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Targeting Cancer Stem Cell Resistance: Limitations of CAR-T Cells and Emerging Approaches

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Abstract

Cancer stem cells (CSCs) play a critical role in tumorigenesis and are key contributors of therapeutic resistance and tumor relapse, a major obstacle to the effective cancer treatment. While the development of Chimeric Antigen Receptor T-cell (CAR-T) therapy has revolutionized oncology, especially with treating hematopoietic cancers, its effectiveness in targeting CSCs in solid tumors (cancerous cells that grow in *specific* organs like the breast or lungs) has been less successful. Here, we cover the several known mechanisms of resistance to CAR-T therapy in CSCs, including how the TME shields CSCs through immune suppression, hypoxia, and ECM rigidity. Moreover, inherent limitations of T-cell persistence, single-antigen targeting, and "on-target off-tumor" toxicity remain major challenges in CAR-T immunotherapy approaches. Recent studies highlight emerging strategies to address these issues, including dual- and multi-antigen CAR-T cells, hypoxia-inducible promoters, ECM-modifying strategies, and TME modulation. Collectively, these developments represent a trend toward versatile, durable, and safer CAR-T therapies with prospects for overcoming CSC-mediated resistance and long-term remission in solid tumors.

Keywords: Cancer stem cells (CSCs), CAR-T therapy, tumor microenvironment (TME), immune evasion, therapy resistance

Introduction

Cancer stem cells (CSCs) are a small subpopulation of cancerous cells within tumors that possess self-renewal, differentiation, and tumorigenicity capabilities when transplanted into an animal host. CSCs play a crucial role in tumor initiation, therapy resistance, and patient relapse, making them a major obstacle to long-term cancer treatment. The resilience and adaptability of CSCs make them difficult to target with conventional therapies. One of the most promising developments in cancer immunotherapy is Chimeric Antigen Receptor T-cell (CAR-T) therapy, which involves engineering a patient's intrinsic T-cells to recognize and attack cancer cells expressing known cancerous antigens. CAR-T cell therapy has shown success in treating hematologic malignancies, particularly B-cell leukemias and lymphomas, and is currently being explored in solid tumors. However, CAR-T therapy faces notable challenges in the context of CSCs despite its potential efforts. Current research shows that in solid tumors, CAR-T therapies highlight issues such as the limited persistence of CAR-T cells, tumor antigen heterogeneity, and immune evasion by CSCs. Furthermore, the tumor microenvironment (TME) plays a critical role in shielding CSCs through mechanisms like immune suppression, hypoxia, and stromal interactions, reducing CAR-T cell effectiveness. Proposed solutions include enhancing CAR-T cell durability and targeting multiple tumor antigens. This review focuses on TME factors that support CSC survival, the obstacles they create for CAR-T cell persistence, and the current strategies being developed to overcome these barriers. We hypothesize that immunosuppressive components of the TME, CSC-driven immune evasion strategies, and intrinsic flaws in CAR-T cell design–such as limited persistence and single-antigen targeting—contribute to therapy failure. We believe that emerging strategies like dual-antigen CAR-T cells and TME-modulating approaches can help address these critical challenges.

Methodology

To conduct this review, we searched through literature archives from PubMed Central, focusing on relevant immunology journals including Frontiers in Immunology, Nature's Blood Cancer Journal, the Journal of Clinical Investigation, etc. Initial literature background consisted of approximately 40 papers filtered by the following keywords: "CAR-T cell therapy," "tumor microenvironment," "TME modulation," "dual-antigen CAR-T," "CAR generations," "extracellular matrix (ECM)," and "cancer stem cells (CSCs).” We prioritized original studies with large sample sizes, a focus on CSCs or TME, and works by respected research groups. Articles that were review articles, non-CSC related, or had little data were not considered. After this filtering process, we selected approximately 20 significant papers that heavily contributed to our understanding of the mechanisms and challenges in CAR-T therapy and potential new strategies to improve it.

Current issues with CAR-T cell therapies

CAR-T cell creation has evolved through four generations of technical advancements, each addressing issues with efficacy and enhancing functionality. The first generation of CAR-T cells, composed of CDζ chains for T-cell activation, solved the problem of targeting but lacked antitumor activity due to the absence of costimulatory signals. The second generation is a single costimulatory signal (CD28 or 4-1BB), enhancing immediate activation and cytotoxicity. The third generation of CAR-T cells carried two costimulatory molecular domains, improving proliferation, tumor clearance, and cytotoxic activity. Finally, the fourth generation consisted of transgenic products delivering T-cells that secreted specific cytokines, overcoming suppression from the tumor immune microenvironment.[3] This progression demonstrates how iterative engineering has addressed early limitations to CAR-T therapy. However, a lack of clinical

applications remains, as *in vitro* methods are still being examined for generations three and four, leaving only the second generation of CAR-T therapy to prevail within *in vivo* applications.

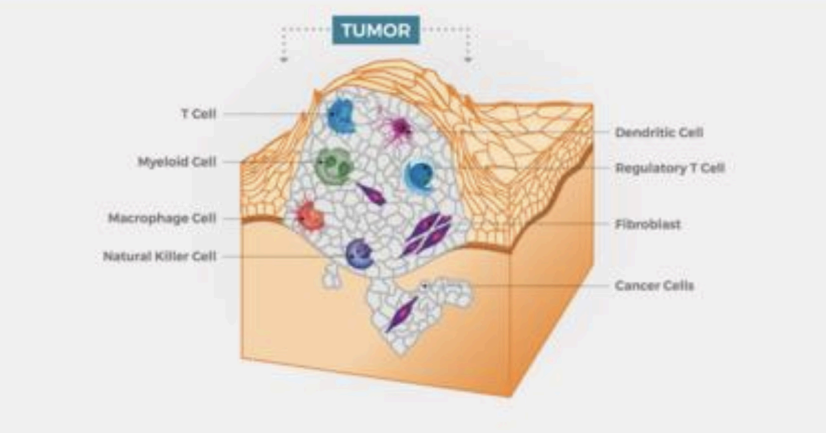
Tumor Microenvironment Contributes to CSC Persistence

Despite significant advancements in CAR-T cell treatments, CSC persistence post-therapy remains a critical barrier to long-term remission. This is due to their resistance mechanisms, which include dormancy, altered metabolism, and immune evasion. Moreover, factors provided by the tumor microenvironment (TME) play an equally important role in protecting CSCs and undermining the therapeutic efficacy of CAR-T cells. The TME forms an immunosuppressive niche that shields the CSCs from immune attack and limits the persistence and activity of therapeutic agents such as CAR-T cells.[14]

The TME comprises a complex network of stromal cells, extracellular matrix components, immune regulatory cells such as regulatory T cells, myeloid-derived suppressor cells, and tumor-associated macrophages, as shown in *Figure 1*.[3] These elements release inhibitory cytokines, which suppress the cytotoxic function of CAR-T cells, creating a biochemical and physical barrier against infiltration.[9] Hypoxia is a major attribute of solid tumors due to their abnormal vasculature and rapid cell growth. The hypoxic nature of many tumor regions alters CSC metabolism, pushing them towards glycolysis rather than oxidative phosphorylation, reducing the immunogenicity of CSCs [6]. Hypoxia-induced factors (HIF) are stabilized through the lack of oxygen, leading to T-cell exhaustion and impairing their differentiation into effector memory cells.[15]

Figure 1.

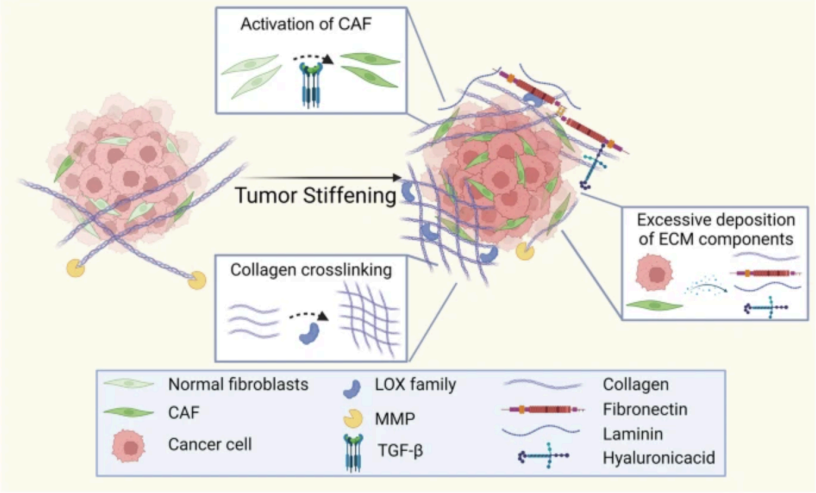
The composition of the TME[20]



The dense extracellular matrix (ECM) impedes CAR-T cell infiltration by forming a physical barrier with high collagen density and increased ECM stiffness [5]. *Figure 2* shows the process of ECM stiffness caused by the accumulation of cancer-associated fibroblasts (CAF), collagen crosslinking, and crosslinking of ECM proteins. This increase in ECM stiffness restricts CAR-T cell movement, blocking their interactions with the CSCs. Lastly, the creation of CAFs is a response to fibroblast transition caused by growth factors secreted by CSCs, typically transforming growth factor-beta (TGF-β) [12]. This leads to the formation of CSC niches and the growth of CSCs through nutrients, resulting in CSC metabolism reprogramming and reduced CAR-T cell therapy efficacy [15]. Ultimately, these factors allow CSCs to hide in protective niches, avoid detection, and reinitiate tumor growth, contributing to relapse after a successful initial therapy.

Figure 2.

Factors Driving ECM Stiffness

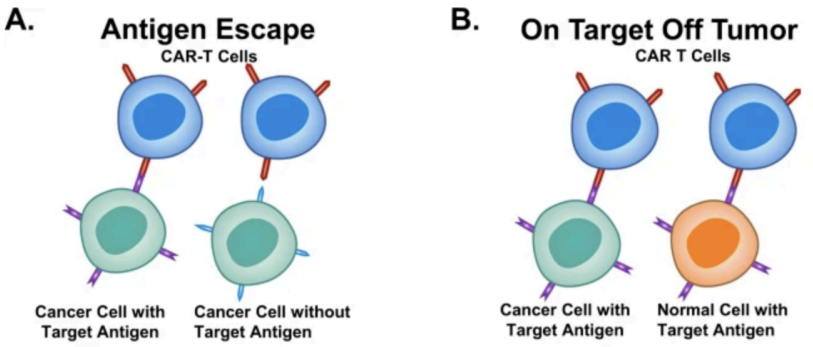


Non-adaptive Mechanisms Limit CAR-T Cell Therapy Flexibility in Solid Tumors

Another critical challenge is that many CAR-T therapies are non-adaptive and lack the flexibility to navigate and survive within the highly immunosuppressive TME. A major issue arises in shared antigens between CSCs and healthy stem cells, causing CAR-T cells to inadvertently attack non-cancerous stem cells that possess similar antigens to the CSCs, a phenomenon called "on target off tumor" [4]. Specifically, CAR-T cells targeting common CSC antigens such as CD44, CD133, or EpCAM may attack healthy stem cells expressing these markers. For example, CD133 is present in hematopoietic stem cells and neural progenitors, leading to myelosuppression or neurotoxicity in preclinical models.[17] *Figure 3* represents the mistargeting of healthy and CSCs by CAR-T cells due to the pre-engineering of CAR-T cell antigen bonding.

Figure 3.

CAR-T cells with Antigen Escape and On Target Off Tumor Interactions[17]



CSCs within the TME can also downregulate target antigens or undergo phenotypic shifts, escaping CAR recognition altogether.[13] Phenotypic plasticity hinders CAR-T cells, which are engineered to target a specific antigen, making them vulnerable to the TME and CSC plasticity. Solid tumors exhibit clonal evolution and genomic instability, leading to variable antigen expression across tumor sites. CAR-T cells targeting a specific antigen often fail in solid tumors as tumor cells downregulate or mutate the target antigen [16]. For instance, ovarian CSCs shift between $EpCAM^+$ and $EpCAM^-$ states through epithelial-mesenchymal transition, driven by TME-derived exosomal miR-200c. $EpCAM^+$ consists of an epithelial phenotype with intact cell-cell adhesion, high proliferation capacity, and susceptibility to EpCAM-targeted CAR-T therapies. $EpCAM^-$ consists of mesenchymal phenotype acquired through epithelial-mesenchymal transition (EMT), characterized by enhanced motility, invasion, and therapy resistance [8]. CAR-T cells are engineered to demonstrate potent cytotoxicity against $EpCAM^+$ tumors in preclinical models, however, CSCs can switch into $EpCAM^-$ states, evading CAR-T cell recognition. This allows the evasion of EpCAM-targeted CAR-T cells while preserving tumor multiplication. The plasticity of CSCs within the TME restricts the application of CAR-T cells, initiating CSC relapse and multiplication.

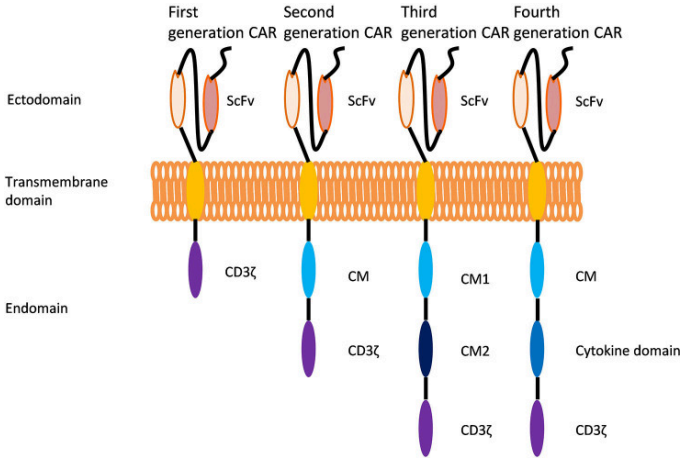
CAR-T Cell Persistence Issues

CAR-T cell therapy represents an effective treatment for cancers such as B-cell acute lymphoblastic leukemia (B-ALL) and B-cell lymphoma, particularly in patients who are resistant to conventional treatments. However, 30–60% of patients relapse, with a common reason being the lack of persistence of CAR-T cells and CD19 antigen loss, a key marker for B-cell development and immune homeostasis (Kotani et al., 2015). Relapses in B-ALL can be broadly categorized into two groups: CD19-positive relapse, which is associated with insufficient CAR-T cell persistence, low activity, or transient B-cell aplasia; and CD19-negative relapse, where the tumor cells evade recognition by CAR-T cells through the loss of CD19 expression. Relapses with CD19-positive cells are uncommon in CAR-T therapy but are usually due to CAR-T cell exhaustion or activation-induced cell death, which emphasizes the necessity to enhance CAR-T cell persistence. Researchers also say that relapses happen because CAR-T cells do not destroy all the tumor cells. According to Mohammad Rashidian, assistant professor of radiology at Harvard Medical School, a few tumor cells can remain after initial CAR-T cell activity. Once CAR-T cells no longer detect cancer cells to kill, they shut down, creating a window of opportunity for the remaining tumor cells to cause relapse [2].

There are four generations of CARs in CAR-T Cells explored in a recent study. The evolution of chimeric antigen receptors (CARs) is portrayed in Figure 4.

Figure 4.

Four generations of CAR design[10]



First-generation CARs consist of a single-chain variable fragment (scFv) joined to the CD3ζ signaling domain, which activates T cells. They lack costimulatory molecules and hence cause poor T cell activation and moderate antitumor activity in vivo. In the absence of long-duration activation and survival signals, the CAR-T cells quickly become exhausted or die, their persistence is shortened, and their therapeutic potential is reduced. To surpass this, second-generation CARs were developed by incorporating a costimulatory domain (CD28 or 4-1BB) that provides T cells with a second signal of activation. The secondary signal enhances T cell expansion, cytokine production, and survival, preventing exhaustion and enhancing their cytotoxicity. The character of the costimulatory domain, nevertheless, significantly influences CAR-T cell activity and persistence. Clinical trials have shown that CAR-T cells containing a CD28ζ domain persist for approximately 30 days, with rapid but short-lived responses. In contrast, 4-1BBζ CAR-T cells persist for over four years, causing long-term surveillance but with decreased early cytotoxicity. These differences are explained by how each domain controls the metabolism, memory, and activation thresholds of T cells. To overcome the trade-offs between power and persistence, third-generation CARs were developed by the addition of more

than one costimulatory signal (CD28 and 4-1BB or OX40) to increase both short-term activity and long-term persistence. While promising, these CARs can cause off-target activity and excessive cytokine release, and clinical experience is limited. In response, fourth-generation CARs—alternately called TRUCKs (T cells Redirected for Universal Cytokine Killing)—build on the second-generation design and add genetic elements for definite cytokine or costimulatory molecule expression. These designs aim to increase antitumor activity and reduce systemic toxicity, with encouraging early results.[10]

Recent studies suggest that overcoming these barriers will require a multifaceted approach: combining CAR-T therapy with TME-modulating agents, engineering CAR-T cells with improved persistence and resistance to exhaustion, and leveraging technologies to reprogram TME-resident cells or deliver localized stimulatory signals.

Counteracting TME Shielding

TME shielding impacts the effectiveness of CAR-T cells in reducing CSCs. The limitations of oxygen within CSCs limit the efficacy of CAR-T cell therapy. However, CAR-T cells can be engineered with hypoxia-inducible promoters, which activate in low-oxygen TMEs, reducing tumor toxicity. Hypoxia-inducible promoters include HIF-1α-regulated systems, where prolyl hydroxylase domain proteins (PHDs) hydroxylate HIF when oxygen levels become low and inactivate at high oxygen levels [15]. However, the influence of HIF remains unknown due to a lack of clinical trials. Expanding CAR-T cells under physiological hypoxia, 0.02–5% O_2, *in vitro* conditions, enriches memory-like T cells with superior persistence. Hypoxia-induced S-2-hydroxyglutarate (S-2HG) promotes this phenotype, providing stability and adaptability to CAR-T cells through application [15].

To overcome ECM barriers, the ECM must be weakened, and CAR-T cells must be exposed to ECM environments *in vitro*, allowing the T-cells to infiltrate into the TME. Exposing T-cells to synthetic ECM-mimicking hydrogels during manufacturing modulates their phenotype. Elastic hydrogels promote effector-like cells for rapid tumor killing, while viscous hydrogels enhance memory-like cells for sustained activity. Through this process, tissue stiffness and viscoelasticity can be manipulated, enhancing T-cell memory and allowing for the addition of other activation agents, limiting ECM strength [19]. Moreover, ECMs can be degraded through various processes, enhancing CAR-T cell infiltration into the TME. CAR-T cells can be engineered to express heparanase, which degrades heparin sulfate proteoglycans, or hyaluronidase to improve tumor infiltration by disturbing ECM components such as hyaluronan and promote deeper tumor infiltration [7]. Furthermore, targeting cancer-associated fibroblasts (CAFs) through FAP-α-specific CARs reduces ECM production and stromal density [1]. These new employments will enhance the persistence of CAR-T cells against the TME, allowing for adaptability and infiltration.

Solutions to CAR-T Cell Persistence

One of the biggest complications of CAR-T cell therapy is a lack of persistence of the engineered T cells in the body, frequently leading to tumor relapse. When CAR-T cells do not last long enough, they are not able to give continuous immune surveillance, and remaining cancer cells will recur. Third-generation CAR-T cells attempt to get around this by having multiple costimulatory sites (such as CD28 with 4-1BB or OX40) on the CAR, which deliver additional and longer activation signals. These secondary signals support T cell expansion, cytokine production, and survival, promoting the in vivo persistence of CAR-T cells [10]. Recently,

Rashidian and others at Dana-Farber engineered the CAR-E platform, which exogenously supports CAR-T cells by engaging a fused molecule of weakened interleukin-2 (IL-2) and a target antigen. This mixture targets the activation of CAR-T cells without activating normal T cells, driving their growth and memory development while limiting toxicity. In animal models, CAR-E not only diversified CAR-T cells but also eliminated tumor cells. Its ability to resensitize CAR-T cells with multiple doses presents a hopeful solution to maximize therapeutic duration, reduce relapse risk, and possibly decrease the dose of CAR-T cells given initially, lowering treatment cost and side effects such as cytokine release syndrome [2]. By inducing a complete and long-standing immune response, these developments point toward great advancements toward better efficiency and affordability of CAR-T therapy.

Cytokine release syndrome (CRS) is usually a life-threatening adverse effect of exaggerated immune stimulation. Because of this, fourth-generation CAR-T cells have been designed with advanced genetic manipulation that provides greater control of cytokine release and costimulatory molecule expression. These regulatory elements help to mature the immune response, minimize the risk of CRS, and improve the overall safety of CAR-T therapy, making it an even more accessible choice for a greater patient population [10]. Antigen escape is the second main challenge besides persistence and safety. This is observed when cancer cells lose CD19, the primary antigen targeted by most CAR-T therapies, rendering them unavailable to the engineered T cells. In response to this, researchers are engineering multi-antigen targeting strategies, engineering CAR-T cells to target not just CD19 but other antigens such as CD22 or CD20 as well. This approach limits immune evasion potential and maximizes CAR-T cell function to destroy tumor cells even if one target is lost [11]. Finally, improving CAR-T cell production efficiency is still essential to expand treatment access. Traditional ex vivo manufacturing is labor

and resource intensive, costly, and often limits the delivery of therapy in a timely manner. New technologies such as in vivo CAR-T cell production, use of bio-instructive materials, and potentially platforms such as CAR-E that reduce the amount of CAR-T cells per patient needed aim to accelerate production, reduce costs, and enhance clinical availability [2]. These new technologies aim to optimize both the efficacy and scalability of CAR-T therapy.

Increasing Adaptability of CART-T Cells

To improve CAR-T cell persistence and resistance to exhaustion, researchers have optimized intracellular signaling domains, cytokine support, and genetic modifications. Compared to CD28-based CAR-T cells, 4-1BB domains have enhanced T cell longevity and memory formation. Current research shows that newly armored CAR-T cells are engineered to secrete cytokines like IL-15 and IL-21 to better survive and function in immune-suppressive tumor environments. Overexpression of transcription factors like c-Jun is a gene editing strategy that has shown advancements in increasing CAR-T resistance to exhaustion while preserving robust anti-tumor activity. A major obstacle in targeting cancer stem cells is shown in the heterogeneous expression of tumor-associated antigens in CAR-T therapy. This limits the efficiency of single-antigen targeting strategies since CSCs lose the antigen expression entirely, leading to antigen escape, allowing them to interrupt immune recognition and disease relapse. Current solutions in place are research exploring dual or multi-antigen targeting approaches, such as Perna et al. (2017), which develops bi-specific CAR-T cells targeting CD33 and CLL-1 in acute myeloid leukemia CSC. In turn, this improved tumor clearance and decreased antigen escape, unlike single-target CAR-T therapies. Another approach includes Hegde et al. (2013)

engineering tandem CAR constructs recognizing two glioblastoma antigens (both HER2 and IL13Rα2), demonstrating enhanced recognition of heterogeneous tumor populations in the cell.

Along with dual-antigen targeting, switchable CAR-T systems are in place to allow simultaneous modulation of CAR specificity. This involves using adaptor molecules that bind both the CAR and the tumor antigen, which decouple antigen recognition from CAR expression. A current model is the SUPRA CAR system, which emphasizes leucine zipper interactions to reversibly engage tumor antigens. This allows flexible targeting of CSC-associated antigens and fine-tuning of CAR-T activity (Cho et al., 2018). These systems are important for overcoming antigenic variability in CSCs and for minimizing off-tumor toxicity by providing temporal control over targeting, allowing for dynamic retargeting by simply altering the external adaptor rather than re-engineering the T cell itself. In turn, this enables real-time modulation of CAR-T specificity in response to tumor evolution. These targeting strategies are integrated using inducible control systems such as hypoxia-responsive or drug-regulated promoters, which enable CAR-T cells to function selectively in specific tumor niches, including hypoxic microenvironments common in CSC-rich tumors. This adaptability not only improves selectivity but also ensures that CAR-T cells remain responsive to the evolving tumor landscape and reduce toxicity in the environment. All of these strategies represent a paradigm shift from static CAR designs to more efficient programs, environmentally safe and sensitive therapies that are more likely to overcome both antigenic diversity and immune evasion in CSC-driven cancers.

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