Molecular Therapy
Review

From concept to cure: The evolution of CAR-T cell therapy

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Chimeric antigen receptor (CAR)-T cell therapy has revolutionized cancer immunotherapy in the 21st century, providing innovative solutions and life-saving therapies for previously untreatable diseases. This approach has shown remarkable success in treating various hematological malignancies and is now expanding into clinical trials for solid tumors, such as prostate cancer and glioblastoma, as well as infectious and autoimmune diseases. CAR-T cell therapy involves harvesting a patient's T cells, genetically engineering them with viral vectors to express CARs targeting specific antigens and reinfusing the modified cells into the patient. These CAR-T cells function independently of major histocompatibility complex (MHC) antigen presentation, selectively identifying and eliminating target cells. This review highlights the key milestones in CAR-T cell evolution, from its invention to its clinical applications. It outlines the historical timeline leading to the invention of CAR-T cells, discusses the major achievements that have transformed them into a breakthrough therapy, and addresses remaining challenges, including high manufacturing costs, limited accessibility, and toxicity issues such as cytokine release syndrome and immune effector cell-associated neurotoxicity syndrome. Additionally, the review explores future directions and advances in the field, such as developing next-generation CAR-T cells aiming to maximize efficacy, minimize toxicity, and broaden therapeutic applications.

INTRODUCTION

Chimeric antigen receptor (CAR)-T cell therapy has revolutionized targeted immunotherapy, enabling the treatment of both blood and solid cancers as well as nononcologic conditions. This groundbreaking therapy emerged from decades of iterative advancements in cell-based treatments and continues to evolve to overcome significant challenges. CAR-T cell therapy combines adoptive cell transfer (ACT) with sophisticated engineering, leveraging the ability to harvest T cells and introduce synthetic constructs. The first generation of CAR-T cells combined a single-chain variable fragment (scFv) from a monoclonal antibody with a CD3ζ intracellular signaling domain (ICD), allowing target cell lysis in a major histocompatibility complex (MHC)-independent manner. Through the discovery of co-stimulatory molecules, subsequent generations of CAR-T cells were produced, allowing for increased proliferation, activation, and longevity of CAR products. This progress translated in numerous U.S. Food and Drug Administration (FDA) approvals for treating hematological malignancies. The success of CAR-T cell therapy in treating blood cancers paved the way for applications in solid tumors and other diseases, while also revealing new challenges inherent to T cells and the tumor microenvironment (TME). These include issues related to infiltration, antigen engagement, exhaustion, and on-target/off-tumor toxicity. Each challenge has driven the creation of next-generation CAR-T cell technologies, further refining their efficacy and safety. Reflecting on the milestones that shaped this field provides critical insights into the ongoing advancements, which promise to expand the potential of CAR-T cell therapy, ultimately aiming to cure patients with challenging disease conditions and improve their quality of life.

History of CAR-T cell development

In this section, we discuss the major milestones in immunotherapy that led to the development of CAR-T cell therapy, beginning with vaccination in the 18th century and culminating in successful clinical trials for treating solid tumors and applications beyond cancer in 2024. These milestones are summarized in a timeline shown in Figure 1.

The emergence of immunotherapy

Immunotherapy represents a promising strategy for treating several challenging diseases, involving the modulation of the patient's immune system by either strengthening it to combat cancer and infections or inducing immune tolerance to prevent autoimmune diseases. Immunotherapy offers renewed hope for patients with refractory, metastatic, or late-stage diseases for whom conventional treatments have failed. The first effective form of immunotherapy, which began in the 18th century, was vaccination against often lethal smallpox infection using the related cowpox virus. This approach was based on the observation that farmers who were frequently infected with cowpox virus, were resistant to smallpox due to

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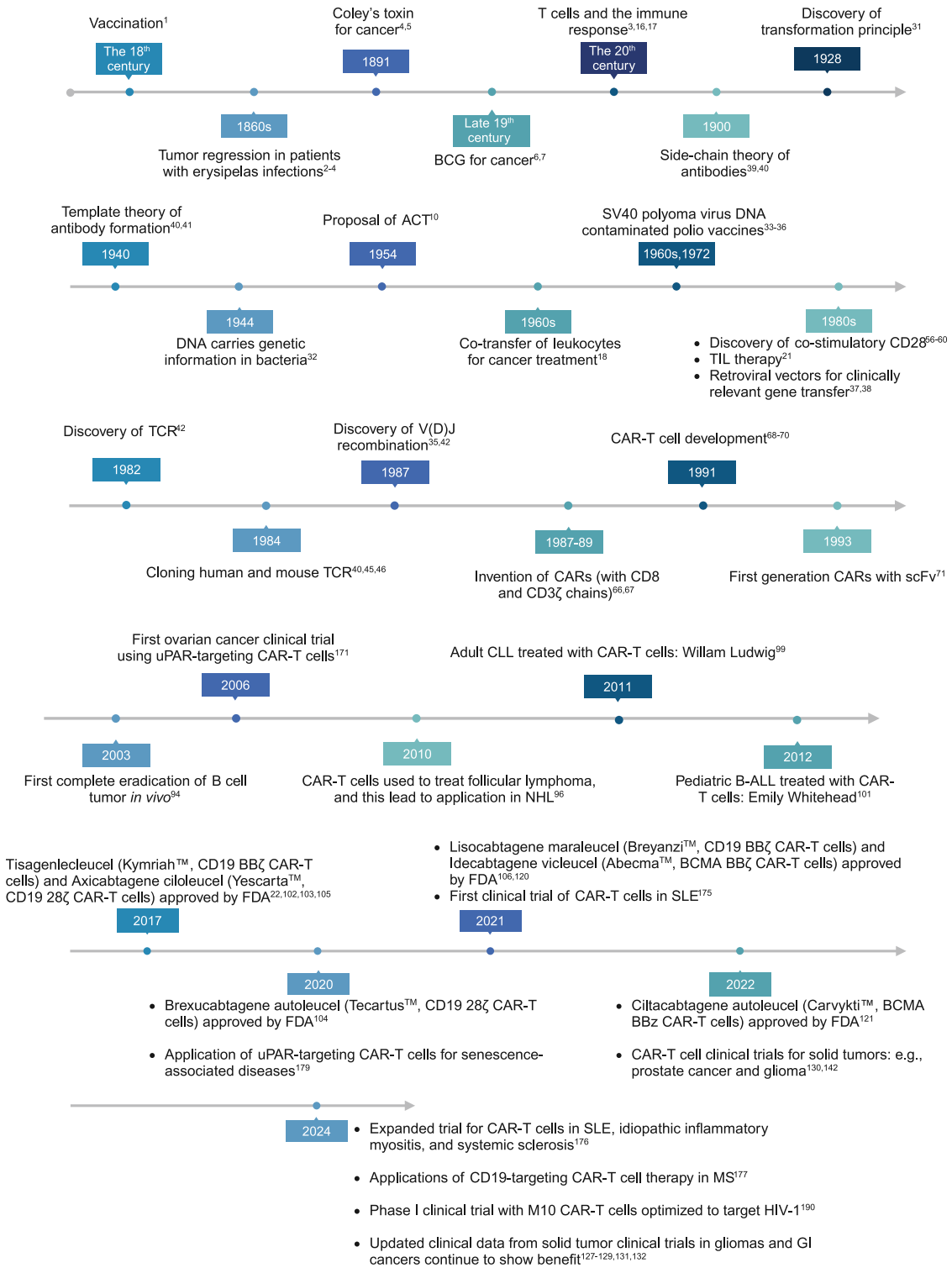

Figure 1. Timeline of key milestones in the CAR-T cell field

This figure represents a timeline of key milestones and discoveries in immunotherapy and CAR-T cell evolution, from the past to the present described in the review. B-ALL, B cell acute lymphoblastic leukemia; BCMA, B-cell maturation antigen; GI, gastrointestinal; NHL, non-Hodgkin's lymphoma; SV40, simian virus 40; uPAR, urokinase-type plasminogen activator receptor; V(D)J recombination, variable–diversity–joining rearrangement.

cross-immunity.[1] In the 1860s, two German physicians, Busch and Fehleisen, independently observed significant tumor regression in patients with erysipelas infections.[2–4] In 1891, William Coley, known today as the "father of immunotherapy," became the first to attempt harnessing the immune system to treat bone cancer.[2–4] His treatment, known as "Coley's toxins," led to significant cancer regression in more than 1,000 patients who received it.[4,5] These early scientific efforts provided initial insights into the correlation between the immune system and cancer. However, the lack of clear mechanistic understanding dampened enthusiasm for these approaches as chemotherapy and radiotherapy gained prominence later on.[4] The cancer-preventing effects of bacterial infections reignited interest in immunotherapy, particularly the use of the attenuated live bacterial vaccine, Bacillus Calmette-Guérin (BCG), as a cancer treatment.[6,7] First used in the late 19th century, BCG remains a standard of care for high-risk non-muscle invasive bladder cancer,[8] further highlighting the transformative potential of immunotherapy. BCG induces "trained immunity," the epigenetic reprogramming of innate immune cells, leading to an enhanced response upon subsequent exposures to various pathogens.

ACT

Immunotherapy can be achieved through the administration of drugs or therapeutic vaccination to induce systemic immune modification or via an established approach known as passive immunization, which is the infusion of pre-formed cells or antibodies. or what has been known as adoptive cell therapy (ACT).[9] This method induces "cell-mediated immunity," an adaptive immune response independent of antibodies or systemically administered drugs, involving immune cells (e.g., mature T lymphocyte subsets) that specifically recognize, target, and destroy infected host cells or tumor cells. The term "adoptive cell transfer" was first proposed in 1954 by Rupert Billingham, Leslie Brent, and Peter Medawar, who demonstrated that transferring immune cells from a donor to a recipient can generate immunity.[10] ACT is now considered a personalized medicine approach that has great potential in inducing targeted anti-tumor immunity, enhancing vaccine efficacy, and overcoming graft-versus-host disease (GvHD).[9]

Among the various types of immune cells, T cells have been predominantly used in ACT due to several advantages: (1) their ability to specifically target and eliminate tumor cells by recognizing antigens that are differentially expressed on the cell surface, (2) their long lifespan and capacity for immunologic memory, (3) their role in amplifying broader immune responses, and (4) their suitability for genetic engineering.[11–13] In 1957, Thomas and Burnet proposed the groundbreaking theory of cancer immunosurveillance, which is a concept that implies that the immune system can identify and destroy most precursors of tumor cells.[3,14–16] Later, in the 20th century, Schreiber, Dunn, Old, and their teams demonstrated that T cells play a crucial role in anti-tumor surveillance and in initiating anti-tumor immune responses.[3,16,17] In the 1960s, Chester Southam and colleagues demonstrated that the subcutaneous growth of human tumor autografts in patients with advanced types of cancers was inhibited by the transfer of autologous leukocytes in approximately one-half of the patients.[18] Although the studies lacked sufficient sample sizes to achieve statistical significance, they highlighted that leukocytes (or lymphocytes) from cancer patients exhibit a specific inhibitory effect on the growth of cancer cells originating from the same individual. These experiments were conducted without informed consent, raising significant ethical concerns.[19] While Southam's experiments provided early insights into the role of immune cells in eliminating cancer, they also represent an example of unethical human experimentation and its potential negative consequences, raising awareness and emphasizing the importance of patient's understanding and acceptance of therapy, and resulting in releasing polices such as the Belmont Report and patient's informed consent.[20]

The source of autologous (self) or allogeneic (donor) T cells can be either polyclonal, referring to a diverse population of T cells derived from multiple immune cell clones that recognize various epitopes (specific parts of an antigen) on the tumor or infected cells, or monoclonal, which refers to a uniform population of T cells derived from a single cell clone that specifically targets a single epitope on the antigen.[21–23] Polyclonal T cells offer broader antigen recognition, potentially enhancing the overall effectiveness of therapy and reducing the risk of immune escape, while monoclonal T cells provide highly targeted immune responses with exceptional specificity and precision, potentially minimizing off-target effects.

Tumor-infiltrating lymphocyte therapy

One specific form of ACT utilizes tumor-infiltrating lymphocytes (TILs), which are naturally occurring T cells that infiltrate a patient's tumor. While ACT is a broad term of cell therapy that uses a patient's own immune cells to fight cancer and other diseases, TIL therapy uses T cells that have already migrated into the tumor itself. The TIL therapy approach involves harvesting TILs from the tumor, activating and expanding them *ex vivo*, and then reinfusing the activated T cells back into the patient. These T cells are not genetically modified, and the process assumes they are enriched for tumor antigen-specific populations, allowing them to specifically target and eliminate tumor cells. TIL therapy was first clinically investigated and developed as a cancer treatment, particularly for melanoma, in the late 1980s by Dr. Steven Rosenberg and his colleagues at the National Cancer Institute.[21] TIL therapy has primarily been used for melanoma, which is considered an immunogenically hot tumor type, primarily due to the ease of obtaining melanoma biopsies. It has shown success in treating advanced metastatic melanoma.[24] On February 16, 2024, the FDA granted accelerated approval to Lifileucel, the first TIL therapy approved for unresectable or metastatic melanoma, marking a milestone as the first commercially available TIL therapy for solid tumors.[25–27] Several factors can influence the outcome of either ACT or TIL therapy, including *ex vivo* culture conditions and host pre-conditioning with lymphodepletion therapies such as chemotherapy and radiotherapy. As for TIL therapy, it also relies on the quality of tumor harvest during surgery and the efficiency of growth and expansion *ex vivo*.[28] Despite its promising potential, TIL therapy faces several significant barriers: (1) the difficulty of obtaining tumor biopsies, which limits patient

eligibility, particularly for certain solid tumor types; (2) insufficient TIL populations in some tumors, making this approach less viable; (3) the complex and expensive manufacturing process, creating logistical and financial hurdles beyond biological limitations; (4) toxicity associated with interleukin-2 (IL-2) administration following TIL infusion, leading to systemic and off-target side effects; and (5) T cell exhaustion and dysfunction, which can further compromise the overall therapeutic efficacy of TIL therapy.[29,30]

DNA integration in T cells

As the role of T cells in the immune system was being explored and ideas were being tested to use T cells to treat diseases, one of the monumental challenges to the field was improving antigen specificity. The discovery of TILs promised to overcome this; however, the discovery of how the T cell achieved specificity remained as one of the holy grails of immunology. Fortunately, decades of research were underway in other fields to understand how genetic information is transmitted and how to harness that technique. This started with Griffith's experiments with pneumococcal types, which introduced the phenomenon of bacterial transformation, revealing that genetic traits could be transferred between strains.[31] This discovery hinted at the existence of a transferable transforming principle.[31] Building on this, Avery and his colleagues, through meticulous experimentation, demonstrated that DNA was the molecule responsible for heredity, refuting previous beliefs that proteins were the genetic material.[32] In the 1960s, experiments on mammalian cells demonstrated that foreign DNA could be incorporated and expressed in cells. Pioneering studies focused on papovaviruses, such as simian virus 40, integrating genetic material into host genomes. These viruses showed the potential for stable and heritable genetic modification.[33–35] In 1972, Theodore Friedmann and Richard Roblin formally proposed using modified viruses for therapeutic gene transfer[36]; below we detail why it took nearly 50 years for the concept of gene therapy to enter the practice of medicine. By the 1980s, the ability to generate retroviruses with highly efficient cell lines that produce high-titer vectors that can introduce therapeutically relevant genes was achieved.[37,38] This set the stage for introducing synthetically developed receptor genes into T cells to improve antigen specificity.

T cell receptor therapy

In 1900, the German medical scientist and Nobel laureate Paul Ehrlich proposed the side-chain theory, suggesting that cells produce antibodies to fight diseases. He referred to these antibodies as "magic bullets" because they specifically target pathogens without causing unintended harm to the body.[39,40] In 1940, Linus Pauling introduced the template theory of antibody formation, arguing that antigens serve as templates that shape the formation of antibodies. These antibodies then acquire a structure complementary to the antigen, resulting in precise antibody-antigen binding.[40,41] These discoveries paved the way for subsequent experiments and the discovery of the T cell receptor (TCR).

In 1982, James Allison and his team used monoclonal antibodies and inbred mice to identify a clonally expressed T cell surface epitope on murine T lymphoma cells, which is now recognized as the TCR.[42] Building on these significant discoveries, Ellis Reinherz made a groundbreaking contribution in 1983 by defining the structure of the human TCR using specific monoclonal antibodies targeting individual T cell clones.[43] At the same time, Philippa Marrack and John Kappler conducted complementary studies in mice, offering crucial insights into the function of TCR.[44] In 1984, Tak Mak and Mark Davis identified the complementary DNA clones encoding the human and mouse TCR.[40,45,46] In 1987, Susumu Tonegawa was awarded the Nobel Prize in Physiology or Medicine for his discovery of V(D)J recombination (variable–diversity–joining rearrangement), the genetic mechanism responsible for the vast diversity of antibodies.[40,47] These landmark discoveries enabled scientists to study TCRs, often referred to as the Holy Grail of immunology. Furthermore, these studies underscored the crucial interactions between TCRs and antigens, significantly advancing our understanding of immune responses.

Unfortunately, not all patients possess T cells capable of recognizing their tumor antigens. In some cases, patients may have tumor-specific T cells; however, these cells may be insufficiently activated or poorly expanded, preventing them from reaching the numbers necessary for effective tumor rejection. For such patients, a therapeutic strategy known as TCR therapy may be used. This approach involves isolating T cells from the patient and equipping them with a synthetic or modified TCR, enabling precise recognition and elimination of cancer antigens. Unlike TIL therapy, which focuses on activating and expanding naturally occurring anti-tumor T cells, TCR therapy allows for the selection of optimal cancer-specific targets and the engineering of specific T cell subtypes.

For effective antigen recognition, T cells require the presence of suitable MHC molecules, also known as human leukocyte antigens (HLAs).[40,48] A significant mechanism of tumor immune evasion is the low affinity of TCRs for self-antigens compared with foreign antigens.[49] This challenge can be addressed using TCR therapy. By tailoring the treatment to the unique characteristics of each patient's tumor (e.g., targeting a known tumor-associated antigen [TAA]), TCR therapy could provide more personalized and effective strategies, offering hope for improved clinical outcomes. For example, T cells engineered with a TCR targeting the New York esophageal squamous cell carcinoma 1 antigen have shown promising outcomes in treating patients with multiple myeloma (MM) and synovial cell sarcoma.[50,51] Furthermore, the first engineered T cell therapy for solid tumors, using T cells modified to express a TCR targeting the HLA-A2-restricted peptide from human melanoma antigen A4, was recently approved by the FDA for the treatment of unresectable or metastatic synovial sarcoma, after encouraging outcomes in clinical trials.[52] Despite the promise of TCR therapy, a major limitation of this type of therapy is the difficulty in identifying candidate patients who express both the target tumor antigen and the corresponding HLA molecule, and this significantly restricts patient eligibility.

Unveiling the co-stimulatory role of CD28

T cell activation requires multiple signals. Signal 1 involves the TCR recognizing a specific antigen presented by MHC molecules on

antigen-presenting cells (APCs). This interaction is associated with the CD3 complex. However, signal 1 alone is insufficient for full T cell activation. Complete T cell activation requires signal 2, mediated by co-stimulatory molecules such as CD28, and signal 3, mediated by cytokines like IL-2.[53–55] In the late 1980s, Paul Martin, John Hansen, and Shu Man Fu first described the agonistic and stimulatory properties of a monoclonal antibody referred to as "clone 9.3," which later became known as CD28.[56–60] CD28 is a membrane glycoprotein and a co-stimulatory receptor that plays an essential role in TCR-mediated T cell activation. It is the primary co-stimulatory molecule for T cell activation in both mice and humans.[61] We utilized CD28 agonistic stimulation to expand T cells *in vitro* and launched several studies to explore the adoptive transfer of engineered T cells in patients with HIV/acquired immunodeficiency syndrome (AIDS).[60] Further details about developing therapies for HIV/AIDS will be provided in subsequent sections. The discovery of CD28 was crucial for advancing our understanding of T cell activation, signaling, function, and cytokine production. Moreover, it played a substantial role in the development and enhancements of cell therapies, which commonly leverage CD3/CD28 bead stimulation and IL-2 treatment for *ex vivo* T cell growth, expansion, and optimal T cell activation.

Development of CAR-T cell therapy

Despite the potential success of TCR therapy, tumors can evade and escape endogenous antigen recognition by downregulating MHC proteins essential for antigen presentation and recognition. Additionally, the rarity of finding candidate patients who express both the target tumor antigen and the corresponding HLA, as mentioned in previous sections, presents a significant challenge. An alternative strategy involves the use of CARs, which can enhance T cell specificity by targeting TAAs in an MHC-independent and unrestricted manner. This strategy circumvents the reliance on TCR-mediated antigen recognition (signal 1) and CD28 stimulation (signal 2) by incorporating both signaling functions directly into the CAR design. CAR-T cells are most commonly generated *ex vivo* using viral transduction to accomplish gene transfer. Retroviruses and lentiviruses are used with all currently approved CAR-T cell therapies.[62–64] The term "chimaera" originates from ancient Lycia and refers to a mythical beast that is part lion, part goat, and part serpent.[65] Similarly, the chimaera in CARs represents a hybrid molecule, part antibody and part TCR. This concept which also combines antibody-derived variable heavy and light regions with TCR-derived constant regions, was first described by Kurosawa and his team in 1987.[66] In 1989, Eshhar and colleagues reported on redirecting T cells to recognize antigens in an MHC-unrestricted manner.[67] Later on, CAR-T cells were independently developed in 1991 by three laboratories: Irving and Weiss,[68] Letourneur and Klausner,[69] and Romeo and Seed.[70] While CARs composed of CD8 hinge and transmembrane domain (TMD) along with a CD3ζ ICD were sufficient to activate T cells,[68] Eshhar and colleagues further refined the design in 1993 by incorporating an antibody domain (known now as the scFv) alongside the signaling domain, creating a first-generation CAR. This innovation enabled the creation of a diverse library of CAR-T cells targeting different antigens, marking a significant advancement in CAR-T cell therapy.[71]

Innovations in CAR-T cell generations

As mentioned earlier, Eshhar described the first generation of CARs in 1993, which were based on scFv derived from a monoclonal antibody and the immunoreceptor tyrosine-based activating motifs of the CD3ζ chain to mediate T cell activation.[71] This resulted in TCR-like signaling, target cell lysis, and cytokine secretion in an MHC-independent manner, both *in vitro* and *in vivo*. However, the modest activation mediated by CD3ζ led to insufficient therapeutic potency due to poor cytokine secretion, limited proliferation, and subsequent cell death via apoptosis.[72,73]

The second generation of CARs was developed to address these challenges by incorporating a co-stimulatory domain. The most well-studied co-stimulatory domain, CD28, contains a signaling domain that, when fused with CD3ζ, provides a secondary activation signal, as described earlier. This combined activation signal enhanced the ability of CAR-T cells to control tumors, attributed to increased proliferation, improved cytokine secretion, upregulation of anti-apoptotic proteins, and delayed activation-induced cell death.[74–78] Other co-stimulatory domains have also been reported, each offering different advantages compared with CD28. Examples are 4-1BB (CD137), the inducible T cell co-stimulator, and OX-40 (CD134), which are commonly used co-stimulatory domains in second-generation CARs. Several reports suggest a hierarchy in the function of these co-stimulatory domains.[79–82] Most studies indicate that incorporating either a CD28 or 4-1BB co-stimulatory domain results in greater potency for treating various cancers. Neither co-stimulation has been consistently superior, but both strategies have demonstrated similar efficacy across different settings. A notable difference observed in some studies is that CD28 co-stimulated cells exhibit higher cytokine release, while 4-1BB co-stimulated cells show greater persistence.[79,80,83–85]

The third generation of CARs was designed to combine the benefits of multiple co-stimulatory domains into a single, more potent version. The CD28-4-1BB-CD3ζ construct was predicted to exhibit the combined benefits of CD28ζ and 4-1BBζ CARs. Consistently, several preclinical studies have demonstrated the enhanced function of third-generation CARs with comparable safety profiles and anti-cancer effects. A CD19-targeting third-generation CAR-T cell clinical trial showed higher expansion and circulation compared with second-generation CAR-T cells. Some data have suggested that CAR-T cell exhaustion may result from overstimulation by multiple co-stimulatory domains.[86] More recently, a separate phase 1/2 clinical trial demonstrated a lack of the exhaustion marker CD39 in patients who responded to the third-generation CAR-T cell treatment, and higher expression in non-responders.[87]

Fourth-generation CAR-T cells (T cells redirected for antigen-unrestricted cytokine-initiated killing, also called TRUCKs) aim to improve upon second-generation CAR-T cells by incorporating an inducible cytokine that is selectively produced upon CAR-T cell activation.[88] These CAR-T cells are engineered with an additional nuclear factor of activated T cell-responsive expression cassette for the

Table 1. Various generations of CARs and their investigational status

CAR generations	Cytoplasmic domain	Key features	Clinical stage	Approved therapies
First generation	CD3ζ only.[71]	it provides T cell activation but not enough to sustain the therapeutic response.[72,206]	not under investigation.	none.
Second generation	CD28/4-1BB/ICOS/OX-40 co-stimulatory domains and CD3ζ.[79–82]	co-stimulatory signal leads to higher activation, proliferation, cytokine secretion, and survival.[79,80,83,84]	approved in some blood cancers,[102,120,153] under investigation for solid tumors.[127,128,131]	therapies targeting CD19 and BCMA with CD28 or 4-1BB co-stimulatory domains.
Third generation	multiple co-stimulatory domains with CD3ζ.[86]	multiple stimulatory signals enhance the activity profile without compromising safety—inconclusive long-term effects.	various phase 1/2 studies in blood cancers.[87]	none.
Fourth generation	CD28 and 4-1BB co-stimulatory domains, NFAT inducible immune modulators, and CD3ζ.[88]	accompanying traditional CAR-T cell activity, the inducible secreted cytokines enhance proliferation and persistence, leading to better anti-tumor capacity possible systemic cytotoxicity due to cytokine secretion in healthy tissues.	phase 1/2 clinical studies.[90]	none.
Fifth generation	CD28 and 4-1BB co-stimulatory domain, additional IL-2Rβ cytoplasmic domains, with STAT recruitment motif and CD3ζ additional development in logic gated CARs.[169–171]	it has a higher stimulation capacity for T cells by activating the JAK-STAT pathway.	pre-clinical.	none.

ICOS, inducible T-cell co-stimulator; BCMA, B-cell maturation antigen; NFAT, nuclear factor of activated T cells; JAK, Janus kinase; STAT, signal transducer and activator of transcription.

inducible expression of various cytokines, including IL-7, IL-12, IL-15, IL-18, IL-23, and combinations of these cytokines, to enhance CAR-T cell cytotoxicity and efficacy.[88,89] The first-in-human clinical trial for the treatment of refractory/relapsed MM using a fourth-generation B cell maturation antigen (BCMA) CAR-T cell engineered to secrete IL-7 and C-C motif chemokine ligand 19 (NCT03778346) showed encouraging safety and efficacy in the first two enrolled patients. Patients treated responded effectively within 1 month, experienced no adverse events higher than grade 2, and showed no relapse for more than 12 months.[90]

Fifth-generation CAR-T cells, or next-generation CAR-T cells, encompass a variety of strategies aimed at improving the safety and efficacy of CAR-T cell therapies. Building on fourth-generation CAR-T cells, next-generation CAR-T cells can incorporate membrane receptors into their design to function through a different mechanism. More examples are shown in the Harnessing synthetic biology for CAR-T cell regulation section. Table 1 summarizes the various generations of CARs, their cytoplasmic domains, key features, and clinical stages, if available, as well as approved therapies.

TCR fusion constructs

In 2019, Baeuerle et al. described TCR fusion constructs (TRuCs), in which they created a novel TCR by fusing the scFv domain of a CAR to various subunits of the TCR. They reported that TRuCs integrated into the TCR complex and were expressed whenever the TCR complex was present on the cell surface. They also noted a better safety profile than traditional CAR-T cells due to reduced cytokine release.[91] More recently, a study on mesothelin-targeting TRuCs, the first clinical trial of its kind, showed that a single infusion led to radiological tumor regression in 93% of patients. In the same study, the use of TRuCs after lymphodepletion resulted in an overall response rate (ORR) of 21% and 29% in patients with mesothelioma and ovarian cancer, respectively. While this was a milestone trial, the therapy response was limited, which was attributed to T cell exhaustion and antigen escape.[92] This line of therapy shows incredible promise for both hematological and solid tumors.

Pivotal developments in CAR-T cell treatment for cancer

CD19-targeting CAR-T cells

The first CAR-T cell trial for B cell malignancies targeted CD20.[93] However, studies by Brentjens and Sadelain indicated that CD19 is a better target for B cell malignancies and is preferred due to its higher expression compared with other lineage restricted antigens.[94] Second generation CD19-targeted CAR T cells containing CD28 or CD137 (4-1BB) signaling domains were developed and tested in preclinical models.[77,83,95] In early-phase clinical trials, CD19-targeted CAR-T cells demonstrated remarkable therapeutic efficacy in relapsed or refractory B cell tumors, such as acute and chronic leukemia and lymphoma.[96–98] William Ludwig was the first adult patient to receive CAR-T cell therapy at the University of Pennsylvania. He was diagnosed with refractory chronic lymphocytic leukemia (CLL) and achieved remission, remaining leukemia free for more than 10 years.[99] Sadly, he passed away in January 2021 due to complications

from a COVID-19 infection.[100] Emily Whitehead was the first pediatric patient with B cell acute lymphoblastic leukemia to receive CD19 CAR-T cell therapy in April 2012.[101] Emily experienced severe cytokine release syndrome (CRS), which was treated with tocilizumab, a monoclonal antibody that blocks the inflammatory protein IL-6. This treatment led to her being cancer free for more than 10 years. Subsequent large-scale clinical trials led to the FDA's approval of Kymriah and Yescarta in 2017, Tecartus in 2020, and Breyanzi in 2021.[22,102–106] Many studies have provided long-term follow-up data for patients from these trials. The ORR ranged from 44% to 91% for B cell lymphoma and CLL, with complete response (CR) rates of 28%–68%. These studies identified multiple groups of patients who maintained a response for more than 2 years. For acute leukemia, the CR rate exceeded 80% in most studies.[84,105,107–115] The role of allogeneic stem cell transplantation after CAR-T cell therapy depends on risk factors, minimal residual disease and whether the patient maintains B cell aplasia, which indicates that the CAR-T cells have continued activity.[116]

BCMA-targeting CAR-T cells

BCMA is a member of the tumor necrosis factor family and plays a role in the proliferation and maturation of B cells.[117] It is expressed by B cells but is more abundantly expressed by MM cells, although its expression can sometimes be lower.[118,119] Approved by the FDA in 2021, Abecma was first tested in the KarMMa clinical trial, where patients with relapsed/refractory MM were treated with BCMA-41BB-CD3ζ CAR-T cells. Carvykti, approved in 2022, was evaluated in the CARTITUDE-1 phase 1b/2 clinical trial. Long-term follow-up data for commercially available BCMA CAR-T cells showed that Abecma achieved a CR or better in 33% of patients, with a response duration of 19 months.[120] For Carvykti, a CR or better was achieved in 83% of patients, with a progression-free survival rate of 55%.[121]

CAR-T cells in solid tumors

As shown, CAR-T cell therapy has demonstrated remarkable success in treating hematological malignancies; however, translating this success to solid tumors has faced substantial challenges. Key obstacles include tumor heterogeneity, poor trafficking, and T cell dysfunction driven by factors in the TME.[122–124] Despite these challenges, recent clinical trials have shown encouraging safety and efficacy of CAR-T cells in multiple solid tumor types. A comprehensive review of ongoing CAR-T cell trials in solid tumors has been recently reported, and we briefly summarize the key clinical trial results here.[125,126]

Several recent clinical studies have demonstrated the potential of locally administered CAR-T cell therapy in treating aggressive malignant gliomas. In a phase 1 study involving 65 patients with recurrent high-grade glioma, the locoregional delivery of IL-13 receptor α 2 (IL13Rα2) CAR-T cells was evaluated. Among the 58 patients who received at least three CAR-T cell infusions, 50% achieved stable disease or better, including two partial responses (PRs) and two CRs. While no dose-limiting toxicities (DLTs) were reported, grade 3 or higher toxicities possibly or probably related to CAR-T cell therapy occurred in 35% of patients.[127] In another phase 1 study, six patients with recurrent glioblastoma (GBM) received intrathecal administration of bivalent CAR-T cells targeting both epidermal growth factor receptor (EGFR) and IL13Rα2. Tumor size reductions were observed in all six patients on the first magnetic resonance imaging scan performed 24–48 h after CAR-T cell administration. One patient at dose level 2 experienced DLTs, presenting with grade 3 anorexia, generalized muscle weakness, and fatigue.[128] Additionally, Choi et al. reported interim results from a phase 1 trial of three patients with recurrent GBM treated with intraventricular CARv3-TEAM-E T cells, a CAR-T cell product designed to target EGFRvIII while secreting T cell-engaging antibody molecules (TEAMs) against wild-type EGFR. All three patients experienced rapid tumor regression, with one patient (patient 2) achieving a durable response lasting more than 150 days. No adverse events exceeding grade 3 or DLTs were observed.[129] In a study involving children and young adults with H3K27M-mutated diffuse midline gliomas, 11 patients received an initial intravenous infusion of GD2-targeted CAR-T cells. Nine of these patients experienced clinical benefit and proceeded to receive additional intraventricular infusions of GD2 CAR-T cells. Among these nine patients, seven demonstrated reductions in tumor size after CAR-T cell therapy. Remarkably, one patient achieved a CR that has persisted for 30 months.[130,131] The study also compared locoregional and systemic CAR-T cell administration. The researchers found a higher presence of regulatory T cells and immunosuppressive myeloid cells after systemic intravenous CAR-T cell administration compared with locoregional intracerebroventricular administration. Additionally, higher-grade CRS was observed after systemic CAR-T cell infusion.[130]

CAR-T cells have shown promising results beyond brain cancers. For instance, Claudin18.2 (CLDN18.2)-targeted CAR-T cells demonstrated an ORR of 38.8% in 98 patients with CLDN18.2-positive gastrointestinal tumors. Notably, no grade 3 or CRS, immune effector cell-associated neurotoxicity syndrome (ICANS), treatment-related deaths, or DLTs were reported.[132] Additionally, a clinical trial evaluating CAR-T cells targeting the oncofetal antigen CLDN6, combined with a CAR-T cell amplifying RNA vaccine, in CLDN6-positive solid tumors reported an unconfirmed ORR of 33%, including one CR. One patient (5%) experienced grade 3 CRS.[133] These trial results underscore the significant advancements being made in the development and application of CAR-T cell therapies for solid tumors.

Next-generation CAR-T cell technologies

The next generation of CAR-T cells under development utilizes innovative techniques to enhance T cell potency, overcome tumor heterogeneity and antigen escape, and improve safety and specificity.[134] These advances and novel technologies are summarized in Figure 2.

Enhancing CAR-T cell potency

CAR-T cells can become exhausted or dysfunctional after prolonged exposure to persistent antigens and immunosuppressive factors in the solid TME, resulting in reduced anti-tumor efficacy and necessitating advancements to enhance their potency.[135,136] One approach to

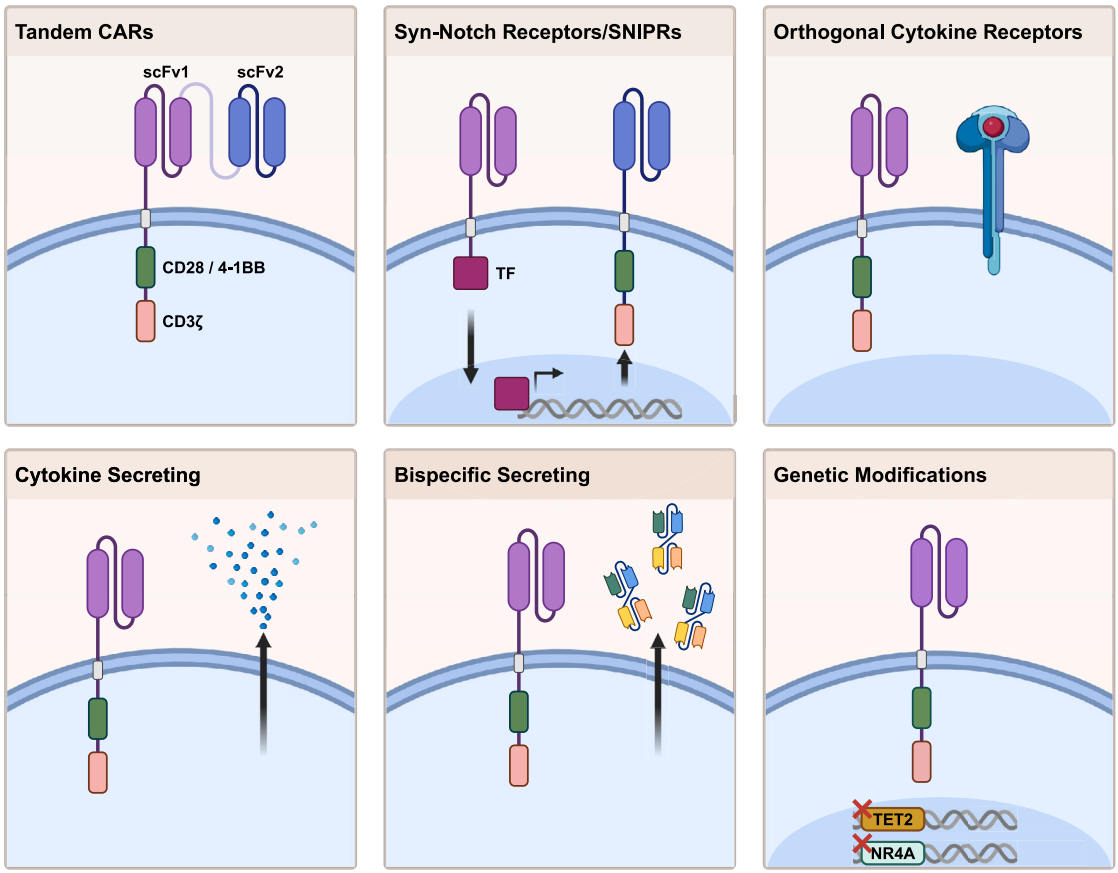

Figure 2. Next-generation CAR-T cell technologies

Next-generation CAR-T cell technologies are being developed to overcome key challenges in solid tumors, such as antigen heterogeneity, immune evasion, and the immunosuppressive TME. Examples include bispecific tandem CAR-T cells that target two tumor antigens simultaneously, reducing the risk of antigen escape and improving efficacy in heterogeneous tumors. SynNotch receptors and SNIPRs introduce a layer of control by requiring an initial tumor antigen signal to activate CAR expression or a therapeutic payload, enhancing specificity and minimizing off-target toxicities. Orthogonal cytokine receptors allow CAR-T cells to selectively respond to engineered cytokines, providing a boost without the systemic toxicities of traditional cytokine therapy. Armored CAR-T cells, which secrete cytokines like IL-18 or bispecifics, enhance T cell infiltration and persistence, improving their ability to function in immunosuppressive TME. The deletion of negative regulators of T cell persistence such as TET2 and NR4A1/2/3 has been shown to mitigate CAR-T cell exhaustion and enhance their antitumor function.

SNIPR, synthetic intramembrane proteolysis receptor; SynNotch, synthetic Notch; TME, tumor-microenvironment.

enhancing CAR-T cell potency involves engineering armored CAR-T cells capable of secreting pro-inflammatory cytokines. Steffin et al. demonstrated that the addition of IL-15 secretion can enhance the anti-tumor activity of CAR-T cells. In their study, patients were treated with either Glypican-3 (GPC3)-targeted CAR-T cells alone or GPC3 CAR-T cells engineered to secrete IL-15. Among the six patients treated with the standard GPC3 CAR-T cells, no anti-tumor responses were observed. In contrast, 33% of patients receiving the IL-15-secreting CAR-T cells achieved a PR.[137] Additionally, Svoboda et al. demonstrated that in patients with non-Hodgkin lymphomas who had relapsed after prior CD19 CAR-T cell therapy, treatment with CD19-targeted CAR-T cells engineered to secrete IL-18 achieved a 3-month ORR of 80%, with 50% of patients achieving a CR.[138,139] Another approach to enhance CAR-T cell potency is to engineer CAR-T cells with decoy receptors that bind to immunosuppressive factors within the solid TME, such as transforming growth factor (TGF)-β.[140] For example, CAR-T cells have been engineered to co-express a dominant-negative TGF-β receptor II (dnTGF-βRII), a truncated receptor that lacks the ICD required for downstream signaling. This strategy has been successfully validated in metastatic prostate cancer, which is characterized by elevated levels of TGF-β. Prostate-specific membrane antigen and six-transmembrane epithelial antigen of prostate-2 CAR-T cells engineered to express a dnTGF-βRII have enhanced anti-tumor responses in patients with metastatic and castration-resistant prostate cancer.[141–143] Another method to boost CAR-T cell potency involves leveraging CRISPR knockout screens to identify and target negative regulators of CAR-T cell proliferation, cytotoxicity, and persistence. These screens have uncovered several genes (e.g., Tet methylcytosine dioxygenase (TET2) and nuclear orphan receptors NR4A1/2/3) that limit CAR-T cell fitness, revealing

that their deletion can significantly enhance the anti-tumor activity of CAR-T cells.[136,144–152]

Overcoming antigen escape and heterogeneity

One of the key factors limiting long-term responses to CAR-T cell therapy is antigen escape. This occurs when cancer cells either downregulate or lose the expression of the target antigen, or when an antigen-negative population overgrows, serving as a resistance mechanism to the targeted therapy. Approximately 30% of patients who relapse after CD19 CAR-T cell therapy will present with CD19-negative disease.[22,153,154] Dual-targeted therapy is a promising strategy to overcome antigen escape and heterogeneity. For example, bispecific CAR-T cells targeting both CD19 and CD20 have demonstrated the potential to prevent CD19-negative relapse in preclinical models and are currently being evaluated in clinical trials.[155,156] Similarly, CD19/CD22 and CD19/BCMA bispecific CAR-T cells are under investigation for their ability to mitigate antigen escape and achieve more durable responses.[157,158] In solid tumors, where tumor heterogeneity and antigen escape are more pronounced, dual-targeting CAR-T cells have shown significant promise. For example, in GBM, bivalent CAR-T cells targeting both EGFR and IL13Rα2 address antigen escape by simultaneously engaging two distinct tumor antigens. The EGFR epitope is expressed in 50%–60% of patients, while IL13Rα2 is expressed in 50%–75% of cases, highlighting the potential of this strategy in improving therapeutic outcomes.[128] Another strategy to overcome antigen escape involves using CAR-T cells engineered to secrete bispecifics. This approach enables CAR-T cells to target one antigen while simultaneously engaging a second tumor antigen via bispecific-mediated bystander T cell activation. For example, CARv3-TEAM-E T cells are designed to target EGFRvIII while secreting TEAMs against wild-type EGFR, as described earlier.[129] STAb-T cells, or secreting T cell engagers, represent an innovative approach to overcome antigen escape and decrease exhaustion. Stab-T cells are genetically engineered to secrete bispecific antibodies that simultaneously target a TAA on cancer cells and the CD3 molecule on T cells. This dual targeting facilitates the formation of an immunological synapse between the engineered T cells and endogenous T cells and the cancer cell, enhancing the immune system's ability to recognize and eliminate malignant cells.[159]

Harnessing synthetic biology for CAR-T cell regulation

Several CAR-T cell therapies have shown promising results in solid tumors; however, on-target/off-tumor toxicities remain a significant challenge due to the scarcity of specific and homogeneous tumor antigens. Next-generation CAR-T cells leverage synthetic biology tools for precise functional regulation. For example, Roybal et al. developed synthetic Notch (synNotch) receptors composed of an extracellular binding domain, a cleavable Notch-based TMD, and a transcriptional activation domain. In this system, CAR-T cells recognize one antigen, which then triggers the expression of a secondary CAR molecule or therapeutic payload. A recent study demonstrated that a synNotch receptor designed to bind BCAN, an extracellular matrix protein localized to the brain, induced the expression of an anti-EphA2 and IL13Rα2 CAR locally, leading to the complete clearance of GBM patient-derived xenograft tumors.[160–162] The Baker and Roybal groups have recently developed an advanced, engineered receptor for soluble cellular communication and disease sensing, called the synthetic intramembrane proteolysis receptor. This receptor can be activated by both natural and synthetic soluble ligands, such as TGF-β and vascular endothelial growth factor. They successfully used the technology to direct CAR-T cells to specifically target and destroy solid tumors, where soluble disease-associated factors are present, thereby minimizing on-target/off-tumor toxicities.[163] Another example is the use of orthogonal IL-2 cytokine-receptor pairs. IL-2 is a potent cytokine that promotes the expansion and function of adoptively transferred T cells.[164] However, the systemic administration of IL-2 can cause severe, life-threatening toxicities.[165] This toxicity is also observed after the administration of IL-2 following TIL therapy, as described in previous sections.[29,30] Sockolosky et al.[166] developed orthogonal IL-2 cytokine-receptor pairs to selectively activate engineered T cells. In this system, T cells are modified to express a mutated IL-2 receptor that exclusively binds to a corresponding mutated IL-2 ligand. In preclinical models, CD19 CAR-T cells engineered to express the orthogonal IL-2 receptor and administered systemic orthogonal IL-2 exhibited enhanced proliferation and anti-tumor activity while avoiding IL-2-associated toxicities.[166–168] Another approach being tested involves adding a full-length or truncated IL-2Rβ cytoplasmic domain between CD28 and CD3ζ, along with a motif for signal transducer and activator of transcription 3 recruitment at the C-terminus of CD3ζ, to activate the Janus kinase/signal transducers and activators of transcription pathway in an antigen-dependent manner. This enhances T cell activity and promotes memory T cell generation.[169,170] Other exciting approaches include switch receptors and the development of lenalidomide-gated CARs, which contain an OFF switch that is responsive to lenalidomide.[171]

Broadening the applications of CAR-T cell therapy

CAR-T cell therapy, having shown remarkable promise to treat cancer, is increasingly being explored for its potential in treating a variety of non-cancerous chronic conditions, particularly autoimmune conditions.[172] CAR-T cells offer a novel approach to resetting dysfunctional immune system through selectively targeting and eliminating malfunctioning T or B cells (Figure 3).

CAR-T cells for autoimmunity

As shown above, CD19 has been successfully used as a target for CAR-T cells in the treatment of B cell malignancies. Researchers have sought to apply the same technology to target autoimmune conditions such as systemic lupus erythematosus (SLE), which develops through the loss of self-tolerance driven by the dysregulation of interferon pathways and the development of autoantibodies.[173] The lack of response from monoclonal antibody therapies, such as rituximab targeting CD20, has further prompted the use of CD19 CAR-T cells to target autoreactive B cells.[174] A single patient pilot was performed in 2021 and showed a substantial expansion of CAR-T cells within a week of infusion followed by a decrease in anti-double-stranded DNA autoantibodies.[175] These promising results were further

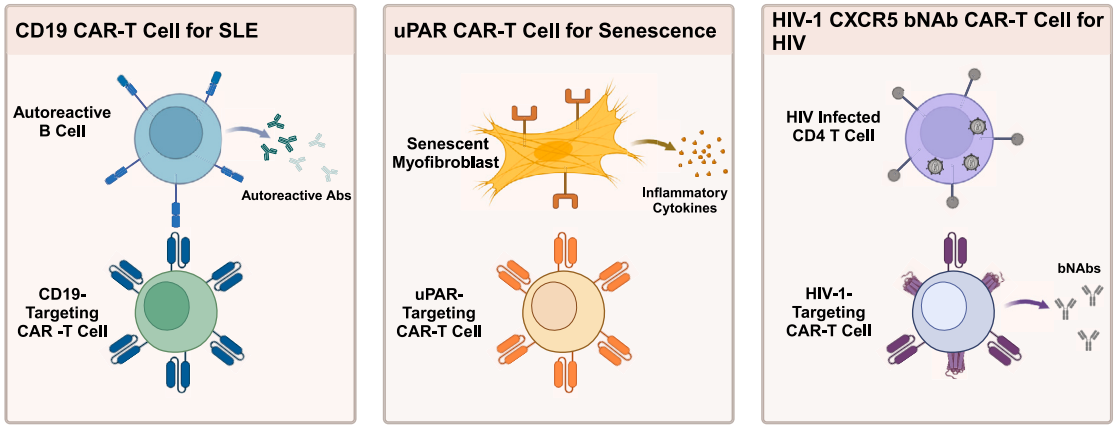

Figure 3. Expanding the horizon of CAR-T cell therapies to non-cancer diseases With the success of CAR-T therapy in blood cancers and promise in solid cancer, this technology has been expanded to autoimmune conditions, senescence-associated diseases, and HIV/AIDS

Whether through direct, applications of CAR therapies used in blood cancers, such as CD19-targetd CAR-T cells for novel treatment of SLE, or the generation of new antigen-targeting CAR-T cell products, CAR-T cell therapy has been shown to not only be a treatment for cancers, but truly a platform to be applied broadly to several other diseases. AIDS, acquired immunodeficiency syndrome.

substantiated with a follow-up study in 15 patients presenting with SLE, idiopathic inflammatory myositis, and systemic sclerosis.[176] Patients displayed similar CAR-T cells expansion, depletion of autoreactive antibodies, and improvement of clinical symptoms.[176] Responses were durable for up to 2 years after infusion and no high-grade CRS cases were seen throughout treatment.[176] These results showed promise for various disease pathologies and are currently being expanded to larger patient cohorts; long-term efficacy is being assessed. Similarly, KYV-101, a CD19-directed CAR-T cell platform, has been successfully used for the treatment of multiple sclerosis (MS), a condition that develops through autoreactive cells targeting myelin sheath proteins.[177] A case report of two patients with progressive MS was published in 2024 demonstrating reduction in autoreactive antibodies while showing no signs of ICANS despite effective CAR-T cell proliferation and trafficking.[177]

CAR-T cells for senescence-associated diseases

Cellular senescence is a state of irreversible cell-cycle arrest that occurs when cells experience stress or damage, such as DNA damage, telomere shortening, or oncogenic activation. Rather than undergoing apoptosis, senescent cells remain metabolically active but lose their ability to proliferate. This process plays a protective role by preventing the uncontrolled division of damaged cells, which could contribute to cancer. However, the accumulation of senescent cells over time is associated with age-related diseases. Senescent cells also secrete a variety of pro-inflammatory factors which can contribute to chronic inflammation and tissue dysfunction. Researchers have developed a platform to successfully ablate senescent cells through using CAR-T cells targeting urokinase-type plasminogen activator receptor, commonly upregulated on the surface of senescent cells and previously targeted for treatment of ovarian cancer.[178,179] Therapeutic efficacy was demonstrated within lung adenocarcinoma and liver fibrosis mouse models. Despite tolerance of low dose therapy, mice administered higher doses developed symptoms associated with lethal CRS.[179]

Recently, the use of CAR-T cells for the treatment of senescence-associated diseases has been expanded through targeting natural killer group 2 membrane D ligands (NKG2DL), including MICA, MICB, ULBP1, ULBP2, and ULBP3, in mouse and nonhuman primate models.[180] Researchers demonstrated successful clearance of mouse embryonic fibroblasts by mouse NKG2DL directed CAR-T cells and increased transcripts of inflammatory cytokines including interferon γ, tumor necrosis factor, and IL-6 within effector CAR-T cells.[180] Findings were further corroborated in a mouse aging model in which a decrease in expression of NKG2DL transcripts in various tissues confirmed effective targeting of senescent cells.[180] Similar efficacious results were seen in a macaque model in which senescence-associated B-galactosidase staining was significantly decreased post CAR-T cell infusion in adipose tissues.[180] These results have shown promise for future translation into clinical trials upon further toxicity and efficacy validation. As with other CAR-T cell applications, there is a potential concern for toxicity associated with CRS and long-term persistence although these can be modulated through kill-switch circuits and drug-mediated immunosuppression.[181]

CAR-T cells for HIV/AIDS

HIV has been difficult to cure due to its ability to evade the immune system, high mutational rate, and latent persistence. CAR-T cells have been seen as a promising avenue for this challenging disease for their rapid proliferation and targeted cytotoxicity.[182,183] We discovered CD28-mediated antiviral effects which were associated with the downregulation of C-C chemokine receptor 5 (CCR5), the HIV-1 co-receptor, on CD4 T cells.[60,184–186] The team went on to show that infused CD4 T cells, rendered HIV-1 resistant by CCR5 deletion using zinc finger nucleases, persisted in HIV-1-infected patients

during interruptions in antiretroviral therapy. These modified T cells were relatively resistant to HIV-1 infection *in vivo*.[28,187] The team also successfully developed a good manufacturing practice-compliant cell culture system, an advancement that facilitated the first clinical trials involving the adoptive transfer of CD4 T cells in patients with late-stage HIV/AIDS.[60,188,189] In other studies, upon repeated administration of CD4/CD3-ζ CAR-T cells to patients presenting with HIV infection, proliferation of CAR-T cells was seen through an increase in the copy number of CAR transcripts 1 day after infusion and overall persistence of CAR-T cell product was seen for more than 1 year.[182] Results showed a significant decrease in HIV transcripts in two patients with baseline plasma viremia 10 weeks into treatment.[182] Although clinical results were modest, the trial further motivated a need to bolster antiviral efficacy. Recent efforts have worked to combine HIV-1 envelope protein directed CAR construct with the follicle-homing C-X-C chemokine receptor type 5 (CXCR5) and broadly neutralizing antibodies (bNAbs), termed M10 cells, to increase cytolytic effects and target latent viral reservoirs.[190] Through the incorporation of bNAbs and CXCR5, cell-free viral particles can also be neutralized to prevent downstream entry into host cells and T cells migration to B cell follicles can be improved respectively. Upon administration of chidamide to stimulate the latent viral reservoir, treatment with M10 cells showed 10 out of 18 patients had significant reduction in HIV-1 RNA transcripts compared with baseline and all patients tolerated therapy without clinical signs of toxicity from treatment.[190] HIV-targeting CAR-T cells face several challenges, including the risk of infection within the engineered cells, low levels of target antigens, off-target toxicities, and the inability to effectively target latent HIV reservoirs. Recent advances aim to overcome these obstacles through improvements in the design and functionality of next-generation HIV-targeted CAR-T cells.

Scaling-up CAR-T cell production

As the CAR-T cell field has seen a rise in efficacy and scope, there is a growing need to increase manufacturing scale and decrease costs to increase accessibility. Key challenges include cell collection via apheresis, T cell expansion techniques, integration of CAR constructs via viral methods, cryopreservation, and overall cost of infrastructure. Allogeneic CAR-T cell therapies have shown promise to increase scalability. Through the development of these off-the-shelf therapies, apheresis is not required from each patient and rather healthy donor cells can be engineered to create a therapeutic product to be administered to multiple patients. The primary challenges in this approach are preventing GvHD and avoiding host immune rejection. Several strategies such as TCR gene editing, HLA modification, CD52 deletion, and alemtuzumab administration have been described.[191,192] To further increase accessibility and efficacy of off-the-shelf CAR-T cell products, induce pluripotent stem cells have been suggested to be used as a source of healthy donor T cells with common HLA variants present to accommodate a wide array of patients, allowing them to be used as universal treatments equipped with genetic engineering to avoid immune rejection and GvHD.[193,194] To improve cell quality and decrease manufacturing costs, researchers have optimized a 24-h protocol for manufacturing CAR-T cells with improved anti-tumor efficacy.[195] To decrease costs and risks associated with viral gene delivery, lipid nanoparticle (LNP) delivery of mRNA is being used to engineer CAR-T cells *ex vivo* and *in vivo*.[196,197] Through mRNA delivery of CAR constructs via LNPs, transient expression can be induced, obviating the need for viral vectors. The anti-tumor efficacy and proliferation capacity of this platform was further confirmed through B cell leukemia mouse models.[196] Perhaps the most exciting development is the use of targeted LNPs loaded with modified mRNA encoding a CAR and the LNP decorated with antibodies to specifically target the LNP to T cells.[197] Weissman and colleagues have recently published a report describing conjugation of a CD4-specific antibody to LNPs loaded with mRNA that specifically and efficiently targets T cells upon intravenous injection into mice.[198] In collaboration with the Weissman group, we have recently shown that T cell-targeted LNPs can deliver mRNA encoding an anti-activated fibroblast CAR in mice, producing functional CAR-T cells *in vivo*.[197] *In vivo* CAR-T cell engineering would allow for an increase in efficiency while decreasing overhead costs of expanding CAR-T cells *ex vivo*, allowing for an increased accessibility to patients. To address challenges with CAR-T cell proliferation and longevity, researchers have utilized CAR-T cell amplifying mRNA vaccines in conjunction with administration of CAR-T cells. A phase 1/2 trial has shown efficacy of boosting CLDN6 targeting CAR therapy through utilization of mRNA technology to deliver tumor antigen to APCs resulting in an overall boost in tumor clearance.[199] Furthermore, to boost immunological response to solid tumors, researchers have utilized oncolytic viruses in conjunction with CAR-T cell administration. Currently, a phase 1 trial is underway combining HER2-specific CAR-T cells combined with intratumoral injection of CAdVEC, containing an adenovirus that produces proinflammatory molecules including IL-12p70 and anti-programmed cell death ligand 1 antibody.[200] Many other methods are being used to increase scalability of CAR-T cell therapy to oncologic malignancies and beyond. Through further optimization, the promising results seen through the treatment of more than 30,000 patients with CAR-T cell therapy can be expanded to treat many more.

Long-term safety of engineered T cells

CAR-T cell therapies have revolutionized cancer treatment; however, concerns regarding their long-term safety persist, particularly the risks of insertional mutagenesis and cellular transformation. T cell homeostasis *in vivo* is controlled at the level of the TCR by clonal competition and T cells are relatively resistant to genotoxicity.[201] In the case of allogeneic T cell products with deleted TCRs, T cell homeostasis is no longer controlled at the clonal level, thus increasing the potential for second hit mutations (i.e., the Knudson hypothesis) and clonal transformation.

Insertional mutagenesis occurs when viral vectors used to introduce CAR constructs integrate into the host genome at sites that may disrupt normal gene function or activate oncogenes, potentially leading to malignant transformation. For instance, we reported that CAR DNA was inadvertently integrated into the genome of a single leukemic B cell during the manufacturing process, resulting in disease

relapse 9 months after treatment.[202] We further documented a case during treatment of CLL in which lentiviral vector integration into the TET2 gene was linked to CAR-T cell expansion and benign clonal outgrowth.[144]

In addition to these theoretical risks, clinical observations have identified actual adverse events. In November 2023, the FDA released a report describing T cell malignancies identified in patients treated with CAR-T cell therapies targeting BCMA or CD19.[203] These malignancies have been observed to develop as early as weeks following infusion and have included fatal outcomes. Consequently, the FDA has mandated updates to the box warnings for all approved CAR-T cell therapies to include the serious risk of T cell malignancies. We recently evaluated the safety outcomes in 783 patients over more than 2,200 total patient-years of observation from 38 T cell therapy trials at the University of Pennsylvania.[203] The trials used integrating gammaretroviral or lentiviral vectors to deliver engineered receptors to target HIV-1 infection or cancer. We found no evidence of high-level marking or other indications of insertional mutagenesis. Thus, the infusion of autologous engineered T cells has a low risk of transformation, as most cases are related to DNA damage from previous chemotherapy.[204] In contrast, infusions of allogeneic T cells have been reported to result in transformation, particularly when manufactured at very high insertion copy numbers using a transposon system for CAR gene delivery.[205]

CONCLUSION

In this review, we have discussed the evolution of CAR-T cell therapy from the past, through the present, to the future. From humble beginnings with the discovery of adoptive cell therapy to the ability to robustly engineer T cells to express synthetic receptors and gene circuits, the field has shown ever-growing promise for the treatment of various malignancies, infections and autoimmunity. While second-generation CAR-T cell therapies have shown significant clinical success, challenges such as improving persistence, reducing toxicity, and overcoming tumor resistance mechanisms have made advancing to the next generation more complex. Additionally, the development of novel CAR-T cell designs often requires standardized manufacturing protocols, determination of the optimal delivery route, dose optimization, and toxicity monitoring, all of which can take considerable time. However, we believe the early clinical data on next-generation CAR-T cells discussed in this review demonstrate a promising path toward regulatory approval of these therapies for standard patient care. Although many challenges await, if the past is any indication of the future, the field will rise to the occasion to continue optimizing the CAR-T cell platform, allowing for safer and more efficacious treatment options for patients.

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AUTHOR CONTRIBUTIONS

All authors contributed to the design and writing of this article and approved the final version of the article.

DECLARATION OF INTERESTS

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