are fused to the CD3 domain or T cell-activating ζ chain of the TCR to create non-MHC-restricted activating receptor molecules. These modified molecules are capable of enhancing T cell antigen detection and cytotoxicity by specifically targeting tumor cells (19). The original 'true' CAR was designed by integrating an scFv antibody receptor directly with the CD3ζ domain. This model was subsequently named the 'T body approach' and these synthetic signaling receptors are now known as CARs or chimeric immune receptors (20).

Second-generation CARs. The success of first-generation CARs in phase 1 clinical trials paved the way for second-generation CAR T cell therapy. This model of CAR T cells was established to elicit a more effective anti-leukemic response in phase I clinical trials with complete remission rates of up to 90% in patients with recurrent B-cell ALL (B-ALL). Here, the second-generation anti-CD19 T cells were integrated into a 4-1BB or CD28 co-stimulatory domain attached to the CD3 domain (21). However, significant concerns remain regarding

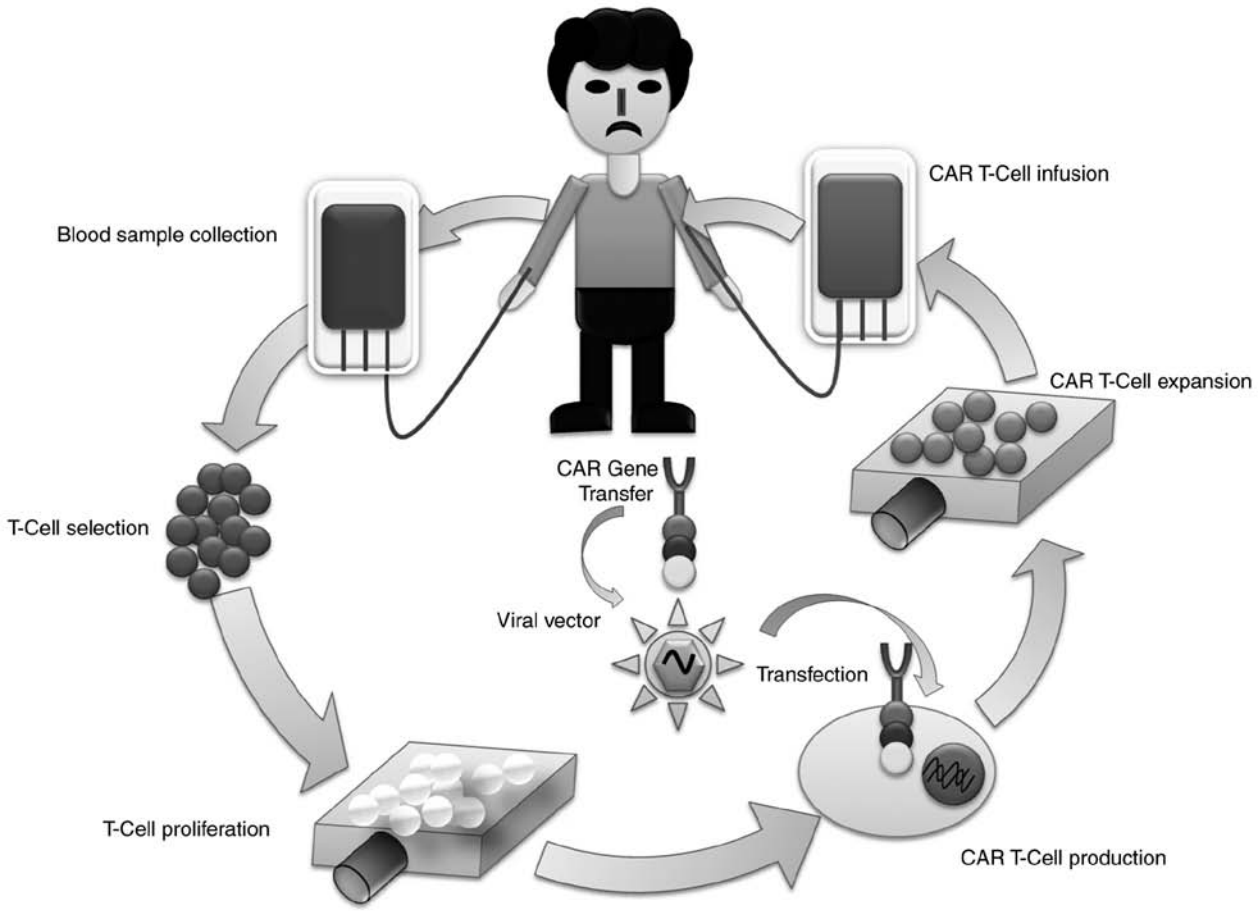

Figure 1. Adoptive CAR T cell therapy. CAR T cell therapy can be defined as a treatment in which a patient's T cells are genetically modified in the laboratory to kill cancer cells. The mechanism of adoptive CART-cell therapy includes the following steps: Collection of patient blood, isolation of T cells from the peripheral blood sample by the process of leukapheresis, transduction of cells by a vector encoding the CAR gene, expansion of CAR T cells *in vitro* and introduction into the patient to fight against cancer. CAR, chimeric antigen receptor.

its efficacy and safety and making it more robust. Anticipating these concerns, second-generation CARs aimed at integrating intracellular signaling domains from different co-stimulatory molecules, such as CD28, 4-1BB or CD137, inducible T cell costimulator (ICOS) or CD278, OX40 or CD134 fused to the cytoplasmic tail of the CAR, thus amplifying the signal (22). First-generation CARs contain a CD3ζ chain as a key transmitter of signals from endogenous TCRs, whereas second-generation CARs contain a CD3ζ chain and a single costimulatory molecule, which is why the receptor is known as a second-generation CAR. For example, anti-CD19 CARs consisting of 4-1BB or CD28 signaling domains produced notable complete response (CR) rates in patients with relapsed and refractory B-cell malignancies (23). CR represents complete response or disappearance of all signs of cancer in response to treatment [The United States Food and Drug Administration (FDA) 2007]. Consequently, CD28-based CARs have a rapid proliferative reaction, thus enhancing T effector cell functions, whereas 4-1BB-based CARs lead to improved T cell accumulation (24).

Third-generation CARs. To extend the antitumor efficacy, third-generation CARs comprise two signaling domains and the CD3ζ chain, such as CD3ζ-CD28-OX40 and CD3ζ-CD28-4-1BB, to achieve improved activation signal, prolonged proliferation, elevated cytokine production and effective function (25). For example, a third-generation CAR consisting of α-CD19-CD3ζ-CD28-4-1BB reported complete remission rates by infiltrating and lysing cancer tissue in patients with chronic lymphocyte leukemia (26). In addition, certain CAR T cells function as memory cells, thus preventing tumor relapse. Despite the significant curative effect, the irrepressible activity of CARs and their increased antitumor efficacy is associated with life-threatening and unfavorable outcomes, with increased secretion of pro-inflammatory cytokines, multi-organ dysfunction, pulmonary failure and death (27).

Fourth-generation CARs. All earlier CARs were based on a precise stratagem and helped in mediating the T cell anti-cancer response. However, these suffered from limitations, including a lack of antitumor activity against solid tumors owing to large phenotypic heterogeneity and deterioration attributed to antigen-negative cancer cells. These shortcomings led to the development of a novel CAR stratagem (28). The fourth-generation CAR was introduced to establish the tumor background via the inducible expression of transgenic immune modifiers, such as interleukin (IL)-12, which activate innate immune cells and enhance T cell activation to reduce antigen-negative cancer cells in the marked lesion (29,30) (Fig. 2).

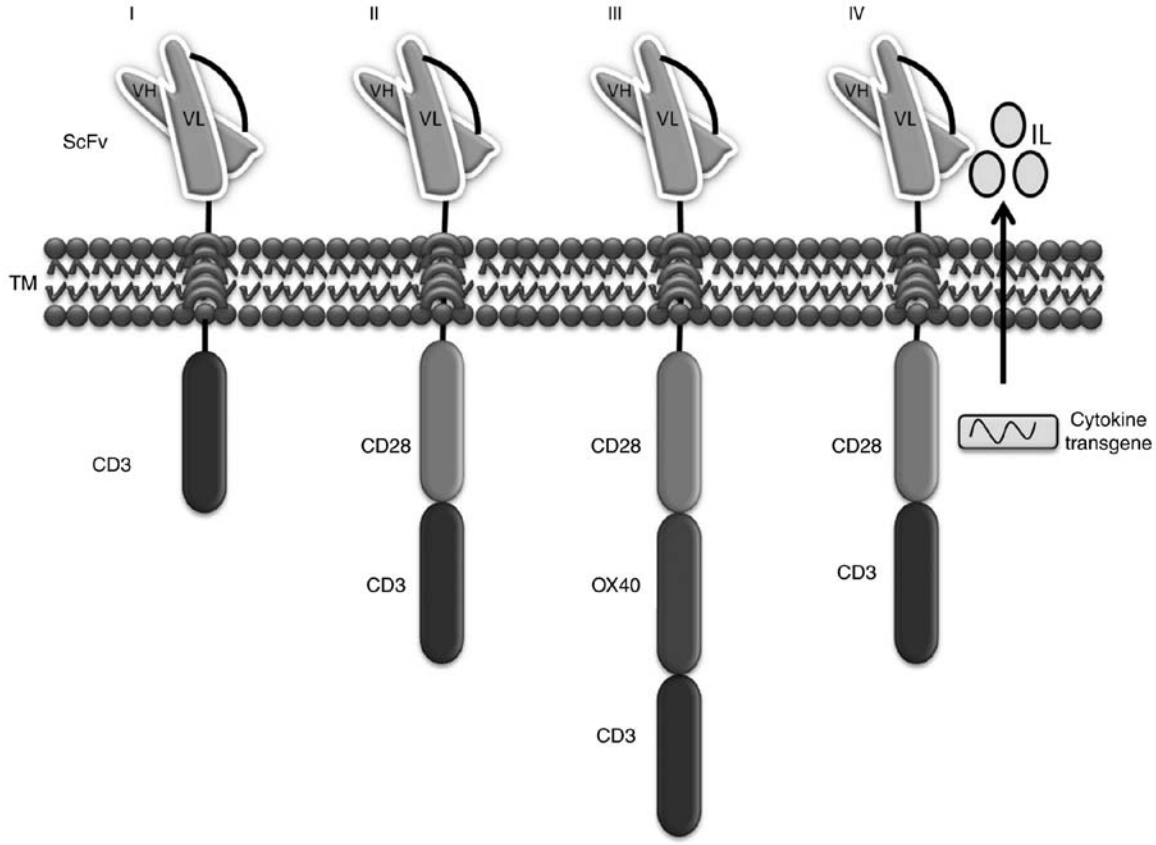

Figure 2. Architectural evolution of CAR T cell design. The CAR is constructed from the extracellular antigen-binding domain derived from monoclonal antibody (scFv) and intracellular signaling domains, linked by a hinge and a transmembrane domain. First-generation CARs contain the CD3ζ chain of the T cell receptor complex, whereas second-generation receptors contain one costimulatory molecule (CD28) and third-generation CARs contain two costimulatory molecules, respectively, such as CD28 and OX40. CARs are usually introduced into primary T cells using a vector. CD3ζ usually maintains the cytotoxic effector function of CAR-T cells. CD28 (blue) is important for T cell proliferation and cytokine secretion. 4-1BB (purple) is another co-stimulatory domain that promotes T cell survival and *in vivo* T cell persistence. Each CAR generation increases in complexity relating to the addition of costimulatory molecules or the induced promoter for cytokine IL-12 in fourth-generation CAR, CAR, chimeric antigen receptor; scFv, single-chain variable fragment; IL, interleukin; TM, transmembrane.

4. Mode of delivery

Gene therapy involves the delivery of DNA into cells and this can be accomplished using a number of methods summarized below. The most traditional method utilizes recombinant viruses (also known as viral vectors), biological nanoparticles and non-viral methods based on the direct delivery of naked DNA.

Viral vector. Viral vectors, such as γ-retrovirus, lentivirus and adenovirus vectors are generally used in gene therapy. Retrovirus transduction is one of the commonly used delivery methods in gene therapy. The method involves a reverse transcriptase that promotes the stable integration of artificial genes into the host genome (31). To create a vector, γ-retroviral coding sequences are substituted by a gene of interest. The retroviral vectors possess an innate capability to disturb the genomic section and results in neoplastic transformation. Thus, γ-retroviral vectors have been used in gene therapy applications (32). Lentiviral vectors are retroviruses derived from human immunodeficiency virus-1, which serve as a major tool for the delivery of transgenes into mammalian cells. The advantage of using lentivirus vectors is their efficient transduction and their stable integration and expression into non-dividing and dividing cells both *in vitro* and *in vivo* (33).

Non-viral delivery methods

Transposon transfection. The mechanism of transposon transfection is different from that of viral transfection. Delivery via transposons is a non-viral process that uses transposon DNA and a transposase enzyme for stable gene transfer. Two important vectors, namely piggyBac (PB) and sleeping beauty (SB), are frequently used (34). Transposons shift from one gene position to another position via a cut-and-paste mechanism. The mechanism of the transposon system using SB and PB involves four steps: i) The transposase enzyme helps in recognition and binding to the transposon; ii) a synaptic complex is produced by the coupling and binding of the repeat elements at both ends of the transposon; iii) the transposon excises the genetic element to be transposed, and iv) the excised element is reintegrated into the target location (35). The transposase for both vectors (SB and PB) consists of the DNA-binding domain, transposable catalytic domain and nuclear localization signal. The SB vector is a safer substitute for viral vectors owing to its innately low enhancer activity, non-pathogenic source and minimum epigenetic alterations at the incorporation site (36). By contrast, the PB vector has been shown to provide a large load capacity and

additional competent transposition action in *in vivo* studies. PB vector-mediated CAR T therapy is considered safer than that based on the SB vector. Therefore, use of the PB vector results in greater CAR production, as evident from the production of CAR targeting CD19 using a PB vector (37).

Electroporation. Electroporation has evolved as a useful technique for modifying genes of diverse cell types. The target cells are exposed to electric fields to temporarily disrupt their cell membranes. This allows the charged molecules to enter the cells. Square-wave and pulse-based systems are new electroporation devices (38). The Lonza Nucleofector II Electroporator performs effective genetic alteration of T cells using certain electric parameters and electroporation buffers (39). The electroporation of human T cells has been reported to be associated with ~40-60% of gene expression and 80% of cell viability (40). One of the limitations of electroporation is the low transfection efficiency and redundant cell damage (41).

Nanoparticles. The most critical step in CAR T cell therapy is T cell activation, for which cells need to be incubated with the viral vector including a CAR gene. However, stable insertional mutagenesis can be dangerous as its possible effects on humans remain to be fully elucidated (42). Retroviral or lentiviral vectors offer permanent gene integration into host cells, with the potential integration in close proximity with a proto-oncogene which can result in an oncogene. The various disadvantages associated with these transfer methods suggest that a safer alternative is needed (43).

The study by Smith *et al* elucidated the use of nanotechnology to resolve the tumor-targeting problem and make the therapy cost-effective. They reported that polymeric nanocarriers can efficiently deliver leukemia-specific CAR genes targeted to specific ligands on the host T cells *in situ* (44). A number of nanoparticles used for gene delivery have been investigated preclinically and reported. For example, magnetic nanoparticles, such as Fe_3O_4, integrate into the cell-penetrating peptide complexes. These oligonucleotides maintain stable cell transfection and plasmid transfection in addition to gene silencing and splice correction (45). A recently published study by Smith *et al* at the Fred Hutchinson Cancer Research Center, Seattle, confirmed that polymeric nanoparticles carrying DNA effectively introduced CAR genes into T cell nuclei and recognized leukemia cells distinctively (46). The use of nanoparticles to replace viral vectors for gene delivery has proved advantageous, as evident from the restriction of unspecific pro- or anti-inflammatory effects or pro- or antiproliferation effects (47).

5. Challenges of CAR T cell therapy

Although CAR therapy has emerged as a promising anticancer approach, it is not free from challenges that require optimization. For example, the enhanced persistence and improved cytotoxic profile of CAR T cell therapy are active areas of research requiring long-term follow-up in clinical trials (48). In addition, a number of serious side effects have been known to be frequently associated with CAR T cell therapy, including neurological toxicity, cytokine release syndrome (CRS), B cell aplasia, tumor lysis syndrome and anaphylaxis (49). The proliferation of CAR T cells produces cytokines in the body which kill cancer cells. The symptoms of CRS-associated toxicity range from mild symptoms of fatigue, nausea, headache, fever and chills to serious symptoms including a lowering of blood pressure, tachycardia and capillary leakage. Another side effect is the presence of CAR T cells targeting antigens on the surface of B cells or T cells that not only target cancer cells but also normal cells (50), resulting in B cell aplasia. Therefore, a thorough investigation is essential to measure the properties of B cell aplasia. Similarly, tumor lysis syndrome can result in toxicity by the collapse of dead cells generally in the beginning of cancer treatment. It could also cause organ damage and be life-threatening to the patient (51).

6. Advanced features of CAR T cell therapy

Although cancer cells have multiple lineages and heterogeneity, they possess common target antigens, such as CD19, CD20, CD22 and numerous others that allow CAR T cells to recognize tumor cells irrespective of cell lineage. Therefore, recent advances in this technique include more precise target antigens expressed by tumor cells (13,52). This review places additional emphasis on new studies of CAR T cell therapy that distinguish diverse CAR T cells which can enhance tumor cell death. Certain models are already being implemented and others are in the clinical investigation status, including NK-CAR and clustered regularly interspaced short palindromic repeats (CRISPR)-CAR therapy (Fig. 3A-E).

Bispecific CARs. A bispecific receptor is one that contains two distinct antigen recognition domains attached and placed with two distinct intracellular signaling domains that are expressed as two different CARs on a single cell surface. At present, bispecific CAR CD19/CD20 has been introduced as a novel synthetic molecule that can recognize and bind to more than one targeted tumor antigen on the cancer cell surface. Therefore, it can create a synergistic cascade of effector molecules when it encounters two tumor antigens (53). Additionally, the bispecific CAR conserves the cytolytic capacity of T cells, i.e., if one of the objective molecules is not accessible to CAR T cells due to a cellular hindrance such as mutation of a target antigen or loss of the target antigen commonly found in malignant cells, a bispecific CAR can counterbalance the tumor evasion (54). Investigated therapeutics include CD3 of T cells and tumor antigens, such as CD19, on malignant cells. The curative ability is evident from blinatumomab, an approved bispecific T cell-engager against relapsed/refractory B-ALL (55). Positive results have been reported in patients <15 years of age with relapsed/refractory ALL, with a heightened response in 26/36 (72%) patients in 9 months. Blinatumomab was discovered while studying a patient who was negative for minimal residual disease (MRD) and who was found to be MRD-positive following consolidated chemotherapy (56). Numerous other bispecific CARs have been investigated preclinically by several research organizations, including CD20/CD19 and CD20/CD3 (57,58). Targeting T cells with bispecific CARs was shown to eliminate transplanted pediatric ALL in a study, whereas T cells targeted by CD20 CAR did not control the disease (59).

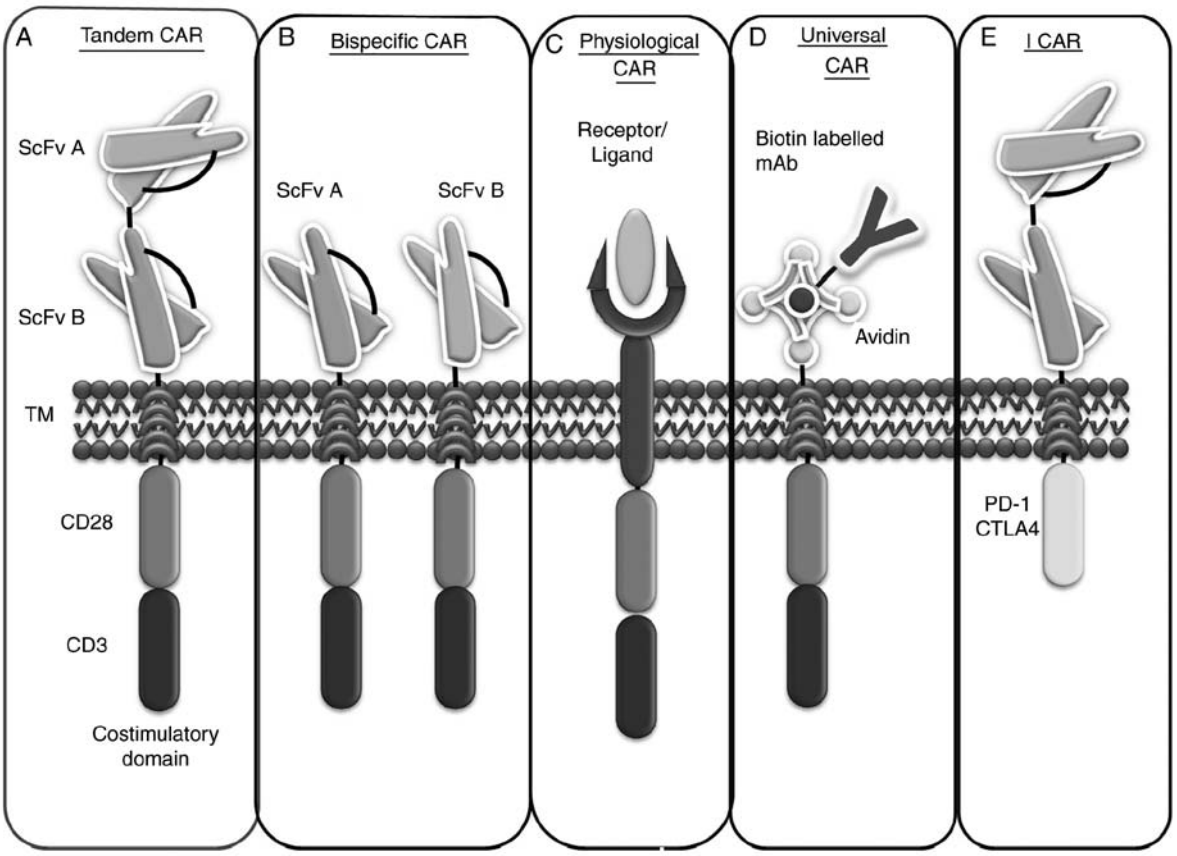

Figure 3. Advanced models of CAR T cells. (A) Tandem CAR: A single CAR structure targets two tumor antigens with a distinct antigen recognition domain (scFv) linked consecutively with a single intracellular domain. (B) Bispecific CAR: Two CARs are expressed simultaneously targeting T cells, with two distinct antigen-recognition domains targeting two tumor antigens. (C) Physiological CAR: CAR structure comprising a ligand and receptor molecule, rather than scFv, to recognize the paired molecule on the tumor cell. (D) Universal CAR: Extracellular domain expresses the avidin-biotin labeled mAb, instead of scFv, which can recognize almost all target antigens specific to mAb. (E) I-CAR: CTLA-4 and PD-1 are two inhibitor receptors that can reversibly regulate the signaling of TCR and can enhance the function of chimeric receptors. CTLA-4 or PD-1 are intracellular domains in I-CARs, which trigger inhibitory signals on T cells, such as a reduction of target cell lytic action and cytokine production. The most important factor of I-CAR-T is its ability to distinguish normal and abnormal antigens on target cancer cells. CAR, chimeric antigen receptor; scFv, single-chain variable fragment; mAb, monoclonal antibody; PD-1, programmed death 1; CTLA-4, cytotoxic T lymphocyte-associated protein 4; I-CAR, inhibitory CAR; TM, transmembrane.

Tandem CARs (Tan-CARs). Conventional CARs cannot meet higher expectations under certain circumstances, such as the downregulation or alteration of targeted antigens that can occur in cancerous cells. These conditions subsequently lead to antigenic loss or escape variations (60). To overcome this shortcoming, scientists are attempting to develop advanced technology, in which two particular antigen recognition sites are joined by a linker, placed in tandem on a single intracellular domain and expressed as a single CAR on a cell surface, termed a Tan-CAR. This model enables the synchronized targeting of both antigens on a single cancer cell or tumor microenvironment. In this manner, it enhances the activation and stimulation of T cells by increasing their avidity and expanding their therapeutic properties (61). Tan-CARs have several significant curative implications as they are as potent as standard disease models with single antigen-specific CARs. Additionally, they are more efficient and less noxious in a higher disease load setting (62). This may be attributed to optimized cytokine production and limited cell killing, as evident from preclinical studies on Tan-CAR T cells in a mouse tumor model, which confirmed its possibility for remedial implication in human disease consisting of B-cell antigen CD19 and human epidermal growth factor receptor 2 (HER2) (63). Recently, a trivalent CAR T cell was designed by a group of scientists by simultaneously co-targeting multiple antigens, such as HER2, IL-13 receptor α2 and ephrin A2, to overcome interpatient changeability with a propensity to target almost 100% of tumor cells (64).

Inhibitory CARs (I-CARs). It has been reported that certain novel immunoinhibitory receptors are involved in T cell activation and the attenuation or termination of T cell responses, such as programmed death-1 (PD-1), programmed death-ligand 1 (PD-L1) and cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4). These pathways are regarded as cancer immunotherapy breakthroughs (65). Recently, two immunologists won the Novel Prize in Physiology/Medicine, Dr James Allison and Dr Tasuku Honjo, for their profound discovery in the field of cancer immunotherapy. Dr Allison's work focused on the T cell surface protein CTLA-4; he found that the inhibition of immune cells and antibodies against CTL-4 eliminated malignant growth and prevented new tumor formation (66). This finding was tested on 14 patients with metastatic melanoma, with relapse observed in three patients. In 2011, the FDA approved an anti-CTLA-4 (ipilimumab) antibody as a treatment for high-grade melanoma (67).

Similarly, Dr Honjo's team investigated a novel T cell protein, PD-1, which was discovered in 1992 (68). They observed that PD-L1 presenting on healthy cells and malignant cells bind to PD-1. They reported another similar molecule, PD-L2, that also binds to PD-1. Based on these findings, they published a report that malignant cells produced PD-L1 and blocked PD-L1 using a counteracting antigen to inhibit tumor growth (69). The first clinical trials to target malignancy were launched in 2006, which indicated significant viability in a number of patients in 2012. The FDA approved the main PD-1 checkpoint inhibitors, nivolumab and pembrolizumab (70,71), for treating melanoma in 2014 (72). However, a single treatment may not be sufficient, which is the reason that a consolidated treatment targeting CTLA-4 and PD-1 is currently being investigated. Both Dr Allison and Dr Honjo encouraged the merging of various strategies to generate more advanced forms of the drug for the immune system to inhibit tumor cells more effectively (73).

Certain tumor cells contain a high level of PD-L1, which assists in evading immune attack. It has already been investigated that PD-1- or CTLA-4-based I-CARs can efficiently control the cytotoxicity, secretion of cytokines, and proliferation and cytotoxicity stimulated by endogenous TCR or an activating chimeric receptor (74). I-CARs are designed to control the actions of CAR T cells through inhibitory receptors. This advanced feature unites the action of two chimeric receptors; one of these generates a dominant-negative signal that restricts the responses of activated CAR T cells by the activating receptor. I-CARs can inhibit the activator CAR response to antigens expressed only by normal cells, thus differentiating between cancer and normal cells (75). Therefore, using genetic engineering to inhibit T cell inhibition physiology and regulate T cell response can be harnessed in an antigen-selective manner. This has been experimentally confirmed preclinically in a mouse model by designing an I-CAR using surface antigen recognition domains CTLA-4 and PD-1. In mice lacking CTLA-4 receptor, substantial T cell activation and proliferation were observed, ultimately leading to rigorous systemic autoimmune disease (76). Similarly, PD-1 is another the inhibitory receptor, which was found to be particularly expressed by activated T cells causing glomerulonephritis and arthritis in C57BL/6 mice and certain non-obese diabetic mice affected by insulitis (77).

Physiological CARs. Initially, several CAR constructions contained scFv of murine origin. This is associated with the risk of an immune response to the modified cells, and the resulting anaphylaxis of CAR T cell transfer cannot be avoided. These disadvantages limit the persistence of the infused cells. Therefore, besides conventional CAR, a physiological CAR has been developed, also known as a receptor-ligand CAR, which can recognize and bind to tumor antigens, such as HER3 and HER4 (78). The physiological CAR consists of an antigen receptor and a CD3ζ intracellular signaling domain with or without a transmembrane region, which can also be engineered into immune cells to target the ligands expressed on tumor cells. This approach increases the capability of T lymphocytes to distinguish tumor-related targets and eliminate cancer cells (79). This physiological CAR is an emerging field of CAR T cell therapy with limited published reports. Experimental trials may have been initiated but the outcomes have not yet been published.

Universal CARs (uCARs). Although scFv is specifically directed against tumor-associated antigens, the recognition specificity potential of CAR T cells is inadequate. Therefore, uCARs were developed to overcome this limitation. To construct a universal CAR, biotin or anti-fluorescein isothiocyanate (FITC) scFv is used as a targeting region, which is fused to a transmembrane domain with one or two endodomains (80). The uCAR-expressing T cells can efficiently recognize and remove cancer cells through the binding of FITC-labeled or biotinylated antigen-specific mAbs, which, in turn, activate the T cells and stimulate their proliferation and production of cytokines (81). The mSA2 CAR T cell is a uCAR, which can be fused to a biotinylated tumor-specific antibody to specifically target various types of tumor. As with interferon γ, it is well equipped for interceding malignant cell lysis and cytokine production (82). Human clinical trials involving uCAR T cells are ongoing. Two children with relapsed and refractory B-ALL achieved molecular and cytogenic remission following uCAR T therapy. Clinical trials have been initiated on uCAR T cell therapy specific to CD19-positive cells (NCT03166878 and NCT03229876). However, detailed information and conclusions are not yet available (83).

Natural killer (NK)-CARs. NK cells are a type of cytotoxic T cell that are essential for natural immunity. The function of NK cells is similar to that of cytotoxic T cells in the adaptive immune response of vertebrates. Immune cells generally identify MHC complexes expressed on infectious cell surfaces to elicit cytokine production, thus generating an immune response, culminating in the apoptosis or death of infectious cells (84). NK cells are the only immune cells that identify infected cells in the absence of MHC and antibodies, thus eliciting a rapid immune response. These are known as 'natural killers' as they do not require activation to destroy cells that are devoid of 'self' MHC class I molecule markers (85), making them important for destructive cells with missing MHC I markers.

Cancer cells that do not cause any inflammation are treated as self by the immune system and do not stimulate a T cell response. NK cells produce a number of cytokines, including tumor necrosis factor α, interferon γ and IL-10, which act as immune suppressors (86). The activation of NK cells leads to the gradual formation of cytolytic effectors cells, such as dendritic cells, macrophages and neutrophils, which consequently facilitate antigen-specific T and B cell responses. NK cell-mediated tumor cell lysis involves several receptors, including NKp44, NKp46, NKG2D, NKp30 and DNAM. Malignant cells usually express NKG2D in addition to ULBP and MICA (87,88). The clinical effectiveness of CAR T cells has been shown for ALL; however, this therapeutic approach has not been confirmed for acute myeloid leukemia (AML), suggesting the need for other therapeutic options (89). Hypothetically, automotive NK cells have an additional favorable toxic effect in comparison to CAR T cells, particularly for avoiding unfavorable effects such as CRS. Additionally, in contrast to T cells, donor NK cells do not target non-hematopoietic cells, indicating that NK cell-mediated antitumor activity may be activated in the absence of graft-vs.-host disease (90) (Fig. 4).

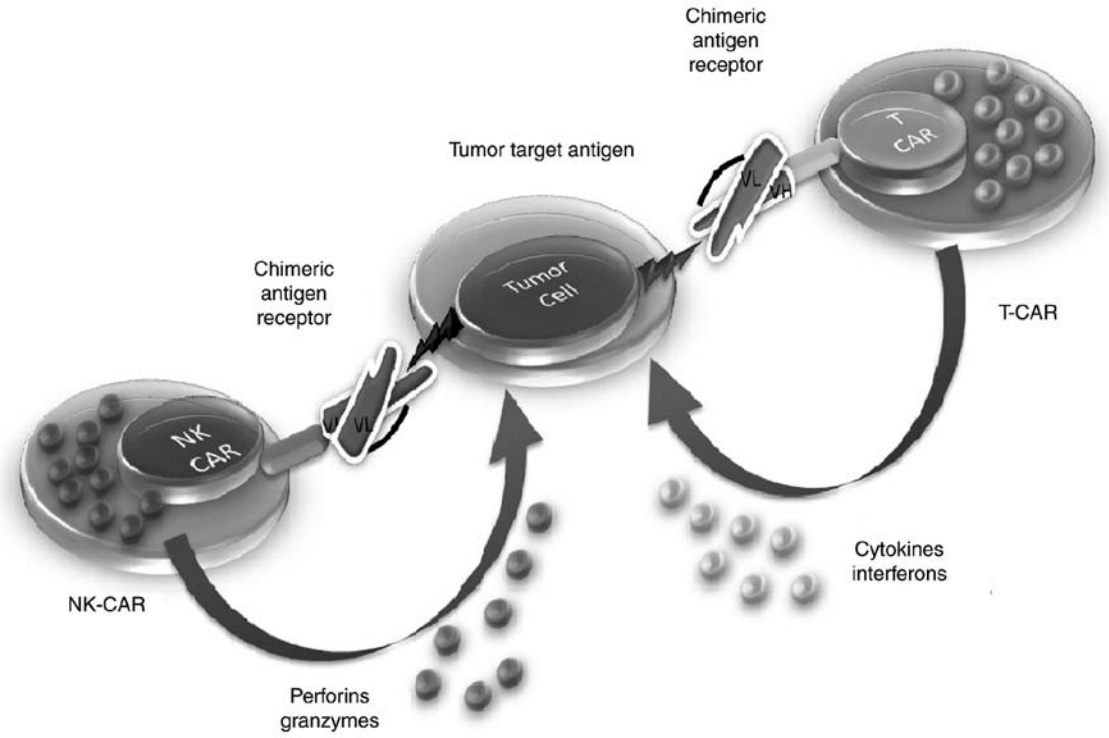

Figure 4. T cells and NK cells in the CAR platform. NK cells are fundamental components of the innate immune system and serve an essential role in host immunity against cancer. Currently, CAR-engineered NK cells are targeted to NKGD2, CD16 antigens; upon encounter with a tumor antigen on the cancer cell, the NK-CAR secretes perforins and granzymes, which directly mediate the cytotoxicity of the targeted tumor cell. CAR T cells secrete cytokines, such as interferons and TNF-a, which also mediate cell cytotoxicity and eventually cancer cell death. CAR, chimeric antigen receptor; NK, natural killer; NKGD2, natural killer group 2 member D.

CRISPR CARs. CRISPR is a gene modifying tool that uses a guide gRNA to modify a DNA sequence. As this technology is an integration-free gene incorporation system, it offers a foolproof and competent gene knock-in process. Following the significant progress associated with CRISPR technology, it has the potential to emerge as a promising immunotherapy (91). The CRISPR system can be directly applied to mammalian cells via transfection using a plasmid that contains both nuclease and sgRNA. Cas9 encodes a large molecule with a multifunctional DNA endonuclease and is known to excise dsDNA from 3-bp upstream of the protospacer adjacent motif (PAM) (92). Once the nuclease binds to its gRNA, the compound scans for an integral target DNA sequence (93). The PAM sequence has a significant role in recognizing self and non-self sequences. The local PAM sequence is usually used as a 'spy' on the nuclease sequence 5-NGG-3, in which N is any of the four deoxyribonucleic acid bases (94).

Recently published reports suggest that CRISPR technology can deliver the CAR gene to the TRAC locus of T cells. In this regard, CRISPR-edited universal-CAR T cell therapy has been used in humans (NCT03166878 and NCT03229876). This technology is rapidly developing with the potential for gene correction (82). It is reported that anti-CD19 CAR to the TCR α constant locus (TRAC locus) not only results in increased T cell potency but also in the consistent expression of CAR in peripheral blood T cells (95). CRISPR-modified cells have been shown to perform well in generally constructed CAR T cells in a mouse model of ALL (96). It has also been demonstrated that targeting the TRAC locus turns on CAR signaling, thereby initiating successful internalization and stimulation of the CAR for single and repeated exposure to a tumor antigen. It also delays effector T cell differentiation and exhaustion (97). Multiplex genome editing is another attractive application of the CRISPR/Cas9 tool. Proficient genomic disturbance of multiple gene loci to create a universal donor cell and a potent effector T lymphocyte targeted to different inhibitory pathways, such as PD-1 and CTLA4, is established by incorporating several gRNAs into a CAR vector. Furthermore, two-fold knockout of the TCR gene and HLA class I can effectively permit the generation of an allogeneic uCAR T cell, in addition to CAR T cells that are universally Fas-resistant via three-fold gene disruption (98).

7. Advantages of CAR therapy over other therapies

The most notable advantage of CAR T cell therapy over other cancer therapies is the abrupt time intervention and single infusion of CAR T cells. Additionally, 2-3 weeks of proper care and observation is sufficient for the patient. CAR T cell therapy is regarded as a 'drug of the present day' and its efficacy may persist for decades as the cells can survive in the host body in the long term, with a constant ability to find and destroy cancer cells during relapse (2,3). Currently, CAR T cell therapy is licensed for use in patients for whom transplantation has not been curative and who relapse following transplant. CAR T cell therapy is expected to be a substitute for different types of transplant (99). Clinical trials on blood cancer have shown that, even in patients with a refractory

Table I. List of chimeric antigen receptor therapy clinical trials.

Target antigen	Type of cancer	Clinical trial ID
CD19	BALL	NCT01044069
CD19	B-CLL	NCT00466531
CD19	Leukemia	NCT01416974
CD19	Lymphoma	NCT00586391
CD19	B-NHL/CLL	NCT00608270
CD20	Mantel cell leukemia/B-NHL	NCT00621452
CD22	Non-Hodgkins Lymphoma	NCT02315612
CD19/CD20	B-Non Hodgkins Lymphoma	NCT00621452
CD19/CD22	B-cell malignancy	NCT03185494
CD133	Hepatocellular carcinoma	NCT02541370
CD171	Neuroblastoma	NCT02311621
PMSA	Prostate cancer	NCT001140373
CEA	Breast cancer	NCT00673829
CEA	Colorectal cancer	NCT00673827
CEA	Lungs cancer	NCT00673322
HER-2	Lungs cancer	NCT00889954
HER-2	Osteosarcoma	NCT00902044
HER-2	Glioblastoma	NCT01109095
CD30	Lymphoma	NCT02274584
FAP	Malignant pleural mesothelioma	NCT01722149
NKGD2	Leukemia	NCT02203825
GD2	Neuroblastoma, osteosarcoma	NCT03356795
EGFRVIII	Glioblastoma	NCT02309373
Mesothelin	Pancreatic cancer	NCT02706782
CD38/CD123	B-Cell Malignancies	NCT03125577
ROR1	Chronic lymphocytic leukemia	NCT02194374
MUC16	Ovarian carcinoma	NCT02498912
GPC3	Lung squamous cell carcinoma	NCT03198546
VEGFRII	Renal cancer	NCT01218867

PMSA, prostate-specific membrane antigen; ROR1, receptor tyrosine kinase-like orphan receptor 1; NKGD2, natural killer group 2 member D, expressed in natural killer cells; GD2, disganglioside molecule expressed on tumors of neuroectodermal origin; EGFRVIII, epidermal growth factor receptor variant III; Mesothelin, differentiation antigen expressed on mesothelial cells and overexpressed in numerous human tumors; MUC16, also known as CA125, is a biomarker for ovarian cancer; GPC3, glypican 3, is a cell surface protein overexpressed in numerous solid tumors; VEGFRII, vascular endothelial growth factor receptor II.

condition in which cancer reverted following several transplants, CAR T cell therapy was successful in completely eradicating the disease (100). Additionally, with CAR T cells, patients can live life without the risk of relapse and benefit from a sanatory treatment, such as stem cell transplantation. Therefore, CAR T cell therapy can be referred to as a 'living drug' (101).

8. CAR T cell therapy trials for solid tumors

Several clinical trials are currently examining the use of CAR T cell therapy against solid tumors and other diseases. Reports suggest that mesothelin-specific CAR mRNA-engineered T cells can induce antitumor activity in solid malignancies (102,103). Furthermore, CAR technology has been used in organ transplantation using two novel HLA-A2-specific CARs, one representing a CD28-CD3d signaling domain (CAR) and the other missing an intracellular signaling domain (dCAR). The adoptive transfer of allospecific regulatory T cells (Tregs) provides a better safeguard from graft rejection compared with that of polyclonal Tregs (104). CAR comprising the ICOS signaling domain liaises with the effective anti-tumor effect on epidermal growth factor receptor variant III (EGFRvIII)-expressing glioma (105). The preclinical evaluation of CAR T cell therapy targeting the tumor antigen 5T4 in ovarian cancer has been associated with a successful outcome (106). An unexpected evolutionary finding was reported during the investigation of CAR targeting autoimmune diseases; therapy lacking autoimmune diseases that purposely target only the disease-causing cells, chimeric autoantibody receptor (CAAR), contain pemphigus vulgaris autoantigen, desmoglein (Dsg3), combined to CD137-CD3d signaling

domains. Dsg3 CAAR-T cells exhibit accurate toxicity against anti-Dsg3-expressing cells (107). Certain solid tumor CAR targets are undergoing research with varied genetic products arising from gene mutations (EGFRvIII) (108) or modified glycosylation patterns (MUC1) (109), and cancer-testis antigen-derived peptides (MAGE), CAR specifically targets certain overexpressed antigens in breast cancer, lung cancer and pancreatic cancer, such as carcinoembryonic antigen (110) GD2, prostate-specific membrane antigen, HER2/ERBB2, MUC16 (111) and mesothelin or tumor-affiliated stoma (fibroblast activation protein and vascular endothelial growth factor receptor) (112).

9. Success rates of approved therapies

As per the 2018 records on the total clinical trials conducted in the immuno-oncology domain, 220 trials involving CAR T therapy were performed to identify specific targets. Successfully developed CAR T cell drugs that are available to the market include CTL019 (Kymriah) (113), KTE-C19 (Yescarta) (114) and JCAR015 (115). These have been developed by companies known to be antecedents of CAR T cell therapy development, such as Novartis in association with the University of Pennsylvania, Kite Pharma with National Cancer Institute, and Juno Therapeutics with Sloan Kettering, respectively, and are used to treat ALL, NHL and ALL. These CAR T therapies represent a defining moment in 2017 in the field of oncology. The first two therapies specific to CD19 and approved by the FDA included Kymriah (tisagenlecleucel-T) and Yescarta (axicabtageneciloleucel) by Novartis and Kite Pharma/Gilead Sciences, respectively (116).

The global ELIANA trial reported a high success rate, with a 3-month complete remission rate of 83% with tisagenlecleucel, and a 6-month survival rate of 89% (117). Another trial, ZUMA-1, reported an equivalent results with an 82% overall response rate and 54% of complete remission rate following a single infusion of the therapeutic regime in 8 months. This indicates that the remission rate was more pronounced in pediatric B-ALL than that in adult relapsed/refractory DLBCL; however, reactivity was high in both diseases (118).

Furthermore, multiple clinical investigations have been implemented by Cellectis following UCART19. Cellectis is currently leading with two successful FDA Investigational New Drug (IND)-approved allogeneic CAR T approaches (119): UCART123 for patients with blastic plasmacytoid dendritic cell neoplasm and AML (120) and UCART22 IND for patients with B-ALL (121). Two other ongoing clinical trials are UCARTCS1 for suppressing CS1-expressing hematologic malignancies (122) and UCART38 for CD38-expressing hematologic malignancies. UCART38 is specifically developed to target T cell ALL, multiple myeloma, mantle cell lymphoma and NHL (123,124). A list of CAR therapy clinical trials are listed in Table I.

10. Future prospects

Although immunotherapy has achieved clinical success in treating blood cancer, the same success rates have not been observed for solid tumors. The various challenges associated with its safety, cost-effectiveness and quality require thorough investigation in order to implement this therapy for all cancer types. Another approach is to integrate CAR T cells with different types of immunotherapy to enhance its effectiveness. For example, CARs may be combined with certain checkpoint inhibitors, which limit tumor defense mechanisms against T cells. It is expected that future CAR T cell therapy regimens will target several diverse molecules for a particular type of tumor, such that CAR T cells can efficiently recognize cancerous cells even if these undergo mutations in their target molecules.

A number of CAR T therapies are already available to the market, but these are expensive, for example, $475,000 (€400,000) for Yescarta and $373,000 (€316,000) for Kymriah. According to experts, when hospitalization expenses and the costs of other drugs required for the treatment are also considered, the cost increases to almost $1,500,000 per cancer patient. Therefore, a possible solution is to reduce the cost of allogeneic CAR T treatment by supplying T lymphocytes from a healthy individual that can be readily utilized when a patient requires it, rather than genetically modifying each patient's T cells individually. Considerable scientific challenges also exist with regard to immunology, in addition to manufacturing, transportation and banking solutions to enhance the extensive treatment of patients. Therefore, scientists are investigating measures to overcome clinical challenges in terms of regulations. CAR T cells are available in several scientific frameworks, which may vary widely in different countries. These combined challenges and technology require standardization; however, CAR T cells offer patients hope of advanced treatment. As the first therapy is already available in the market, there is potential for a specific and improved alternative becoming available in upcoming decades.

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Authors' contributions

RM and NG conceptualized and co-wrote the manuscript. CRC, SA, PS and PG contributed to the literature review, organization and writing of various sections of the manuscript. NG is the PI and grant holder.

Ethical approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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