Limitations and recent developments of CAR-T cell therapy

Abstract

Chimeric Antigen Receptor-T cell (CAR-T cell) therapy has become a revolutionary immunotherapeutic strategy for treating hematologic malignancies. However, despite its early success, CAR-T cell therapy continues to experience critical limitations, like limited cell persistence of CAR-T cells, susceptibility to immune escape by tumor cells, and poor efficacy in solid tumors. This review investigates the role of CAR-T cell therapy in cancer treatment and how recent developments, such as dual-antigen targeting, re-engineering of the tumor microenvironment, introduction of enhancers, and combinatorial delivery systems, are addressing ongoing limitations in the field. Referencing recent developments in both clinical and preclinical research, this review highlights that through the combination of molecular engineering and targeted modulation of the immune system's response to tumors, CAR-T cell therapy is transitioning from a narrowly focused therapy to a broader, more widely applicable therapy across cancer types, potentially reshaping the future of cancer treatment.

Introduction

Chimeric Antigen Receptor-T cell (CAR-T cell) therapy has become a revolutionary therapeutic strategy in the realm of cancer treatment. (June et al., 2018) Designed to allow lymphocytes such as T cells to target and eliminate cells expressing a specific surface antigen, Chimeric Antigen Receptors (CARs) are engineered artificial receptors that, unlike normal T cell receptors, don't rely on major histocompatibility complex (MHC) molecules to recognize antigens. (Sadelain et al., 2013) Because of CAR's ability to stimulate strong immune responses and effectively attack tumors, the US Food and Drug Administration (FDA) approved anti-CD19 CAR-T cell therapy against B-cell tumors in 2017 – an important milestone in the development of immunotherapies. (Maude et al., 2018; Neelapu et al., 2017; Schuster et al., 2017) Moreover, in spite of prior success, CAR-T cell therapy still faces many key challenges: most notably regarding the limited cell persistence of CAR-T cells, the evolution of cancer cells to avoid detection and destruction by immunotherapies, and the poor effectiveness of the therapy in solid tumors. Hence, in an effort to shape CAR-T cell therapy into a broader and more applicable cancer treatment, ongoing innovations such as bispecific CAR-T cells targeting both CD19 and CD20 antigens, inhalable nanovesicles carrying a STING agonist, integration of a CAR enhancer, and the directing of A1R expression to tumor sites are being developed to address current limitations. This review – focusing on how innovations are addressing its critical limitations – examines current applications and emerging strategies in CAR-T cell therapy.

Structure of CAR (Fig. 1)

For CARs to perform their function, they require these 4 main components: antigen-binding domain, hinge region, transmembrane domain, and intracellular signaling domain(s).

Antigen-binding domain

Produced from monoclonal antibodies, specifically their variable heavy (VH) and variable light (VL) chains, the antigen-binding domain is the part of a CAR that directly interacts with cancer cells by allowing the CAR to detect and bind to specific antigens on them. Joined together by a flexible linker, the VH and VL chains are joined together to form a single-chain variable fragment (scFv). CARs use scFvs to recognize and bind to antigens found on the extracellular surface of cancer cells and activate the T cell without the use of major histocompatibility complex (MHC) molecules. (G. Zhang et al., 2014) Moreover, beyond binding, how strongly and specifically the CAR binds to its target can also be altered by the way the VH and VL chains interact and the position of the specific parts that bind to epitopes called complementary-determining regions (CDRs). (Chailyan et al., 2011) To activate a CAR-T cell, the affinity of the antigen-binding domain to the epitope of the antigen needs to be high enough. With that said, if the affinity is too high, it will cause an activation-induced cell death or other harmful side effects in the patient. (Caruso et al., 2015; Liu et al., 2015)

Hinge region

Connecting the antigen-binding domain to the transmembrane domain, the hinge region, also known as the spacer region, is the part of a CAR that extends and positions the binding domain far enough from the surface of the T cell, allowing for the binding domain to reach and bind to the epitope of a specific antigen. Although the hinge region can provide flexibility, which helps the CAR overcome steric hindrance and reach epitopes that might otherwise be hard to access, a CAR's efficacy depends on the length and composition of the hinge. Potentially, it can affect how flexible the receptor is, how well a CAR is expressed on a T cell, how strongly a CAR signals after binding an antigen, how effectively a CAR recognizes an epitope, and the strength of the activation of the T cell. (Hudecek et al., 2015; Jensen & Riddell, 2015) As it controls the distance between the CAR-T cell and the target cell, the hinge is also important for the formation of an immunological synapse, which is needed for proper T cell activation. (Srivastava & Riddell, 2015) Generally, longer hinges are more suitable for reaching epitopes closer to the surface of the target cell or those that are part of bulky, glycosylated antigens, while shorter hinges are more suitable for epitopes that are farther away from the surface of the target cell. (Guest et al., 2005; Hudecek et al., 2015; James et al., 2008; Wilkie et al., 2008) Because different hinge lengths are commonly required for each antigen and epitope, researchers often have to test different spacer lengths to determine what functions best with a specific antigen-binding domain. Most hinge regions come from parts of CD8, CD28, IgG1, or IgG4 proteins. However, deriving hinge regions from parts of IgG proteins can be risky, as they may

interact with Fcγ receptors in the body, which not only results in CAR-T cell depletion but also reduced CAR-T cell persistence. (Almåsbak et al., 2015; Hombach et al., 2010)

Transmembrane domain

Apart from anchoring it into the T cell membrane, the transmembrane domain is a part of the CAR that can also influence how much a CAR is expressed on the surface of a T cell, affect the stability of the CAR, aid with signaling or forming of the immunological synapse and dimerize with other natural signaling proteins in a T cell. (Bridgeman et al., 2010; Guedan et al., 2018; T. Zhang et al., 2012) Because different transmembrane domains are often used depending on the hinge or signaling domains being used in a specific CAR, researchers have yet to fully understand how switching out one transmembrane domain for another affects the function of CARs. Moreover, scientists have discovered that since it can result in CARs forming dimers and joining with the cell's natural T cell receptors (TCRs), using the CD3ζ transmembrane domain could possibly help more effectively activate T cells. (Bridgeman et al., 2010) With that said, using a CD3ζ transmembrane domain in a CAR could result in it being less stable than those with CD28 transmembrane domains. (Dotti et al., 2014) Furthermore, studies have shown that depending on the combination of the transmembrane and hinge regions, CAR-T cells could behave differently.

Intracellular signaling domain(s) (Fig. 2)

To figure out how to design CARs that trigger strong and lasting T cell responses, researchers have placed immense emphasis on studying the intracellular signaling domain, also known as the endodomain. Produced in the late 1990s, the first-generation CARs only consisted of a CD3ζ or FcRγ signaling domain that contained immunoreceptor tyrosine-based activation motifs (ITAMs), which helped activate T cells when the CAR binds to its target antigen. (Gross et al., 1989; Rafiq et al., 2020) However, with just CD3ζ signaling alone, many issues arose: the T cells didn't multiply or survive well after they recognized antigens, the CARs didn't produce strong responses in the lab, and early clinical trials showed that these CARs had little to no therapeutic effect. (Brocker & Karjalainen, 1995; Hege et al., 2017a; Till et al., 2008) Hence, to address these limitations, researchers added a co-stimulatory domain to the first-generation CAR. (Imai et al., 2004; Maher et al., 2002) The two most commonly used co-stimulatory domains are CD28 and 4-1BB (CD137). Because of the addition of these domains, the second-generation CARs demonstrated improved T cell persistence, cytokine production (ie, IL-2), and response to repeated antigen exposure. (Maher et al., 2002) Ultimately, these CARs functioned well in many blood cancers such as Chronic lymphocytic leukemia (CLL), B-cell acute lymphoblastic leukemia (B-ALL), Diffuse large B-cell lymphoma (DLBCL), and multiple myeloma. (van der Stegen et al., 2015) They are now being tested in solid tumors like Glioblastoma, advanced sarcoma, liver metastases, mesothelioma, ovarian cancer, and pancreatic cancer. (van der Stegen et al., 2015) With that said, as some scientists believed that one co-stimulatory domain may not fully activate the T cell, third-generation CARs were developed to include two co-stimulatory

domains (CD28 and 4-1BB) in a row along with CD3ζ. (Pulè et al., 2005) Moreover, the effectiveness of this generation of CARs depends on the cancer type, proving that more research and testing are required. (Abate-Daga et al., 2014; Milone et al., 2009; Zhong et al., 2010)

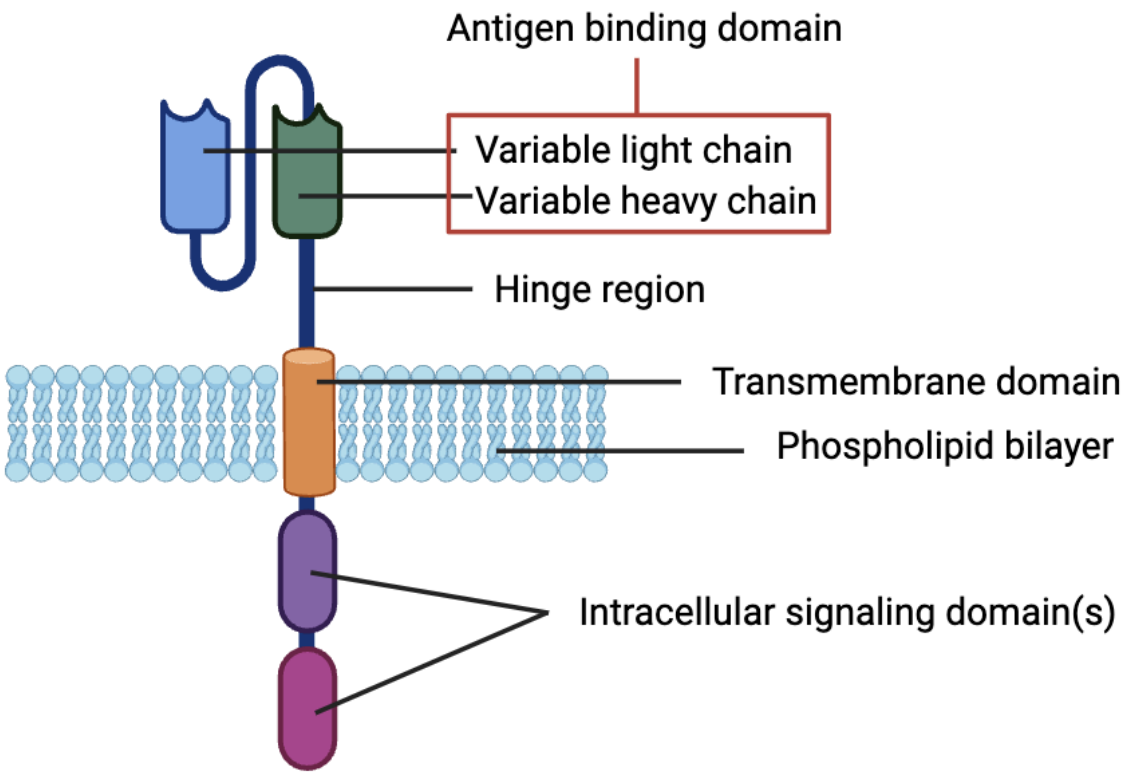

Fig. 1 Structure of CAR. CARs contain 4 main components: (1) antigen binding domain, (2) hinge region, (3) transmembrane domain, and (4) intracellular signaling domain(s)

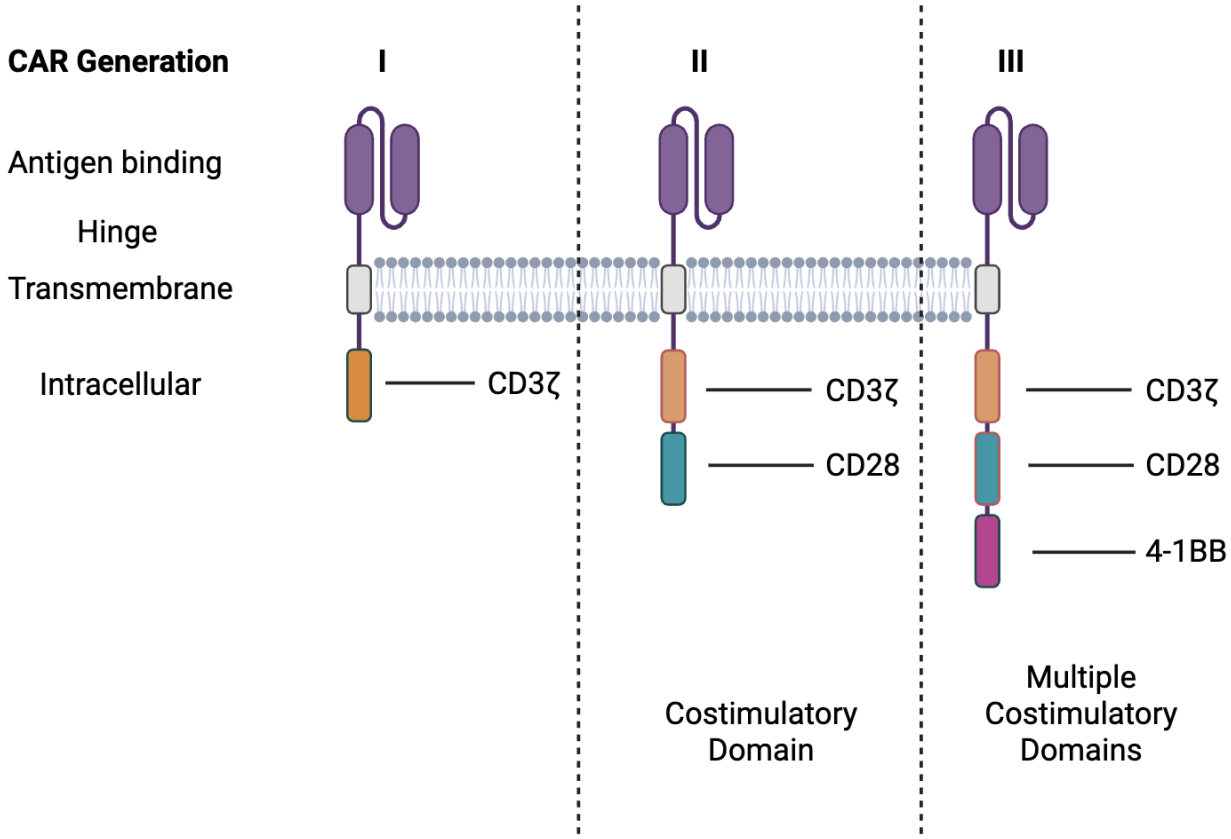

Fig. 2 Generations of CARs. The first generation of Chimeric antigen receptors (CARs) consisted of only a CD3ζ signaling domain. In addition to the CD3ζ signaling domain, the second generation of CARs consisted of a CD28 costimulatory domain, and the third generation of CARs consisted of both CD28 and 4-1BB costimulatory domains.

Introduction to CAR-T cell therapy: What is it and how is it administered to patients?

Chimeric Antigen Receptor T cell therapy (CAR-T cell therapy) is an immunotherapy that trains one's body's own T cells (a type of white blood cell) to better recognize and destroy cancer cells. Especially since other treatments like chemotherapy or stem cell transplants have failed, CAR-T cell therapy has shown remarkable success in treating blood cancers. While normal T cells can recognize and attack infected or abnormal cells, cancer cells often escape detection by sending out "off signals" or hiding their identity. (Perales et al., 2018)

To solve this problem, instead of relying on major histocompatibility complexes (MHCs), CAR-T cells are genetically modified to carry a synthetic receptor (CAR) that directly binds to antigens, which are specific proteins found on cancer cells (ie, CD19 in B-cell cancers). (Fig. 3) This allows for CAR-T cells to have a stronger targeting ability as they don't rely on other immune cells, longer-lasting memory to help them fight off relapsing cancer, and enhanced killing ability even in advanced or cancers that are harder to treat. (Maurya et al., 2025) Once inside the body, CAR-T cells continue to survive and multiply. They "patrol" the bloodstream and look for any cancer cells that return over time, providing patients with long-term protection. Furthermore, some CAR-T cells even form memory T cells and stay in the body permanently, and attack relapsed cancer. Because of its effectiveness, CAR-T cell therapy has been FDA-approved to treat several blood cancers like Acute Lymphoblastic Leukemia (ALL), Non-Hodgkin Lymphoma (NHL), and Multiple Myeloma. (Maude et al., 2018; Neelapu et al., 2017; Schuster et al., 2017) Some of the approved CAR-T products include Kymriah (tisagenlecleucel) by Novartis, Yescarta (axicabtagene ciloleucel) by Kite Pharma, Breyanzi, Abecma, and other newer products that are still under clinical trials. Especially for patients who had relapsed after several treatments, CAR-T cell therapy has shown incredibly high success rates. For example, the ELIANA trial showed an 83% complete remission at 3 months with Kymriah, and the ZUMA-1 trial showed a 54% complete remission rate in patients treated with Yescarta. (Almond et al., 2017; Locke et al., 2017)

While effective, the process for CAR-T cell therapy is personalized and takes several weeks (Fig. 4). First, through Leukapheresis, doctors collect a patient's blood and, using a machine that separates blood components, isolate the patient's T cells. (Miliotou & Papadopoulou, 2018; Mirzaei et al., 2019) Then, the isolated T cells are modified to express CARs. This can be done using viral vectors (ie. γ-retroviruses or lentiviruses) that are engineered to carry the CAR gene into the T cell's DNA (Kidd et al., 2012; Yi et al., n.d.), transposon systems (ie. Sleeping Beauty or piggyBac) which use "cut-and-paste" DNA methods with a transposase enzyme (Munoz-Lopez & Garcia-Perez, n.d.), electroporation that uses electric pulses to open cell membranes so DNA can enter (Yarmush et al., 2014), or nanoparticles which are tiny carriers that deliver CAR genes safely without the use of viruses (Smith et al., 2017). Once modified, the T cells are grown in large numbers in the lab until there are millions of CAR-T cells prepared for treatment. To lower their existing immune cells, called lymphodepletion, the patient may first receive a short round of chemotherapy. This helps the CAR-T cells work better. Following this, the CAR-T cells are infused through an intravenous

(IV). After the treatment is complete, for the first 2-3 weeks, patients are observed closely for side effects.

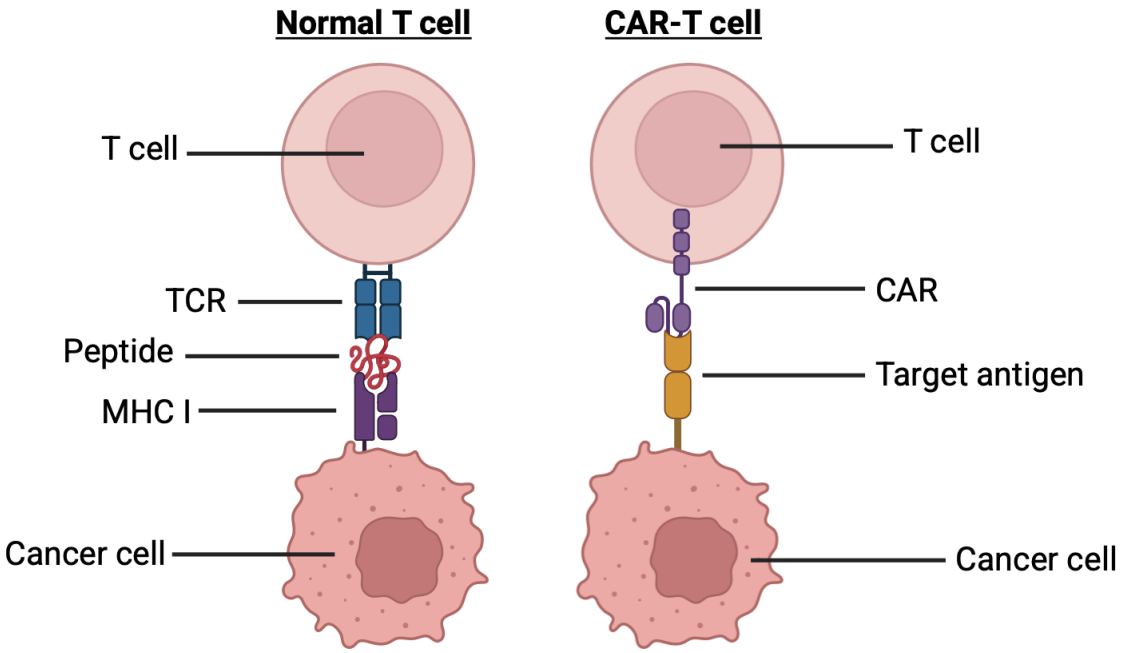

Fig. 3 Normal T cell vs CAR-T cell. Normal T cells rely on the Major Histocompatibility Complex (MHC) of the cancer cell to present the antigen for the T cell receptor (TCR) to bind to it, whereas CAR-T cells can directly bind to the target antigen using their Chimeric Antigen Receptor (CAR).

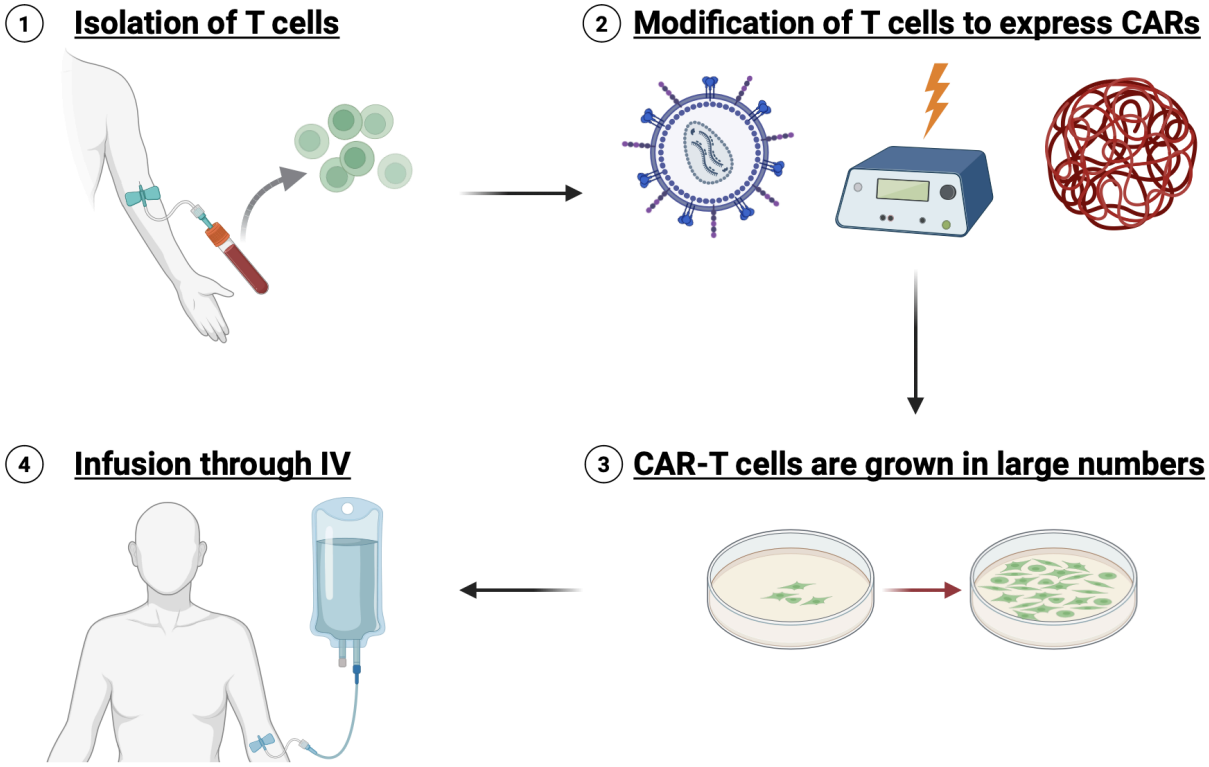

Fig. 4 Administration of CAR-T cells to patients. Patients first undergo Leukapheresis to isolate their T cells from other blood components. After isolation, the T cells are modified to express Chimeric Antigen Receptors (CARs) through viral vectors, transposon systems, electroporation, or nanoparticles. Once modified, the CAR-T cells are grown in large numbers in labs to be used for treatment. The CAR-T cells are then infused into the patient's bloodstream through an intravenous (IV).

Key limitations in current CAR-T cell therapy

Despite the initial success of CAR-T cell therapy, it continues to face many limitations – namely, the limited cell persistence of CAR-T cells, the evolution of cancer cells to avoid detection and destruction, and poor efficacy of the therapy in solid tumors.

Limited cell persistence of CAR-T cells

With the inability to persist for long periods of time, sustained anti-tumor activity and durable patient responses are hindered. Although CAR-T cells can expand and mediate tumor clearance at first, many patients are susceptible to relapse due to a decline in CAR-T cell levels over time. Because of this diminished persistence, this will cause problems specifically in hostile tumor microenvironments where immunosuppressive signals like TGF-β, IL-10, and checkpoint pathways like PD-1/PD-L1 rapidly exhaust CAR-T cells and impair their survival. (Kloss et al., 2018; Yin et al., 2018) Furthermore, the short life span of CAR-T cells may be due to inadequate co-stimulatory signaling or poor T cell fitness at the time of infusion. Given that early-generation CARs lacked co-stimulatory domains, they failed to provide the necessary signals for robust memory formation, which contributed to rapid attrition in vivo. (Brocker & Karjalainen, 1995; Hege et al., 2017b; Till et al., 2008) Even with newer generations of CARs being developed, the persistence of CAR-T cells remains variable and patient-dependent. (Abate-Daga et al., 2014; Milone et al., 2009; Zhong et al., 2010)

Evolution of cancer cells to avoid detection and destruction

Because of the adaptive evolution of tumor cells, this can lead to antigen escape. Antigen escape is a process where cancer cells downregulate or completely lose expression of the target antigen recognized by CAR-T cells. Although initial single-antigen CAR-T therapies – such as CD19 or BCMA targeting – achieved high response rates in hematologic cancers, over time, a significant number of patients relapse as the tumor cells modify themselves to avoid immune recognition. For example, due to loss or mutation of the CD19 antigen, up to 30-70% of relapsed B-ALL patients experience recurrence. (Majzner & Mackall, 2018; Maude et al., 2015) Likewise, following anti-BCMA CAR-T treatment, BCMA downregulation has been observed in multiple myeloma. (Brudno et al., 2018; Cohen et al., 2019; Green et al., 2018) Additionally, solid tumors exhibit this immune escape pattern, like reduced IL13Ra2 expression following CAR-T therapy in glioblastoma. (Brown et al., 2016) This could lead to an undermining of long-term treatment success because of the ability of tumor cells to effectively "hide" from the immune attack.

Poor efficacy in solid tumors

Due to several interrelated biological and structural barriers, the effectiveness of CAR-T cell therapy in solid tumors remains limited. Firstly, solid tumors lack ideal antigen targets that are both tumor-specific and not expressed on normal tissues. Because many antigens targeted in solid tumors are also found on healthy cells, although at low levels, this leads to on-target

off-tumor toxicity and reduced therapeutic windows. Additionally, in solid tumors, CAR-T cells face significant trafficking and infiltration challenges. Unlike blood cancers, solid tumors have dense physical barriers like the extracellular matrix and tumor stroma that impede T cell penetration. (B.-L. Zhang et al., 2016) Finally, the immunosuppressive tumor microenvironment (TME), which is rich in regulatory T cells, myeloid-derived suppressor cells (MSDCs), and Tumor-associated macrophages (TAMs) that secrete inhibitory cytokines and engage immune checkpoint pathways (ie, PD-1/PD-L1, CTLA-4), results in T cell exhaustion and poor persistence of the CAR-T cells. (Quail & Joyce, 2013; Yin et al., 2018)

Targeting poor efficacy in solid tumors: Re-engineering of the tumor microenvironment and combinatorial delivery systems

Although CAR-T cell therapy has been successful for some blood cancers such as leukemia and lymphoma (Albelda, 2024; Flugel et al., 2023), it has not yet shown the same success in treating solid tumors like lung, breast, and ovarian cancers. When treating solid tumors, the main challenge lies in the fact that the tumor microenvironment (TME) (Hou et al., 2021; Landoni et al., 2024; Lee et al., 2023), which is the space around tumor cells, is a hostile environment for immune cells. In solid tumors, the TME creates multiple layers of defense that shut down immune responses. It consists of multiple components:

Component	Example(s)	Function
Checkpoint proteins	PD-L1	They act as regulators of the immune system and prevent CAR-T cells from attacking tumor cells. While they function as "off switches" for the immune system and ensure it doesn't damage healthy tissues, they allow for tumors to escape immune destruction.
Suppressive immune cells	Regulatory T cells (Tregs) and Myeloid-derived suppressor cells (MDSCs)	They release anti-inflammatory signals to further weaken CAR-T cells.
Adenosine	–	High levels of it build up in the low-oxygen TME and signal through the A2AR receptor on CAR-T cells to slow them down.

Overall, these components shorten the lifespan and reduce the efficacy of CAR-T cells when destroying solid tumor cells.

Hence, to tackle the limited efficacy of CAR-T cell therapy in solid tumors, the most direct approach taken by scientists was to target modifying the TME to make it more suitable for CAR-T cell activity. For example, scientists engineered inhalable nanovesicles to overcome the hostile TME (Fig. 5) (Zhu et al., 2025). Inhalable nanovesicles are small, biodegradable vesicles that contain therapeutic molecules that could be directly inhaled into the lungs. Each nanovesicle carries an anti-PD-L1 single-chain variable fragment (scFv) and a STING agonist (cGAMP). The scFv is a small antibody piece that binds to PD-L1 on tumor cells and blocks the PD-1/PD-L1 interaction, allowing for the CAR-T cells to remain active. The cGAMP is a molecule that activates the Stimulator of Interferon Genes (STING) pathway and leads to the production of type I interferons such as IFN-β and the attraction of antigen-presenting cells such as dendritic cells to help amplify the immune response. Success of these inhalable nanovesicles was observed in mouse models of lung cancer where they triggered local immune activation without systemic

toxicity, increased levels of molecules (ie. IFN-β, IL-2, and CXCL9) that help attract and activate more CAR-T cells, reduced exhaustion markers (ie. PD-1, LAG3, and TIGIT) on CAR-T cells, promoted the formation of $TCF1^+$ memory-like T cells which are known for their persistence and self-renewal properties, and resulted in stronger tumor shrinkage, delayed relapse and improved survival of CAR-T cells.

While scientists can target the TME, they can also focus on changing the CAR-T cells themselves to resist suppressive signals from within the TME. For example, scientists discovered that adenosine, which is released in high amounts by tumors, functions by signaling through the A2AR receptor to deactivate CAR-T cells. In response to this, because A1R signals in the opposite direction of A2AR and enhances T cell activation and survival when activated, they used CRISPR-Cas9 gene editing to insert the gene for the A1R receptor into CAR-T cells(Sek et al., 2025). These newly engineered CAR-T cells with the A1R receptor resulted in higher production of immune-stimulating cytokines (ie, IFNγ, TNFα, and IL-2), reduced expression of exhaustion markers (ie, PD-1 and TIM-3), and increased expression of CD69 and IRF8, which indicated stronger immune readiness and potential for longer-lasting memory. Furthermore, to make these effects even more stable, scientists added a transcription factor called NR4A2, which acts like a program that helps CAR-T cells, especially in environments full of stress and inflammation, maintain anti-suppressive gene expression over time. In mice with tumors, CAR-T cells engineered with A1R and NR4A2 resulted in sustained tumor disappearance, preserved stem-like and central memory T cell populations that are essential for long-term immunity, and eliminated the need for repeated dosing of CAR-T cells.

In combination, these strategies could potentially revolutionize CAR-T cell therapy for increased efficacy in solid tumors, which make up the vast majority of cancers.

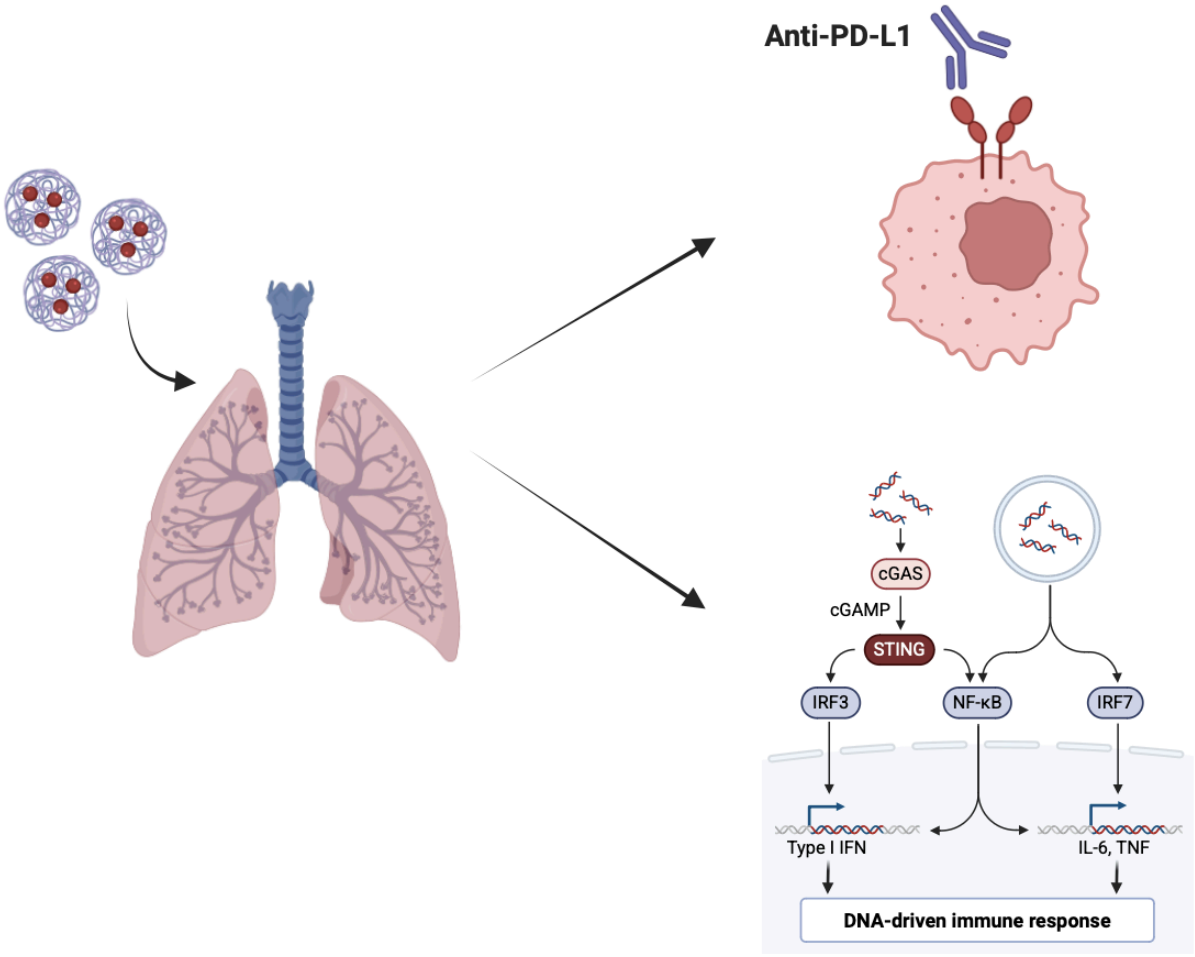

Fig. 5 Using inhalable nanovesicles to overcome the hostile tumor microenvironment (TME). Inhalable nanovesicles that contain therapeutic molecules are inhaled into the patient's lungs. Upon inhaling these nanovesicles, they block the PD-1/PD-L1 interaction, allowing for CAR-T cells to remain active, and they activate the Stimulator of Interferon Genes (STING) pathway, initiating a strong immune response.

Targeting susceptibility to immune escape by tumor cells: Dual-antigen targeting

Another major limitation in CAR-T cell therapy is the downregulation or loss of the target antigen on tumor cells (Neelapu et al., 2017; Tong et al., 2020). This facilitates tumor cells escaping from immune cells, leading to relapse. This phenomenon is well-documented in B-cell malignancies (ie. diffuse large B cell lymphoma (DLBCL) and B cell acute lymphoblastic leukemia (B-ALL) where mechanisms such as missense mutations, frameshifts, or alternative splicing of target antigens like CD19, can evade recognition by CARs (*Convergence of Acquired Mutations and Alternative Splicing of CD19 Enables Resistance to CART-19 Immunotherapy | Cancer Discovery | American Association for Cancer Research*, n.d.).

Hence, to reduce the likelihood of relapse due to the loss of target antigens on tumor cells, dual-antigen targeting strategies – such as by simultaneously engaging two distinct antigens associated with tumors – have been developed (Dai et al., 2020; Grada et al., 2013; Hamieh et al., 2019; Hegde et al., 2016; Majzner & Mackall, 2018; Zah et al., 2016).

For example, in the phase I/II of a clinical trial, scientists developed a tandem CAR construct with the ability to bind to both CD19 and CD20 (Wang et al., 2024). CD19 and CD20 surface proteins are ideal targets for cancer therapy as they are highly expressed almost exclusively on B lymphocytes and absent on most other cell types. The construct, connected via a flexible linker, integrated single-chain variable fragments (scFvs) specific for CD19 and CD20 and incorporated the hinge and transmembrane domains of CD8 with 4-1BB and CD3ζ signaling domains (Fig. 6). In preclinical assays, the CD19/20 CAR-T cells demonstrated efficacy in destroying cancer cells that expressed either CD19, CD20, or even both, and they released similar levels of cytokines as single-target CAR-T cells. The release of cytokines is important as they function as chemical messengers that enable immune cells to communicate and coordinate an immune attack against harmful substances such as cancer cells.

Clinically, this dual-targeting approach showed promising efficacy. In the group of patients with relapsed or refractory (R/R) B-cell non-Hodgkin lymphoma (NHL), with a 12-month overall survival rate of 81.82% and progression-free survival of 60%, 81.8% of patients experienced complete remission. Importantly, even in the presence of antigen loss for one target, the bispecific CAR-T cells remained robust. This suggests a decreased risk of immune escape in these CAR-T cells compared to single-target CAR-T therapy. Through single-cell RNA sequencing, results showed that the main CAR-T cell groups after infusion contained high levels of genes that are involved in immune signaling, especially those that help activate the body's first-line immune defenses and those that aid in the destruction of cancer cells. Despite changes in the way cancer cells display their surface antigens to allow them to avoid detection from the immune system, likely due to the mentioned features, these CAR-T cells sustained anti-tumor activity.

Furthermore, beyond mitigating relapse driven by antigen loss, this dual-antigen targeting strategy also broadens the therapeutic window for patients with heterogeneous antigen expression profiles. Through the maintenance of targeting capability against tumor cells that

have downregulated one antigen, these CD19/20 CAR-T cells exemplify how rational CAR design can directly address a critical resistance mechanism in immunotherapy.

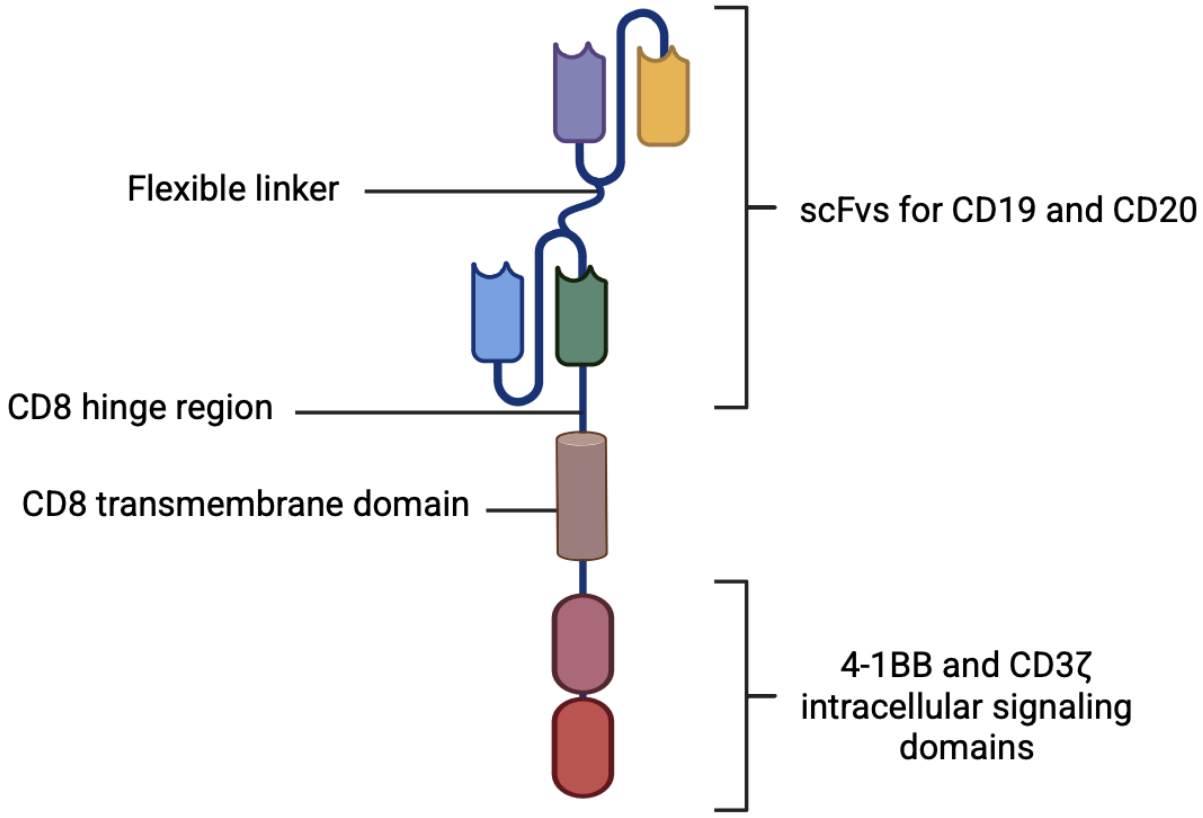

Fig. 6 Structure of CD19/20 CAR-T cell. The CD19/20 CAR-T cells have CAR constructs that contain an antigen-binding domain with single-chain variable fragments (scFvs) for CD19 and CD20, a CD8 hinge and transmembrane domain, and 4-1BB and CD3ζ intracellular signaling domains.

Targeting limited cell persistence: CAR enhancer (CAR-E)

After the initial CAR-T cell treatment, CAR-T levels often deplete, resulting in patients being vulnerable to relapse. Hence, new ways to target the limited cell persistence of CAR-T cells are being developed to improve the long-term effectiveness of the therapy.

For example, scientists have designed a CAR enhancer (CAR-E) to prolong CAR-T durability without broad cytokine exposure (Rakhshandehroo et al., 2025). CAR-E functions by coupling a tumor-antigen binder to a low-affinity Interleukin 2 (IL-2) dimer that engages only antigen-bound CAR-T cells. This ensures that the cis IL-2R signaling is delivered precisely where it's required. While not compromising its efficacy by preserving cytotoxicity and not activating other cells, such as non-transduced T cells that might hurt the safety and efficacy of the therapy, CAR-E switches on STAT5 and early activation in CAR-T cells (Fig. 7). By switching on STAT5 and early activation in CAR-T cells, this will result in bigger, longer-lived, and more effective CAR-T responses. The CAR-E only functions when both the CAR endodomain and the IL-2 receptor signal, and it's pulled inside the CAR-T cell itself via the IL-2 receptor (IL-2R), which helps keep the effect contained.

In vivo, dose-dependent multiplication and enhanced cell persistence of CAR-T cells in blood, spleen, and bone marrow, as well as enrichment of memory phenotypes such as central memory (T_CM) and stem-like memory (T_SCM), are produced through brief, pulsed dosing (serum t½ ~1.5 h). Importantly, CAR-T cells that are treated with CAR-E can be maintained even at lower CAR-T doses or in the absence of a tumor and can re-proliferate upon interaction with cancer cells that reappear. This supports an on-demand "booster" paradigm to sustain CAR-T cell levels after they peak and drop.

Overall, CAR-E is a safe method that improves cell persistence of CAR-T cells, as its low-affinity IL-2 is weak enough that it prevents activation of nearby, non-CAR-T cells, and it clears from the body quickly, allowing doctors to fine-tune the dosage. Furthermore, since the CAR-E mainly consists of components derived from humans, it minimizes predicted immunogenicity. The design of the CAR-E is also modular, meaning the binding piece to use CAR-E with other CAR targets (ie, CD19/CD22 or GD2) can be swapped, potentially even in solid tumors. In practice, scientists can initially carry out manufacturing that starts with memory-like T cells (T_SCM/T_CM) and keeps the culture time short to ensure cells stay youthful and durable. Then, CAR-E provides targeted IL-2 signals after infusion to maintain and expand those memory-leaning CAR-T cells in patients. Additionally, as CAR-E clears fast, clinicians can briefly give small pulses of CAR-E when CAR-T cell levels start to dip or when tumor signals reappear, then stop. This ensures the maintenance of cell persistence while minimizing negative side effects like cytokine toxicity, where the immune system releases too many cytokines all at once and triggers undesirable outcomes, or unwanted T cell activation.

When taken together, it's a targeted, scalable method to ensure CAR-T cell persistence while ensuring maintenance of control over systemic cytokine exposure.

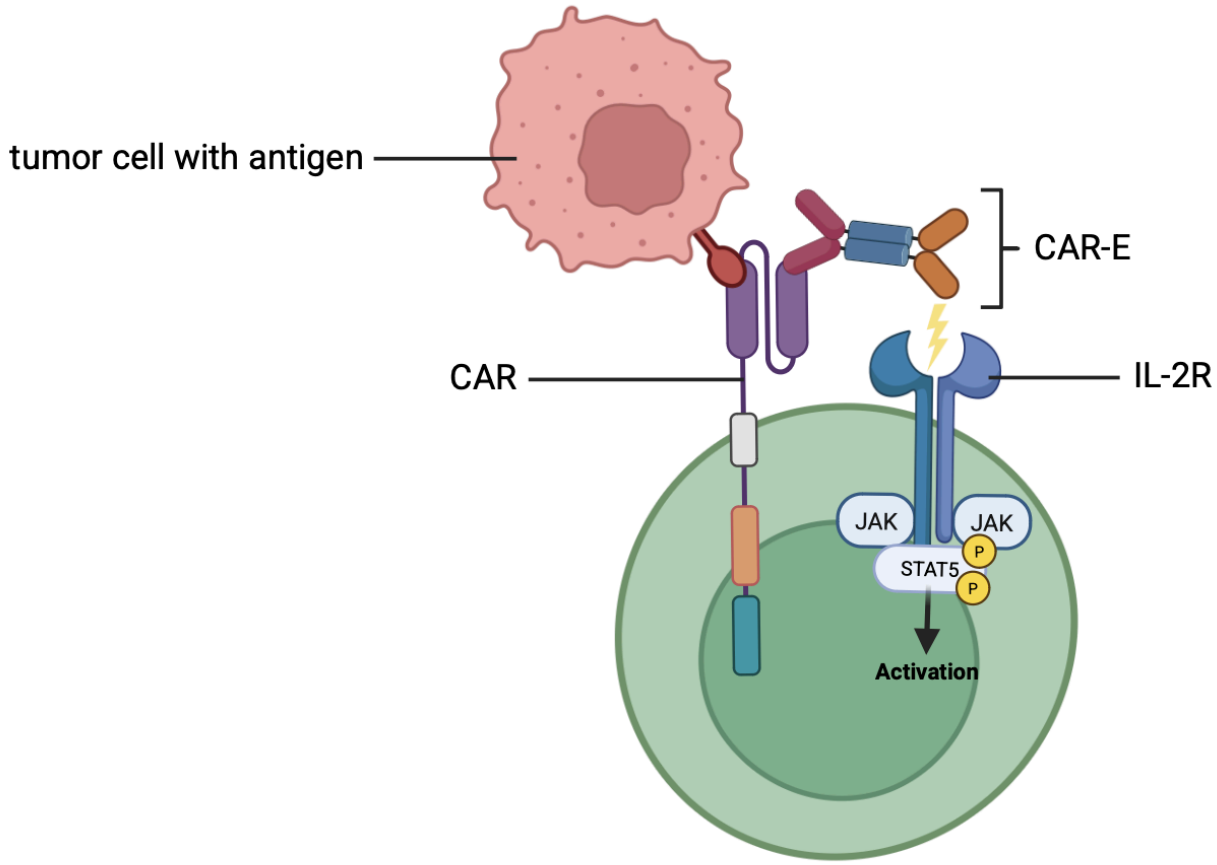

Fig. 7 CAR enhancer (CAR-E) mechanism. CAR-E couples tumor-antigen recognition with a low-affinity IL-2 dimer that engages the IL-2 receptor (IL-2R) only in CAR-T cells bound to antigens. This activates the JAK/STAT5 pathway, promoting early activation, enhanced persistence, and stronger cytotoxic responses without broad cytokine exposure.

Conclusion

Offering unprecedented remission rates in hematologic malignancies and giving rise to personalized, immune-based treatment for cancer, CAR-T cell therapy is one of the most transformative innovations in modern oncology. However, because of challenges such as limited cell persistence, susceptibility to immune escape, and poor efficacy in solid tumors, its potential to be used as a treatment in clinical practice has been hindered. Recent advances, such as re-engineering the tumor microenvironment through the use of inhalable nanovesicles and A1R-directed CAR-T cells, developing dual-antigen targeting constructs to counter antigen loss, and integrating CAR enhancers to sustain memory-like T cell populations, demonstrate that these limitations are neither overcome nor insurmountable. Together, the rise of these strategies indicates a shift in the therapy from a reactive treatment toward an increasingly proactive, precision-focused CAR design where CAR-T cell therapy is tailored to the tumor's biology and individual patients' differing needs. As deeper immunological insights are being discovered and innovations are arising, CAR-T cell therapy is anticipated to evolve from a niche intervention method for specific blood cancers into a more versatile and adaptable platform that's capable of overcoming heterogeneous antigen expression, hostile microenvironments, and short-term durability. Whether CAR-T cell therapy fulfills its promise of becoming a universal, durable, and safe cancer treatment depends on the continued integration of preclinical breakthroughs into clinical practices accessible for patients worldwide.

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The abstract is well-written and provides a comprehensive summary of the review's arguments. The introduction is succinct, but is able to lay out the necessary questions of why it's necessary to discuss the future of CAR T cells. However, there are two things I would ask the author to consider. First, potentially make the overarching research topic more focused – it's currently very general, and maybe it would be good to have a more specific research topic that seems novel in the field. Second, it would be good to touch a bit more on what a CAR T cell is in the introduction. I know this is discussed later in the review, but it would be good to bring it here now.

Overall, the body of the review has good content, there is quite a lot of summarizing of the information, and a lack of analysis of this info. I would urge the author to think about the 'so what' question when going through the body, and thinking about whether some critical analysis can be included in different parts. There are also a few specific comments that I've made on a few sections of the body which I've put below.
Overall, the style of writing is good, and what you would see in a journal publication. However, there are occasions where the writing becomes slightly informal. The author should consider the audience they're writing to ensure this consistency, and do not use conversational language for writing. For example, there are moments when this is aimed at scientific communication for people that are not in science, "While normal T cells can recognise and attack infected or abnormal cells, cancer cells often escape detection by sending out "off signals" or hiding their identity." While this is okay, I would recommend maintaining a professional style of writing.
The figures need references to the papers which they were inspired from. Furthermore, I would suggest revising the image in Figure 6. First of all, it should be mentioned in the figure legend that this is a tandem CAR – all figure legends should be able to stand on their own, and not require context from the rest of the writing. Second, the flexible linker in the figure is incorrect, as the way it's drawn is scientifically not possible (coming from a protein engineering perspective). I would suggest referring to this link for a nicer representation: https://www.frontiersin.org/journals/immunology/articles/10.3389/fimmu.2025.1546172/full.

The conclusion summarises the review well, but it would be great for the author to think critically and creatively about what the next steps are going to be for the field. For example, moving beyond cancer towards autoimmune diseases such as multiple sclerosis and lupus. Additionally, what is the potential for developing off-the-shelf allogeneic CAR T cells, or using a more innate-like immune cell such as γδ T cells which has a higher probability of penetrating the solid tumour, and offers other mechanisms such as antibody-dependent cellular cytotoxicity (ADCC). I recommend having a look at Jonathan Fisher's papers from UCL to learn more about γδ T cells.

Overall, the main revisions that will need to be made are adding more critical thinking into the body, minor improvements in writing style, and expanding upon points in more detail.

A few additional notes:
Ensure that the in-text citations are before punctuation, rather than after. Also it would be good to reference according to each point made, rather than adding all of the citations at the end of the sentence.

Page 2, “As it controls the distance between the CAR-T cell and the target cell, the hinge is also important for the formation of an immunological synapse, which is needed for proper T cell activation.”: You need to explain this in more detail. Why is the distance important? What happens if it the linker is too short or too long? The kinetic segregation model is good to discuss here.

Page 2, “Moreover, scientists have discovered that since it can result in CARs forming dimers and joining with the cell’s natural T cell receptors (TCRs), using the CD3ζ transmembrane domain could possibly help more effectively activate T cells. (Bridgeman et al., 2010) With that said, using a CD3ζ transmembrane domain in a CAR could result in it being less stable than those with CD28 transmembrane domains.”: Why, what’s the evidence? Feel like there’s a lot of summarising here without a lot of explanation or analysis.

Page 2, “didn’t multiply or survive well”: Do not use contractions in the review. Also try and use appropriate technical words, instead of multiply, use proliferate. “Survive well” also seems a bit too informal, consider revising this. There are other sentences which are similar which I have not included here, so consider proofreading with this in mind.

Page 2, “The two most commonly used co-stimulatory domains are CD28 and 4-1BB (CD137)”: But why did they decide on these co-stim domains? Probably an important part to discuss.

Page 3, “Moreover, the effectiveness of this generation of CARs depends on the cancer type, proving that more research and testing are required.”: Why might this be the case? What’s the field’s hypothesis for this? Be specific.

Page 6, “sending out “off signals” or hiding their identity”: Use technical words.

Page 6, “This allows for CAR-T cells to have a stronger targeting ability as they don’t rely on other immune cells, longer-lasting memory to help them fight off relapsing cancer, and enhanced killing ability even in advanced or cancers that are harder to treat.”: The author often uses a lot of lists to back up their claim. I would suggest instead that they expand on this in more detail. For example, what immune cells are you talking about – are we talking about the APCs or other cytotoxic immune cells, as I would argue that they will always rely on other immune cells within the TME. Why do they have enhanced killing ability? All of this needs more detail, this cannot be mentioned and then not expanded on later within the paragraph, as it comes off as being vague.

Page 9, “decline in CAR-T cell levels over time…due to inadequate co-stimulatory signalling or poor T cell fitness at the time of infusion.”: For both points – why does this happen? Especially for the second point, think about T cell fitness being dependent on the patient that the T cells come from.

Page 12, A2AR and A1R – the author needs to explain what this is in more detail, and not only mention them within the paragraph.

Page 16, The CAR-E needs to be explained in more detail. Specifically that this is a soluble protein, which is not really clear until looking at the figure and need to explain in more detail how the cis-IL2R signalling works.

I recommend accepting with minor revisions.

Review of Limitations and recent developments of CAR-T cell therapy

Summary: The author writes a strong paper describing the key limitations of CAR-T cell therapy going into detail on cell persistence, tumor escape, and changes in efficacy in different applications. The student emphasizes each of these limitations and draws from the literature to identify developments that circumvent them making strides for a more advanced innovative cancer therapy. The student demonstrated a deep understanding of the literature, and professional tone throughout the paper. Taken together, I recommend this manuscript is accepted with minor revisions.

Major Comments:

Page 6: At the beginning of this section where the author describes that other treatments like chemotherapy and stem cell transplants have failed, there is little information provided comparing the details of these methods compared to CAR-T cell therapy. Chemotherapy and stem cell transplants do not always fail, so it would be helpful if the author could provide details on how often these treatments fail and compare it to how often CAR-T fails.

Page 9: Figures should be spread throughout the text and not reserved for the end of each section. This makes it more difficult for the reader to look at the figure as they are reading the text. The student should integrate the figures close to where they are referred to in the body to allow easy visualization for the reader while reading the text.

Page 11: The structure of the paper is difficult to follow. It is unclear how the manuscript is organized into sections as there are numerous sections that are missing key transitions. It would be useful for the author to either have fewer sections and combine some sections creating subsections, or provide transition paragraphs to explain the shift from limitations to developments.

Overall: The author frequently cites >4 references when introducing new ideas or topics, but under-cites the literature when describing specific studies. The manuscript would benefit from an increase in specific studies with individual citations compared to the more generalized sentences with numerous references. The author should be cautious to include citations where necessary and not add more references that necessary to back up the claims.

Overall: The author must review their references and ensure properly formatted. For example, the reference “Convergence of Acquired Mutations and Alternative Splicing of….” Is formatted like a website with incorrect information. This reference is a journal article, with authors, a year, issue, volume, and page numbers. All references should have authors, year, title, journal, volume, issue (if available), page numbers, and doi.

Minor Comments:

Page 1: The author uses a contraction “don’t” in the introduction. The reviewer suggests avoiding the use of contractions in academic scientific literature. There are other areas throughout the paper that contractions are used and they should also be expanded.

Page 2: The author refers to a figure in the section heading: “Structure of CAR (Fig. 1).” The author should refer to a figure in the body text, not in the heading of a section.

Page 2: The author defines MHC in the section “Antigen-binding domain” but this acronym has already been defined in the introduction. For all acronyms defined, the author only needs to define acronyms once and after that, can just use the acronym for more concise language. There are other instances of redefining CAR and CAR-T that is repetitive.

Page 5: Figure 2 does not need it’s own entire page, the text should immediately follow the figures.

Page 11: The table needs a label (Table 1) and must be referred to directly in the text.

Page 11: References should be placed at the end of the sentence, not in the middle.

Page 12: There is no reference provided for the study discussing the effect of adenosine on CAR-T cell deactivation. Ensure that large paragraphs describing studies are backed by references.

Overall: The author should ensure their formatting of in text citations is consistent throughout the text. Sometimes the period is after the reference, while other times it is before.

Page 14: The end of the first paragraph is a sentence structure that is confusing to the reviewer. Please ensure that the punctuation and citation is correct.

Integrative Molecular Engineering of CAR-T Cells: Emerging Strategies to Enhance Persistence, Targeting, and Tumor Microenvironment Adaptation

Abstract

Chimeric Antigen Receptor-T cell (CAR-T cell) therapy has become a revolutionary immunotherapeutic strategy for treating hematologic malignancies. However, despite its early success, CAR-T cell therapy continues to experience critical limitations, such as limited cell persistence of CAR-T cells, susceptibility to immune escape by tumor cells, and poor efficacy in solid tumors. This review investigates the role of CAR-T cell therapy in cancer treatment and how recent developments, such as dual-antigen targeting, re-engineering of the tumor microenvironment, introduction of enhancers, and combinatorial delivery systems, are addressing ongoing limitations in the field. Referencing recent developments in both clinical and preclinical research, this review highlights that through the combination of molecular engineering and targeted modulation of the immune system's response to tumors, CAR-T cell therapy is transitioning from a narrowly focused therapy to a broader, more widely applicable therapy across cancer types, potentially reshaping the future of cancer treatment.

Introduction

Chimeric Antigen Receptor-T cell (CAR-T cell) therapy has become a revolutionary therapeutic strategy in the realm of cancer treatment (June et al., 2018). Designed to allow lymphocytes such as T cells to target and eliminate cells expressing a specific surface antigen, Chimeric Antigen Receptors (CARs) are engineered artificial receptors that, unlike normal T cell receptors, do not rely on major histocompatibility complex (MHC) molecules to recognize antigens (Sadelain et al., 2013). Because of CAR's ability to stimulate strong immune responses and effectively attack tumors, the US Food and Drug Administration (FDA) approved anti-CD19 CAR-T cell therapy against B-cell tumors in 2017 – an important milestone in the development of immunotherapies (Maude et al., 2018; Neelapu et al., 2017; Schuster et al., 2017). In spite of prior success, CAR-T cell therapy still faces many key challenges: most notably, the limited cell persistence of CAR-T cells, the evolution of cancer cells to avoid detection and destruction by immunotherapies, and the poor effectiveness of the therapy in solid tumors. Hence, in an effort to shape CAR-T cell therapy into a broader and more applicable cancer treatment, ongoing innovations such as bispecific CAR-T cells targeting both CD19 and CD20 antigens, inhalable nanovesicles carrying a STING agonist, integration of a CAR enhancer, and the directing of A1R expression to tumor sites are being developed to address current limitations. This review – focusing on how innovations are addressing its critical limitations – examines current applications and emerging strategies in CAR-T cell therapy.

Structure of CAR

For CARs to perform their function, they require these 4 main components: antigen-binding domain, hinge region, transmembrane domain, and intracellular signaling domain(s) (Fig. 1).

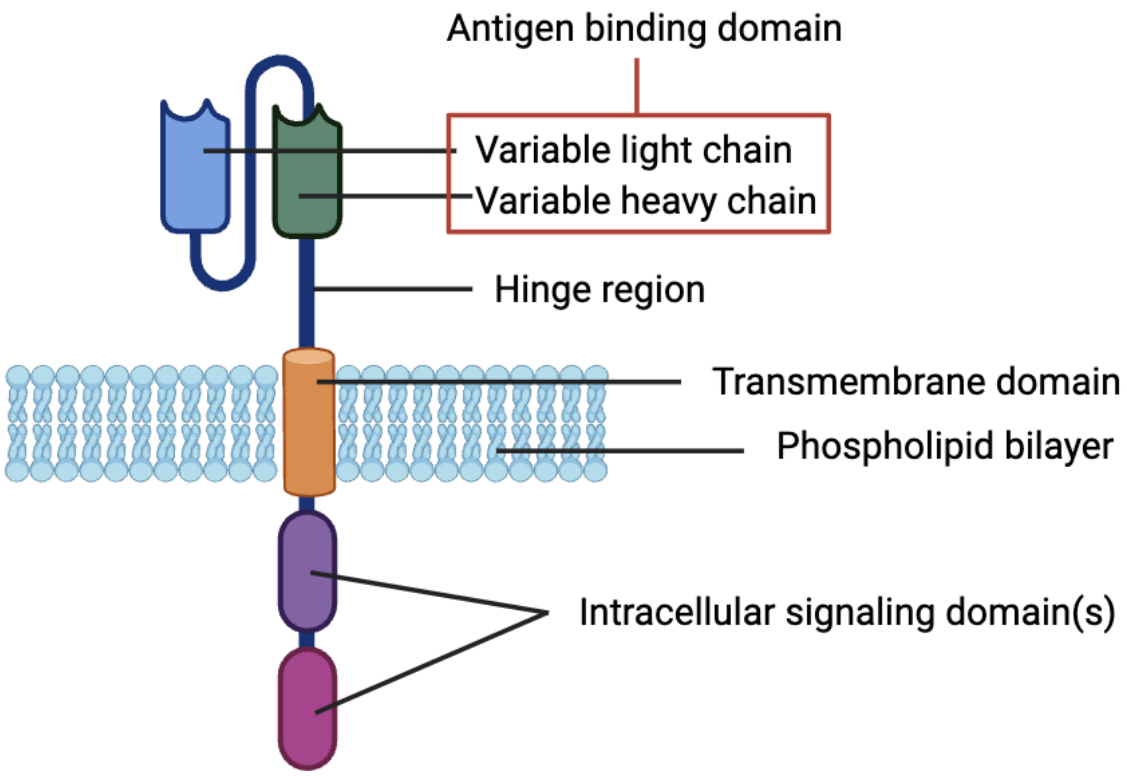

Fig. 1 Structure of CAR. CARs contain 4 main components: (1) antigen binding domain, (2) hinge region, (3) transmembrane domain, and (4) intracellular signaling domain(s)

Antigen-binding domain

Produced from monoclonal antibodies, specifically their variable heavy (VH) and variable light (VL) chains, the antigen-binding domain is the part of a CAR that directly interacts with cancer cells by allowing the CAR to detect and bind to specific antigens on them. Through a flexible linker, the VH and VL chains are connected to form a single-chain variable fragment (scFv) (Fig. 1). CARs use scFvs to recognize and bind to antigens on the extracellular surface of cancer cells and activate the T cell without the use of MHCs (G. Zhang et al., 2014). Moreover, beyond binding, binding specificity and affinity of the CAR to its target can also be altered by the way the VH and VL chains interact and the position of the specific parts that bind to epitopes called complementary-determining regions (CDRs) (Chailyan et al., 2011). To activate a CAR-T cell, the sufficient affinity of the antigen-binding domain to the epitope is required. However, excessively strong affinity will result in activation-induced cell death or other detrimental side effects in the patient (Caruso et al., 2015; Liu et al., 2015).

Hinge region

Connecting the antigen-binding domain to the transmembrane domain, the hinge region, also known as the spacer region, is the part of a CAR that extends and positions the binding domain far enough from the surface of the T cell, allowing for the binding domain to reach and bind to the epitope of a specific antigen (Fig. 1). Although the hinge region can provide flexibility, which helps the CAR overcome steric hindrance and reach epitopes that might otherwise be hard to access, a CAR's efficacy depends on the length and composition of the hinge. Potentially, it can affect how flexible the receptor is, how well a CAR is expressed on a T cell, how strongly a CAR signals after binding an antigen, how effectively a CAR recognizes an epitope, and the strength of the activation of the T cell (Hudecek et al., 2015; Jensen & Riddell, 2015). As it controls the distance between the CAR-T cell and the target cell, the hinge is also important for the formation of an immunological synapse, which is needed for proper T cell activation (Srivastava & Riddell, 2015). According to the kinetic segregation model, T cell activation is dependent on the spatial exclusion of large inhibitory phosphatases like CD45 from the close-contact zone between the T cell and its target. The size of the hinge determines how closely the two cell membranes can approach each other. If the hinge is too short, steric hindrance may prevent optimal receptor-ligand engagement. If the hinge is too long, the intermembrane distance may be too wide to exclude CD45 efficiently. In either case, improper spacing can disrupt the subsecond evacuation of CD45 that initiates signaling. This could lead to reduced activation or signaling errors. Thus, the hinge length directly influences the molecular organization and signaling kinetics of the CAR-T immunological synapse (Taylor, Allard, & Read, 2022). Additionally, longer hinges are generally more suitable for reaching epitopes closer to the surface of the target cell or those that are part of bulky, glycosylated antigens, while shorter hinges are more suitable for epitopes that are farther away from the surface of the target cell (Guest et al., 2005; Hudecek et al., 2015; James et al., 2008; Wilkie et al., 2008). Because different hinge lengths are commonly required for each antigen and epitope, researchers often have to test different spacer lengths to determine what functions best with a specific antigen-binding domain. Most hinge regions come from parts of CD8, CD28, IgG1, or IgG4 proteins. However, deriving hinge regions from parts of IgG proteins can be risky, as they may interact with Fcγ receptors in the body, which not only results in CAR-T cell depletion but also reduced CAR-T cell persistence (Almåsbak et al., 2015; Hombach et al., 2010).

Transmembrane domain

Apart from anchoring it into the T cell membrane, the transmembrane domain is a part of the CAR that can also influence how much a CAR is expressed on the surface of a T cell, affect the stability of the CAR, aid with signaling or forming of the immunological synapse, and dimerize with other natural signaling proteins in a T cell (Bridgeman et al., 2010; Guedan et al., 2018; T. Zhang et al., 2012) (Fig. 1). Because different transmembrane domains are often used depending on the hinge or signaling domains being used in a specific CAR, researchers have yet

to fully understand how switching out one transmembrane domain for another affects the function of CARs. Moreover, scientists have discovered that since it can result in CARs forming dimers and joining with the cell's natural T cell receptors (TCRs), using the CD3ζ transmembrane domain could possibly help more effectively activate T cells. This is because this structural coupling brings the CAR into close proximity with the TCR signaling complex, which allows it to recruit the same intracellular kinases that normally activate T cells (Bridgeman et al., 2010). With that said, due to the possibility of this domain interacting with endogenous CD3ζ chains, it may reduce receptor stability and surface expression, making CARs with a CD3ζ transmembrane domain less stable than those with CD28 transmembrane domains (Dotti et al., 2014). Furthermore, studies have shown that depending on the combination of the transmembrane and hinge regions, CAR-T cells could behave differently.

Intracellular signaling domain(s)

To figure out how to design CARs that trigger strong and lasting T cell responses, researchers have placed immense emphasis on studying the intracellular signaling domain, also known as the endodomain (Fig. 1). Produced in the late 1990s, the first-generation CARs only consisted of a CD3ζ or FcRγ signaling domain that contained immunoreceptor tyrosine-based activation motifs (ITAMs), which helped activate T cells when the CAR binds to its target antigen (Gross et al., 1989; Rafiq et al., 2020) (Fig. 2). However, with just CD3ζ signaling alone, many issues arose: the T cells did not proliferate or maintain functionality after they recognized antigens, the CARs did not produce strong responses in the lab, and early clinical trials showed that these CARs had little to no therapeutic effect (Brocker & Karjalainen, 1995; Hege et al., 2017a; Till et al., 2008). Hence, to address these limitations, researchers added a co-stimulatory domain to the first-generation CAR (Imai et al., 2004; Maher et al., 2002). The two most commonly used co-stimulatory domains are CD28 and 4-1BB (CD137). These two co-stimulatory domains were specifically chosen due to them enhancing CAR-T cell function through distinct signaling pathways. CD28 provides rapid T cell activation and cytokine production by promoting PI3K and NF-κB signaling, which leads to stronger but shorter-lived responses. In contrast, 4-1BB signaling enhances mitochondrial biogenesis and memory formation, which results in more sustained CAR-T cell persistence and reduced exhaustion (Long et al., 2015). Because of the addition of these domains, the second-generation CARs demonstrated improved T cell persistence, cytokine production (ie, IL-2), and response to repeated antigen exposure (Maher et al., 2002). Ultimately, these CARs functioned well in many blood cancers such as Chronic lymphocytic leukemia (CLL), B-cell acute lymphoblastic leukemia (B-ALL), Diffuse large B-cell lymphoma (DLBCL), and multiple myeloma (van der Stegen et al., 2015). They are now being tested in solid tumors like Glioblastoma, advanced sarcoma, liver metastases, mesothelioma, ovarian cancer, and pancreatic cancer (van der Stegen et al., 2015). With that said, as some scientists believed that one co-stimulatory domain may not fully activate the T cell, third-generation CARs were developed to include two co-stimulatory domains (CD28 and 4-1BB) in a row along with CD3ζ (Pulè et al., 2005). Moreover, the

effectiveness of this generation of CARs depends on the cancer type. While hematologic cancers like B-cell malignancies present uniform antigen targets and allow for CAR-T cells to circulate freely, solid tumors often display heterogeneous antigen expression and contain dense extracellular matrices, immunosuppressive cells, and inhibitory cytokines that limit the infiltration and persistence of CARs (Kloss et al., 2018; Yin et al., 2018). Consequently, the field hypothesizes that CAR-T efficacy is determined by the interplay between tumor composition and the design of CAR signaling. This explains why second- and third-generation CARs show strong results in blood cancer but variable success in solid tumors (Abate-Daga et al., 2014; Milone et al., 2009; Zhong et al., 2010).

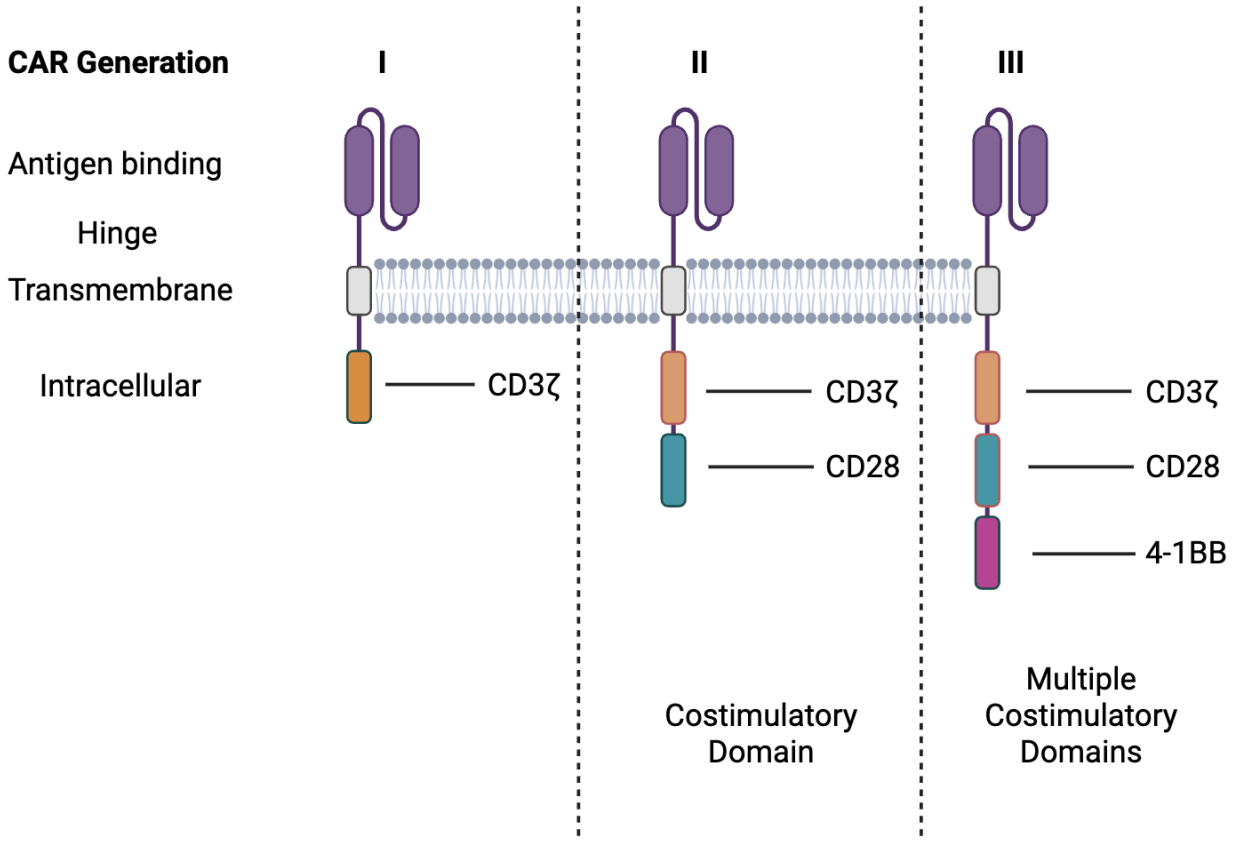

Fig. 2 Generations of CARs. The first generation of CARs consisted of only a CD3ζ signaling domain. In addition to the CD3ζ signaling domain, the second generation of CARs consisted of a CD28 costimulatory domain, and the third generation of CARs consisted of both CD28 and 4-1BB costimulatory domains.

Introduction to CAR-T cell therapy: What is it and how is it administered to patients?

CAR-T cell therapy is an immunotherapy that modifies one's body's own T cells (a type of white blood cell) to better recognize and destroy cancer cells. Although conventional treatments such as chemotherapy and stem cell transplantation have been successful in helping many achieve remission, they often fail in a significant subset of cases. For example, nearly 40% of acute myeloid leukemia (AML) patients experience induction failure after standard chemotherapy that combines cytarabine and anthracyclines (Culver-Cochran et al., 2024). Furthermore, many of these refractory cases do not respond to salvage therapy or allogeneic stem cell transplantation. Similarly, in patients with high-risk leukemia undergoing haploidentical stem cell transplants, despite intensified conditioning regimens, graft failure and relapse are common (Passweg et al., 2000). In contrast, even in patients who have not responded to prior chemotherapy or transplant, CAR-T cell therapy has achieved remarkable success in treating relapsed or refractory blood cancers.

While normal T cells can recognize and attack infected or abnormal cells, cancer cells often escape detection by upregulating inhibitory ligands like PD-L1 or downregulating antigen presentation, which suppresses T cell activation and masks their immunogenic identity (Perales et al., 2018). To address this problem, instead of relying on MHCs, CAR-T cells are genetically modified to carry a synthetic receptor that directly binds to antigens, which are specific proteins found on cancer cells (ie, CD19 in B-cell cancers) (Fig. 3). Because they are genetically engineered to recognize specific antigens independently of MHC presentation, CAR-T cells exhibit enhanced anti-tumor activity. This allows for CAR-T cells to rapidly engage tumor cells, secrete cytotoxic molecules such as perforin and granzyme B, and even induce apoptosis in tumors that evade normal immune detection.

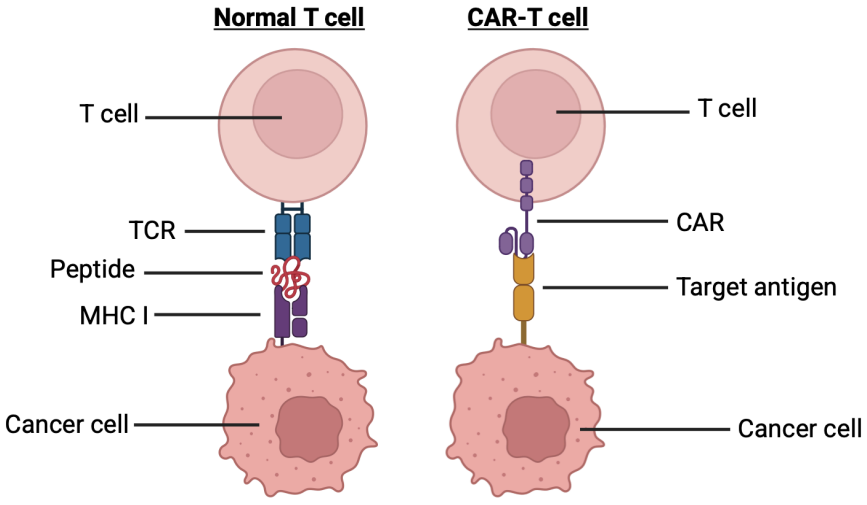

Fig. 3 Normal T cell vs CAR-T cell. Normal T cells rely on the MHCs of the cancer cell to present the antigen for the TCR to bind to it, whereas CAR-T cells can directly bind to the target antigen using their CAR.

Additionally, CAR-T cells can develop memory-like phenotypes, which allow them to persist longer and respond faster to relapsing cancer cells. Although CAR-T cells still interact with other components of the tumor microenvironment like endogenous cytotoxic T cells and natural killer cells, their design provides a direct, high-affinity mechanism for the killing of tumor cells that enhances efficacy even in cancers that are more difficult to treat (Maurya et al., 2025). Once inside the body, CAR-T cells continue to survive and proliferate. They "patrol" the bloodstream and look for any cancer cells that return over time, providing patients with long-term protection. Some CAR-T cells even form memory T cells and stay in the body permanently, and attack relapsed cancer. Because of its effectiveness, CAR-T cell therapy has been FDA-approved to treat several blood cancers like Acute Lymphoblastic Leukemia (ALL), Non-Hodgkin Lymphoma (NHL), and Multiple Myeloma (Maude et al., 2018; Neelapu et al., 2017; Schuster et al., 2017). Some of the approved CAR-T products include Kymriah (tisagenlecleucel) by Novartis, Yescarta (axicabtagene ciloleucel) by Kite Pharma, Breyanzi, Abecma, and other newer products that are still under clinical trials. Especially for patients who had relapsed after several treatments, CAR-T cell therapy has shown incredibly high success rates. For example, the ELIANA trial showed an 83% complete remission at 3 months with Kymriah, and the ZUMA-1 trial showed a 54% complete remission rate in patients treated with Yescarta (Almond et al., 2017; Locke et al., 2017).

While effective, the process for CAR-T cell therapy is personalized and takes several weeks (Fig. 4).

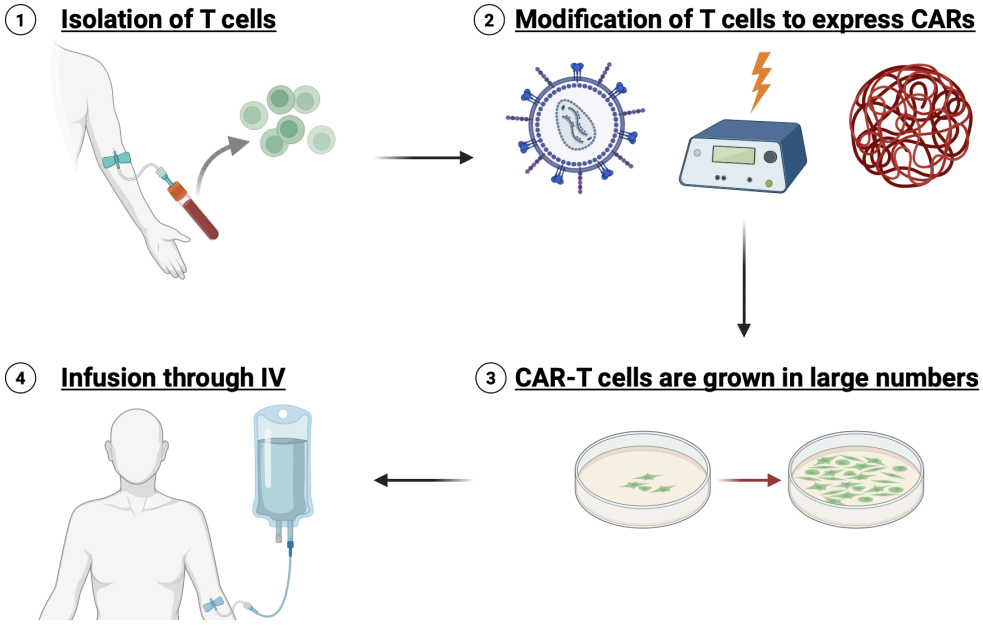

Fig. 4 Administration of CAR-T cells to patients. Patients first undergo Leukapheresis to isolate their T cells from other blood components. After isolation, the T cells are modified to express CARs through viral vectors, transposon systems, electroporation, or nanoparticles. Once modified, the CAR-T cells are grown in large numbers in labs to be used for treatment. The CAR-T cells are then infused into the patient's bloodstream through an IV.

First, through Leukapheresis, doctors collect a patient's blood and, using a machine that separates blood components, isolate the patient's T cells (Miliotou & Papadopoulou, 2018; Mirzaei et al., 2019). Then, the isolated T cells are modified to express CARs. This can be done using viral vectors (ie. γ-retroviruses or lentiviruses) that are engineered to carry the CAR gene into the T cell's DNA, transposon systems (ie. Sleeping Beauty or piggyBac) which use "cut-and-paste" DNA methods with a transposase enzyme, electroporation that uses electric pulses to open cell membranes so DNA can enter, or nanoparticles which are tiny carriers that deliver CAR genes safely without the use of viruses (Kidd et al., 2012; Yi et al., n.d.; Munoz-Lopez & Garcia-Perez, n.d.; Yarmush et al., 2014; Smith et al., 2017). Once modified, the T cells are grown in large numbers in the lab until there are millions of CAR-T cells prepared for treatment. To lower their existing immune cells, called lymphodepletion, the patient may first receive a short round of chemotherapy. This helps the CAR-T cells work better. Following this, the CAR-T cells are infused through an intravenous (IV). After the treatment is complete, for the first 2-3 weeks, patients are observed closely for side effects.

Key limitations in current CAR-T cell therapy

Despite the initial success of CAR-T cell therapy, it continues to face many limitations – namely, the limited cell persistence of CAR-T cells, the evolution of cancer cells to avoid detection and destruction, and poor efficacy of the therapy in solid tumors.

Limited cell persistence of CAR-T cells

With the inability to persist for long periods of time, sustained anti-tumor activity and durable patient responses are hindered. Although CAR-T cells can expand and mediate tumor clearance at first, many patients are susceptible to relapse due to a decline in CAR-T cell levels over time. This decline in cell levels could be due to several reasons. Firstly, suboptimal function of the co-stimulatory domains within the CAR could reduce T cell proliferation, survival, and memory formation after interaction with the antigen. Without sufficient co-stimulation, CAR-T cells will fail to sustain robust activation, leading to limited persistence. Furthermore, due to age, prior chemotherapy, chronic infections, or exhaustion induced by the tumor, the function of patient-derived T cells may already be compromised. This results in lower proliferation capacity, increased susceptibility to apoptosis, and impaired metabolic fitness. The diminished persistence causes issues, specifically in hostile tumor microenvironments (TME) where immunosuppressive signals like TGF-β, IL-10, and checkpoint pathways like PD-1/PD-L1 rapidly exhaust CAR-T cells and impair their survival (Kloss et al., 2018; Yin et al., 2018). Furthermore, the short life span of CAR-T cells may be due to inadequate co-stimulatory signaling or poor T cell fitness at the time of infusion. Given that early-generation CARs lacked co-stimulatory domains, they failed to provide the necessary signals for robust memory formation, which contributed to rapid attrition in vivo (Brocker & Karjalainen, 1995; Hege et al., 2017b; Till et al., 2008). Even with

newer generations of CARs being developed, the persistence of CAR-T cells remains variable and patient-dependent (Abate-Daga et al., 2014; Milone et al., 2009; Zhong et al., 2010).

Evolution of cancer cells to avoid detection and destruction

Because of the adaptive evolution of tumor cells, this can lead to antigen escape. Antigen escape is a process where cancer cells downregulate or completely lose expression of the target antigen recognized by CAR-T cells. Although initial single-antigen CAR-T therapies – such as CD19 or BCMA targeting – achieved high response rates in hematologic cancers, over time, a significant number of patients relapse as the tumor cells modify themselves to avoid immune recognition. For example, due to a loss or mutation of the CD19 antigen, up to 30-70% of relapsed B-ALL patients experience recurrence (Majzner & Mackall, 2018; Maude et al., 2015). Likewise, following anti-BCMA CAR-T treatment, BCMA downregulation has been observed in multiple myeloma (Brudno et al., 2018; Cohen et al., 2019; Green et al., 2018). Additionally, solid tumors exhibit this immune escape pattern, like reduced IL13Ra2 expression following CAR-T therapy in glioblastoma (Brown et al., 2016). This could lead to an undermining of long-term treatment success because of the ability of tumor cells to effectively "hide" from the immune attack.

Poor efficacy in solid tumors

Due to several interrelated biological and structural barriers, the effectiveness of CAR-T cell therapy in solid tumors remains limited. Firstly, solid tumors lack ideal antigen targets that are both tumor-specific and not expressed on normal tissues. Because many antigens targeted in solid tumors are also found on healthy cells, although at low levels, this leads to on-target off-tumor toxicity and reduced therapeutic windows. Additionally, in solid tumors, CAR-T cells face significant trafficking and infiltration challenges. Unlike blood cancers, solid tumors have dense physical barriers like the extracellular matrix and tumor stroma that impede T cell penetration (B.-L. Zhang et al., 2016). Finally, the immunosuppressive TME, which is rich in regulatory T cells, myeloid-derived suppressor cells (MSDCs), and Tumor-associated macrophages (TAMs) that secrete inhibitory cytokines and engage immune checkpoint pathways (ie, PD-1/PD-L1, CTLA-4), results in T cell exhaustion and poor persistence of the CAR-T cells (Quail & Joyce, 2013; Yin et al., 2018).

Advancements and strategies to overcome limitations in CAR-T cell therapy

Hence, to enhance the overall effectiveness of CAR-T cell therapy in cancer treatment, scientists have pursued multiple strategies to overcome poor efficacy in solid tumors, susceptibility to immune escape by tumor cells, and limited cell persistence.

Targeting poor efficacy in solid tumors: Re-engineering of the tumor microenvironment and combinatorial delivery systems

Although CAR-T cell therapy has been successful for some blood cancers such as leukemia and lymphoma, it has not yet shown the same success in treating solid tumors like lung, breast, and ovarian cancers (Albelda, 2024; Flugel et al., 2023). When treating solid tumors, the main challenge lies in the fact that the TME, which is the space around tumor cells, is a hostile environment for immune cells (Hou et al., 2021; Landoni et al., 2024; Lee et al., 2023). In solid tumors, the TME creates multiple layers of defense that shut down immune responses (Table 1).

Component	Example(s)	Function
Checkpoint proteins	PD-L1	They act as regulators of the immune system and prevent CAR-T cells from attacking tumor cells. While they function as "off switches" for the immune system and ensure it does not damage healthy tissues, they allow for tumors to escape immune destruction.
Suppressive immune cells	Regulatory T cells (Tregs) and MDSCs	They release anti-inflammatory signals to further weaken CAR-T cells.
Adenosine	–	High levels of it build up in the low-oxygen TME and signal through the A2AR receptor on CAR-T cells to slow them down.

Table 1 Components of the TME. The TME consists of multiple components: checkpoint proteins, suppressive immune cells, and adenosine. Together, these components allow for the TME to create a strong defense that shuts down immune responses in solid tumors.

Overall, these components shorten the lifespan and reduce the efficacy of CAR-T cells when destroying solid tumor cells.

Hence, to tackle the limited efficacy of CAR-T cell therapy in solid tumors, the most direct approach was to target modifying the TME to make it more suitable for CAR-T cell activity. For example, inhalable nanovesicles have been engineered to overcome the hostile TME (Zhu et al., 2025)(Fig. 5).

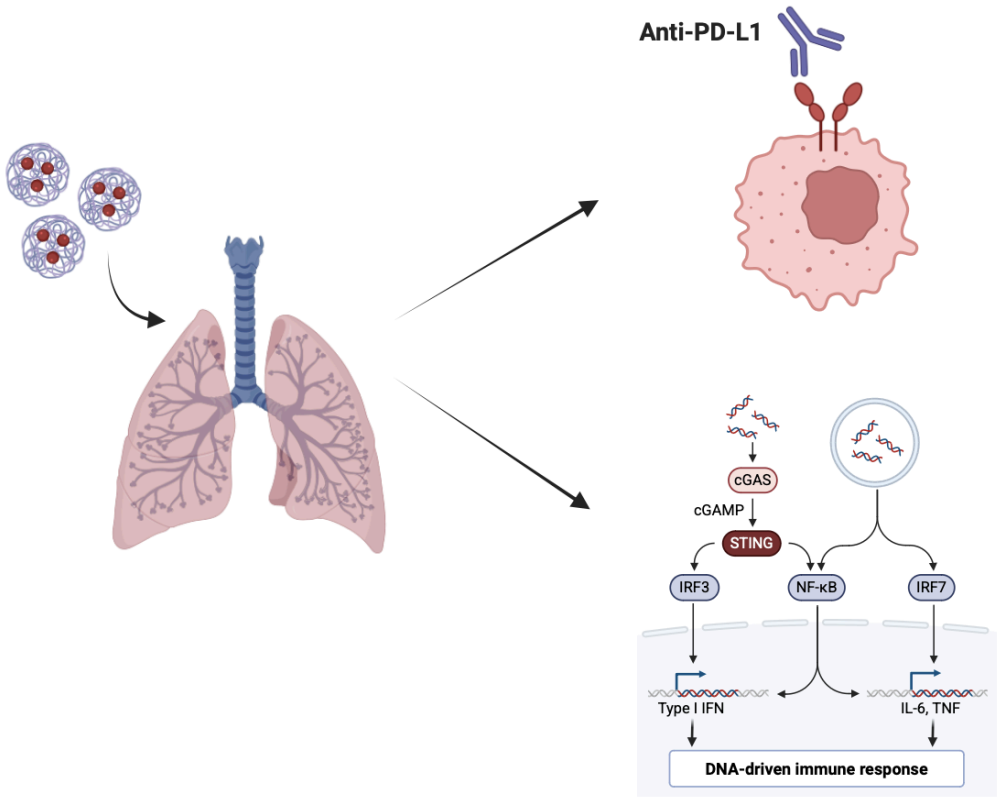

Fig. 5 Using inhalable nanovesicles to overcome the hostile TME. Inhalable nanovesicles that contain therapeutic molecules are inhaled into the patient's lungs. Upon inhaling these nanovesicles, they block the PD-1/PD-L1 interaction, allowing for CAR-T cells to remain active, and they activate the STING pathway, initiating a strong immune response.

Inhalable nanovesicles are small, biodegradable vesicles that contain therapeutic molecules that could be directly inhaled into the lungs. Each nanovesicle carries an anti-PD-L1 single-chain variable fragment (scFv) and a STING agonist (cGAMP). The scFv is a small antibody piece that binds to PD-L1 on tumor cells and blocks the PD-1/PD-L1 interaction, allowing for the CAR-T cells to remain active. The cGAMP is a molecule that activates the Stimulator of Interferon Genes (STING) pathway and leads to the production of type I interferons such as IFN-β and the attraction of antigen-presenting cells such as dendritic cells to help amplify the immune response. Success of these inhalable nanovesicles was observed in mouse models of lung cancer where they triggered local immune activation without systemic toxicity, increased levels of molecules (ie. IFN-β, IL-2, and CXCL9) that help attract and activate more CAR-T cells, reduced exhaustion markers (ie. PD-1, LAG3, and TIGIT) on CAR-T cells, promoted the formation of $TCF1^{+}$ memory-like T cells which are known for their persistence and self-renewal properties, and resulted in stronger tumor shrinkage, delayed relapse and improved survival of CAR-T cells.

Besides targeting the TME, efforts have also been made in changing the CAR-T cells themselves to resist suppressive signals from within the TME. Research has shown that adenosine, which is released in high amounts by tumors, functions by signaling through the A2AR receptor, a G protein-coupled receptor that suppresses T cell proliferation, cytokine production, and cytotoxic activity, to deactivate CAR-T cells (Sek et al., 2025). In response to this, because A1R, another adenosine receptor whose signaling counteracts A2AR, enhances T cell activation and survival when activated, CRISPR-Cas9 gene editing technology was used to insert the gene for the A1R receptor into CAR-T cells. These newly engineered CAR-T cells with the A1R receptor resulted in higher production of immune-stimulating cytokines (ie, IFNγ, TNFα, and IL-2), reduced expression of exhaustion markers (ie, PD-1 and TIM-3), and increased expression of CD69 and IRF8, which indicated stronger immune readiness and potential for longer-lasting memory. To stabilize the beneficial effects, the transcription factor NR4A2 has been added, which helps CAR-T cells, especially in environments full of stress and inflammation, maintain anti-suppressive gene expression over time. In mice with tumors, CAR-T cells engineered with A1R and NR4A2 resulted in sustained tumor disappearance, preserved stem-like and central memory T cell populations that are essential for long-term immunity, and eliminated the need for repeated dosing of CAR-T cells.

In combination, these strategies could potentially revolutionize CAR-T cell therapy for increased efficacy in solid tumors, which make up the vast majority of cancers.

Targeting susceptibility to immune escape by tumor cells: Dual-antigen targeting

Another major limitation in CAR-T cell therapy is the downregulation or loss of the target antigen on tumor cells (Neelapu et al., 2017; Tong et al., 2020). This facilitates tumor cells escaping from immune cells, leading to relapse. This phenomenon is well-documented in B-cell malignancies (ie. DLBCL and B-ALL) where alterations in target antigens like CD19–caused by missense mutations, frameshifts, or alternative splicing–allow tumor cells to evade recognition by CARs (Sotillo et al., 2015).

Hence, to reduce the likelihood of relapse due to the loss of target antigens on tumor cells, dual-antigen targeting strategies – such as by simultaneously engaging two distinct antigens associated with tumors – have been developed (Dai et al., 2020; Grada et al., 2013; Hamieh et al., 2019; Hegde et al., 2016; Majzner & Mackall, 2018; Zah et al., 2016).

For example, in the phase I/II of a clinical trial, researchers developed a tandem CAR construct with the ability to bind to both CD19 and CD20 (Wang et al., 2024). CD19 and CD20 surface proteins are ideal targets for cancer therapy as they are highly expressed almost exclusively on B lymphocytes and absent on most other cell types. The construct, connected via a flexible linker, integrated scFvs specific for CD19 and CD20 and incorporated the hinge and transmembrane domains of CD8 with 4-1BB and CD3ζ signaling domains (Fig. 6).

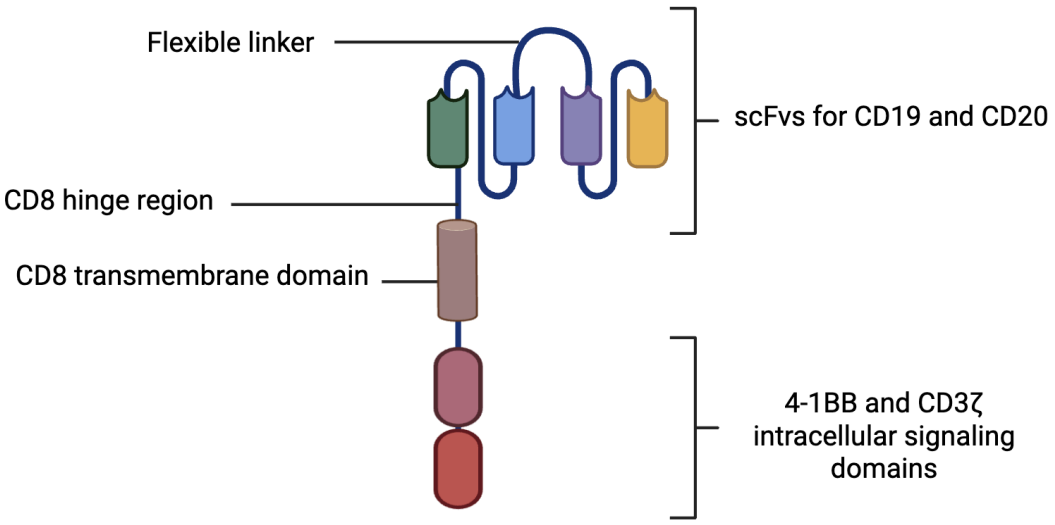

Fig. 6 Structure of CD19/20 CAR-T cell. The CD19/20 CAR-T cells have tandem CAR constructs that contain an antigen-binding domain with single-chain variable fragments (scFvs) for CD19 and CD20, a CD8 hinge and transmembrane domain, and 4-1BB and CD3ζ intracellular signaling domains.

In preclinical assays, the CD19/20 CAR-T cells demonstrated efficacy in destroying cancer cells that expressed either CD19, CD20, or even both, and they released similar levels of cytokines as single-target CAR-T cells. The release of cytokines is important as they function as chemical messengers that enable immune cells to communicate and coordinate an immune attack against harmful substances such as cancer cells.

Clinically, this dual-targeting approach showed promising efficacy. In the group of patients with relapsed or refractory (R/R) B-cell NHL, with a 12-month overall survival rate of 81.82% and progression-free survival of 60%, 81.8% of patients experienced complete remission. Importantly, even in the presence of antigen loss for one target, the bispecific CAR-T cells remained robust. This suggests a decreased risk of immune escape in these CAR-T cells compared to single-target CAR-T therapy. Through single-cell RNA sequencing, results showed that the main CAR-T cell groups after infusion contained high levels of genes that are involved in immune signaling, especially those that help activate the body's first-line immune defenses and those that aid in the destruction of cancer cells. Despite changes in the way cancer cells display their surface antigens to allow them to avoid detection from the immune system, likely due to the mentioned features, these CAR-T cells sustained anti-tumor activity.

Furthermore, beyond mitigating relapse driven by antigen loss, this dual-antigen targeting strategy also broadens the therapeutic window for patients with heterogeneous antigen expression profiles. Through the maintenance of targeting capability against tumor cells that have downregulated one antigen, these CD19/20 CAR-T cells exemplify how rational CAR design can directly address a critical resistance mechanism in immunotherapy.

Targeting limited cell persistence: CAR enhancer (CAR-E)

After the initial CAR-T cell treatment, CAR-T levels often deplete, resulting in patients being vulnerable to relapse. Hence, new ways to target the limited cell persistence of CAR-T cells are being developed to improve the long-term effectiveness of the therapy.

For example, scientists have designed a CAR enhancer (CAR-E) to prolong CAR-T durability without broad cytokine exposure (Rakhshandehroo et al., 2025). CAR-E is a recombinant protein that exists in the extracellular space and can diffuse to interact with CAR-T cells that are bound to the target antigen. It functions by coupling a tumor-antigen binder to a low-affinity Interleukin 2 (IL-2) dimer that engages only antigen-bound CAR-T cells. This ensures that the cis IL-2 receptor (cis IL-2R) signaling is delivered precisely where it is required. In this context, rather than activating neighboring T cells in the environment (trans signaling), "cis" signaling means that the IL-2 dimer acts locally within the same CAR-T cell that has engaged its target antigen. While not compromising its efficacy by preserving cytotoxicity and not activating other cells, such as non-transduced T cells that might hurt the safety and efficacy of the therapy, CAR-E switches on STAT5 and early activation in CAR-T cells (Fig. 7). By switching on STAT5 and early activation in CAR-T cells, this will result in bigger, longer-lived, and more effective CAR-T responses. The CAR-E only functions when both the CAR endodomain and the IL-2 receptor signal, and it's pulled inside the CAR-T cell itself via the IL-2R, which helps keep the effect contained.

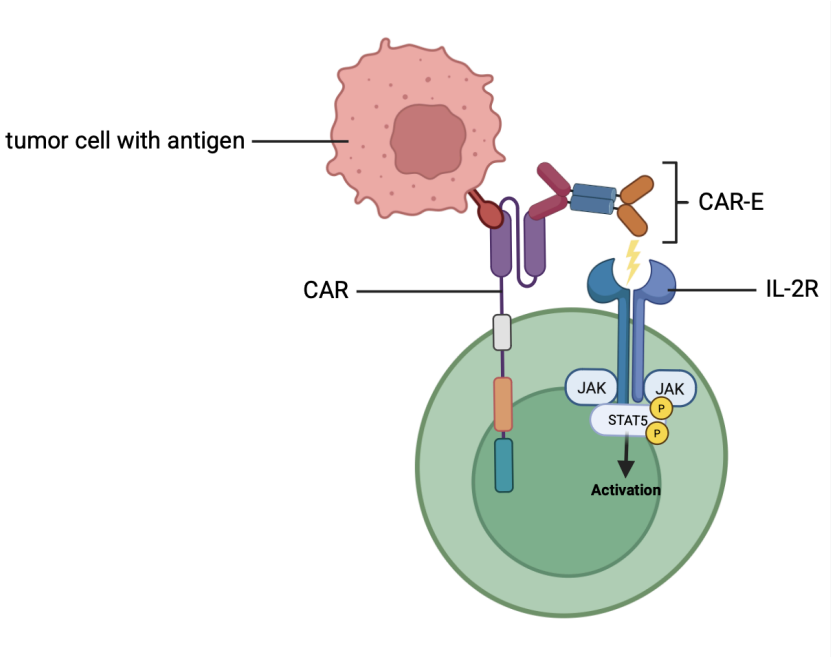

Fig. 7 CAR-E mechanism. CAR-E couples tumor-antigen recognition with a low-affinity IL-2 dimer that engages the IL-2R only in CAR-T cells bound to antigens. This activates the JAK/STAT5 pathway, promoting early activation, enhanced persistence, and stronger cytotoxic responses without broad cytokine exposure.

In vivo, dose-dependent multiplication and enhanced cell persistence of CAR-T cells in blood, spleen, and bone marrow, as well as enrichment of memory phenotypes such as central memory (T_CM) and stem-like memory (T_SCM), are produced through brief, pulsed dosing (serum t½ ~1.5 h). Importantly, CAR-T cells that are treated with CAR-E can be maintained even at lower CAR-T doses or in the absence of a tumor and can re-proliferate upon interaction with cancer cells that reappear. This supports an on-demand "booster" paradigm to sustain CAR-T cell levels after they peak and drop.

Overall, CAR-E is a safe method that improves cell persistence of CAR-T cells, as its low-affinity IL-2 is weak enough that it prevents activation of nearby, non-CAR-T cells, and it clears from the body quickly, allowing doctors to fine-tune the dosage. Furthermore, since the CAR-E mainly consists of components derived from humans, it minimizes predicted immunogenicity. The design of the CAR-E is also modular, meaning the binding piece to use CAR-E with other CAR targets (ie, CD19/CD22 or GD2) can be swapped, potentially even in solid tumors. In practice, scientists can initially carry out manufacturing that starts with memory-like T cells (T_SCM/T_CM) and keeps the culture time short to ensure cells stay youthful and durable. Then, CAR-E provides targeted IL-2 signals after infusion to maintain and expand those memory-leaning CAR-T cells in patients. Additionally, as CAR-E clears fast, clinicians can briefly give small pulses of CAR-E when CAR-T cell levels start to dip or when tumor signals reappear, then stop. This ensures the maintenance of cell persistence while minimizing negative side effects like cytokine toxicity, where the immune system releases too many cytokines all at once and triggers undesirable outcomes, or unwanted T cell activation.

When taken together, it is a targeted, scalable method to ensure CAR-T cell persistence while ensuring maintenance of control over systemic cytokine exposure.

Conclusion

Offering unprecedented remission rates in hematologic malignancies and giving rise to personalized, immune-based treatment for cancer, CAR-T cell therapy is one of the most transformative innovations in modern oncology. However, because of challenges such as limited cell persistence, susceptibility to immune escape, and poor efficacy in solid tumors, its potential to be used as a treatment in clinical practice has been hindered. Recent advances, such as re-engineering the tumor microenvironment through the use of inhalable nanovesicles and A1R-directed CAR-T cells, developing dual-antigen targeting constructs to counter antigen loss, and integrating CAR enhancers to sustain memory-like T cell populations, demonstrate that these limitations are neither overcome nor insurmountable. Together, the rise of these strategies indicates a shift in the therapy from a reactive treatment toward an increasingly proactive, precision-focused CAR design where CAR-T cell therapy is tailored to the tumor's biology and individual patients' differing needs. As deeper immunological insights are being discovered and innovations are arising, CAR-T cell therapy is anticipated to evolve from a niche intervention method for specific blood cancers into a more versatile and adaptable platform that is capable of overcoming heterogeneous antigen expression, hostile microenvironments, and short-term

durability. Whether CAR-T cell therapy fulfills its promise of becoming a universal, durable, and safe cancer treatment depends on the continued integration of preclinical breakthroughs into clinical practices accessible for patients worldwide.

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Integrative Molecular Engineering of CAR-T Cells: Emerging Strategies to Enhance Persistence, Targeting, and Tumor Microenvironment Adaptation

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Abstract

Chimeric Antigen Receptor-T cell (CAR-T cell) therapy has become a revolutionary immunotherapeutic strategy for treating hematologic malignancies. However, despite its early success, CAR-T cell therapy continues to experience critical limitations, such as limited cell persistence of CAR-T cells, susceptibility to immune escape by tumor cells, and poor efficacy in solid tumors. This review investigates the role of CAR-T cell therapy in cancer treatment and how recent developments, such as dual-antigen targeting, re-engineering of the tumor microenvironment, introduction of enhancers, and combinatorial delivery systems, are addressing ongoing limitations in the field. Referencing recent developments in both clinical and preclinical research, this review highlights that through the combination of molecular engineering and targeted modulation of the immune system's response to tumors, CAR-T cell therapy is transitioning from a narrowly focused therapy to a broader, more widely applicable therapy across cancer types, potentially reshaping the future of cancer treatment.

Introduction

Chimeric Antigen Receptor-T cell (CAR-T cell) therapy has become a revolutionary therapeutic strategy in the realm of cancer treatment (June et al., 2018). Designed to allow lymphocytes such as T cells to target and eliminate cells expressing a specific surface antigen, Chimeric Antigen Receptors (CARs) are engineered artificial receptors that, unlike normal T cell receptors, do not rely on major histocompatibility complex (MHC) molecules to recognize antigens (Sadelain et al., 2013). Because of CAR's ability to stimulate strong immune responses and effectively attack tumors, the US Food and Drug Administration (FDA) approved anti-CD19 CAR-T cell therapy against B-cell tumors in 2017 – an important milestone in the development of immunotherapies (Maude et al., 2018; Neelapu et al., 2017; Schuster et al., 2017). In spite of prior success, CAR-T cell therapy still faces many key challenges: most notably, the limited cell persistence of CAR-T cells, the evolution of cancer cells to avoid detection and destruction by immunotherapies, and the poor effectiveness of the therapy in solid tumors. Hence, in an effort to shape CAR-T cell therapy into a broader and more applicable cancer treatment, ongoing innovations such as bispecific CAR-T cells targeting both CD19 and CD20 antigens, inhalable nanovesicles carrying a STING agonist, integration of a CAR enhancer, and the directing of A1R expression to tumor sites are being developed to address current limitations. This review – focusing on how innovations are addressing its critical limitations – examines current applications and emerging strategies in CAR-T cell therapy.

Structure of CAR

For CARs to perform their function, they require these 4 main components: antigen-binding domain, hinge region, transmembrane domain, and intracellular signaling domain(s) (Fig. 1).

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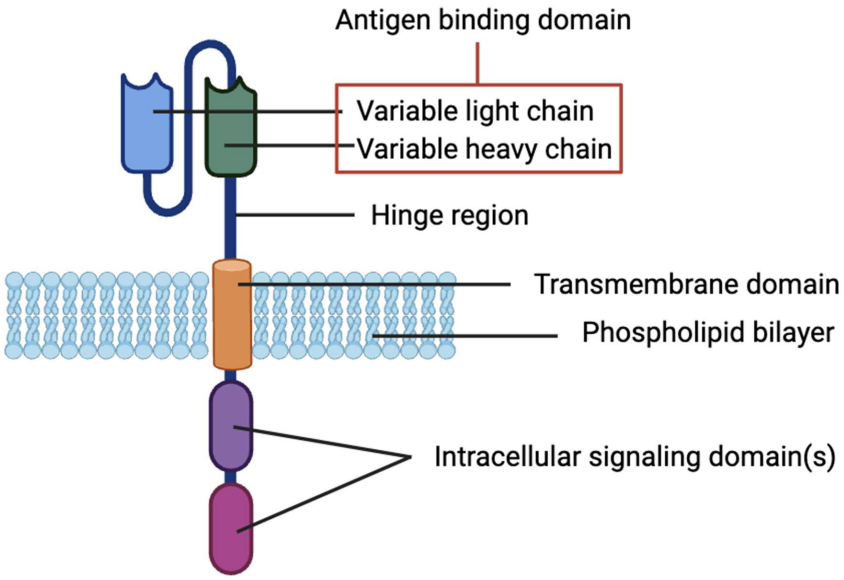

Fig. 1 Structure of CAR. CARs contain 4 main components: (1) antigen binding domain, (2) hinge region, (3) transmembrane domain, and (4) intracellular signaling domain(s)

Antigen-binding domain

Produced from monoclonal antibodies, specifically their variable heavy (VH) and variable light (VL) chains, the antigen-binding domain is the part of a CAR that directly interacts with cancer cells by allowing the CAR to detect and bind to specific antigens on them. Through a flexible linker, the VH and VL chains are connected to form a single-chain variable fragment (scFv) (Fig. 1). CARs use scFvs to recognize and bind to antigens on the extracellular surface of cancer cells and activate the T cell without the use of MHCs (G. Zhang et al., 2014). Moreover, beyond binding, binding specificity and affinity of the CAR to its target can also be altered by the way the VH and VL chains interact and the position of the specific parts that bind to epitopes called complementary-determining regions (CDRs) (Chailyan et al., 2011). To activate a CAR-T cell, the sufficient affinity of the antigen-binding domain to the epitope is required. However, excessively strong affinity will result in activation-induced cell death or other detrimental side effects in the patient (Caruso et al., 2015; Liu et al., 2015).

Hinge region

Connecting the antigen-binding domain to the transmembrane domain, the hinge region, also known as the spacer region, is the part of a CAR that extends and positions the binding domain far enough from the surface of the T cell, allowing for the binding domain to reach and bind to the epitope of a specific antigen (Fig. 1). Although the hinge region can provide flexibility, which helps the CAR overcome steric hindrance and reach epitopes that might otherwise be hard to access, a CAR's efficacy depends on the length and composition of the hinge. Potentially, it can affect how flexible the receptor is, how well a CAR is expressed on a T cell, how strongly a CAR signals after binding an antigen, how effectively a CAR recognizes an epitope, and the strength of the activation of the T cell (Hudecek et al., 2015; Jensen & Riddell, 2015). As it controls the distance between the CAR-T cell and the target cell, the hinge is also important for the formation of an immunological synapse, which is needed for proper T cell activation (Srivastava & Riddell, 2015). According to the kinetic segregation model, T cell activation is dependent on the spatial exclusion of large inhibitory phosphatases like CD45 from the close-contact zone between the T cell and its target. The size of the hinge determines how closely the two cell membranes can approach each other. If the hinge is too short, steric hindrance may prevent optimal receptor-ligand engagement. If the hinge is too long, the intermembrane distance may be too wide to exclude CD45 efficiently. In either case, improper spacing can disrupt the subsecond evacuation of CD45 that initiates signaling. This could lead to reduced activation or signaling errors. Thus, the hinge length directly influences the molecular organization and signaling kinetics of the CAR-T immunological synapse (Taylor, Allard, & Read, 2022). Additionally, longer hinges are generally more suitable for reaching epitopes closer to the surface of the target cell or those that are part of bulky, glycosylated antigens, while shorter hinges are more suitable for epitopes that are farther away from the surface of the target cell (Guest et al., 2005; Hudecek et al., 2015; James et al., 2008; Wilkie et al., 2008). Because different hinge lengths are commonly required for each antigen and epitope, researchers often have to test different spacer lengths to determine what functions best with a specific antigen-binding domain. Most hinge regions come from parts of CD8, CD28, IgG1, or IgG4 proteins. However, deriving hinge regions from parts of IgG proteins can be risky, as they may interact with Fcγ receptors in the body, which not only results in CAR-T cell depletion but also reduced CAR-T cell persistence (Almåsbak et al., 2015; Hombach et al., 2010).

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Transmembrane domain

Apart from anchoring it into the T cell membrane, the transmembrane domain is a part of the CAR that can also influence how much a CAR is expressed on the surface of a T cell, affect the stability of the CAR, aid with signaling or forming of the immunological synapse, and dimerize with other natural signaling proteins in a T cell (Bridgeman et al., 2010; Guedan et al., 2018; T. Zhang et al., 2012) (Fig. 1). Because different transmembrane domains are often used depending on the hinge or signaling domains being used in a specific CAR, researchers have yet to fully understand how switching out one transmembrane domain for another affects the

function of CARs. Moreover, scientists have discovered that since it can result in CARs forming dimers and joining with the cell's natural T cell receptors (TCRs), using the CD3ζ transmembrane domain could possibly help more effectively activate T cells. This is because this structural coupling brings the CAR into close proximity with the TCR signaling complex, which allows it to recruit the same intracellular kinases that normally activate T cells (Bridgeman et al., 2010). With that said, due to the possibility of this domain interacting with endogenous CD3ζ chains, it may reduce receptor stability and surface expression, making CARs with a CD3ζ transmembrane domain less stable than those with CD28 transmembrane domains (Dotti et al., 2014). Furthermore, studies have shown that depending on the combination of the transmembrane and hinge regions, CAR-T cells could behave differently.

Intracellular signaling domain(s)

To figure out how to design CARs that trigger strong and lasting T cell responses, researchers have placed immense emphasis on studying the intracellular signaling domain, also known as the endodomain (Fig. 1). Produced in the late 1990s, the first-generation CARs only consisted of a CD3ζ or FcRγ signaling domain that contained immunoreceptor tyrosine-based activation motifs (ITAMs), which helped activate T cells when the CAR binds to its target antigen (Gross et al., 1989; Rafiq et al., 2020) (Fig. 2). However, with just CD3ζ signaling alone, many issues arose: the T cells did not proliferate or maintain functionality after they recognized antigens, the CARs did not produce strong responses in the lab, and early clinical trials showed that these CARs had little to no therapeutic effect (Brocker & Karjalainen, 1995; Hege et al., 2017a; Till et al., 2008). Hence, to address these limitations, researchers added a co-stimulatory domain to the first-generation CAR (Imai et al., 2004; Maher et al., 2002). The two most commonly used co-stimulatory domains are CD28 and 4-1BB (CD137). These two co-stimulatory domains were specifically chosen due to them enhancing CAR-T cell function through distinct signaling pathways. CD28 provides rapid T cell activation and cytokine production by promoting PI3K and NF-κB signaling, which leads to stronger but shorter-lived responses. In contrast, 4-1BB signaling enhances mitochondrial biogenesis and memory formation, which results in more sustained CAR-T cell persistence and reduced exhaustion (Long et al., 2015). Because of the addition of these domains, the second-generation CARs demonstrated improved T cell persistence, cytokine production (ie, IL-2), and response to repeated antigen exposure (Maher et al., 2002). Ultimately, these CARs functioned well in many blood cancers such as Chronic lymphocytic leukemia (CLL), B-cell acute lymphoblastic leukemia (B-ALL), Diffuse large B-cell lymphoma (DLBCL), and multiple myeloma (van der Stegen et al., 2015). They are now being tested in solid tumors like Glioblastoma, advanced sarcoma, liver metastases, mesothelioma, ovarian cancer, and pancreatic cancer (van der Stegen et al., 2015). With that said, as some scientists believed that one co-stimulatory domain may not fully activate the T cell, third-generation CARs were developed to include two co-stimulatory domains (CD28 and 4-1BB) in a row along with CD3ζ (Pulè et al., 2005). Moreover, the effectiveness of this generation of CARs depends on the cancer type. While hematologic cancers

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like B-cell malignancies present uniform antigen targets and allow for CAR-T cells to circulate freely, solid tumors often display heterogeneous antigen expression and contain dense extracellular matrices, immunosuppressive cells, and inhibitory cytokines that limit the infiltration and persistence of CARs (Kloss et al., 2018; Yin et al., 2018). Consequently, the field hypothesizes that CAR-T efficacy is determined by the interplay between tumor composition and the design of CAR signaling. This explains why second- and third-generation CARs show strong results in blood cancer but variable success in solid tumors (Abate-Daga et al., 2014; Milone et al., 2009; Zhong et al., 2010).

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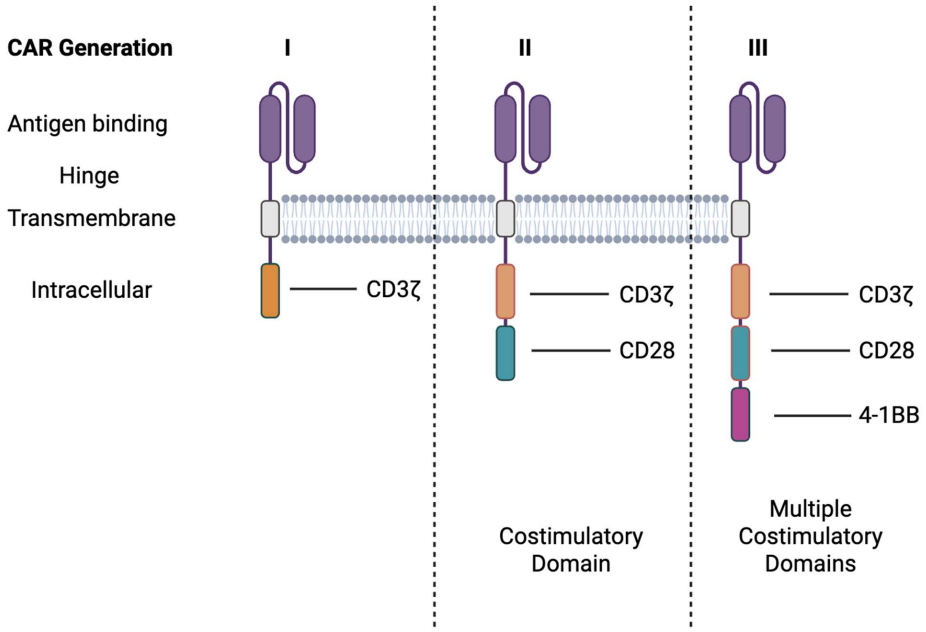

Fig. 2 Generations of CARs. The first generation of CARs consisted of only a CD3ζ signaling domain. In addition to the CD3ζ signaling domain, the second generation of CARs consisted of a CD28 costimulatory domain, and the third generation of CARs consisted of both CD28 and 4-1BB costimulatory domains.

Introduction to CAR-T cell therapy: What is it and how is it administered to patients?

CAR-T cell therapy is an immunotherapy that modifies one's body's own T cells (a type of white blood cell) to better recognize and destroy cancer cells. Although conventional treatments such as chemotherapy and stem cell transplantation have been successful in helping many achieve remission, they often fail in a significant subset of cases. For example, nearly 40% of acute myeloid leukemia (AML) patients experience induction failure after standard chemotherapy that combines cytarabine and anthracyclines (Culver-Cochran et al., 2024). Furthermore, many of these refractory cases do not respond to salvage therapy or allogeneic stem cell transplantation. Similarly, in patients with high-risk leukemia undergoing haploidentical stem cell transplants, despite intensified conditioning regimens, graft failure and relapse are common (Passweg et al., 2000). In contrast, even in patients who have not responded to prior chemotherapy or transplant, CAR-T cell therapy has achieved remarkable success in treating relapsed or refractory blood cancers.

While normal T cells can recognize and attack infected or abnormal cells, cancer cells often escape detection by upregulating inhibitory ligands like PD-L1 or downregulating antigen presentation, which suppresses T cell activation and masks their immunogenic identity (Perales et al., 2018). To address this problem, instead of relying on MHCs, CAR-T cells are genetically modified to carry a synthetic receptor that directly binds to antigens, which are specific proteins found on cancer cells (ie, CD19 in B-cell cancers) (Fig. 3). Because they are genetically engineered to recognize specific antigens independently of MHC presentation, CAR-T cells exhibit enhanced anti-tumor activity. This allows for CAR-T cells to rapidly engage tumor cells, secrete cytotoxic molecules such as perforin and granzyme B, and even induce apoptosis in tumors that evade normal immune detection.

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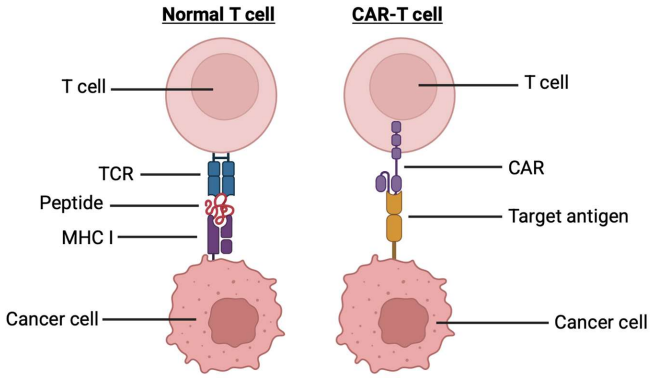

Fig. 3 Normal T cell vs CAR-T cell. Normal T cells rely on the MHCs of the cancer cell to present the antigen for the TCR to bind to it, whereas CAR-T cells can directly bind to the target antigen using their CAR.

Additionally, CAR-T cells can develop memory-like phenotypes, which allow them to persist longer and respond faster to relapsing cancer cells. Although CAR-T cells still interact with other components of the tumor microenvironment like endogenous cytotoxic T cells and natural killer cells, their design provides a direct, high-affinity mechanism for the killing of tumor cells that enhances efficacy even in cancers that are more difficult to treat (Maurya et al., 2025). Once inside the body, CAR-T cells continue to survive and proliferate. They "patrol" the bloodstream and look for any cancer cells that return over time, providing patients with long-term protection. Some CAR-T cells even form memory T cells and stay in the body permanently, and attack relapsed cancer. Because of its effectiveness, CAR-T cell therapy has been FDA-approved to treat several blood cancers like Acute Lymphoblastic Leukemia (ALL), Non-Hodgkin Lymphoma (NHL), and Multiple Myeloma (Maude et al., 2018; Neelapu et al., 2017; Schuster et al., 2017). Some of the approved CAR-T products include Kymriah (tisagenlecleucel) by Novartis, Yescarta (axicabtagene ciloleucel) by Kite Pharma, Breyanzi, Abecma, and other newer products that are still under clinical trials. Especially for patients who had relapsed after several treatments, CAR-T cell therapy has shown incredibly high success rates. For example, the ELIANA trial showed an 83% complete remission at 3 months with Kymriah, and the ZUMA-1 trial showed a 54% complete remission rate in patients treated with Yescarta (Almond et al., 2017; Locke et al., 2017).

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While effective, the process for CAR-T cell therapy is personalized and takes several weeks (Fig. 4).

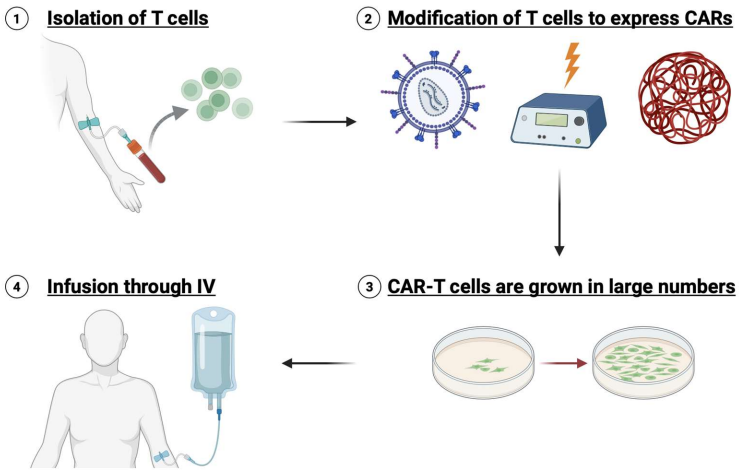

Fig. 4 Administration of CAR-T cells to patients. Patients first undergo Leukapheresis to isolate their T cells from other blood components. After isolation, the T cells are modified to express CARs through viral vectors, transposon systems, electroporation, or nanoparticles. Once modified, the CAR-T cells are grown in large numbers in labs to be used for treatment. The CAR-T cells are then infused into the patient's bloodstream through an IV.

First, through Leukapheresis, doctors collect a patient's blood and, using a machine that separates blood components, isolate the patient's T cells (Miliotou & Papadopoulou, 2018; Mirzaei et al., 2019). Then, the isolated T cells are modified to express CARs. This can be done using viral vectors (ie. γ-retroviruses or lentiviruses) that are engineered to carry the CAR gene into the T cell's DNA, transposon systems (ie. Sleeping Beauty or piggyBac) which use "cut-and-paste" DNA methods with a transposase enzyme, electroporation that uses electric pulses to open cell membranes so DNA can enter, or nanoparticles which are tiny carriers that deliver CAR genes safely without the use of viruses (Kidd et al., 2012; Yi et al., n.d.; Munoz-Lopez & Garcia-Perez, n.d.; Yarmush et al., 2014; Smith et al., 2017). Once modified, the T cells are grown in large numbers in the lab until there are millions of CAR-T cells prepared for treatment. To lower their existing immune cells, called lymphodepletion, the patient may first receive a short round of chemotherapy. This helps the CAR-T cells work better. Following this, the CAR-T cells are infused through an intravenous (IV). After the treatment is complete, for the first 2-3 weeks, patients are observed closely for side effects.

Key limitations in current CAR-T cell therapy

Despite the initial success of CAR-T cell therapy, it continues to face many limitations – namely, the limited cell persistence of CAR-T cells, the evolution of cancer cells to avoid detection and destruction, and poor efficacy of the therapy in solid tumors.

Limited cell persistence of CAR-T cells

With the inability to persist for long periods of time, sustained anti-tumor activity and durable patient responses are hindered. Although CAR-T cells can expand and mediate tumor clearance at first, many patients are susceptible to relapse due to a decline in CAR-T cell levels over time. This decline in cell levels could be due to several reasons. Firstly, suboptimal function of the co-stimulatory domains within the CAR could reduce T cell proliferation, survival, and memory formation after interaction with the antigen. Without sufficient co-stimulation, CAR-T cells will fail to sustain robust activation, leading to limited persistence. Furthermore, due to age, prior chemotherapy, chronic infections, or exhaustion induced by the tumor, the function of patient-derived T cells may already be compromised. This results in lower proliferation capacity, increased susceptibility to apoptosis, and impaired metabolic fitness. The diminished persistence causes issues, specifically in hostile tumor microenvironments (TME) where immunosuppressive signals like TGF-β, IL-10, and checkpoint pathways like PD-1/PD-L1 rapidly exhaust CAR-T cells and impair their survival (Kloss et al., 2018; Yin et al., 2018). Furthermore, the short life span of CAR-T cells may be due to inadequate co-stimulatory signaling or poor T cell fitness at the time of infusion. Given that early-generation CARs lacked co-stimulatory domains, they failed to provide the necessary signals for robust memory formation, which contributed to rapid attrition in vivo (Brocker & Karjalainen, 1995; Hege et al., 2017b; Till et al., 2008). Even with

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newer generations of CARs being developed, the persistence of CAR-T cells remains variable and patient-dependent (Abate-Daga et al., 2014; Milone et al., 2009; Zhong et al., 2010).

Evolution of cancer cells to avoid detection and destruction

Because of the adaptive evolution of tumor cells, this can lead to antigen escape. Antigen escape is a process where cancer cells downregulate or completely lose expression of the target antigen recognized by CAR-T cells. Although initial single-antigen CAR-T therapies – such as CD19 or BCMA targeting – achieved high response rates in hematologic cancers, over time, a significant number of patients relapse as the tumor cells modify themselves to avoid immune recognition. For example, due to a loss or mutation of the CD19 antigen, up to 30-70% of relapsed B-ALL patients experience recurrence (Majzner & Mackall, 2018; Maude et al., 2015). Likewise, following anti-BCMA CAR-T treatment, BCMA downregulation has been observed in multiple myeloma (Brudno et al., 2018; Cohen et al., 2019; Green et al., 2018). Additionally, solid tumors exhibit this immune escape pattern, like reduced IL13Ra2 expression following CAR-T therapy in glioblastoma (Brown et al., 2016). This could lead to an undermining of long-term treatment success because of the ability of tumor cells to effectively "hide" from the immune attack.

Poor efficacy in solid tumors

Due to several interrelated biological and structural barriers, the effectiveness of CAR-T cell therapy in solid tumors remains limited. Firstly, solid tumors lack ideal antigen targets that are both tumor-specific and not expressed on normal tissues. Because many antigens targeted in solid tumors are also found on healthy cells, although at low levels, this leads to on-target off-tumor toxicity and reduced therapeutic windows. Additionally, in solid tumors, CAR-T cells face significant trafficking and infiltration challenges. Unlike blood cancers, solid tumors have dense physical barriers like the extracellular matrix and tumor stroma that impede T cell penetration (B.-L. Zhang et al., 2016). Finally, the immunosuppressive TME, which is rich in regulatory T cells, myeloid-derived suppressor cells (MSDCs), and Tumor-associated macrophages (TAMs) that secrete inhibitory cytokines and engage immune checkpoint pathways (ie, PD-1/PD-L1, CTLA-4), results in T cell exhaustion and poor persistence of the CAR-T cells (Quail & Joyce, 2013; Yin et al., 2018).

Advancements and strategies to overcome limitations in CAR-T cell therapy

Hence, to enhance the overall effectiveness of CAR-T cell therapy in cancer treatment, scientists have pursued multiple strategies to overcome poor efficacy in solid tumors, susceptibility to immune escape by tumor cells, and limited cell persistence.

Targeting poor efficacy in solid tumors: Re-engineering of the tumor microenvironment and combinatorial delivery systems

Although CAR-T cell therapy has been successful for some blood cancers such as leukemia and lymphoma, it has not yet shown the same success in treating solid tumors like lung, breast, and ovarian cancers (Albelda, 2024; Flugel et al., 2023). When treating solid tumors, the main challenge lies in the fact that the TME, which is the space around tumor cells, is a hostile environment for immune cells (Hou et al., 2021; Landoni et al., 2024; Lee et al., 2023). In solid tumors, the TME creates multiple layers of defense that shut down immune responses (Table 1).

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Component	Example(s)	Function
Checkpoint proteins	PD-L1	They act as regulators of the immune system and prevent CAR-T cells from attacking tumor cells. While they function as "off switches" for the immune system and ensure it does not damage healthy tissues, they allow for tumors to escape immune destruction.
Suppressive immune cells	Regulatory T cells (Tregs) and MDSCs	They release anti-inflammatory signals to further weaken CAR-T cells.
Adenosine	–	High levels of it build up in the low-oxygen TME and signal through the A2AR receptor on CAR-T cells to slow them down.

Table 1 Components of the TME. The TME consists of multiple components: checkpoint proteins, suppressive immune cells, and adenosine. Together, these components allow for the TME to create a strong defense that shuts down immune responses in solid tumors.

Overall, these components shorten the lifespan and reduce the efficacy of CAR-T cells when destroying solid tumor cells.

Hence, to tackle the limited efficacy of CAR-T cell therapy in solid tumors, the most direct approach was to target modifying the TME to make it more suitable for CAR-T cell activity. For example, inhalable nanovesicles have been engineered to overcome the hostile TME (Zhu et al., 2025)(Fig. 5).

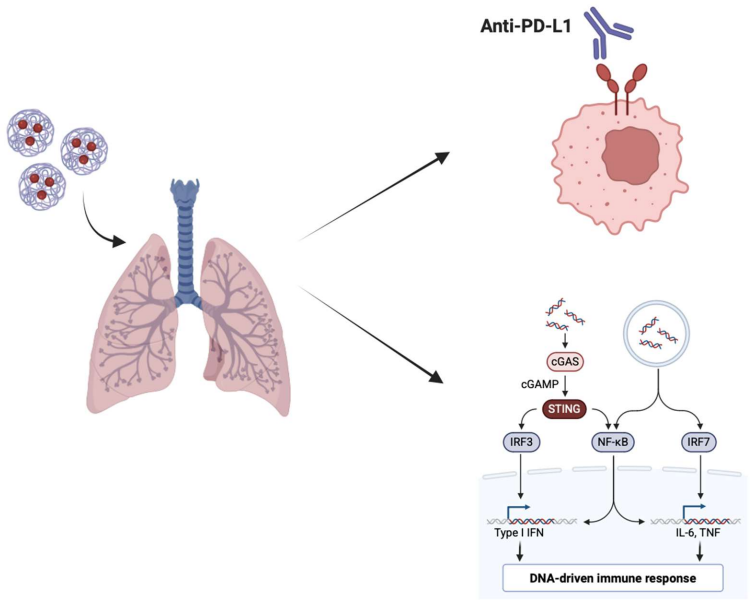

Fig. 5 Using inhalable nanovesicles to overcome the hostile TME. Inhalable nanovesicles that contain therapeutic molecules are inhaled into the patient's lungs. Upon inhaling these nanovesicles, they block the PD-1/PD-L1 interaction, allowing for CAR-T cells to remain active, and they activate the STING pathway, initiating a strong immune response.

Inhalable nanovesicles are small, biodegradable vesicles that contain therapeutic molecules that could be directly inhaled into the lungs. Each nanovesicle carries an anti-PD-L1 single-chain variable fragment (scFv) and a STING agonist (cGAMP). The scFv is a small antibody piece that binds to PD-L1 on tumor cells and blocks the PD-1/PD-L1 interaction, allowing for the CAR-T cells to remain active. The cGAMP is a molecule that activates the Stimulator of Interferon Genes (STING) pathway and leads to the production of type I interferons such as IFN-β and the attraction of antigen-presenting cells such as dendritic cells to help amplify the immune response. Success of these inhalable nanovesicles was observed in mouse models of lung cancer where they triggered local immune activation without systemic toxicity, increased levels of molecules (ie. IFN-β, IL-2, and CXCL9) that help attract and activate more CAR-T cells, reduced exhaustion markers (ie. PD-1, LAG3, and TIGIT) on CAR-T cells, promoted the formation of $TCF1^{+}$ memory-like T cells which are known for their persistence and self-renewal properties, and resulted in stronger tumor shrinkage, delayed relapse and improved survival of CAR-T cells.

Besides targeting the TME, efforts have also been made in changing the CAR-T cells themselves to resist suppressive signals from within the TME. Research has shown that adenosine, which is released in high amounts by tumors, functions by signaling through the A2AR receptor, a G protein-coupled receptor that suppresses T cell proliferation, cytokine production, and cytotoxic activity, to deactivate CAR-T cells (Sek et al., 2025). In response to this, because A1R, another adenosine receptor whose signaling counteracts A2AR, enhances T cell activation and survival when activated, CRISPR-Cas9 gene editing technology was used to insert the gene for the A1R receptor into CAR-T cells. These newly engineered CAR-T cells with the A1R receptor resulted in higher production of immune-stimulating cytokines (ie, IFNγ, TNFα, and IL-2), reduced expression of exhaustion markers (ie, PD-1 and TIM-3), and increased expression of CD69 and IRF8, which indicated stronger immune readiness and potential for longer-lasting memory. To stabilize the beneficial effects, the transcription factor NR4A2 has been added, which helps CAR-T cells, especially in environments full of stress and inflammation, maintain anti-suppressive gene expression over time. In mice with tumors, CAR-T cells engineered with A1R and NR4A2 resulted in sustained tumor disappearance, preserved stem-like and central memory T cell populations that are essential for long-term immunity, and eliminated the need for repeated dosing of CAR-T cells.

In combination, these strategies could potentially revolutionize CAR-T cell therapy for increased efficacy in solid tumors, which make up the vast majority of cancers.

Targeting susceptibility to immune escape by tumor cells: Dual-antigen targeting

Another major limitation in CAR-T cell therapy is the downregulation or loss of the target antigen on tumor cells (Neelapu et al., 2017; Tong et al., 2020). This facilitates tumor cells escaping from immune cells, leading to relapse. This phenomenon is well-documented in B-cell malignancies (ie. DLBCL and B-ALL) where alterations in target antigens like CD19–caused by missense mutations, frameshifts, or alternative splicing–allow tumor cells to evade recognition by CARs (Sotillo et al., 2015).

Hence, to reduce the likelihood of relapse due to the loss of target antigens on tumor cells, dual-antigen targeting strategies – such as by simultaneously engaging two distinct antigens associated with tumors – have been developed (Dai et al., 2020; Grada et al., 2013; Hamieh et al., 2019; Hegde et al., 2016; Majzner & Mackall, 2018; Zah et al., 2016).

For example, in the phase I/II of a clinical trial, researchers developed a tandem CAR construct with the ability to bind to both CD19 and CD20 (Wang et al., 2024). CD19 and CD20 surface proteins are ideal targets for cancer therapy as they are highly expressed almost exclusively on B lymphocytes and absent on most other cell types. The construct, connected via a flexible linker, integrated scFvs specific for CD19 and CD20 and incorporated the hinge and transmembrane domains of CD8 with 4-1BB and CD3ζ signaling domains (Fig. 6).

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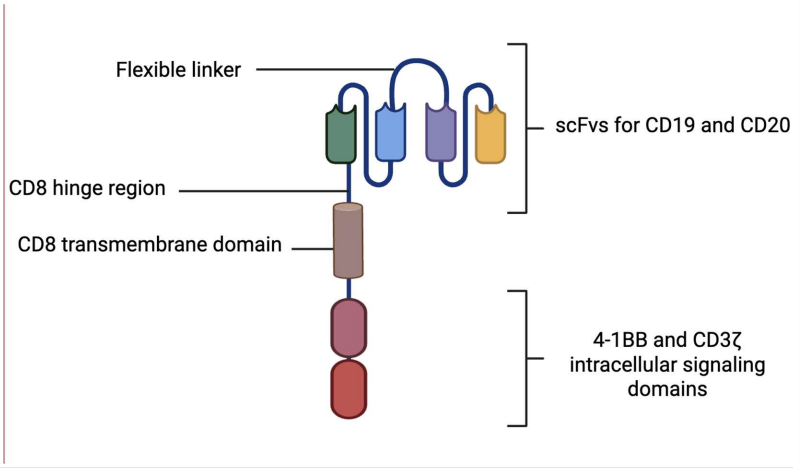

Fig. 6 Structure of CD19/20 CAR-T cell. The CD19/20 CAR-T cells have tandem CAR constructs that contain an antigen-binding domain with single-chain variable fragments (scFvs) for CD19 and CD20, a CD8 hinge and transmembrane domain, and 4-1BB and CD3ζ intracellular signaling domains.

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In preclinical assays, the CD19/20 CAR-T cells demonstrated efficacy in destroying cancer cells that expressed either CD19, CD20, or even both, and they released similar levels of cytokines as single-target CAR-T cells. The release of cytokines is important as they function as chemical messengers that enable immune cells to communicate and coordinate an immune attack against harmful substances such as cancer cells.

Clinically, this dual-targeting approach showed promising efficacy. In the group of patients with relapsed or refractory (R/R) B-cell NHL, with a 12-month overall survival rate of 81.82% and progression-free survival of 60%, 81.8% of patients experienced complete remission. Importantly, even in the presence of antigen loss for one target, the bispecific CAR-T cells remained robust. This suggests a decreased risk of immune escape in these CAR-T cells compared to single-target CAR-T therapy. Through single-cell RNA sequencing, results showed that the main CAR-T cell groups after infusion contained high levels of genes that are involved in immune signaling, especially those that help activate the body's first-line immune defenses and those that aid in the destruction of cancer cells. Despite changes in the way cancer cells display their surface antigens to allow them to avoid detection from the immune system, likely due to the mentioned features, these CAR-T cells sustained anti-tumor activity.

Furthermore, beyond mitigating relapse driven by antigen loss, this dual-antigen targeting strategy also broadens the therapeutic window for patients with heterogeneous antigen expression profiles. Through the maintenance of targeting capability against tumor cells that have downregulated one antigen, these CD19/20 CAR-T cells exemplify how rational CAR design can directly address a critical resistance mechanism in immunotherapy.

Targeting limited cell persistence: CAR enhancer (CAR-E)

After the initial CAR-T cell treatment, CAR-T levels often deplete, resulting in patients being vulnerable to relapse. Hence, new ways to target the limited cell persistence of CAR-T cells are being developed to improve the long-term effectiveness of the therapy.

For example, scientists have designed a CAR enhancer (CAR-E) to prolong CAR-T durability without broad cytokine exposure (Rakhshandehroo et al., 2025). CAR-E is a recombinant protein that exists in the extracellular space and can diffuse to interact with CAR-T cells that are bound to the target antigen. It functions by coupling a tumor-antigen binder to a low-affinity Interleukin 2 (IL-2) dimer that engages only antigen-bound CAR-T cells. This ensures that the cis IL-2 receptor (cis IL-2R) signaling is delivered precisely where it is required. In this context, rather than activating neighboring T cells in the environment (trans signaling), "cis" signaling means that the IL-2 dimer acts locally within the same CAR-T cell that has engaged its target antigen. While not compromising its efficacy by preserving cytotoxicity and not activating other cells, such as non-transduced T cells that might hurt the safety and efficacy of the therapy, CAR-E switches on STAT5 and early activation in CAR-T cells (Fig. 7). By switching on STAT5 and early activation in CAR-T cells, this will result in bigger, longer-lived, and more effective CAR-T responses. The CAR-E only functions when both the CAR endodomain and the IL-2 receptor signal, and it's pulled inside the CAR-T cell itself via the IL-2R, which helps keep the effect contained.

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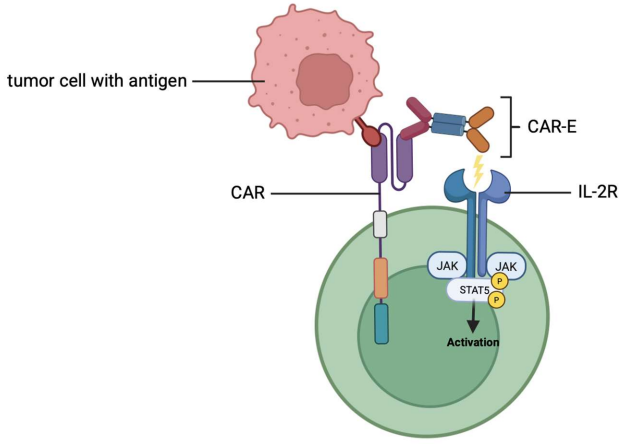

Fig. 7 CAR-E mechanism. CAR-E couples tumor-antigen recognition with a low-affinity IL-2 dimer that engages the IL-2R only in CAR-T cells bound to antigens. This activates the JAK/STAT5 pathway, promoting early activation, enhanced persistence, and stronger cytotoxic responses without broad cytokine exposure.

In vivo, dose-dependent multiplication and enhanced cell persistence of CAR-T cells in blood, spleen, and bone marrow, as well as enrichment of memory phenotypes such as central memory (T_CM) and stem-like memory (T_SCM), are produced through brief, pulsed dosing (serum t½ ~1.5 h). Importantly, CAR-T cells that are treated with CAR-E can be maintained even at lower CAR-T doses or in the absence of a tumor and can re-proliferate upon interaction with cancer cells that reappear. This supports an on-demand "booster" paradigm to sustain CAR-T cell levels after they peak and drop.

Overall, CAR-E is a safe method that improves cell persistence of CAR-T cells, as its low-affinity IL-2 is weak enough that it prevents activation of nearby, non-CAR-T cells, and it clears from the body quickly, allowing doctors to fine-tune the dosage. Furthermore, since the CAR-E mainly consists of components derived from humans, it minimizes predicted immunogenicity. The design of the CAR-E is also modular, meaning the binding piece to use CAR-E with other CAR targets (ie, CD19/CD22 or GD2) can be swapped, potentially even in solid tumors. In practice, scientists can initially carry out manufacturing that starts with memory-like T cells (T_SCM/T_CM) and keeps the culture time short to ensure cells stay youthful and durable. Then, CAR-E provides targeted IL-2 signals after infusion to maintain and expand those memory-leaning CAR-T cells in patients. Additionally, as CAR-E clears fast, clinicians can briefly give small pulses of CAR-E when CAR-T cell levels start to dip or when tumor signals reappear, then stop. This ensures the maintenance of cell persistence while minimizing negative side effects like cytokine toxicity, where the immune system releases too many cytokines all at once and triggers undesirable outcomes, or unwanted T cell activation.

When taken together, it is a targeted, scalable method to ensure CAR-T cell persistence while ensuring maintenance of control over systemic cytokine exposure.

Conclusion

Offering unprecedented remission rates in hematologic malignancies and giving rise to personalized, immune-based treatment for cancer, CAR-T cell therapy is one of the most transformative innovations in modern oncology. However, because of challenges such as limited cell persistence, susceptibility to immune escape, and poor efficacy in solid tumors, its potential to be used as a treatment in clinical practice has been hindered. Recent advances, such as re-engineering the tumor microenvironment through the use of inhalable nanovesicles and A1R-directed CAR-T cells, developing dual-antigen targeting constructs to counter antigen loss, and integrating CAR enhancers to sustain memory-like T cell populations, demonstrate that these limitations are neither overcome nor insurmountable. Together, the rise of these strategies indicates a shift in the therapy from a reactive treatment toward an increasingly proactive, precision-focused CAR design where CAR-T cell therapy is tailored to the tumor's biology and individual patients' differing needs. As deeper immunological insights are being discovered and innovations are arising, CAR-T cell therapy is anticipated to evolve from a niche intervention method for specific blood cancers into a more versatile and adaptable platform that is capable of overcoming heterogeneous antigen expression, hostile microenvironments, and short-term

durability. Whether CAR-T cell therapy fulfills its promise of becoming a universal, durable, and safe cancer treatment depends on the continued integration of preclinical breakthroughs into clinical practices accessible for patients worldwide.

Acknowledgments

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Responses to Referee 1

Summary: The author greatly appreciates the referee for their thoughtful and constructive feedback on the manuscript. The referee raised several key points to be addressed, including refining the overarching research focus, strengthening the critical analysis within the body of the review, and expanding on the introduction and conclusion for improved clarity and balance. In response, the author has refined the scope of the review to emphasize a more specific and novel aspect of CAR-T cell therapy, revised multiple sections (pages 2, 3, 6, 9, 12, and 16) to include deeper analysis, clearer reasoning, and stronger scientific context, and ensured formal consistency throughout the text. Figure 6 has been corrected and clarified with an updated legend, and citation formatting has been standardized in accordance with editorial guidance. While the conclusion remains focused on CAR-T therapy to maintain coherence with the manuscript's central theme, all comments have been carefully considered and addressed. These revisions have strengthened the manuscript's clarity, specificity, and scientific rigor in direct response to the referee's suggestions.

Responses to the referee's comments:

1) First, potentially make the overarching research topic more focused – it's currently very general, and maybe it would be good to have a more specific research topic that seems novel in the field.

Response: The title has been revised to reflect a more focused and novel research direction. The new title, *"Integrative Molecular Engineering of CAR-T Cells: Emerging Strategies to Enhance Persistence, Targeting, and Tumor Microenvironment Adaptation,"* narrows the overarching topic to emphasize recent molecular innovations aimed at improving CAR-T cell persistence, specificity, and adaptability within the tumor microenvironment. This refinement clarifies the manuscript's central focus while aligning it with emerging advancements in CAR-T cell engineering.

2) Second, it would be good to touch a bit more on what a CAR T cell is in the introduction. I know this is discussed later in the review, but it would be good to bring it here now.

Response: The author sincerely appreciates this thoughtful suggestion. Upon careful consideration, the author has decided to retain the current form of the introduction, as it already provides a concise yet sufficient explanation of CAR-T cells to establish the necessary background without introducing redundancy. Specifically, the existing text — *"Designed to allow lymphocytes such as T cells to target and eliminate cells expressing a specific surface antigen, Chimeric Antigen Receptors (CARs) are engineered artificial receptors that, unlike normal T cell receptors, do not rely on major histocompatibility complex (MHC) molecules to recognize antigens"* — effectively introduces the concept early in the manuscript. This level of

detail ensures clarity while maintaining focus and avoiding repetition with later sections where CAR-T cell mechanisms are discussed in greater depth.

3) However, there are occasions where the writing becomes slightly informal. The author should consider the audience they're writing to ensure this consistency, and do not use conversational language for writing. For example, there are moments when this is aimed at scientific communication for people that are not in science, "While normal T cells can recognise and attack infected or abnormal cells, cancer cells often escape detection by sending out "off signals" or hiding their identity." While this is okay, I would recommend maintaining a professional style of writing.

Response: The author sincerely appreciates this valuable feedback regarding writing style and tone. In response, the author has carefully re-read and revised the entire manuscript to ensure consistency in formality and maintain a professional scientific tone throughout. Informal or conversational phrasing has been replaced with more precise and discipline-appropriate language, aligning the manuscript with the expectations of a scientific audience.

4) First of all, it should be mentioned in the figure legend that this is a tandem CAR – all figure legends should be able to stand on their own, and not require context from the rest of the writing. Second, the flexible linker in the figure is incorrect, as the way it's drawn is scientifically not possible (coming from a protein engineering perspective).

Response: The author greatly appreciates this detailed and insightful feedback. In response, Figure 6 has been revised to accurately depict the tandem CAR structure, and the flexible linker has been corrected to reflect a scientifically accurate configuration based on protein engineering principles. Additionally, the figure legend has been updated to clearly specify that the figure represents a tandem CAR, ensuring that it can stand independently without requiring additional context from the main text.

5) The conclusion summarises the review well, but it would be great for the author to think critically and creatively about what the next steps are going to be for the field.

Response: The author appreciates this thoughtful comment. After careful consideration, the author has chosen to retain the current focus of the conclusion on CAR-T therapy. While discussing broader directions in cancer therapy would indeed be of interest, such an expansion could shift the manuscript's emphasis away from CAR-T cells, which remain the central topic of this review. Maintaining this focus ensures coherence and thematic consistency throughout the paper.

6) Ensure that the in-text citations are before punctuation, rather than after. Also it would be good to reference according to each point made, rather than adding all of the citations at the end of the sentence.

Response: The author appreciates this helpful comment. In response, all in-text citations have been revised to appear before punctuation marks as recommended. Citations have also been adjusted to correspond directly to individual points made within sentences, ensuring accuracy, clarity, and consistency in referencing throughout the manuscript.

7) "As it controls the distance between the CAR-T cell and the target cell, the hinge is also important for the formation of an immunological synapse, which is needed for proper T cell activation.": You need to explain this in more detail. Why is the distance important? What happens if it the linker is too short or too long? The kinetic segregation model is good to discuss here.

Response: The author thanks the referee for this suggestion. In response, the manuscript now includes a detailed explanation of how hinge length affects CAR-T cell activation. According to the kinetic segregation model, the hinge determines the distance between the T cell and its target, which is critical for proper exclusion of inhibitory phosphatases and efficient signaling. If the hinge is too short or too long, this spacing is disrupted, potentially reducing activation or causing signaling errors.

8) "Moreover, scientists have discovered that since it can result in CARs forming dimers and joining with the cell's natural T cell receptors (TCRs), using the CD3ζ transmembrane domain could possibly help more effectively activate T cells. (Bridgeman et al., 2010) With that said, using a CD3ζ transmembrane domain in a CAR could result in it being less stable than those with CD28 transmembrane domains.": Why, what's the evidence? Feel like there's a lot of summarising here without a lot of explanation or analysis.

Response: The author thanks the referee for this comment. In response, the manuscript now provides additional explanation and analysis regarding the CD3ζ transmembrane domain. It is clarified that this domain brings the CAR into close proximity with the TCR signaling complex, enabling recruitment of intracellular kinases that activate T cells. However, interaction with endogenous CD3ζ chains may reduce receptor stability and surface expression, making CARs with a CD3ζ transmembrane domain less stable than those with CD28 transmembrane domains.

9) "didn't multiply or survive well": Do not use contractions in the review. Also try and use appropriate technical words, instead of multiply, use proliferate. "Survive well" also

seems a bit too informal, consider revising this. There are other sentences which are similar which I have not included here, so consider proofreading with this in mind.

Response: The author appreciates this valuable suggestion. In response, all contractions have been removed from the manuscript, and informal expressions, including terms such as “multiply” and “survive well,” have been revised to use more precise and technically appropriate language. The manuscript has been carefully proofread to ensure a consistent formal scientific tone throughout.

10) “The two most commonly used co-stimulatory domains are CD28 and 4-1BB (CD137)”: But why did they decide on these co-stim domains? Probably an important part to discuss.

Response: The author thanks the referee for this suggestion. In response, the manuscript now explains that CD28 and 4-1BB were chosen because they enhance CAR-T cell function through distinct signaling pathways. CD28 promotes rapid T cell activation and cytokine production, leading to strong but shorter-lived responses, whereas 4-1BB signaling supports mitochondrial biogenesis and memory formation, resulting in more sustained persistence and reduced exhaustion.

11) “Moreover, the effectiveness of this generation of CARs depends on the cancer type, proving that more research and testing are required.”: Why might this be the case? What’s the field’s hypothesis for this? Be specific.

Response: The author thanks the referee for this insightful comment. In response, the manuscript now clarifies that CAR-T efficacy varies by cancer type due to differences in tumor characteristics. Hematologic cancers, such as B-cell malignancies, present uniform antigen targets and allow CAR-T cells to circulate freely, whereas solid tumors exhibit heterogeneous antigen expression and possess dense extracellular matrices, immunosuppressive cells, and inhibitory cytokines that limit CAR-T infiltration and persistence. Consequently, the field hypothesizes that the effectiveness of CAR-T therapy is determined by the interplay between tumor composition and CAR design, explaining the strong results in blood cancers compared with the variable success observed in solid tumors.

12) “sending out “off signals” or hiding their identity”: Use technical words.

Response: The author appreciates this suggestion and has carefully re-read the manuscript to replace informal expressions with more precise and technical terminology throughout. Phrases such as “sending out off signals” and “hiding their identity” have been revised to reflect appropriate scientific language, ensuring clarity and professionalism in the manuscript.

13) “This allows for CAR-T cells to have a stronger targeting ability as they don’t rely on other immune cells, longer-lasting memory to help them fight off relapsing cancer, and enhanced killing ability even in advanced or cancers that are harder to treat.”: The author often uses a lot of lists to back up their claim. I would suggest instead that they expand on this in more detail. For example, what immune cells are you talking about – are we talking about the APCs or other cytotoxic immune cells, as I would argue that they will always rely on other immune cells within the TME. Why do they have enhanced killing ability? All of this needs more detail, this cannot be mentioned and then not expanded on later within the paragraph, as it comes off as being vague.

Response: The author thanks the referee for this suggestion. In response, the manuscript has been revised to provide additional detail regarding CAR-T cell targeting and efficacy. It is clarified that CAR-T cells can develop memory-like phenotypes, allowing for prolonged persistence and faster responses to relapsing cancer. While they continue to interact with other components of the tumor microenvironment, such as endogenous cytotoxic T cells and natural killer cells, their design provides a direct, high-affinity mechanism for tumor cell killing, enhancing efficacy even in cancers that are more difficult to treat.

14) “decline in CAR-T cell levels over time…due to inadequate co-stimulatory signalling or poor T cell fitness at the time of infusion.”: For both points – why does this happen? Especially for the second point, think about T cell fitness being dependent on the patient that the T cells come from.

Response: The author thanks the referee for this insightful comment. In response, the manuscript now provides additional explanation for the decline in CAR-T cell levels over time. Suboptimal co-stimulatory signaling can reduce T cell proliferation, survival, and memory formation, limiting persistence. Additionally, patient-derived T cells may already exhibit compromised function due to factors such as age, prior chemotherapy, chronic infections, or tumor-induced exhaustion, resulting in lower proliferation capacity, increased apoptosis, and impaired metabolic fitness.

15) A2AR and A1R – the author needs to explain what this is in more detail, and not only mention them within the paragraph.

Response: The author thanks the referee for this comment. In response, the manuscript now provides additional explanation of these receptors. Adenosine, released in high amounts by tumors, signals through the A2AR receptor, a G protein-coupled receptor that suppresses T cell proliferation, cytokine production, and cytotoxic activity, thereby deactivating CAR-T cells. Conversely, A1R, another adenosine receptor whose signaling counteracts A2AR, enhances T cell activation and survival when activated. To exploit this, CRISPR-Cas9 gene editing has been

used to insert the A1R gene into CAR-T cells, improving their function in the immunosuppressive tumor microenvironment.

16) The CAR-E needs to be explained in more detail. Specifically that this is a soluble protein, which is not really clear until looking at the figure and need to explain in more detail how the cis-IL2R signalling works.

Response: The author thanks the referee for this suggestion. In response, the manuscript now clarifies that CAR-E is a recombinant soluble protein that exists in the extracellular space and can diffuse to interact with CAR-T cells bound to their target antigen. Additionally, the concept of “cis” IL-2 receptor signaling has been explained: rather than activating neighboring T cells (trans signaling), the IL-2 dimer acts locally within the same CAR-T cell that has engaged its target, ensuring precise and targeted activation.

Responses to Referee 2

Summary: The author greatly appreciates the referee for their thoughtful and constructive feedback on the manuscript. The referee raised several key points, including maintaining a formal academic tone, defining acronyms consistently, standardizing citation formatting, and improving the structure and flow of the manuscript. In response, the author has removed all contractions, ensured each acronym is defined only once and used consistently, and revised all in-text citations to appear before punctuation marks while standardizing the reference list. Structural adjustments include consolidating sections on strategies to enhance CAR-T cell therapy into a single comprehensive section with three clear subsections, and integrating images throughout the text to complement the discussion. These revisions have improved the clarity, readability, and overall quality of the manuscript in accordance with the referee's suggestions.

Responses to the referee's comments:

1) At the beginning of this section where the author describes that other treatments like chemotherapy and stem cell transplants have failed, there is little information provided comparing the details of these methods compared to CAR-T cell therapy. Chemotherapy and stem cell transplants do not always fail, so it would be helpful if the author could provide details on how often these treatments fail and compare it to how often CAR-T fails.

Response: The author thanks the referee for this suggestion. In response, the manuscript now provides additional context comparing conventional treatments to CAR-T therapy. While chemotherapy and stem cell transplantation have successfully induced remission in many patients, a substantial subset still experiences treatment failure—for example, nearly 40% of acute myeloid leukemia patients fail induction chemotherapy, and graft failure or relapse remains common in high-risk leukemia patients undergoing haploidentical stem cell transplants. In contrast, CAR-T cell therapy has demonstrated remarkable efficacy even in patients who have not responded to prior chemotherapy or transplantation, highlighting its potential in relapsed or refractory blood cancers.

2) Figures should be spread throughout the text and not reserved for the end of each section. This makes it more difficult for the reader to look at the figure as they are reading the text. The student should integrate the figures close to where they are referred to in the body to allow easy visualization for the reader while reading the text.

Response: The author thanks the referee for this suggestion. In response, all figures have been repositioned and integrated throughout the text close to where they are discussed, allowing readers to easily reference visual material while reading the corresponding sections.

3) The structure of the paper is diU̓icult to follow. It is unclear how the manuscript i organized into sections as there are numerous sections that are missing key transitions. It would be useful for the author to either have fewer sections and combine some sections creating subsections, or provide transition paragraphs to explain the shift from limitations to developments.

Response: The author has made structural adjustments to improve the flow of the manuscript, consolidating multiple sections on strategies to enhance CAR-T cell therapy into a single comprehensive section titled “Advancements and strategies to overcome limitations in CAR-T cell therapy,” now organized into three clear subsections to maintain logical progression and reduce redundancy.

4) The author must review their references and ensure properly formatted. For example, the reference “Convergence of Acquired Mutations and Alternative Splicing of....” Is formatted like a website with incorrect information. This reference is a journal article, with authors, a year, issue, volume, and page numbers. All references should have authors, year, title, journal, volume, issue (if available), page numbers, and doi.

Response: The author thanks the referee for this comment. In response, all references have been carefully reviewed and corrected to ensure proper formatting, including authors, year, title, journal, volume, issue (if available), page numbers, and DOI, in accordance with journal guidelines.

5) The author uses a contraction “don’t” in the introduction. The reviewer suggests avoiding the use of contractions in academic scientific literature. There are other areas throughout the paper that contractions are used and they should also be expanded.

Response: The author thanks the referee for this suggestion. In response, all contractions throughout the manuscript have been removed or expanded to maintain a formal academic tone.

6) The author refers to a figure in the section heading: “Structure of CAR (Fig. 1).” The author should refer to a figure in the body text, not in the heading of a section.

Response: The author thanks the referee for this suggestion. In response, all figure references have been moved from section headings to the body text to ensure proper placement and clarity.

7) The author defines MHC in the section “Antigen-binding domain” but this acronym has already been defined in the introduction. For all acronyms defined, the author only needs to define acronyms once and after that, can just use the acronym for more concise language. There are other instances of redefining CAR and CAR-T that is repetitive.

Response: The author thanks the referee for this comment. In response, all acronyms have been reviewed to ensure they are defined only once upon first mention, and subsequent uses consistently employ the acronym, eliminating redundant definitions throughout the manuscript.

8) Figure 2 does not need it's own entire page, the text should immediately follow the figures.

Response: The author thanks the referee for this suggestion. In response, Figure 2 has been repositioned so that the text immediately follows the figure, improving readability and flow.

9) The table needs a label (Table 1) and must be referred to directly in the text.

Response: The author thanks the referee for this comment. In response, the table has been labeled as Table 1 and is now directly referred to within the text to ensure proper referencing and clarity.

10) References should be placed at the end of the sentence, not in the middle.

Response: The author thanks the referee for this suggestion. In response, all in-text citations have been adjusted to appear at the end of sentences, ensuring consistency and adherence to proper referencing style.

11) There is no reference provided for the study discussing the eUect of adenosine on CAR-T cell deactivation. Ensure that large paragraphs describing studies are backed by references.

Response: The author thanks the referee for this comment. In response, an appropriate reference has been added to support the discussion of adenosine's effect on CAR-T cell deactivation, and all relevant paragraphs describing studies have been reviewed to ensure they are properly cited.

12) The end of the first paragraph is a sentence structure that is confusing to the reviewer. Please ensure that the punctuation and citation is correct.

Response: The author thanks the referee for this suggestion. In response, the sentence has been rephrased for clarity and correct punctuation: *"This phenomenon is well-documented in B-cell malignancies (ie. DLBCL and B-ALL) where alterations in target antigens like CD19–caused by missense mutations, frameshifts, or alternative splicing–allow tumor cells to evade recognition by CARs."*

The author has done well in integrating most of the comments/feedback that I have given them in the initial review, and have very much enjoyed reading the author's responses to my comments. This includes the revision of the title which is much more focused now, in addition to the explanation of the use of adapting hinges to suit the target antigen – this is a big area in the field, with a company that's based on the idea of KS and hinge adaptations, called MatchBio. I have only a few minimal comments to add onto this.

Firstly, to the author's response of my 5th comment regarding what the next steps are the field are in the conclusion – I am not asking the author to stray away from the CAR-T cell field to discuss other possible strategies of cancer immunotherapy, which I think the author misunderstood. Instead, I want the author to consider within the CAR-T therapy field, based on the additional limitations which are coming out of clinical trials including relapse after CAR-T treatment 10 years later, as well as limited efficacy in solid tumours for a variety of reasons, what does the author think will come next for the CAR-T field? I would like specifics, for example, will there be increasing use of soluble proteins alongside CAR-T cell therapies, either to improve specificity or efficacy? What other modifications will they add to the CAR-T cell – such as a PD-1 switch receptor. These are the things I'm looking for, as it should be that by the end of the review, the author has a good understanding of the CAR-T field, and in some sense, it is good to see what the author's opinion is on what's next – instead of potentially what's already in the literature.

Secondly, in Table 1 on page 10 – within the examples column, the author needs to write the long form of MDSCs, and ideally Tregs and MDSCs are also mentioned somewhere within the text and not only in the table. Besides this, the function column requires writing in a more formal manner, similar to my comments last time. For example, "immune destruction", "further weaken CAR-T cells", "signal through A2AR receptor on CAR-T cells to slow them down" – there are more technical, more scientific words which can be used to express the above, and so I would recommend revising this.

Lastly, also on page 10 regarding the inhalable nanovesicles – the explanation of what this is and the signalling pathways to improve CAR-T cell efficacy is good and detailed. However, it would be good for the author to specify where this is most likely effective. From the figure, and the idea that it is inhalable, it sounds like it would be most effective for lung cancer CAR-T cell treatment because it becomes highly bioavailable in the lungs specifically. Or is it because this increases bioavailability compared to intravenous delivery of anti-PD-L1 mAbs?

Besides the above comments, this is a very good piece of writing, clearly showing a lot of time has been dedicated to the research and understanding of this topic, so well done to the author. These last few comments are just to ensure that the paper is just a small step above from the level that it's currently at. Overall, I would recommend this paper be accepted with the minimal revisions above.

The author's changes to the manuscript strengthen it greatly. There are only a few minor suggestions remaining for the manuscript that warrant consideration prior to acceptance:

- Table 1 – the table label should be above the table. Unlike a figure's caption where it is found below the figure, a table has a caption that is above it. This caption is normally short and less descriptive than a figure caption.
- The section heading "Introduction to CAR-T cell therapy: What is it and how is it administered to patients?" is a long heading. The reviewer would advise making this more concise and avoiding the word introduction in a section that is not the introduction.