Current strategies to improve CAR-T cell persistence

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Abstract
Chimeric antigen receptor T (CAR-T) cell therapy has transformed the field of immunology by redirecting T lymphocytes toward tumor antigens. Despite successes in attaining remission rates as high as 70%, the performance of CAR therapy is limited by the survival of T cells. T cell persistence is crucial as it sustains immune response against malignancies, playing a critical role in cancer treatment outcomes. This review explores various approaches to improve CAR-T cell persistence, focusing on the choice between autologous and allogeneic cell sources, optimization of culture conditions for T cell subsets, metabolite adjustments to modify T cell metabolism, the use of oncolytic viruses, and advancements in CAR design. While these approaches are promising on their own, combining them could further enhance the persistence of T cells, particularly in targeting solid tumors. Understanding the underlying mechanisms behind these strategies is essential for maximizing the potential of CAR-T therapy in treating cancer. Further research is needed to improve safety and efficacy and seamlessly integrate the discussed strategies into the manufacturing process.

Introduction
Chimeric antigen receptor T (CAR-T) cell therapy has transformed the field of immunology with outstanding success in treating hematological cancers. CARs are cell-surface receptors designed to navigate T cells toward a specific antigen. The extracellular domain of the CAR originates from an antibody fragment, allowing the cell to recognize tumor-associated antigens (TAAs). Tumors with low or no major histocompatibility complex (MHC) expression can be challenging for T cells to attack. However, CAR-T cells target cancer cells independently of MHC presentation and bind directly to TAAs. Once T cells are harvested from the donor's blood, the CAR construct is introduced into T cells through viral vector transduction. The engineered CAR-T cells are then cultured and expanded *ex vivo* with supplementation of cytokines before being injected into the patient. Since the first FDA-approved CAR therapy, Kymriah was approved in 2017, many similar therapeutics, such as Yescarta, Tecartus, and Breyanzi, have been introduced. These treatments have had remarkable success, with patients achieving complete remission (CR) 30–70% of the time and, in some trials, over 90% (1). However, many studies still depict CARs as ineffective because of a lack of persistence.

The clinical successes of CAR-T therapy have been linked to the ability of CAR-T cells to proliferate and survive within the patient's body (2). Favorable outcomes depend on the persistence of CAR-T cells after initial infusion. Sustained persistence ensures that T cells remain active to mount an effective immune response, thus providing long-term protection against malignancies. The longer CAR-T cells can expand and remain cytotoxic, the stronger the anti-tumor response and the higher the likelihood of achieving remission and patient survival. Considering that cancer is a long-term challenge, the duration of the immune response is even more important. Thus, this review paper will explore strategies to increase persistence.

Autologous or Allogeneic: Choosing a Cell Source

Choosing a source of CAR-T cells is important to maximize persistence. The source and components of T cells influence the properties of CAR-T cells, so they should be carefully selected. CAR-T cells are classified as autologous (derived from the host) or allogeneic (derived from a donor); both of these types of cells have certain advantages and limitations that have been observed in clinical trials (Fig. 1).

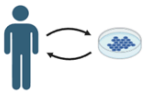
Characteristic	Autologous CAR-T Cells 	Allogeneic CAR-T Cells 
Origin	Patient	Healthy Donor
Manufacturing	Complex logistics; 2-10% failure rate; long and delayed manufacturing;	Streamlined industrial process; many CAR-T cells produced in a single batch; easily accessible and available for patient treatment
Risks	Cytokine release syndrome;	Cytokine release syndrome; Graft-versus-Host disease; rejection of allogeneic cells
Cost	High cost	Moderate cost
Redosing	Limited by number of cells	No limitations for redosing
Persistence	Intermediate to long (months to years)	Short to intermediate (weeks to months)

Figure 1. Comparison between autologous vs allogeneic CAR-T cell.
Autologous cells have longer manufacturing processes, higher costs, and limited redosing but exhibit stronger persistence with a lower risk for cytokine release syndrome. Allogeneic cells are a cheaper, more accessible patient treatment option but are less personalized and display shorter persistence with higher risk for CRS and GvHD.

The main advantage presented by autoCAR-T cells is the diminished risk of immune rejection (3). Donor T cells carry the potential risk of life-threatening Graft versus Host Disease (GvHD) (4). This is because allogeneic T cells may recognize the recipient's tissues as foreign due to human leukocyte antigen (HLA) disparities, triggering an immune response against the host cells. A clinical study observed that 21.4% of patients experiencing relapsed/refractory acute lymphoblastic leukemia (ALL) who underwent alloCAR-T therapy experienced acute GvHD (5).

Additionally, externally sourced T cells may be rapidly destroyed by the patient's immune system, decreasing persistence (4),(6). Hence, autologous CAR-T cell therapy generally corresponds with longer persistence due to the absence of an allogeneic reaction (4). Yet autologous therapies require a tailored manufacturing process with well-known disadvantages. Autologous CAR-T cell manufacturing has 2-10% failure rates resulting from contamination, operator error, and equipment failure in commercial settings (6). Autologous cell collection has a high cost and long manufacturing processes, delaying the availability of the treatment (4). In this context, allogeneic CAR-T cells offer certain advantages due to decreased cost and scale manufacturing ability. Allogeneic therapies allow for many cells to be harvested from the donor that are then cryopreserved, making treatments readily available for use and decreasing time to production (4). The systematic production of allogeneic T cells is expected to reduce cost and expand accessibility for CAR-T cell therapy (4). Autologous T cell collection can only be used to treat the donor patient, whereas allogeneic T cell manufacturing creates multiple batches of product for many patients (4). Allogeneic T cells can be injected again if the initial infusion is insufficient, which is especially crucial for patients whose disease recurs, necessitating a second dose. T cells taken from a patient who has undergone prior medical treatment may be limited in numbers, thus affecting initial administration doses and any reinfusion that may be necessary (3).

Clinical trials demonstrate that autologous T cells persist longer than allogeneic products in treating B-cell acute lymphoblastic leukemia (B-ALL). With allogeneic CAR-T cells, only 14% of patients had detectable CAR-T cells after 42 days; only 1 patient exhibited persistence beyond 120 days (6). In contrast, the median persistence of tisagenlecleucel (autologous CAR-T cell medication) was 168 days, with some CAR-T cells persisting longer than 20 months (6). Other *in vivo* trials of relapsed/refractory ALL in humans demonstrated the presence of cytokine-release syndrome (CRS) after CAR-T cell treatment. CRS is indicated by high amounts of inflammatory cytokines such as interleukin-6 (IL-6) and interferon-γ (IFN-γ) and can become life-threatening (5). In a retrospective study, many more patients experienced severe CRS (Grade ≥ 3) from autoCAR treatment than alloCAR treatment (5). The median peak IL-6 level from the autoCAR cells (161.6 pg/mL) exceeded the peak level from the alloCAR group (38.7 pg/mL) (5). The overall remission rate was similar, with the complete remission (CR) rate being 88.2% for the autoCAR group and 100% CR rate for alloCAR (5). However, a contrasting prospective study found CRS occurring in 57% of patients treated with autoCAR-T cells and 91% of those administered with alloCAR-T cells (6). The study dosed seven children and 14 adults with UCART19, an allogeneic CAR-T therapy used to treat B-ALL (7). Not only did 19 patients (91%) suffer from CRS, but grade 1 acute skin GvHD occurred in 2 patients and grade 1 or 2 neurotoxicity occurred in eight patients (38%) (7). Thus, more research is necessary to determine the relationship between CAR-T cell source and the risk of CRS and other adverse events.

Various gene editing methods (TALENs, Zinc-finger nucleases, and CRISPR/Cas9) have been used with allogeneic transplants to reduce the risk for GvHD and enhance T cell persistence (8). Due to the manufacturing drawbacks of autoCAR-T cells, allogeneic cells should continue to be developed as a cheaper alternative. Gene editing of allogeneic T cells should be explored, especially to minimize life-threatening risks like GvHD and short persistence, which could lead to relapse.

Optimizing culture medium for T cells in vitro

CAR-T cell persistence is heavily dependent on T cell differentiation, of which there are many T cell subsets. Before encountering their cognate antigen, immature T cells are referred to as naïve T (T_n) cells. After an antigen is encountered, T_n cells activate and differentiate into stem cell memory T (T_{SCM}) cells, central memory T (T_{CM}) cells, effector memory T (T_{EM}) cells, or eventually terminal effector T (T_{EFF}) cells, each with varying implications for CAR-T efficacy (1). Recent work concluded that T_{SCM} and T_{CM} cells have longer persistence and stronger anti-tumor activity than T_{EM} and T_{EFF} counterparts (9). Specifically, one clinical trial saw CD19–CAR-modified $CD8^+$ T_{SCM} cells exhibit improved fitness and sustained antitumor responses against systemic ALL (9). As a result, procedures for the expansion of T cells have been optimized to promote more T_{SCM} and T_{CM} cells. This has been accomplished through the strategies explored below.

Before T cell activation, T_n cells mainly use oxidative phosphorylation (OXPHOS) and fatty acid oxidation (FAO) in mitochondria (10). Cell metabolism is optimized for minimal energy consumption for cells in resting states like T_N cells. T_N cells use imported nutrients to produce energy through OXPHOS, which breaks down nutrients like fatty acids, amino acids, and glucose (11). Upon encountering an antigen, T_n cells activate and transform into T_{EFF} cells, triggering an accelerated metabolism and promoting aerobic glycolysis (12). However, memory cell subsets remain similar to T_n cells in that they largely depend on OXPHOS as opposed to glycolysis (13). Recent studies indicate that the sustained elevation of glycolysis hinders the development of long-lived memory cells. Pushing T cells toward an irreversibly differentiated state causes an inability to persist post-adoptive cell transfer (ACT) (14). Therefore, to achieve sustained persistence, strategies should shift the metabolic processes from glycolysis towards OXPHOS to induce more T_{SCM} and T_{EM} cells.

Metabolite adjustments

Multiple studies have explored metabolite adjustments to restrict or interfere with glycolysis and improve the ratio of T_{SCM} and T_{CM} cells. 2-deoxy-D-glucose (2-DG) suppresses glycolysis by replacing the 2-hydroxyl group with hydrogen. Utilizing 2-DG during T cell activation to limit the reliance on glycolysis pushed $CD8^+$ T cells towards a differentiation pathway, favoring the formation of T_{SCM} and T_{CM} memory cells (17). The inhibition process preserves the formation of long-lived memory $CD8^+$ T cells, helping to improve the efficacy and survival of CAR-T cells. Similarly, intracellular L-arginine concentrations enhance the differentiation of T_{SCM} and T_{CM} cells with longer persistence and stronger anti-tumor activity in murine models. One study used L-arginine supplementation in the cell medium to promote a switch from glycolysis to OXPHOS in CAR-T cells. The metabolic shift towards OXPHOS contributed to a higher proportion of memory cells with increased survival ability and stronger antitumor cytotoxicity (18). Moreover, adding 6-diazo-5-oxo-L-norleucine (DON) to the cell culture enhances OXPHOS and reduces glycolytic activity (19). This resulted in more T_{CM} subsets and stronger anti-tumor activity *in vivo* (19). Furthermore, recent studies indicate that using PhysiologixTM xeno-free (XF) hGFC (Phx), a human growth factor, facilitates T cell proliferation and CAR-T cell expansion (20). CAR-T cells were compared to cells conditioned in Phx or HS to determine a difference in anti-tumor response in a murine xenograft model (20). T cells cultured in Phx had improved persistence *in vivo* and superior cytotoxic capabilities *in vitro* (20). Specifically, the dipeptide

carnosine found in Phx augments the persistence of T cells and shifts them towards an oxidative state, thus enriching the T_{CM} cell subset.

The common characteristic behind these studies is the metabolic increase of OXPHOS and potential inhibition of glycolysis. This improves T cell differentiation towards T_{SCM} and T_{CM} cells for superior persistence and antitumor potential.

Cytokine Optimization

Additionally, various cytokine combinations have been tested to manipulate metabolic changes and improve the ratio of T_{SCM} and T_{CM} cells during the CAR-T cell expansion phase. IL-2 has been considered the "gold standard" growth factor, though it triggers a shift from OXPHOS to glycolysis (1). Other cytokine combinations have additionally improved persistence *in vivo* by promoting OXPHOS and blocking glycolysis. Using IL-7 and IL-15 in clinical studies led to higher proportions of T_{SCM} and T_{CM} than just IL-2, which saw larger amounts of T_{CM} and T_{EM} (15). A separate study confirmed this, where IL-7 facilitated the highest levels of the T_{SCM} subset compared to other pro-inflammatory cytokines. (16). A trial *in vivo* demonstrated that IL-12 modulates the expression of CD62L on activated T cells, resulting in superior persistence and proliferation compared to IL-2.(15). Additionally, *in vivo* administration of IL-15 and IL-21 treated T cells exhibited the phenomenal persistence and most effective tumor elimination *in vivo*. However, it is interesting to note that IL-2 improved the accumulation of CAR-T cells in vitro (16), even though IL-2-treated CAR-T cells display reduced anti-tumor cytotoxicity. Further experiments are required to determine whether other cytokine conditions can outperform the standard IL-2-manufactured CAR-T.

Oncolytic Viruses

Viral-based gene therapy strategies have attracted scientific and clinical attention. Oncolytic viruses (OVs) mediate anti-tumor effects through direct tumor cell lysis, causing the secretion of tumor-associated antigens and IFNs, which contribute to antitumor immunity (21). As standalone treatments, oncolytic viruses have yielded only modest anti-tumor effects in patients. This is largely due to the restricted entry of oncolytic viruses into tumors, their limited persistence within the host, and the patient's antiviral response (22). However, OVs can be used in combination to enhance the efficacy of other therapies, including CAR-T cell therapy, to induce a stronger anti-tumor immune response (22).

Preclinical studies show that integrating CAR-T cells with OVs directs attention to the tumor-specific expressed tumor antigens limitations that arise from the use of CAR alone. One trial paired CAR-T cells directed towards CA IX and an oncolytic adenovirus delivering chemokine ligand 5 (CCL5) and IL-12 to investigate anti-tumor potential (22). Results demonstrated that the combined therapy, Ad5-ZD55-hCCL5-hIL12, caused a modest suppression of human-derived tumors in murine models. Ad5-ZD55-hCCL5-hIL-12 and CA9-CAR-T cells enhanced CAR-T cell penetration within solid tumors, restrained cancer growth, and extended overall persistence (22). Hence, this study demonstrates effective results in integrating OAV with CAR-T cells for anti-tumor treatment. Another study examined CAR-T cells in conjunction with OVs equipped with CCL5 and the proinflammatory IL-15 (23). Researchers observed that the administration of the OAV accelerated cell death in neuroblastoma tumors treated by CAR-T

therapy (23). The adenovirus caused CCL5 and IL-15 to be secreted, which activated CAR-T cells and increased their persistence, thus lengthening overall mouse survival (23). A different study tested the treatment of OAV with chemokine CXCL11 against glioblastoma models using immunodeficient mice (24). The combination of therapies elicited robust antitumor effects and increased infiltration of $CD8^+$ T lymphocytes (24).

While these preclinical studies have shown favorable results, further investigation should assess the potency of OVs with CAR-T therapy in human immune systems. An ongoing study (NCT03740256) was designed to analyze the safety concerns of using oncolytic viruses in humans. The trial combines HER2-targeted CAR-T cells and an oncolytic adenovirus, CAdVEC, to administer treatment for patients with HER2-positive cancer. This trial is still in the recruitment phase, although its results may provide insight into treating patients with a combination of CAR-T and OVs.

CAR Design

Since the creation of CARs in 1993, the survival of engineered T cells has posed a serious obstacle to successful clinical outcomes. As a result, several generations of CARs have been introduced, each with its own design and attributes that prolong cell survival (25). The first generation was composed of a single-chain variable fragment (scFv) and a cytoplasmic CD3ζ signaling domain (Fig. 2A) (26). However, it was unsuccessful in activating the T cells through the CD3ζ domain. First-generation CARs cannot produce a sustained anti-tumor response or cytokine release due to poor signaling (27).

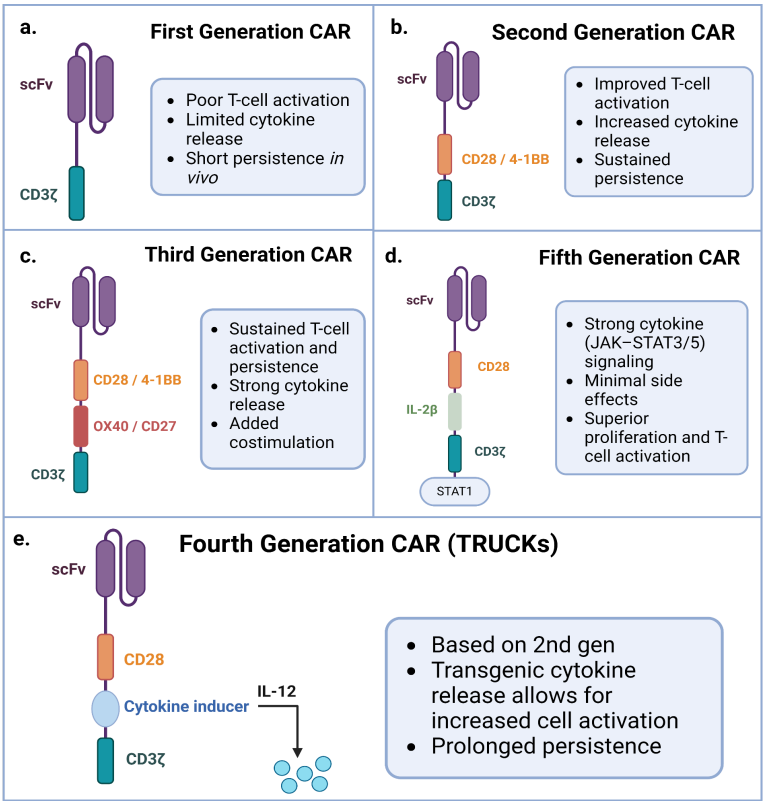


Figure 2. CAR-T construct designs. A) First-generation CARs contained an extracellular domain (scFv) and an additional signaling domain CD3ζ. **(B)** The second generation included a secondary costimulator, CD28 or 4-1BB. **(C)** Third-generation CARs were composed of two secondary costimulatory domains. **(D)** Fourth-generation TRUCKs were modeled after second-generation CARs and contained a gene expression cassette-inducing cytokines. **(E)** The fifth generation of CARs consisted of an IL-2 receptor β-chain domain and a binding location for STAT3.

To strengthen signal transduction, the second generation of CARs included a costimulatory domain, such as CD28 or 4-1BB, to promote T cell activation, survival, and proliferation of the engineered cells (Fig. 2B) (28). Kymriah and Yescarta, both FDA-approved therapies for B-ALL and Large B-cell lymphoma (LBCL), are second-generation CAR-T medications. Second-gen CARs demonstrate the importance of including a costimulatory domain to achieve stronger persistence and overall function of CAR-T cells. A clinical trial administered CAR-T cells containing 4-1BBζ to 14 patients with chronic lymphocytic leukemia, finding that the cells persisted for over 4 years among patients who achieved complete remission (2). Another use involving the 4-1BB costimulatory receptor promoted the development of the T_{CM} subset with enhanced metabolic efficiency through FAO and OXPHOS pathways (29). Additionally, autologous T cells expressing a CD28ζ domain yielded T_{SCM} and T_{CM} cells with increased glycolytic metabolism. Overall, CARs incorporating 4-1BBζ or CD28ζ tend to promote the differentiation of T_{SCM} and T_{CM} cells, respectively, leading to enhanced persistence. The important idea from the second generation is that including a costimulatory domain is crucial for substantive T cell activation, ultimately leading to improved anti-tumor response.

Third-generation CARs improved costimulation by including two co-stimulatory signaling domains (Fig. 2C) (11). Incorporating the OX40, ICOS, or CD27 as secondary costimulatory domains increases the expansion and persistence of resting T cells through proinflammatory pathways (30). In murine models, third-generation CARs mount a more robust antitumor response and contribute to longer-surviving mice (31). Specifically, ICOS signaling improves CAR-T cell persistence in solid tumors compared to a single costimulatory domain by increasing cytokine release, such as IFN-γ and IL-22 (11). A clinical study demonstrated third-generation CARs to perform moderately well, with 2 patients achieving complete remission and 1 patient arriving at a partial response (32). The study observed T cell persistence of up to 12 months with slightly improved antitumor activity, suggesting a benefit from the additional costimulatory domain (32). Hence, the preclinical and clinical outcomes of third-generation CARs remain promising.

The fourth iteration of CARs, termed “TRUCKs,” was modeled after second-generation CARs. TRUCKs feature a gene expression cassette that can be activated to produce a transgenic cytokine (Fig. 2D) (11). Cytokines, including IL-12, are delivered directly to the tumor to improve immune response (33). TRUCKs can minimize systemic side effects commonly associated with widespread cytokine release, such as CRS, by producing cytokines near the tumor. After the initial CAR-T cell response, tumor relapse can occur if CAR-T cells fail to detect cancer cells with minimal receptor expression (33). As a result, secretion of inducible IL-12 from TRUCKs brings immune cells toward the microenvironment to eradicate remaining parts of the tumor (33). Stimulating cytokines alters the tumor microenvironment and leads to prolonged activation of CARs and shields overactive T cells from programmed cell death, thus increasing persistence (34). Finally, fifth-gen CARs include an interleukin-2 receptor β-chain domain and a binding location for STAT3 (Fig. 2E) (11). This structure contributes to strong cytokine (JAK–STAT3/5) signaling within the specific tumor sites while minimizing systemic side effects (34). IL-2 signaling influences two important aspects of immune response: T cell differentiation and memory recall responses (35). Fifth-generation CAR modifications contribute to improved T cell activation, persistence, and proliferation through cytokine-mediated signaling

pathways (34). The 5th generation is the most recent and advanced iteration of CARs, but more clinical trials are necessary to ensure its safety and efficacy.

Conclusion

CAR-T therapy has become recognized as a revolutionary immuno-therapeutic tool that targets a wide variety of cancers. However, obstacles like a lack of T cell persistence must be overcome to achieve superior clinical results. Cancer is a long-term challenge characterized by its ability to evolve and resist treatment over time. Given these circumstances, the sustained activity of T cells becomes more important to attain long-lasting immune responses for complete remission of disease. Fortunately, persistence can be improved through multiple strategies, such as choosing an appropriate source of T cells, optimizing the cell medium with cytokine and metabolite adjustments, using oncolytic viruses, and developing CAR architecture (Fig 3). These strategies have demonstrated significant promise, and their safety and efficacy should continue to be examined.

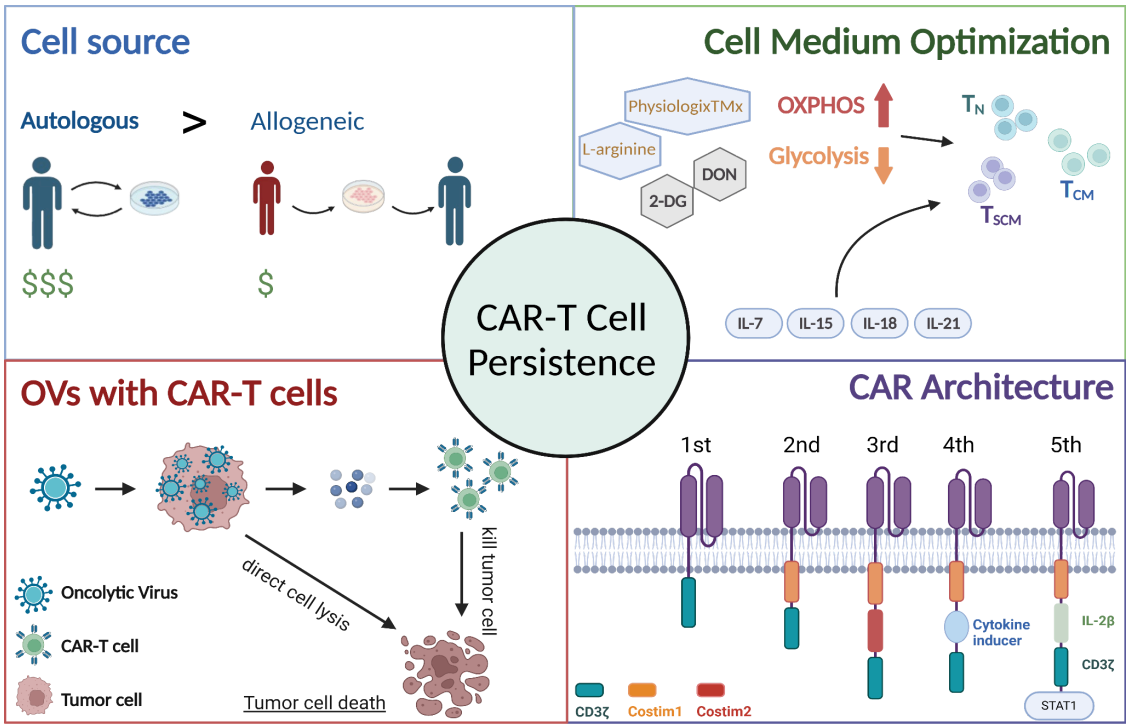


Figure 3. Approaches to increase CAR-T cell persistence.
Upper left: choosing between autologous or allogeneic cells as a source for CARs. Upper right: optimizing cell medium to increase T cell differentiation towards memory cells. Bottom left: oncolytic viruses overcome immunosuppressive mechanisms within the microtumor environment. Bottom right: generations of (CAR-T cell) construct designs.

In addition, while the discussed methods have been incrementally successful on their own, combining them could further extend T cell persistence, especially when treating solid tumors (36). As a result, our knowledge of each approach and its underlying mechanisms is crucial for leveraging these strategies to improve the persistence of CAR-T cells.

The lengthy and complex manufacturing process makes it challenging to implement drastic modifications to the production of CAR-T cells. Changing things like the cell culture medium or adding metabolite adjustments are challenging to incorporate on a large scale. These changes must be carefully evaluated to ensure they do not compromise the quality or safety of the treatment, which would lengthen the already long manufacturing process. Any significant changes made during the process can prolong production time for patients in urgent need of an emergency treatment. The use of oncolytic viruses presents a unique challenge in the context of safety, particularly concerning the risk of CRS. Mitigating the chance of CRS requires careful consideration of treatment timing and dosing specific to the patient, which will extend manufacturing times further. The cost of incorporating changes in T cell production is also a concern. Development of autologous T cells is already expensive, although allogeneic T cell production remains a favorable solution. Regardless, implementing the discussed changes of T cell persistence will require costly upgrades to manufacturing facilities and equipment.
In addition, the absence of biomarkers for CAR-T therapy makes it difficult to tailor CAR treatments to patients. Current biomarkers for baseline characteristics, CAR-T cell function, long-term survival, and toxicities are insufficient, making it challenging to guide treatment decisions properly. In the context of implementing new solutions to improve T cell persistence, biomarkers become even more critical to optimize the treatment. More research should be done to validate current biomarkers and identify new ones.

This review discussed strategies to extend CAR-T cell persistence. Approaches such as selecting an appropriate source of cells, enriching culture conditions, using combinatorial treatments, and optimizing CAR architecture have made significant progress in improving T cell persistence. Future research should focus on refining these strategies to implement them in the CAR manufacturing process.

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