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# Chimeric-Antigen-Receptor (CAR) T Cells and the Factors Influencing their Therapeutic Efficacy

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## Abstract

Immunotherapeutic treatments for malignant cancers have revolutionized the medical and scientific fields. Lymphocytes engineered to display chimeric antigen receptor (CAR) molecules contribute to the exciting advancements that have stemmed from a greater understanding of cell structure and function, biological interactions, and the unique tumor microenvironment. CAR T cells circumvent the unique immune evasion capability of tumors by acting in a major histocompatibility complex (MHC) independent manner. Various factors contribute to the efficacy of CAR therapy, including CAR structure, gene transfer strategies, *in vitro* culture system, target selection, and preconditioning regimens. While recent clinical trials have shown promising success, cytotoxicity and other various challenges need to be addressed before CAR therapy can reach its full clinical potency. This review will discuss factors associated with CAR therapeutic success and the difficulties that continue to be a focus of research around the world.

### Keywords

Cancer immunotherapy; Chimeric antigen receptor; Persistence; Tumor antigen; Chemotherapy

## INTRODUCTION

The National Cancer Institute estimated over 1.5 million new cancer cases and more than five-hundred thousand cancer deaths in the United States during 2016 [1]. As scientists race to find effective treatments for these destructive malignancies, the CAR T cell field proves especially promising. The basic concept of chimeric antigen receptor (CAR) T cell therapy involves directing a patient's own T cells to kill tumor cells which express a specific antigen. Tumor cell recognition by CAR T cells is based on antibody and antigen rather than T cell receptor (TCR) and major histocompatibility complex MHC. CAR T cell therapy has shown



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remarkable clinical results, especially for hematological malignancies. This review will discuss the factors that potentially influence CAR therapeutic efficacy in clinic trials.

## CAR T Cell Subsets

T cells can be divided into many subsets, each expressing varying persistence and functionality. Theoretically, all cell subsets can be used for CAR cell engineering. However, given the clinical feasibility, the most common formulation used in current clinical trials are $CD4^+$ T-helper cells plus cytotoxic $CD8^+$ T cells. CD4/CD8 CAR cell efficacy, cytokine production, antitumor activity, and proliferation depend on subpopulations and ratios used [2]. Preclinical studies indicate that both CD4 and CD8 work together to eliminate tumors. CD8 T cells are the most effective cytotoxic cells in terms of tumor elimination, where as CD4 T cells not only produce the cytokines that are critical for CD8 T cell function, but they also kill tumor cells directly.

Both CD4 and CD8 T cells can be further divided into many subpopulations based on their function or *in vivo* persistence. For example, regulatory T cells (Treg) can suppress immune response by secreting immunosuppressive factors or by delivering negative signals to the T cells. A recent study using CAR engineered Treg cells showed that these cells could be used for autoimmune disease treatment [3]. Based on *in vivo* migration and persistence, T cells can also be divided into central memory and effector memory T cell subsets.

Current studies support the theory that central memory T cells ($T_{cm}$) are a more desirable T cell subset for CAR T cells therapy because of their prolonged *in vivo* persistence [2, 4–6]. Allogeneic CAR T cells are attractive because they are “off-shelf” CAR T cells and can be produced with standard criteria and better quality control.

Several groups are using virus specific T cells for adoptive T cell therapy. Virus specific T-cells (VST) are well tolerated by patients, do not lead to graft versus host disease (GVHD) even if the cells are donor-derived, and have been shown to display antitumor activity [7]. VST cells can be stimulated by viral vaccines and are most effective soon after lymphodepletion when viral infections are most likely to occur [7]. They might persist even longer than autologous T cells because of the persistent antigen signal transduced by TCR. However, due to the prolonged culture time needed to select virus specific T cells, the quality of the cells might be impaired [8–10].

Another prospective CAR host is the Natural Killer T-cell (NKT) [11]. CD1D Va24-invariant NKTs are promising because their monomorphic nature limits toxicity and presents a safe approach to donor derived T cell engineering without GVHD [12]. iNKT CAR engineering faces the challenge of sufficient ex vivo expansion due to the limited amount of cells occurring naturally in the body, but researchers developing a greater knowledge of these cells may prove iNKT CAR engineering very effective [11, 13].

## CAR Structure

CAR engineered constructs commonly include an extracellular domain for antigen recognition, a trans membrane domain, and an intracellular domain that triggers cell function (Figure 1) [14–16]. The structure of these parts plays a crucial role in effective

CAR engineered cancer treatment. The extracellular domain of a CAR construct typically incorporates a single-chain variable fragment (scFv) and a spacer. The antigen specific scFv, cloned from a hybridoma, is made up of monoclonal antibody heavy and light chains connected by a linker [17]. While many studies use murine scFvs, humanized or fully human scFvs have been shown to express similar antitumor activity and enhanced persistence [18, 19]. Preclinical studies suggest that mouse derived scFvs might actually induce an immune response against the T cells themselves, resulting in the depletion of murine based CAR T cells.

Just as the most effective scFv varies with tumor type, optimal spacer design also depends on the specific tumor epitope being targeted [20, 21]. Carefully devised spacers offer flexibility and enhanced antigen binding, but spacers used incorrectly can inhibit CAR cell efficacy *in vivo* [20, 21].

Intracellular signaling domains trigger cell function. Typically, a CD3zeta moiety is used in conjunction with one (second generation) or two (third generation) costimulatory domains [22]. Common costimulatory domains include OX-40, CD-28, and 4-1BB [22, 23]. CD-28 invokes heightened cytokine activity but can contribute to cell exhaustion [24, 25]. Ox-40 and 4-1BB, both members of the tumor necrosis factor (TNF) family, enhance persistence for CD4 cells and CD8 cells, respectively [24, 25]. ICOS based CAR T cells can induce IL-17-like CD4 T cells and mediate strong antitumor activity in humanized mice models [26]. While most studies find second generation CARs are more potent than first generation CARs with no costimulatory domain, results of third generation CAR studies provide conflicting results [19, 22, 23, 27, 28]. Optimal CAR design seems to vary based on the targeted tumor.

Many studies focus on improving CAR construction in order to enhance binding capability, therapeutic safety, and *in vivo* immune stimulation. For example, bispecific OR-gate CARs are a novel method for improving CAR cells' ability to bind to tumor specific antigens [16, 29]. This molecule recognizes two distinct antigens and can be fully activated by either or both, reducing the escape of antigen negative tumor cells and diminishing the risk of relapse [16]. The "TanCAR" molecule utilizes two tandem scFv regions and is able to target two antigens, mitigating the risk of tumor antigen escape [29, 30]. Clinically, many patients who receive CD19 targeted CAR T cell therapy experience CD19 negative relapse. For this reason, such multiple antigen targeting CAR structures could be extremely useful for inhibiting antigen negative relapse.

Many new alterations to the traditional CAR structure enhance the safety of CAR treatment. iCARs have a dominant inhibitory signal that is activated upon recognition of healthy tissue antigen [31]. Masked CARs have an antigen binding domain that is sterically blocked until exposure to the protease-enriched tumor microenvironments, in which the peptide mask is removed and CAR function commences [31]. "Off-target" toxicity can be lethal for cellular therapy, but the risk can be reduced dramatically if redirected T cells can target two different antigens. Roybal et al. developed a novel "precision dual-receptor circuit" CAR using synthetic Notch based structure, in which the activation strictly depends on the presence of two antigens [32]. Trans CARs display two different CAR molecules with distinct

specificities in a single cell, physically separating the CD3z activation molecule from the costimulatory domain. Because optimal CAR function is only activated upon recognition of both antigens simultaneously, this trans signaling approach may prove to limit on-target, off-tumor toxicity while retaining the efficacy of a second generation CAR [33].

Some recently developed CARs have manually controllable persistence in order to enhance safety. Switchable CARs are dependent on the infusion of switch molecules for activation, and cell function can only begin with the formation of a complex between the CAR cell, switch, and tumor antigen [34, 35]. Implementation of suicide genes also enhances the safety of CAR infusion by offering controlled persistence of CAR cells [36]. For example, herpes simplex virus-thymidine kinase (HSV-TK) generates CAR cell susceptibility to antiviral medication; inducible caspase-9 (iCasp-9) prompts apoptosis upon ligation with a dimerizing drug; and a truncated EGFR (tEGFR) gene invokes antibody dependent cellular cytotoxicity [31]. ON-switch CARs were designed to act only in the presence of small, injected molecules, minimizing the risk of toxicity induced by cellular therapy. This approach can be extremely useful in a clinical setting. However, without instant antigen exposure, the *in vivo* persistence of ON-switch CAR T cells has yet to be determined [37]. Another novel modification is the inclusion of Step-tagII in the CAR or TCR structure, which allows for *in vivo* transgenic T cell enrichment, stimulation, and monitoring [38].

Other novel CAR construct ideas focus on stimulating the immune system. T cells integrated with bispecific T cell engagers (BiTEs) are engineered to secrete BiTEs (Blinatumomab) upon tumor antigen recognition. BiTEs have antigen specific scFvs fused to anti-CD3 recognition domains that can stimulate bystander T cells when secreted from the infused cells [39]. T cells redirected for universal cytokine-mediated killing (TRUCKs) express IL-12 upon activation, attracting innate immune cell responses to the tumor lesion [40]. Both BiTEs and TRUCKs incorporate immune stimulating mechanisms that recruit different cells in the immune system to work together while fighting tumors, paving the way for solid tumor CAR therapy.

## Gene Transfer Techniques

Gene transfer technologies allow scientists to engineer lymphocytes with the desired CAR structure. While a variety of these methods, both viral and non-viral, are capable of introducing CAR constructs in T cells, each technique has advantages and disadvantages depending on the investigative purpose.

Non-viral methods for gene transfer include DNA/RNA electroporation and the transposon/transposase system. Relative to viral alternatives, these vectors are cheap and easy to prepare, though they often demand longer culture times [41]. Electroporation makes cells temporarily permeable, allowing genetic material to pass through the membrane. The physical disturbance to the cell utilized in electroporation can cause cell damage, and gene transfer efficiency is only 16–57% for DNA plasmids [41]. However, transgene expression following electroporation can be greater than 90% for mRNA [42]. Electroporated genes are generally only expressed for a short duration due to low genome integration, so this technique can be useful for avoiding on-target/off-tumor toxicities [43]. The Sleeping Beauty (SB) transposon system has a 60% efficiency [44], and a technique combining

electroporation and SB transposon has shown 90% specific killing of target cells *in vitro* [45, 46]. Despite the high transduction efficiency, this system requires a four-week culture [45]. The long culture times associated with non-viral gene transfer methods may impair T cell function and *in vivo* persistence.

While non-viral techniques show great promise, viral vectors, including gamma retrovirus and lentivirus, are the most common methods of gene transfer in CAR research studies (Table 1). These vectors result in long term expression of transferred genes due to successful integration into the genome [41]. Retroviral vectors typically have a transduction efficiency of 50–68% [47–49], but a recent study has shown that an improved protocol can result in a transduction efficiency of greater than 90% in murine models [48]. Lentiviral vectors are able to transduce non-dividing cells [41] and have transduction efficiencies as great as 80% [50]. Moreover, lentiviral vectors are less susceptible to gene silencing because they integrate into transcriptionally active regions [47]. However, previous clinical trials demonstrated that virus mediated gene integration was able to induce clonal expansion of hematopoietic progenitors. This safety issue still needs to be addressed, and additional advancements regarding gene transfer techniques are crucial for reducing the cost in the clinical setting.

## *In vitro* Culture System

CAR T cell *in vitro* culturing can be divided into five steps: T cell collection and purification, activation, transduction, expansion, and reinfusion (Figure 2). Suitable T cells collected from blood or tissue samples are not naturally present in large enough numbers for successful CAR therapy. *In vitro* expansion of these cells is necessary, yet prolonged expansion can generate harmful effects on the cells' *in vivo* persistence [51]. Different expansion protocols present varying strengths and weaknesses, and CAR success depends on the utilization of proper methodology.

The most common component of these methods is the anti-CD3 antibody. This molecule produces a potent proliferative signal, but it requires a costimulatory signal such as anti-CD28 in order to avoid anergy [51]. Anti-CD3/CD28 stimulation is often propagated through the use of magnetic beads coated in these antibodies. Bead stimulation results in extensive proliferation of T cells [51], and when cells are further expanded in interleukin (IL)-7 and IL-15 they display strong effector function and maintain the preferred stem/memory phenotype [52]. For long culture times, IL-21 is considered beneficial due to its role in regulation of telomerase and T cell exhaustion [46, 53, 54]. The frequency of CD4+ and CD8+ memory stem T cells is greatest following a short stimulation, while extended stimulation leads to fewer memory markers and swift differentiation [55]. Additional research warns that high CD3/CD28 bead to cell ratios yield considerably increased levels of activated cell apoptosis [56]. In contrast, culture conditions incorporating soluble anti-CD3 plus irradiated mixed mononuclear cells (MNCs) are highly effective in expanding CD8 cells [51].

Another efficient culture technique involves artificial antigen presenting cells (aAPCs) [46, 57]. aAPCs offer an affordable alternative to bead based expansion while mimicking natural dendritic cell stimulation. This technique is especially useful for long cultures times which

require several rounds of stimulation. Notably, a recent study indicated that T cells from acute lymphoblastic leukemia (ALL) and non-Hodgkin lymphoma (NHL) show different *in vitro* expansion capacity upon anti-CD3/anti-CD28 beads stimulation. However, interleukin-7 (IL-7) and IL-17 can rescue the *in vitro* expansion ability of T cells from NHL patients [58]. Therefore, optimal culture conditions should be carefully considered based on the patient's condition and tumor types. In order to meet the cGMP requirements, standardized culture systems should be set up as soon as possible.

## *In vivo* Persistence

Evidence shows that prolonged patient survival is highly associated with CAR T cell persistence [59]. Different measures prove influential in extending periods of persistence, including variations in cell subsets utilized, the CAR construct itself, and preconditioning regimens employed. T cell subsets from which CAR cells are derived affect both the persistence and function of infused cells [60]. Memory T cells, especially central memory T cells ($T_{cm}$), and less differentiated naïve cells and stem central memory T cells ($T_{scm}$) yield the longest *in vivo* survival [2, 61, 62]. Interestingly, culture systems initiated with antigen-experienced T cells could impair the *in vivo* persistence of an entire cell population [63]. On the other hand, the addition of costimulatory domains such as CD28 or 4-1BB in second generation CAR constructs enhances cell persistence [12, 22, 23, 43]. So far, 4-1BB CAR T cells have shown the best *in vivo* persistence compared to CAR cells with other costimulatory factors, which can be explained by the different metabolic patterns [64–66].

To efficiently eliminate tumor cells, reinfused CAR T cells should be able to: 1) migrate to the tumor site and infiltrate into tumor (for solid tumors); 2) resist immunosuppressive signals and respond to the tumor antigen; and 3) expand locally and differentiate into effector T cells. Fortunately, previous studies have already shown that IV infused CAR T cells are able to migrate to the tumor sites and expanded locally [67]. However, regional or intratumoral delivery of CAR T cells has shown superior therapeutic effects for solid tumor treatment, suggesting that the migration and tumor infiltration capacity of locally infused cells are far more optimal than IV delivered treatment [68–70].

T cell persistence is necessary for tumor elimination, but the immune suppressive microenvironment created by tumor cells can easily induce T cell exhaustion [71]. For example, some leukemia tumor cells can secrete indoleamine 2,3-dioxygenase (IDO), which suppresses CAR T cell function [72]. Similar to chronic virus infection, persistent tumor antigen stimulation induces T cell exhaustion. This impairs T cell persistence, especially for patients who suffer from a high tumor burden or a solid tumor [73]. However, the exhaustion of CAR T cells in recent clinical trials due to strong CD28 costimulatory factor and TCR signaling is most likely the reason severe GVHD development is so rare [74]. Despite this theory, improving the T cells' ability to avoid tumor antigen induced T cell exhaustion is becoming a popular field of study.

## Target Selection

Many factors contribute to the success of CAR cells, but persistence and proliferation are futile and dangerous unless the correct target antigen is chosen. On-tumor/off-target

toxicities caused by unsuitable targets are a serious concern in CAR T cell therapy. Adverse effects are triggered by the expression of low levels of the target antigen on off-target organs. Several promising studies demonstrated that using affinity-tuned scFvs can result in the selective targeting of antigens that are over expressed on tumor cells while sparing normal cells with low expression of the antigen [75, 76]. Lytic activity of CAR cells initiates with ~200 antigen molecules per target cell, and cytokine production commences at an antigen density of a few thousand molecules [77]. For this reason, it is very important that healthy tissue displays a minimal amount of target antigen [75, 76].

In hematologic malignancies, tumor cells and normal cells express the same, specific antigen. Therefore, the on-target toxicity is predictable and generally manageable. For example, the most common CAR T cell target is CD19, which is expressed on both healthy and malignant B cells. Fortunately, anti-CD19 CAR treatment induced Hypogammaglobulinemia has been counteracted by the administration of immunoglobulin replacement therapy [78, 79].

Many types tumor cells share common tumor antigens. For example, pancreatic, prostatic, and urinary tumors all show positive expression of PSCA [19]. Melanoma lesions, sarcomas, astrocytomas, gliomas, neuroblastomas, and leukemias all display MCSP [43]. Some studies are focused on identifying tumor antigens such as cancer-associated Tn-Glyco form of MUC1, which are recently tested as CAR T cell target for adenocarcinoma therapy adenocarcinoma [80]. However, on-target toxicity in solid tumor treatment can be extremely dangerous as most solid tumor antigens are actually normal antigens that are over expressed in tumor cells.

In addition to the lack of adequate tumor specific antigen, the tumor antigens that are known can be highly heterogenetic and can easily escape single antigen targeted therapy. Moreover, some malignant hematological cells can escape CAR T cells via lineage switching [81, 82]. For this reason, CAR T cells that can target multiple antigens are extremely important for inhibiting tumor relapse. For example, CD20 targeting CAR T cells might be effective at treating relapsed CD19 negative B cell leukemias in the future [93]. Neoantigens, which are encoded by mutated genes and do not appear in healthy tissue, are the most desirable antigen due to their tumor specific distribution [83]. In addition, neoantigens include intracellular proteins, opening the possibility of an antigen pool that is not restricted to the surface of the cell. TCR-like antibody based CAR T cells utilize an antibody which can specifically recognize peptides and the MHC complexes. Theoretically, these CAR T cells can recognize intracellular mutant peptides, but dealing with the specificity is still the biggest challenge [84–87].

Given that tumor specific antigens are rare, antigens expressed in tumor and nonessential tissues (such as CD19, CD20, CD22, BCMA, PSMA, and more) can be relatively good candidates for CAR T cell therapy [88, 89]. Regardless of whether a cell has a TCR or CAR engineered molecule, the antigen it recognizes should be abnormally up-regulated in tumor cells. Tissue distribution of a new antigen must be studied extensively before conducting a clinical trial. Moreover, due to the multiple clinical observations of antigen negative relapse, it is clear that identifying additional backup antigens could lead to a greater chance of saving

a patient's life. For example, CD20 and CD22 targeting CAR T cells could be used to treat relapsed CD19 negative B cell leukemia in the in the future [90].

## Patient Preconditioning

After infusion of CAR cells, the engineered lymphocytes compete against native blood cells for endogenous serum cytokines and also fight against suppressive T regulatory cells [91]. This environment can be detrimental to the efficacy of transferred CAR cells. In addition, when treating hematological malignancies with CAR therapy, the expression of a targeted antigen on healthy B cells can be injurious to the proliferation and impact of infused cells against targeted cancer tissue [92].

A more supportive environment can be achieved through the addition of a chemotherapeutic preconditioning regimen. Lymphodepletion and myeloablative therapies pave the way for successful infusion of engineered cells by freeing the environment of competitive native blood cells. Lymphocyte depletion prior to CAR cell infusion has greatly enhanced the ability of new cells to fight cancer. Studies using animal models have shown that CD19 CAR T cells can effectively target and lyse leukemia cells, but this effect is dependent on prior lymphodepletion [92–94]. These results were mirrored in recent clinical trials, where preconditioning regimens played an integral role in treatment success [4, 78, 95–103].

While conditioning regimens have proven successful, their effect lasts for only a few weeks or months [104]. This constricts the therapeutic window during which CAR cell infusion can be efficacious. Prolonging lymphodepletion could extend this window, but doing so is highly likely to deplete infused CAR cells in addition to host blood cells. However, the ability to engineer cells facilitates the development of CAR T cells that are resistant to lymphodepletive therapy. Through the inactivation of genes targeted by chemotherapy, a series of resistant CARs has already been created which displays antitumor activity and proliferation alongside a lymphodepletion regimen [104]. While this research could enable successful combination immunotherapy and lead to large scale utilization of a universal CAR, further research is necessary to ensure these cells can also be potent in a clinical setting. Recently, several patients died from cerebral edema attributable to fludarabine, a chemotherapy drug which has been introduced into preconditioning regimens in several centers [105].

## Toxicities

Despite the success of recent clinical trials, CAR T cell therapy can induce severe toxicity which can be lethal if not managed appropriately. As discussed previously, one such toxicity occurs when the targeted tumor antigen also surfaces on healthy tissue. This threat results in a furtive search for tumor specific antigens during preclinical studies. Diminishing on-target, off-tumor toxicities has also been attempted through the development of trans-signaling CARs discussed previously [33]. Other toxicities include allergic reactions to CAR treatment, which have induced anaphylaxis in treated patients [106].

Neurologic toxicities are linked to the migration of CAR cells to the cerebrospinal fluid and can appear in the form of headaches, confusion, facial nerve palsy, and seizures, among other symptoms [107]. These dangerous side effects may necessitate intubation or

mechanical ventilation [107]. Neurotoxic events show variable incidence rates, between 0–50%, and pose a serious threat to the future of CAR therapy [78, 107]. Recent clinical studies indicated that neurologic toxicity could be lethal and might be associated with the application of the chemotherapy drug fludarabine alongside a high dose of CAR T cells. However, the mechanism of CAR therapy induced neurologic toxicities is still largely unknown.

Tumor lysis syndrome (TLS) is caused by rapid tumor killing that results in the release of intracellular content such as ions and some metabolic byproducts that cause systematic metabolic abnormalities. TLS is most common in patients who respond well to chemotherapy and CAR T cell therapy [108]. Prophylactic allopurinol might be given prior to conditioning chemotherapy [108, 109].

Another common toxicity associated with CAR therapy is cytokine release syndrome (CRS). CRS often corresponds with the T cell proliferation that marks successful treatment [78]. It develops in response to the inflammatory cytokines released by the activation of large numbers of lymphocytes or myeloid cells [110]. Symptoms frequently first appear in the form of high fevers and other constitutional ailments resembling an infection [110]. As CRS advances, additional neurologic, hepatic, hematologic, cardiovascular, pulmonary, renal, gastrointestinal, or musculoskeletal symptoms may occur [107]. While multiple grading scales for CRS exist, most range from grades 1–4 with life-threatening symptoms displayed at grade 4 CRS [110, 111].

Biologically, severe CRS is associated with an elevation of twenty-four known cytokines [111]. Elevated cytokines include interferon-γ, IL-10, and IL-6. Increased levels of IL-10 and IL-6 are also present in patients with macrophage activation syndrome/ hemophagocytic lymphohistiocytosis (MAS/HLH), and some patients with post-CAR treatment CRS display clinical similarities to HLH patients [112]. IL-6 is an inflammatory cytokine produced by macrophages, dendritic cells, T cells, and various other cells in the body. This cytokine is involved in many biological processes, including autoantibody production, B cell maturation, bone and lipid metabolism, and more [113]. It accomplishes these functions through both classical and trans-signaling pathways, binding to the IL-6 receptor and interacting with gp130 in order to induce intracellular signaling [114, 115]. Notably, recent studies indicated that severe CRS might contribute to the lineage switch from ALL to AML, which might result in the escape of tumor cells from CAR T cell surveillance and relapse [82].

Current CRS treatments commonly target the biological pathways of IL-6. Siltuximab, an anti-IL-6 antibody, and Tocilizumab, an IL-6 receptor blocking antibody, have demonstrated success in treating CRS [116]. Tocilizumab has shown impressive clinical results, leading to rapid reversal of severe CRS without affecting long-term T cell survival [78, 112, 117]. Corticosteroids have also been used to control CRS, but they are known to inhibit T cell activation and impede the success of CAR cells against tumor tissue [110, 113, 117]. Other ways to control CRS resulting from CAR therapy include integrating switch molecules or suicide genes into CAR constructs, which would grant clinicians the ability to down regulate or even terminate CAR T responses [34–37, 118]. Additionally, because CRS is associated

with exposure to antigen presenting cells, CRS can be minimized by reducing the amount of antigen positive cells that come in contact with the CAR molecules. This could be accomplished by treating patients with lymphodepletion therapy prior to CAR infusion for B cell malignancies. Administering CAR cells in smaller doses or simply to patients with smaller disease burdens may also be effective in reducing CRS cases [119].

Some research has been conducted on models that are able to predict which patients are at risk for developing severe CRS, possibly paving the way for early intervention strategies [111]. One predictive factor is the disease burden prior to CAR infusion. In addition, cytokine analysis within the first three days after infusion can also indicate a patient's likelihood of developing life-threating CRS. One study has resulted in sixteen regression and decision tree models that offer high sensitivity and specificity in predicting which patients will develop severe toxicity [111]. Whatever the method, cytotoxicity associated with CAR therapy must be controlled before CAR treatment can truly make a difference in cancer care.

## CAR Therapy in the Clinic

Recent advancements in immunotherapy have resulted in an increasing amount of studies exploring the potential of CAR cancer therapy in a clinical setting. Many completed clinical trials show the safety and efficacy of CAR therapy (Table 1), and a multitude of ongoing trials may prove even more successful. The vast majority of clinical CARs are aimed at treating hematological malignancies. CAR molecules are most commonly transferred through viral transduction techniques, and many are cultured in OKT3 (anti-CD3) and IL-2 or CD3/CD28 magnetic beads. General trends in CAR molecule construction are also evident. 4-1BB and/or CD28 costimulatory molecules in conjunction with a CD3z/TCRz signaling domain comprise the vast majority of intracellular domains in clinical trials. Many studies employ preconditioning regiments, the most common of which is cyclophosphamide at various doses and sometimes in conjunction with other drugs. Overall, the wide variability seen in cell dosage and phenotype in addition to differences in preconditioning regimens makes it challenging to determine specific factors involved in therapeutic success. While many studies show promising results, further research must be conducted in order to ensure each patient receives effective, life saving treatment that may very well be possible through CAR cell therapy.

## Challenges

In addition to the prevalence of toxicities, more challenges in CAR therapeutic efficacy still persist. Specifically, targeting solid tumors has proven difficult. A large concern in solid tumor CAR treatment involves the search for a suitable target antigen. Mutated antigens are uncommon, and most tumor markers are also displayed on healthy cells. The mutated antigens that are truly tumor specific are often displayed beneath the cell surface, rendering CAR therapy futile, and even these antigens are not consistently expressed [120]. These difficulties can cause serious adverse effects, even death, if not properly addressed [121]. Moreover, since antigen negative relapse has already been observed repeatedly in clinical trial settings, the identification of secondary tumor antigens is also a crucial need.

The microenvironment shielding tumors is hostile for T cells. Abnormal blood flow, metabolic anomalies, acidosis, and down-regulation of adhesive molecules undermine the potency of CAR T cell therapy by inhibiting the cells' trafficking, cytolytic activity, and survival while favoring tumor growth [122]. Tumor cells are able to produce many kinds of chemokines which can attract other cells and form an immunosuppressive microenvironment [123]. CCL2 is one important chemokine produced by several tumor cells such as mesothelioma, breast cancer and pancreatic cancer. Force expressing of CCL2 receptors can enhance the CAR T cells tumor migration and therapeutic effects [124, 125]. Lymphocytes have an uphill battle in the fight against cancer, and scientists are just beginning to understand the full scope of challenges that must be overcome in treating malignant tumors.

A challenging, yet promising, development is the advancement of universal CAR cells. Universal CAR cells would allow quick and efficient treatment and would further boost the industrialization and standardization of CAR T cell production. This would pave the way for more patients to benefit from the treatment. While much work must be done to reach this state, it would allow the full potency of CAR therapy to truly change the realm of cancer treatment. Given that CAR T cells are able to efficiently migrate to and expand in tumor environments; some pioneer studies are investigating the use of CAR T cells as a carrier to deliver drugs [126, 127]. These studies further expand the potential application of CAR T cell therapy and also bring to light new challenges concerning the development of CAR structure.

## CONCLUSION

Immunotherapies have revolutionized the realm of cancer treatment and research. Specifically, CAR cell therapy has shown potent results and promises even more success in the near future. Variations in structure, gene transfer methods, *in vitro* culture techniques, target selection, and preconditioning regimens greatly affect the efficacy of clinical CAR treatments. Therefore, development of standard operating procedures (SOPs) for CAR T cell therapy, including T cell harvest, engineering, expansion, shipping, preconditioning regimen, toxicity management and so on, has become an emergency. While many challenges still loom on the horizon, CAR T cell therapy will likely become a routine treatment strategy for many kinds of tumors in the future.

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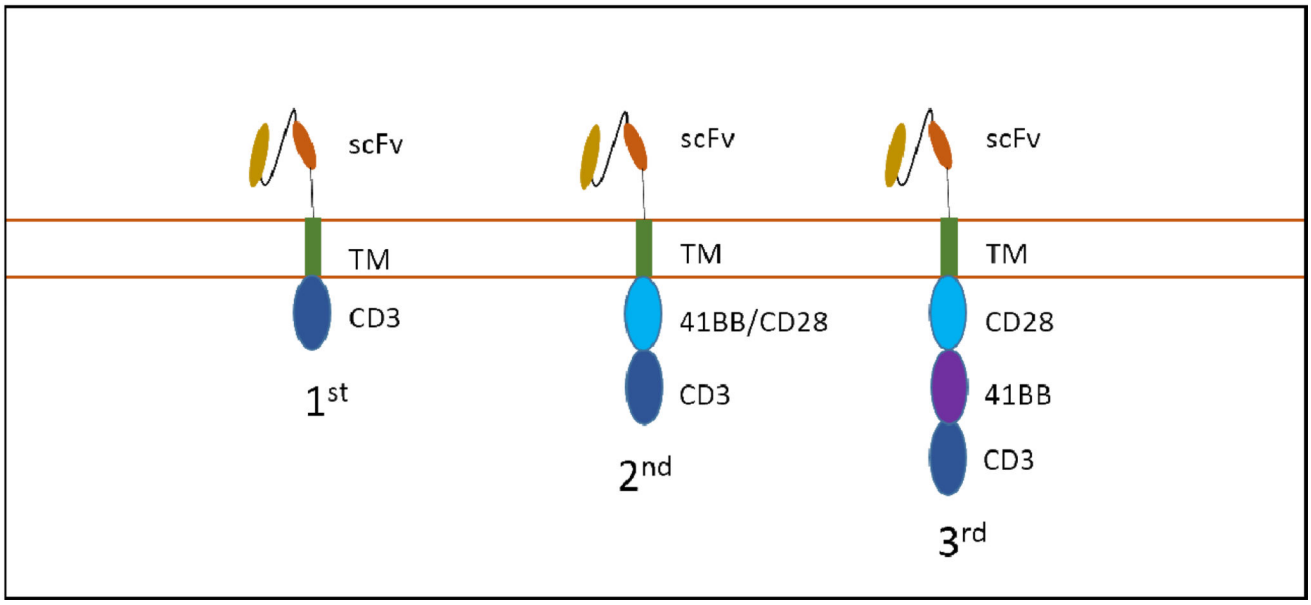


**Figure 1.**

Structures of three different generation CARs. 1st generation CARs possess the basic moieties: extracellular scFv domain, transmembrane domain and intracellular CD3 signal domain. 2nd generation CARs Introduce one costimulatory factor which further enhances the CAR T cell's *in vivo* persistence. 3rd generation CARs combine two intracellular costimulatory factors.

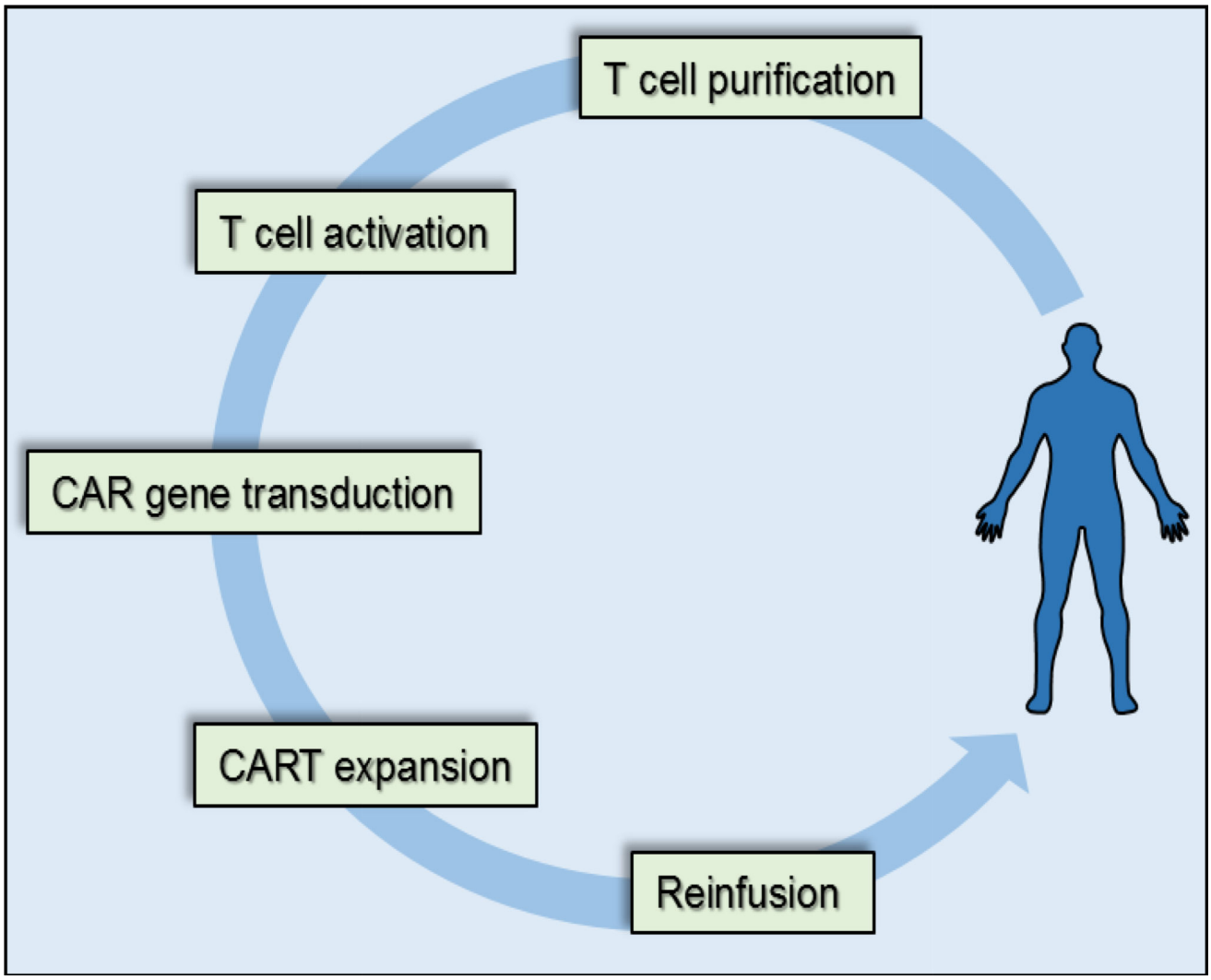


**Figure 2.**

CAR T cell therapy. Patient PBMC is collected with apheresis, and T cell subsets can be further purified with MACS microbeads. CD3/CD28 conjugated Dynobeads are used for T cell activation, and retrovirus or lentivirus infection is done during the following two days. T cells are then expanded in a large scale semi-closed culture system (Wave bioreactor) for about 10 days. After removing the beads, the CAR T cells need to be carefully formulated and cryopreserved for further quality control assays or infused back into patients.

**Table 1**

List of representative clinical trials of CAR T cell therapy.

| REF | NUMBER OF PATIENTS | DISEASE | SIGNALING DOMAIN | PRE-CONDITIONING | PRIOR ALLO-HSCT | RESPONSE | PERSISTENCE | TOXICITIES |
|---|---|---|---|---|---|---|---|---|
| (*128*) | 8 | B-cell NHL | CD28.CD3ζ | SC | Yes (n=8) | CR (n=6); PD (n=2) | Peak: ~14d, diminished by ~28d | Grade 4 hematologic toxicities; grade 3 nonhematologic toxicities |
| (*4*) | 29 | B-ALL | 41BB.CD3ζ | Cy (n=11); Cy +etoposide (n=2); Cy +Flu (n=17) | Yes (n=11) | BM remission (n=27) | Peak between 7–14d; duration at least 28d | CRS; neuro toxicities |
| (*100*) | 15 | DLBCL; lymphoma; CLL | CD28.TCRζ | Cy | N/A | CR (n=8); PR (n=4); SD (n=1); N/A (n=2) | Peak between 7–17d followed by rapid decrease | > grade 3 toxicity |
| (*96*) | 9 | B-ALL | 41BB.CD3ζ | SC (n=2); None (n=7) | Yes (n=3) | Regression (n=6); PD (n=3) | Peak 2–3w; maintained 6–12w | CRS; GVHD; neurological injury |
| (*129*) | 21 | ALL | CD28.TCRζ | Cy + Flu | Yes (8) | CR (n=14); SD (n=3); PD (n=4) | Peak at 14d, persist until ~42d | CRS; fever; hypokalemia; fever and neutropenia |
| (*82*) | 7 | B-ALL | 41BB.CD3ζ | Cy; Cy+Flu | N/A | CR (n=7), related AML relapse (n=2) | N/A | N/A |
| (*66*) | 14 | CLL | 41BB.CD3ζ | Cy + Flu (n=3); Cy/ pentostatin (n=5); Bendamustine (n=6) | None | CR (n=4); PR (n=4); NR (n=6) | Persist up to 4 years in CR patients | CRS |
| (*130*) | 20 | CLL; DLBCL; MCL; ALL | CD28.TCRζ | None | Yes (n=20) | CR (n=6); PR (n=2); SD (n=8); PD (n=4) | Peak within 2w of infusion followed by rapid decline | Grade 3–4 toxicities |
| (*95*) | 12 | MM | CD28.CD3ζ | Cy + Flu | N/A | CR (n=1); PR (n=3); SD (n=8) | Persist less than 3 months | Grades 2–4 toxicities |
| (*102*) | 16 | NHL/CLL; MM | CD28.TCRζ | Cy | N/A | CR (n=2); PR (n=1); SD (n=6); NR (n=8) | Peak: 1–2w; Duration: 6+ w | None |
| (*105*) | 32 | NHL | 41BB.CD3ζ | Cy (n=12); Cy+Flu (n=20) | 18/32 | CR (10/30) | 10d expansion, 1–3 month peak | sCRS (4/32), grade 3–4 neurotoxicity (9/32) |

SC = salvage chemotherapy; Cy = cyclophosphamide; Flu = fludarabine; NHL = non-Hodgkin lymphoma; ALL = acute lymphoblastic leukemia; DLBCL = diffuse large B-cell lymphoma; CLL = chronic lymphocytic leukemia; MM = multiple myeloma; CR = complete remission; PR = partial remission; PD = progressive disease; BM = bone marrow; SD= stable disease; NR = no response.