J Cell Immunol. 2024;6(1):51-63.

Journal of Cellular Immunology Review Article

Mechanism, Challenges, and Progresses of Chimeric Antigen Receptors T-cell Cancer Therapy

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Received date: February 14, 2024, **Accepted date:** February 27, 2024

Citation: Hussein A, Mammo B. Mechanism, Challenges, and Progresses of Chimeric Antigen Receptors T-cell Cancer Therapy. J Cell Immunol. 2024;6(1):51-63.

Abstract

Cancer is a deadly disease and affects everyone at any level of age. Many people lose hope once they are diagnosed with cancer. This is because there is no effective treatment for it. Till the end of the 19th century, researchers across the world were eager to design effective remedies for this decapitating phenomenon and designed so many interventions even though nothing is compensatory for a malignant tumor; in some cases, the side effects overwhelm the benefits. These interventions were surgery, irradiation, and chemotherapy like immune checkpoint inhibitors. The research in the early 20th century has come up with a very promising remedy for cancer at clinical trials and even some of which have been approved by various drug quality control agencies such as the Food and Drug Administration (FDA). This is the Chimeric Antigen Receptor T-cell (CAR T-cell) Therapy. For this, T-cells are collected from the patient and genetically engineered, with the use of a viral vector, to express a CAR that is specific to an antigen expressed on a tumor cell. Then, the modified T-cells are expanded in the laboratory and re-infused into the patient to target and destroy all cells that express the tumor-associated antigen, to act as surveillance after it is fully transplanted. Its clinical trial attests a very good efficacy, safety, complete remission, and perdurability of the engineered CAR T-cells. However, there is still undiscovered knowledge regarding the full applications of CAR T-cell therapy especially for solid cancers, indicating the need for researchers' efforts to end cancer globally.

Keywords: CAR T-cell therapy, Hybridoma, Immunotherapy, Intracellular co-stimulatory signals, mAb, MHC, Phage display library, TAAs, T-cell, TME, Tumor

Introduction

Cancer is a serious health problem in both animals and humans. In 2023 only, 1,958,310 new cancer cases and 609,820 cancer deaths are recorded to occur in the United States [1]. The most frequently diagnosed and expected to be the leading cause of cancer related death is lung cancer, accounting for 24.3% of all cancer deaths, followed by colorectal (11.0%), pancreatic (6.7%) and breast cancers (6.5%) in Canada [2]. Furthermore, Worldwide, prostate cancer is another most commonly diagnosed male malignancy (7.5%) and the fourth leading cause of cancer death in men and pets.

For a long time, many cancers were treated by different modes of therapeutics like surgery, chemotherapy, and irradiation, but none has prevented relapsing, and its side effects outweigh the importance. But history's path is unimaginable when it's not yet past, but present, when we are still standing in the middle of it. As the best option for cancer therapy, immunology has been employed to treat malignant tumors. These are monoclonal antibodies (mAbs), tumor vaccines, immune checkpoint blockade, Immunomodulators, and recently ongoing chimeric antigen receptor T-cells [3].

T-cell, as an anti-tumors cell has a TCR–CD3 complex with two polypeptides chains: α and the β, consisting of three CD3 dimers subunits, the CD3γε and CD3δε heterodimers and a CD3ζζ homodimer [4]. Each chain is composed of a

variable region three complementarity-determining regions that recognize peptides derived from the target antigen presented on the major histocompatibility complex (MHC) and a constant region domain. As the T-cell receptor (TCR) itself possesses no intracellular signaling components, its binding to the peptide–MHC epitope interface and forming a complex with multiple CD3 signaling subunits, represents the key interaction for signal transduction to fully activate T-cells. These indicate that TCR-peptide-MHC binding only does not activate T-cells [5, 6]. Recently, T-cell-based- immunotherapy, the CAR T-cell therapy has come of age as a feasible, safe, and efficacious approach to treat cancer. The T-cell-based immunotherapy is designed by mimicking the principles and organization of TCR and activation signal cooperation, with svFv serving as a recognition of an antigen and CD3ζζ and inducible T-cell costimulator (ICOS) like CD28 as activation signals [6].

The importance of CAR T-cell therapy over all cancer therapies is that it is immediate, and a single infusion of CAR T-cells can result in a good prognosis. A complete response rate of up to 100% was reported by Milone *et al.* [7] across 16 different trials for various B cell neoplasms with trend higher response rates in patients with acute lymphoblastic leukemia (ALL) than chronic lymphocytic leukemia (CLL). The field echoes with stories of lives extended: The woman with a grapefruit-sized tumor in her lung from melanoma, survived and became healthy after 13 years; the 6-year-old child near death from leukemia survived; the man with metastatic kidney cancer whose disease continued fading away even after treatment stopped [8]. Secondly, 2–3 weeks of proper care is enough for the patient. At the same time, the use of highly personalized, engineered cell therapeutics capable of undergoing division poses multiple challenges and requires an additional level of optimization [9]. So, this paper summarizes the current understanding of the key principles of CAR-T-cell therapy, its applications, challenges, and its prospects.

T-cell

T-cell types and functions

Naïve T lymphocytes, both $CD8^+$ and $CD4^+$ T-cells, once in the thymus, interact with antigens lodged on MHC on antigen presenting cells (APCs) through their αβ TCR and differentiated into different surface CD proteins to be trained for effector function and memory [6,10]. They are powerful components of acquired immunity, which mainly contributes to the clearance of tumors [11].

T-cells can also be classified based on the TCR chains they bear, whether heterodimers consisting of either disulfide-linked α- and β- chains (αβ TCR) or γ- and δ-chains (γ δ) [12]. γδ T-cells are unusual lymphocytes that express TCRs rearranged from TCR γ and δ genes and have a pivotal role in cancer immunosurveillance through its receptor and natural killer activation [13]. The γδ T-cells develop earlier than αβ T-cells in the thymus and directly recognize antigens via their γδ TCRs without the need for MHC molecules, similar to B-cells unlike αβ T-cells [14]. The γδ T-cell population consists of tissue-resident and peripheral blood γδ T-cells and accounts for about 0.5% to 5% of all peripheral blood T-cells [13].

Cytotoxic T-cell ($CD8^+$): $CD8^+$ T lymphocytes will become cytotoxic T lymphocytes. These T-cells are an essential immune defense against intracellular microorganisms, like viruses and bacteria, and for cancer surveillance. $CD8^+$ T-cells recognize peptides presented by MHC Class I molecules, found on all nucleated cells. Once activated, $CD8^+$ has three major mechanisms to kill infected or malignant cells [10]. These are through cytotoxins stored in specialized lytic granules, cytokines (TNF and IFN-γ), Granzyme B, perforin, and macrophage inflammatory protein 1β [6].

T-helper lymphocyte ($CD4^+$): $CD4^+$Tcells recognize peptides in the context of MHC class II molecules expressed on the surface of APC and activated to play an important role in antitumor immunity, by releasing cytokines, and helping B cells to produce antibodies as regulatory function [15] and activate and attract macrophages to the site of infection [6].

Mechanism of tumor immune escape

Tumor immune escape mechanisms are the ways by which a particular tumor cell evades immune surveillance [16]. They acquire these either through genetic mutation leading to a change in surface receptors or the release of metabolically active enzymes [17]. For instance, as depicted in **Figure 1**, ALL escapes immune surveillance through the expansion of immunosuppressive cells, secretion of anti-inflammatory cytokines, over-expression of immune checkpoint ligands, down-regulation of MHC-I and MHC-II, production of immunosuppressive enzymes, reduce the secretion of pro-inflammatory cytokines, and induction of NK and T-cell exhaustion. These help the tumor cells to survive and replicate in the tumor microenvironment.

Chimeric Antigen Receptor T-cell Design and Expansion

Tumor associated antigens (TAAs) or tumor specific antigen (TSA) identification and CAR design

Identification of proper TAAs is the first step and critical to the successful use of CAR T-cell therapy for cancer treatment. The ideal TAA for CAR T-cells has the following key characteristics: expression on all tumor cells including the cancer stem cell, the expression on the tumor cell surface, playing a key role in tumor cell survival, and lack of expression on normal tissues (**Table 1**) [19]. However, few TAAs are in fact ideal as many are also expressed on one or more normal tissues. The is responsible for on-target-off-tumor toxicity as in the case of

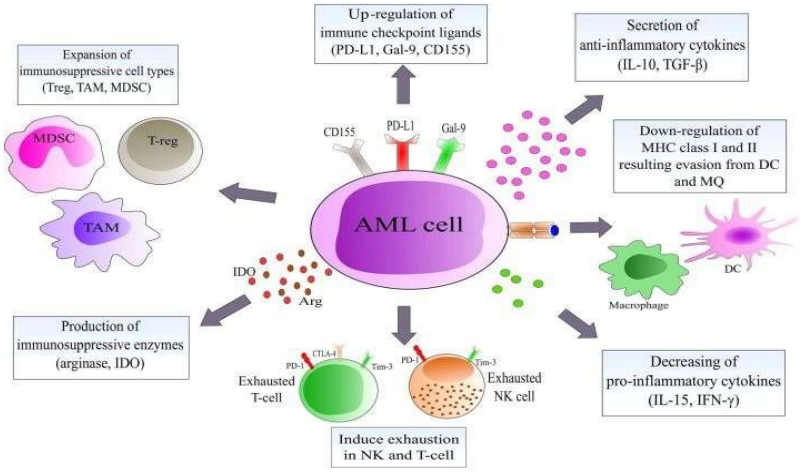

Figure 1. Immune escapes mechanisms of acute lymphoblastic leukemia. Source: [18].

Table 1. Tumor-associated antigens of CAR T-cell target. Adapted from [23].

Antigen	Full name	Disease
EGFR	Epidermal growth factor receptor	NSCLC, epithelial carcinoma, glioma
EGFRvIII	Variant III of the epidermal growth factor receptor	Glioblastoma
HER2	Human epidermal growth factor receptor 2	Ovarian cancer, breast cancer,glioblastoma, colon cancer, osteosarcoma, medulloblastoma
MSLN	Mesothelin	Mesothelioma, ovarian cancer, pancreatic adenocarcinoma
PSMA	Prostate-specific membrane antigen	Prostate cancer
CEA	Carcinoembryonic antigen	Pancreatic adenocarcinoma, breast cancer, colorectal carcinoma
GD2	Disialoganglioside 2	Neuroblastoma, melanoma

carbonic anhydrase IX–specific CAR T-cell infusion resulted in severe high grade cytokinase releasing syndrome in a patient with renal cell carcinoma or colon cancer (CRS) [20].

Regarding solid tumors, no such cell surface antigen with comparable properties of CD19 has yet been identified. Currently, TAAs, including mesothelin (MSLN), human epidermal growth factor receptor 2 (HER2), epidermal growth factor receptor (EGFR), prostate-specific membrane antigen (PSMA), prostate stem cell antigen (PSCA), and several overexpressed antigens are extensively investigated in CAR T-cell therapy for solid tumors at clinical trials [21,22].

The clinical benefit of CAR T-cells has now been reported by several researchers, targeting the CD19 antigen driving the growth of B cell lymphoma in patients with hematologic malignancies [19,24].

CARs are engineered membrane fusion proteins. The basic design of CAR T-cells consists of two fundamental domains [25]. An extracellular antigen-recognition domain consisting of an antigen-specific single-chain variable fragment derived from a monoclonal antibody specific for TAAs and intracellular T-cell signaling domains necessary for T-cell activation [26,27]. Kudva and Modak [28], indicated that the internal fusion of CD28, 4-1BB (CD137), and OX40 (CD134) costimulatory signals enhance single-chain fragment variable (scFv), creates different generations of CARs and enhance the function and persistence of CAR T-cells, and reduce toxicity during the tumoricidal process.

Physiologically T-cells have TCR co-localized with CD3, having hetero-dimers comprising four subunits ζ, δ, ε, and γ - which then initiates the first TCR signal upon binding to an antigen. In CAR modeling, CD3ζ is the most common immune receptor

tyrosine-based activation motif of the CD3-zeta chain alone proved to be efficient to induce signals with similar potency to the normal TCR/CD3 signaling in the first-generation CARs. But naive T-cells depend upon more than one stimulus for full activation, and ligation of TCR to CD3 ζ alone is insufficient and results in transient persistence, inefficient tumor toxicity, and low-level cytokine secretion [6,29]. This indicates the need for co-stimulatory signals 4-1BB, CD28, or OX40 (**Figure 2 right side**).

svFv (binding moiety) generation and phage display screening: Antigen-specificity for a T-cell is encoded by the TCR [31]. However, CAR-modified T-cells recognize tumor cells via CAR, independent of TCR-MHC-Peptide interactions. This binding moiety consists of scFv, a TAA-specific monoclonal antibody derived from humanized mice [11]. For this, a Phage display encoding monoclonal antibody fragment against an antigen of interest will be selected, isolated, and amplification after being cloned with a scFv DNA sequence from hybridoma as depicted in **Figure 3**.

Intracellular signaling: The intracellular signaling domain of CAR T-cells determines the strength, quality, and persistence of a T-cell response to tumor antigens and is frequently manipulated to enhance the potency of CAR T-cell therapy [33]. So, the incorporation of both the primary and costimulatory signaling domains, CD28, CD134, and CD137 in a single gene product will enhance the persistence and efficacy of T-cells against tumors [23] by enhancing T-cell proliferation, glucose metabolism, and self-limited T-cell persistence, with $CD28^+$ and stimulation of lipid oxidation and support greater T-cell persistence with 4-1BB [34].

CAR T-cell therapy process

In CAR T-cell therapy, T-cells will be genetically modified for TAA recognition through the expression of a chimeric antigen receptor (CAR) [29]. Its processes commence with the collection of mononuclear (lymphocyte, specifically the $CD3^+$ T-cells) cells from the patient's blood through leukapheresis. Then $CD3^+$ T-cells will be engineered by either viral or non-viral vector loading CAR gene, artificially [35]; and modified to express a transgene encoding a tumor-specific CAR, followed by *in vitro* expansion in optimized T-cell culture conditions (**Figure 4**). Next harvested and formulated at a specified dose. Finally, quality and release testing are performed on the

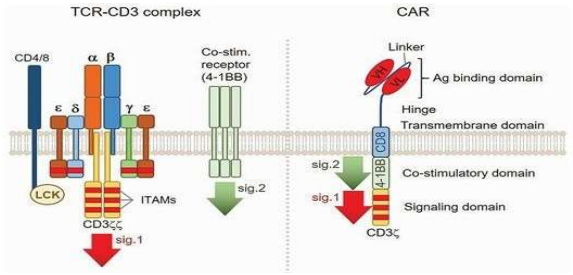

Figure 2. TCR organization and CAR T-cell construct. Source: [30].

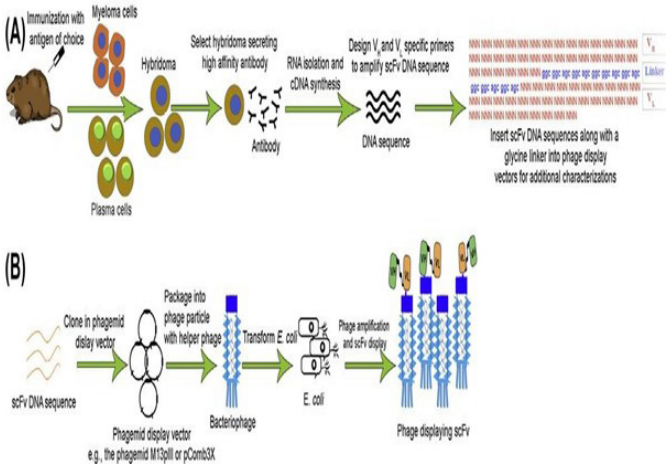

Figure 3. Production of phage display library of scFv gene for CAR design. Source: [32].

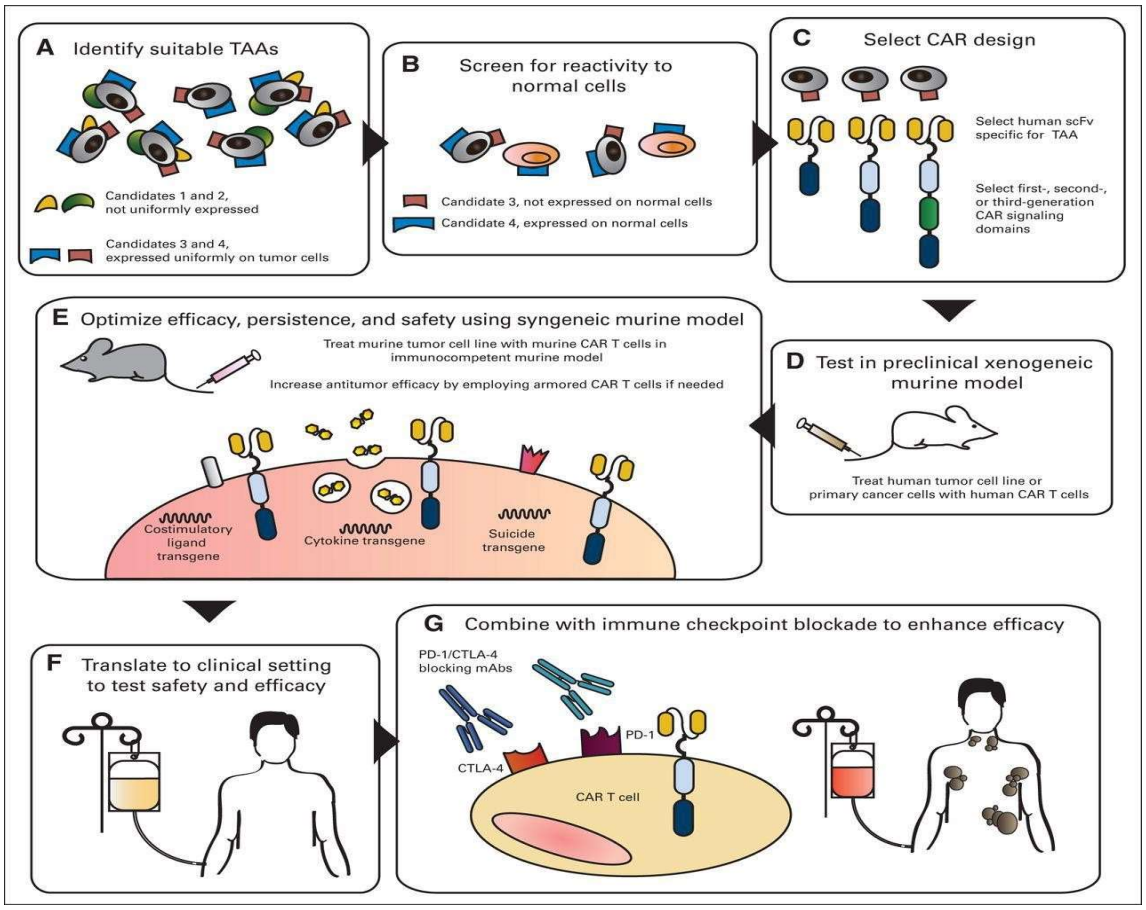

Figure 4. Pathway for designing optimal CAR T-cells for tumor immunotherapy. Source: [37]. **(A)** TAA identification, with **(B)** secondary selection if it is expressed on non-malignant cells **(C)** CAR (scFv) generation from humanized mice ►hybridoma, expansion with phage display libraries. **(D)** Preclinical studies in xenotransplantation murine tumor models to validate potential efficacy. **(E)** Whenever possible, murine-derived CAR T-cells should be assessed in clinically relevant syngeneic tumor models thereby providing a platform for the exploration of additional genetic modifications (eg, armored CAR T-cells) to optimally enhance *in vivo* antitumor efficacy, persistence, and safety. Incorporate suicide gene (PD-1) to enhance safety regarding unforeseen on-target/off-tumor toxicity before initial clinical trial application. Finally, Clinical trial application with either CAR T-cells or armored CAR T-cells with immune checkpoint inhibitors (i.e., PD-1/PD-L1 and/or CD80/CTLA4 blockade) to protect CAR T-cells and further recruit endogenous immune effectors.

product, to ensure the safety of the infusible product [35,36]. These processes require approximately 3 weeks, and the preparation of CAR T-cells takes approximately 2 weeks.

Precondition and clinical trials

Patient-derived xenograft (animal modeling) for preclinical testing: Though the real reason is the pre-clinical experiments were not rigorously designed, for more than 80% of potential therapeutic failures in people, animal models were taking the blame [38]. So, recent advances in the development of new methods of cancer immunotherapy require the production of complex cancer animal models that reliably reflect the exact physiological, anatomical, and genotypic complexity of the patient's tumor and its microenvironment, to evaluate the effectiveness of immunotherapy [39].

PDXs (patient derived xenografts) are produced by orthotopically or subcutaneously implanting fresh human tumor tissue of 2 mm^3 into immunodeficient gamma-irradiated mice in which the chance of rejection is lower [39-41]. Normally the immune-deficient mice, the SCID mice used for the generation of PDXs have combined T, B, and NK cell deficiency, and tolerance for human cell grafts. This almost accurately reflects the complexity mediated by the natural development of the tumor, including genomic heterogeneity, tumor architecture, and microenvironment factors [40].

For hematological tumors, the mice will be humanized by intravascular co-transplantation of human peripheral blood

mononuclear cells or hematopoietic stem cells five weeks after irradiation and monitored by flow cytometry for determining the percentage of differentiated human CD45$^+$cells in its peripheral blood to overcome innate immunity limitation [42].

Pretreatment of the patient: According to Zhao *et al.* [43] the use of some chemotherapeutic drugs, such as fludarabine, cyclophosphamide, gemcitabine, lenalidomide, and docetaxel, as transient lymphodepletion will remove existing lymphocytes in lymphoid tissue or bone marrow tissue, facilitate implantation space for newly implanted CAR T-cells, and induce the production of bone marrow cytokines, promoting the recovery and proliferation of immune cells including CAR T-cells.

T cell dosage, administration, and persistence: Expansion and persistence of CAR-T cells determine the outcome of cancer patients after CAR T-cell therapy, CAR T-cell dose, and the number of dose regimens. Zhang *et al.* [44] found that CAR T-cells could be detected and expanded in both peripheral blood and tumor tissues of those patients receiving a high dose of CAR T-cell therapy. According to Porter *et al.* [45], patients who received CAR T-cells of a median 1.6×10^8 T-cells for refractory CLL had a response rate of 57% with 50% complete remission with no relapse that functionally persists beyond 4 years.

However, the major problems of current CAR T-cell immunotherapy are limited replicative lifespans of T lymphocytes, from a few days to 4 weeks [46] and B cell aplasia in all responding patients with CRS coincident with T-cell proliferation [45]. Fortunately, to increase the tolerability of the treatment and persistence, the latest generation of CAR T-cells is used, and the dose is often split over multiple injections. For instance, Ahmed *et al.* [47] showed administration of 1×10^4–1×10^8 HER2 specific CAR T-cells in up to 9 infusions through different routes were being investigated to have improved outcomes. So, CAR T-cell generation, administration route, and number of regimens could have a dramatic impact on the efficacy of the treatment. However, Fraietta *et al.* [48] indicated that the progeny of a single CAR T-cell was sufficient to mediate potent anti-tumor effects in advanced leukemia.

Antitumor Mechanism, Challenges, and Control Mechanisms

Antitumor mechanism

As demonstrated by Porter *et al.* [45] and Sadelain *et al.* [49], CAR T-cells are potent antitumor agents against lymphoma through CD19+ targeted CAR T-cells. To be effective after infusion, CAR T-cells must expand, persist, exhibit enduring antitumor cytotoxicity, withstand and/or counteract an immunosuppressive tumor microenvironment, and overcome targeted tumor antigen escape [37]. So, for these, immune surveillance CAR T-cells recognize specific tumor antigens in an MHC-independent manner; this helps the CAR T-cells to recognize a broad range of foreign antigens on surface cancer cells that do not express MHC which leads to the activation and execution of its antitumor function [11]. Once CAR specifically binds with TAAs, T-cells are activated through the phosphorylation of immune receptor tyrosine-based activation motifs and subsequently induce cytokine secretion, T-cell proliferation, and cytotoxicity [50].

Chimeric immune receptor-activated T lymphocytes perform cytotoxicity through two predominant pathways: (1) secretion of perforin and granzyme granules and (2) activation of death receptor signaling via Fas/Fas-ligand or TNF/TNF-R. CD8$^+$ T-cells kill tumor cells through those two pathways. CD4$^+$ T cells destroy target cells primarily via perforin or granzyme, while death receptor-mediated apoptosis is believed to function as a compensatory pathway [51].

Side effects

Cytokine release syndrome: Acute toxicity, especially cytokinase-releasing syndrome CRS is thought to result from the systemic activation of CAR T-cells following the engagement of CD19$^+$ malignant (on-target) and healthy (off-target) cells [52]. These symptoms are mostly self-limiting and range from mild to severe. These are fever, myalgia, and nausea; and in severe cases, it escalated to hypotension, capillary leak, and hypoxia [45]. Neurotoxicity symptoms can include headaches, seizures, and cerebral edema. These symptoms usually occur within the first two weeks, but more severe cases can present within 72 hours [53].

These side effects are commonly associated with pro-inflammatory cytokines such as IL-6 and, IL-1β, during CAR T-cell therapy, and these cytokines are significantly elevated in the blood following CAR T-cell infusion [54]. Post-infusion and following target cell recognition, CAR T-cells are activated and secrete various inflammatory factors, such as GM- CSF, which can activate myeloid cells that serve as a source of pro-inflammatory cytokines taking part in toxicity [55].

The problem of target loss/antigen escape: CD19-targeting CAR T-cells effectively kill both CD19$^+$ tumor cells and CD19$^+$ normal B cells resulting in prolonged B cell aplasia [56]. Long-term B cell aplasia can be detrimental and can lead to an increased risk of infections [56]. So, the expression of target antigen in normal cells can also pose a significant safety risk but can effectively be managed by the infusion of replacement therapy gamma-globulins. On the way, it serves as quite a useful marker for CAR T-cell persistence and functionality [6].

Challenge in CAR T-cell therapy in solid tumors: Besides hematological malignancy treatment with CAR T-cells, various solid tumors were also targeted by CAR T-cells. However, many obstacles need to be solved to improve the safety

and efficacy of CAR T-cells against solid tumors [57]. The challenges in developing CAR therapy for solid tumors lie in the tumor microenvironment, which may dampen CAR T-cell function [30]. The extracellular environment is suboptimal for T-cell function, owing to hypoxia, necrosis, acidification, nutrient shortage (glucose, glutamine, l-arginine), and an array of immunosuppressive molecules (PD-L1, IL-10, TGFβ, indolamine-2-3-dioxygenase [58].

So, the limited success of CAR T-cell therapy against solid tumors may be due to many factors. These include the lack of unique TAAs in most cancers, the inability of *ex vivo* expanded CAR T-cells to persist and proliferate following adoptive transfer, inefficient trafficking of CAR T-cells to tumor sites, heterogeneous expression of the targeted antigens leading to an outgrowth of antigen-negative tumor variants, the presence of immunosuppressive molecules and cells and the metabolically hostile tumor microenvironment [59].

The control mechanism of side effects and improvement of challenges

The clinical implementation of CAR T-cell therapy requires the establishment of a safe, efficient, and reproducible CAR T-cell manufacturing process [49]. Several medical interventions have been established to control or prevent on-target/off-tumor toxicity and others, by focusing on rational CAR design and genetic manipulation of T-cells to prevent inflammatory toxicities (**Figure 5**) [60]. These aim to either rapidly eliminate the engineered T-cells or better constrain their function [30].

CARs can include molecular switches that can be controlled post-infusion to modulate the level of CAR-T activation [60]. To reduce the toxicity associated with CAR T-cell therapy, suicide genes or transient mRNA CAR were applied to remove CAR T-cells or shorten their lifespan when necessary [61]. The increase in specificity and localization of tumors can reduce unwanted CAR T-cell activation by reducing on-target/off-tumor activity, which may result in lower levels of inflammatory factors released by CAR T-cells [60].

How to improve CAR T-cell therapy for solid tumors: To date, some overexpressed endogenous molecules in tumor tissues, especially those that promote tumor proliferation and persistence, have been selected as targets for CAR T therapy [47, 62]. As some normal tissues may also express nonspecific tumor antigens, there will be potential damage to normal tissue triggered by on-target and off-tumor toxicity. To overcome the challenges associated with solid tumors, various strategies have been developed involving optimized CAR structures, innovative combination therapy aimed at enhancing the specificity, infiltration, and efficacy of CAR T cells and reprogramming the inhibitory conditions [63], and conditioning regimen before T cell infusion for graft rejection inhibition (**Figure 6**) [30].

Applications and Clinical Trials of CAR T-cell Therapy

Hematological malignancies

Kymriah: From the FDA approval of tisagenlecleucel (Kymriah; Novartis), on 30th August 2017 for patients with relapsed ALL, targeting $CD19^+$ B cells, several efforts have been undertaken by clinical trials showing highly promising results using CAR T-cells against different types of cancer. The approval of Kymriah was supported by more than 80% complete- response rates for relapsing B-ALL [64].

Yescarta: The second approved CAR T-cell product is Axicabtagene Ciloleucel (Yescarta®). It has got marketing

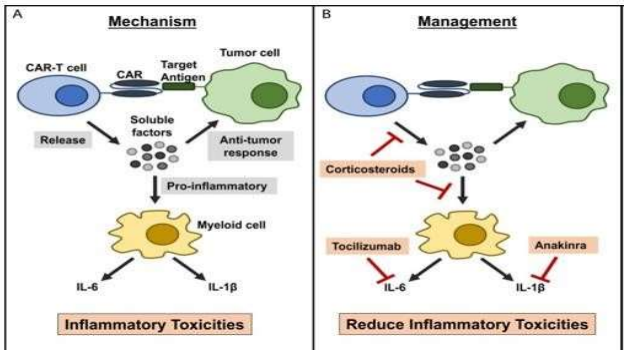

Figure 5. The mechanism for inflammatory toxicities, management strategies, and future strategies for prevention. Source: [60]. **(A) Mechanism**: When a CAR interacts with a target antigen, activated CAR T-cells produce soluble factors which can either help or hurt the anti-tumor response by activating myeloid cells resulting in inflammatory cytokines, such as IL-6 and IL-1. These are secretions responsible for inflammatory toxicities seen in CAR T-cell recipients. **(B) Management:** Anti-inflammatory drugs, such as corticosteroids, reduce inflammatory cytokine release by CAR T-cells or myeloid cell activation. Targeting specific cytokine receptors, such as IL-6R by tocilizumab and IL-1R by anakinra, are also currently used to reduce inflammatory cytokines or cytokine signaling pathways.

legalization in Europe parallel to Kymriah in August 2018. For its approval, a second-generation CAR T-cells targeting $CD19^+$ B-cells similar to Kymriah but with different intracellular co-stimulatory domains derived from CD28 are used in 108 adult patients with diffused large B-cell lymphoma (LBCL) and primary mediastinal large B-cell lymphoma with 1-year follow-up for its response, survival, and safety in the USA. In this study, an 82% response rate and 58% complete remission was recorded. Jacobson et al. [65], also showed in their study that Axicel treatment demonstrated a manageable safety profile and meaningful clinical benefit of 82% overall response rate with 54% complete remission.

Tecartus (brexucaptagene): In a single-group clinical trial of 60 patients with refractory mantle cell lymphoma who received one infusion of $CD19^+$ targeting Tecartus and followed up for at least 6 months, 87% responded to the drug with 62% complete remission [66].

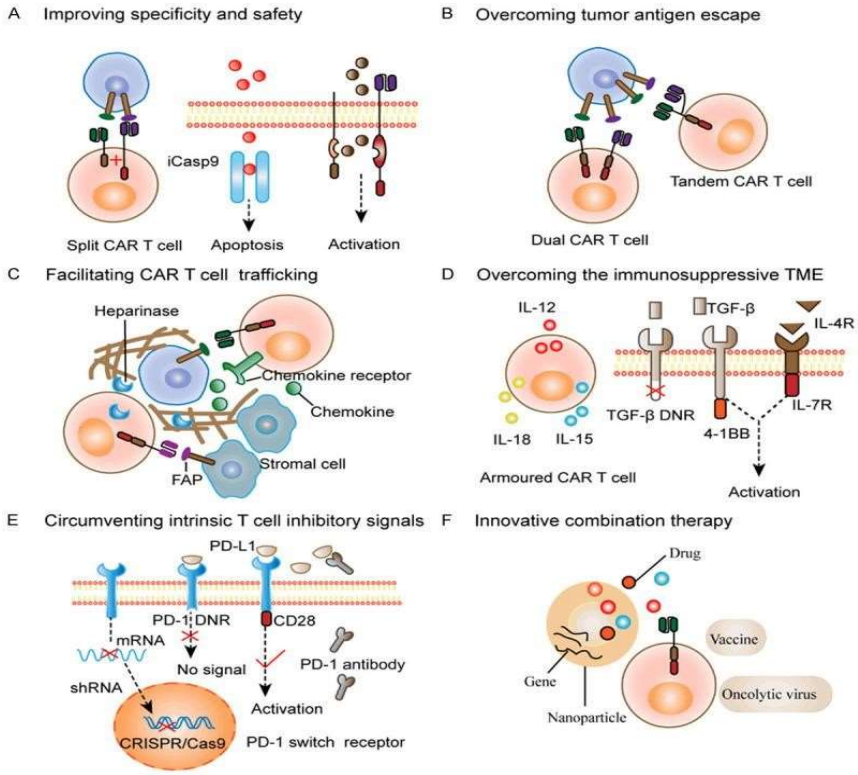

Figure 6. Innovative strategies to circumvent the challenges of CAR T-cell therapy. Source: [63]. **A. Improving specificity and safety**: Indicates using CAR T-cells containing separate co-stimulatory signals and activation signals, which require the presence of two different tumor antigens for full activation. On-switch CAR-T cells or off-switch CAR-T cells are forced to undergo activation or apoptosis. **B. Overcoming tumor antigen escape**: Multi-target CAR T-cells are designed to target different tumor antigens on single cells, this can be dual CAR T-cells with two CARs or tandem CAR T-cells with two or more scFv in one CAR. **C. Facilitating CAR T-cell trafficking**: This can be achieved by optimizing CAR T-cells to express appropriate CCR chemokine, including, CCR2b, and CCR4, which bind to chemokine ligands secreted by tumors. Alternatively, modifying CAR T-cells to express FAP to remove stromal cells, and to secrete the HPSE enzyme, degrading the tumor matrix to further overcome physical barriers. **D. Overcoming the immunosuppressive tumor microenvironment**: Armoring CAR T-cells with immune stimulatory factors such as IL12, IL15, and IL18 genes to modulate the local cytokine microenvironment for recruiting endogenous immune cells and prolonging CAR T-cell survival. Another strategy is modifying CAR T-cells to inhibit transmission of immunosuppressive signals into the cells, through TGF-β-DNRs (tumor growth factor-beta dominant negative receptor) expression, rather than converting TGF-β signals into 4-1BB or IL-12 stimulatory signals, and inverting cytokine receptors infused with the extracellular domain of the IL-4R and the endo-domain of the IL-7R in which both resulted in activation of the cells. **E. Circumventing intrinsic T-cell inhibitory signals**: This is through blocking of PD-L1 production, using CRISPR/Cas9 to knock out the PD-L1 gene in CAR T-cells, or using shRNA to degrade mRNA in CAR T-cells. In addition, PD-L1 DNRs (PD-1 dominant negative receptor) lacking the intracellular domain are unable to transmit inhibitory signals. PD-L1 switch receptors with intracellular immune-stimulatory signal CD28 can convert inhibitory signals into stimulatory signals. PD-1 blocking antibodies secreted by CAR T-cells can competitively bind PD-L1. **F. Innovative combination therapy to synergistically enhance CAR T-cell functions**: Oncolytic virus vaccines to activate CAR T-cells *in vivo*. Oncolytic viruses can be modified to carry genes or stimulatory cytokines to enhance the efficacy of CAR T-cells [63].

Idecabtagene vicleucel (Abecma®): In a phase II study designed to evaluate the efficacy of Abecma® (at the dose of 1.5 to 4.5 x 10^7 CAR-T cells) targeting BMCA+ in 127 patients with advanced and pretreated multiple myeloma for over 13 months median follow-up by [67], the overall response rate of 73% with 33% complete remission and 5% and 3% CRS and neurotoxicity respectively was recorded.

CAR T-cell therapy for solid tumors

Carcinoembryonic antigen CAR T-cells: CEA is a sensitive tumor biomarker for gastrointestinal cancer, like CRC in tissues and serum but cannot be detected in most normal adult tissues, except in the gastrointestinal tract at a low level restricted to the apical surface of the epithelial cell membranes facing the lumen, which is invisible to immune cells; being this, Zhang *et al.* [44] carried out a phase I clinical trial with 2nd generation CEA CAR T-cell therapy of CEA^+ CRC patients by systemic delivery through an intravenous infusion (NCT02349724) with lymphodepletion pretreatment. Seven out of 10 patients who experienced the progressive disease in previous treatments had stable disease with slight variation in the tumor diameters after CAR T-cell therapy by PET analyses, in two of them, the tumor growth was inhibited for more than 30 weeks and as an indicator of the efficacy of CEA, and also show dropped Serum CEA to a satisfactory level.

FRα (Folate Receptor alpha)-CAR T-cells: Recently Luangwattananun et al. [68] showed the co-culturing of fourth-generation FRα-CAR T-cells (CD28, 4-1BB, and CD27) with FRα-expressing MDA-MB-231 breast cancer cell line at an effector to target ratio of 20:1, resulted in lysis of 88.7 ± 10.6% of the target cells and even more with higher surface FRα expression. This selective cytolysis of the CAR T-cells has not been seen when co-cultured with FRα-negative MCF10A normal breast-like cell line at an equal ratio.

Mesothelin: Mesothelin is expressed in 90% of malignant epithelioid pleural mesothelioma, 69% of lung adenocarcinoma, 60% of breast cancer, 46% of esophageal carcinoma, and 12.8% of cervical cancers, serves as a specific tumor biomarker treatment. *In vitro* experiment of 4 hours co-culture of MESO-CAR T-cells under different effector/target cell ratios (1:1, 2:1, 5:1, and 20:1) with MESO-expressing SiHa cells as target cells and Caski cells as non-target cells, [69] showed that the rate of MESO-CAR T-cells killing of MESO-expressing SiHa cells reached 9%, 10 %, 18 %, and 22 %, respectively and maximum of 10% Caski cells regardless of the ratio. This indicates that MESO-CAR T-cells could efficiently kill SiHa cells with high expression of MESO but had little effect on MESO-free cells furthermore, several CAR T-cell designs have been under trials for different TAAs and TSAs (**Table 2**).

The veterinary implication of CAR T-cell therapy

Canine cancers are the leading cause of death in dogs over 10 years of age, with 50% of older dogs developing the disease and approximately one in four dogs eventually dying from it. They share histologic and genetic similarities with human cancers concerning architecture and heterogeneity recognized within and between tumor classes [71]. They also follow similar clinical courses and exhibit comparable response patterns to those seen in people treated with multiple modes of similar standard adjuvant anti-cancer therapies [72]. Osteosarcoma is the most common primary tumor of bone in both dogs and humans. It is about 75 times more common in dogs than in

Table 2. List of chimeric antigen receptor therapy clinical trials. Source: [70].

Target antigen	Type of cancer	Clinical trial ID
PSMA	Prostate cancer	NCT001140373
CEA	Breast cancer	NCT00673829
CEA	Lung cancer	NCT00673322
HER-2	Lung cancer	NCT00889954
HER-2	Osteosarcoma	NCT00902044
HER-2	Glioblastoma	NCT01109095
CD30	Lymphoma	NCT02274584
GD2	Neuroblastoma, osteosarcoma	NCT03356795
EGFRVIII	Glioblastoma	NCT02309373
Mesothelin	Pancreatic cancer	NCT02706782
CD38/CD123	B-Cell Malignancies	NCT03125577
MUC16	Ovarian carcinoma	NCT02498912
GPC3	Lung squamous cell carcinoma	NCT03198546

humans, which is about 8.4% and less than 4% respectively followed by Prostate tumors, mast cell tumors, and canine lymphoma, [73].

For the advancement of CAR T-cells into the clinic, the role of rodents and primates was decisive. In the evaluation of CAR T-cells in dogs with spontaneous cancer, Panjwani *et al.* [74] designed a pilot trial using CAR T-cells to treat canine DLBCL. In this experiment he designed and manufactured CD20-targeting 2nd generation canine CAR T-cells for functional evaluation *in vitro* and *in vivo* using lentivectors to parallel human CAR T-cell manufacturing; and infusion resulted in the death of all $CD20^{+}$ targets bearing B-cells in an antigen-specific manner and circulating CAR T-cells post-infusion. He also shows patient survival times correlated with *in vitro* product expansion.

Conclusion and Future Prospects

Genetically, reprogrammed, patient-derived chimeric antigen receptor (CAR)-T lymphocytes with the ability to recognize predefined surface antigens with high specificity in a non-MHC restricted manner have shown increasing and promising anti-tumor efficacy and safety in preclinical and clinical studies depending on some FDA approved CAR T-cell drug result and on-going clinical trial reports of hematological malignancy. This type of therapy is most important in patients in which other therapies have not been effective. However, several obstacles remain to be overcome for a successful application of CAR T-cells in solid tumors, including the lack of ideal TAAs, hostile solid tumor microenvironment, inefficient trafficking of CAR-T cells to tumor sites, and the risk of side effects by utilization of CRISPR/Cas 9 gene-editing technology to turn the cells.

List of Abbreviations

AML: Acute Myeloid Leukemia; APC: Antigen-Presenting Cells; BMCA: B cell Maturation Antigen; Cas9: CRISPR-Associated Protein 9; CCR: Carbon Catabolite Receptor; CD: Cluster of Differentiation; CEA: Carcinoembryonic Antigen; CLL: Chronic Lymphoblastic Leukemia; CRC: Colorectal Cancer; CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats; CRS: Cytokine Releasing Syndrome; CTLA-4: Cytotoxic T-lymphocyte Antigen-4; DC: Dendritic cells; DLBCL: Diffuse Large B cell Lymphoma; EGFR: Epithelial Growth Factor Receptor; FAP: Fibroblast Activation Protein; FDA: Food and Drug Administration; Gal-9: Galectin-9; GD2: Disialoganglioside 2; GM-CSF: Granulocyte-Macrophage Colony Stimulating Factor; GPC3: Glypican 3; HER2: Human Epidermal Growth Factor 2; HPSE: Heparinase Enzyme; iCasp9: Inducible Caspase 9; ICOS: Intracellular Co-stimulatory Signals; IDO: Indoleamine 2, 3-dioxygenase; IFN-γ: Interferon Gamma; MCF-7: Michigan Cancer Foundation-7; MDA MB 231: M.D. Anderson Metastasis Breast Cancer 231; MDSC: Myeloid-Derived Suppressor Cells; MHC: Major Histocompatibility Complex; MIB-1β: Macrophage Inflammatory Protein-1β; MQ: Macrophage; MUC16: Mucin 16; NK: Natural Killer Cells; NOD: Non-Obese Diabetes; NSCLC: Non-Small Cell Lung Cancer; NT: Neurotoxicity; PD-1: Programmed Death-1; PD-L1: Programmed Death-Ligand 1; PSMA: Prostate-Specific Membrane Antigen; SCID: Severely Combined Immune-Deficient; shRNA: Short Hairpin RNA; TAA: Tumor-Associated Antigen; TAM: Tumor-Associated Macrophages; TCR: T cell Receptor; TGF-β: Transforming Growth Factor-β; TGF-β-DNRs: Dominant-Negative Transforming Growth Factor-beta Receptors; Tim-3: T-cell immunoglobulin and mucin domain-containing protein; TRAC: TCR Alpha Constant; T-reg: T-regulatory cells

Conflicts of Interest

The authors declare no conflict of interest.

Funding Statement

This manuscript received no financial funding.

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