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CARs are sharpening their weapons

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ABSTRACT

The tumor microenvironment hinders CAR T-cell access, activation, and persistence at the tumor site, thereby impacting on the therapeutic efficacy. To tackle these obstacles, ongoing efforts are focusing on further engineering CAR T-cells to enhance their homing, fitness, long-term persistence, and antitumor activity. Advances in genetic modification have prompted the development of armored CAR T-cells equipped with a combination of synergistic elements strengthening their function. These include cytokine release, chemokine receptor expression, immune checkpoint inhibition, gene-editing of inhibitory molecules, or metabolic reprogramming, among others. Multiarmored CAR T-cells may allow addressing the unmet clinical needs of patients with solid tumors or hard-to-treat hematological malignancies who do not benefit from conventional CAR T-cell therapy. Accordingly, several clinical trials are currently assessing the safety and efficacy of these novel CAR constructs.

INTRODUCTION

Factors hindering CAR T-cell activity in the tumor microenvironment

Chimeric antigen receptor (CAR) T-cell therapy has demonstrated remarkable efficacy in B-cell malignancies leading to the approval of six products by the Food and Drug Administration.[1] However, many patients still relapse or do not benefit from CAR T-cell therapy, especially in the context of solid tumors and acute myeloid leukemia. While antigen escape and tumor heterogeneity are the primary causes of diminished efficacy, the tumor microenvironment (TME) has been found to play a critical role in affecting CAR T-cell trafficking, activation, proliferation, and persistence.[1]

Researchers have, therefore, been developing several approaches to armor CAR T-cells to protect them from a hostile TME. Both in solid and hematological malignancies the TME comprises tumor-supportive stromal cells, myeloid-derived suppressor cells, regulatory T-cells (Treg), and tumor-associated macrophages. These components release immunosuppressive soluble factors, express inhibitory receptors and ligands, and engage a close interplay with cancer cells, supporting their maintenance, survival, and therapy resistance.[1] Currently, several engineering strategies are under development to ensure the functionality of CAR T-cells in these hostile circumstances (figure 1 and online supplemental table 1).

T-cells redirected toward universal cytokine killing to enhance CAR T-cell activity in the immunosuppressive TME

CAR T-cells redirected toward universal cytokine killing (TRUCKs) are engineered to secrete stimulatory cytokines playing the dual role of sustaining CAR T-cell activity and persistence in an autocrine fashion while stimulating the endogenous immune response via paracrine effects.[2] CAR T-cells secreting pro-inflammatory interleukins (IL) such as IL-7, IL-12, IL-15, IL-18, and IL-23 have been tested in preclinical models whereby they exhibited enhanced expansion, heightened antitumor activity, and prolonged persistence while stimulating the host immunity. However, TRUCKs may carry the risk of severe side effects if common γ-chain cytokines are constitutively released. Their systemic accumulation can potentially result in neurotoxicity and cytokine release syndrome.[3] Therefore, the development of inducible and selective cytokine-engineering strategies is critical for the successful clinical implementation of TRUCKs.

To surmount this hurdle, Ma *et al* designed inducible IL-23-engineered CAR T-cells whereby IL-23 production is CAR-dependent and restricted to the tumor site, demonstrating improved antitumor activity, while granting safety.[4] Shum *et al* proposed another innovative approach by engineering CAR T-cells with a constitutively active IL-7 cytokine receptor (C7R) that boosts T-cell expansion, survival, and antitumor activity in multiple in vivo models without the risk of toxic side effects.[5] This study has led to the approval of a phase I clinical trial (NCT03635632) assessing C7R-Disialoganglioside (GD2) CAR T-cells safety and efficacy in patients with GD2$^+$ tumors, including neuroblastoma, sarcoma, uveal melanoma, and breast cancer.

Several early-phase clinical trials are investigating TRUCKs in cancer treatment.

IL-18-secreting CD19.CAR T-cells are under evaluation (NCT04684563) in CD19$^+$ hematological tumors. IL-18 induces Th1 and NK-cells to release interferon-γ stimulating both the CAR and the bystander immune cells.[3] HuCART19-IL18, a fourth-generation CAR T-cell therapy, displayed a good safety profile and durable responses in refractory or relapsed lymphoma patients. At 82% objective response rate, with a median response duration not reached, current ongoing studies aim to validate efficacy and safety, by continuing to increase the cell dose.[6] The employ of IL-12 is being tested in a phase I/II trial (NCT03542799) using an inducible system by epidermal growth factor receptor (EGFR) CAR T-cells in metastatic colorectal cancer. Likewise, IL-12 enhances both innate and adaptive immunity synergizing with CAR-mediated antitumor activity. Two phase I clinical trials are evaluating IL-15 armored Glypican-3 (GPC-3) CAR T-cells for the treatment of GPC-3$^+$ solid tumors in adults (NCT05103631) and pediatric patients (NCT04377932). IL-15 regulates T-cell homeostasis prolonging survival and increasing their effector functions but can potentially lead to systemic toxicity. For safety purposes, the CAR has been equipped with an inducible caspase-9 suicide gene preventing multiorgan failure.[3]

Overall, TRUCKs show promise as therapeutic agents by incorporating immunostimulatory cytokines or constitutively active cytokine receptors into the CAR construct, aiming at sustaining T-cell activation, expansion, and persistence.[4 5] Nevertheless, larger-scale investigations are needed to establish the safety implications of delivering cytokine signals to CAR T-cells.

Cytokine-modulating CARs to bypass soluble inhibitors

Cytokine-modulating CARs are engineered to modify CAR T-cell response to cytokines, circumventing the challenging TME. Transforming growth factor-β (TGF-β) promotes Treg differentiation, which, in turn, induces TGF-β secretion, generating a positive feedback loop that abates CAR T-cell activity. One strategy to block the TGF-β effect involves equipping the CAR with the TGF-β dominant-negative receptor II (dnTGF-βRII) functioning as a decoy receptor for the cytokine, thus preventing the activation of its downstream signaling pathway.[7] CAR T-cells coexpressing dnTGF-βRII displayed reduced exhaustion and improved expansion and antitumor activity in preclinical models of multiple myeloma (MM) and prostate cancer.[1 3] A phase I study (NCT03089203) is currently enrolling patients to establish the clinical benefit of this armored CAR.

Another promising approach to manipulate the cytokine effect on CAR T-cells combines the CAR with CD40 ligand (CD40L), a transmembrane protein upregulated on activated T-cells. CD40L binding to its receptor on dendritic cells (DCs) promotes pro-inflammatory cytokine production by T-cells boosting their proliferation and cytotoxicity. Furthermore, when interacting with CD40$^+$ cancer cells, CD40L contributes to tumor apoptosis. Exploiting the proapoptotic effects of CD40-CD40L interaction, Curran *et al* developed a CD40L armored CD19.CAR which extended mice survival, licensed antigen-presenting cells, improved endogenous immune response, and mobilized tumor-specific T-cells in a systemic lymphoma model.[8] Encouraged by these promising results, a phase I/II clinical study (NCT05693844) is currently recruiting advanced/metastatic solid cancer patients to assess CD40L-expressing mesothelin CAR T-cell safety and efficacy.

Taken together, the presented approaches are both promising. The first, coexpressing dnTGF-βRII in CAR T-cells provides T-cells with surprisingly improved proliferative capacity, along with enhanced cytokine signaling and antitumor activity.[7] The second, combining the cytotoxic activity of the CAR with the immunomodulatory molecule CD40L on T-cells represents an effective strategy to orchestrate a persistent antitumor response, involving not only the CAR itself but mobilizing also the endogenous immune response.[8] However, both studies pose some safety concerns: the increased proliferation of dnTGF-βRII CAR T may lead to a lymphoproliferative syndrome as previously described in mice,[7] while CD40L-expressing CAR T-cells may trigger massive release of pro-inflammatory cytokines. Overall, clinical trials will confirm the feasibility of cytokine-modulating CARs in clinics.

CARs coupled with immune checkpoint blockade to surmount checkpoint inhibition

CAR T-cells equipped with immune checkpoint (IC) blockade are designed to overcome inhibitory pathways, with programmed-cell-death-protein 1 (PD-1) being one of the most studied ones. The PD-1/PD-L1 axis contributes to T-cell exhaustion and disrupting this interaction holds the potential to induce a synergistic effect on both CAR T-cells and the host immune response.[1] One approach employs a PD-1 dominant-negative receptor, which similar to dnTGF-βRII, is a decoy receptor for PD-L1. CAR T-cells expressing dnPD-1 demonstrated improved antitumor activity in several preclinical models of solid tumors.[9] Another option is the “switch receptor”, where the extracellular PD-1 is linked to the intracellular CD28 activating domain, providing costimulation to the CAR on PD-L1 engagement. A phase II clinical trial (NCT03258047) has evaluated this approach in B-cell non-Hodgkin’s lymphoma. Nevertheless, to draw definitive and conclusive outcomes, a larger patient cohort is required.

Furthermore, genome-editing strategies are being explored to knockdown PD-1, incorporating a PD-1 short hairpin RNA-expressing cassette in the CAR construct (NCT03208556) or adopting Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/Cas9 system (NCT04213469, NCT05812326).[10] Other studies are combining CAR expression with PD-1-blocking single-chain fragment variables (scFvs) secretion. The feasibility of EGFR.CAR T-cells secreting anti-PD-1 scFv is under evaluation in advanced solid tumors (NCT02862028).

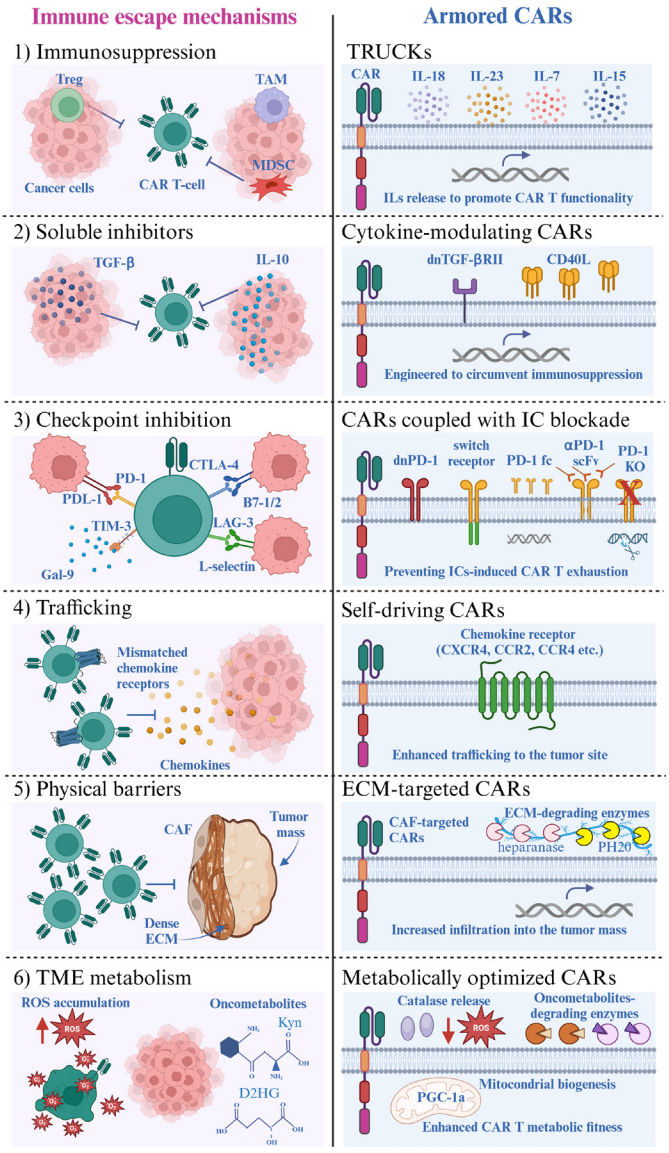

Figure 1 Armored CAR T-cells to counteract tumor microenvironment immune escape mechanisms. CAR T-cell therapy is encumbered by many challenges like tumor heterogeneity, antigen escape, cell exhaustion, on-target/off-tumor effects, and CAR-associated toxicities, like cytokine-release syndrome and neurotoxicity, but above all by the immunosuppressive tumor microenvironment (TME). Regulatory T-cells (Tregs), tumor-associated macrophages (TAMs), myeloid-derived suppressor cells (MDSCs), and stromal cells support the tumor, suppressing immunity. T-cells redirected toward universal cytokine killing (TRUCKs) release interleukins (IL) to enhance CAR T-cell activity and persistence while reactivating host immunity (A). Soluble inhibitors, such as TGF-β and IL-10, abate CAR T-cell expansion and effector functions. Cytokine-modulating CARs can make the CAR resistant to these inhibitory signals or enhance tumor clearance, pairing CAR-mediated activity with CD40 ligand (CD40L) release (B). Tumors upregulate immune checkpoint (IC) ligands that interact with receptors, like programmed-cell-death 1 (PD-1), cytotoxic T-lymphocyte associated protein 4 (CTLA-4), T-cell immunoglobulin mucin 3 (TIM-3), and lymphocyte activation-gene 3 (LAG-3) on CAR T-cells leading to exhaustion. CARs coupled with IC blockade were designed to overcome this hurdle. Different strategies have been tested, like the expression of dominant-negative forms of the receptors (dnPD-1), the equipment with a PD-1/CD28 switch receptor, the release of PD-1-Fc proteins or anti-PD-1 scFv, and the knock-out of the inhibitory receptor (C). Tumors hijack common chemokine axes to promote disease maintenance, progression, and resistance. The mismatch between tumor-derived chemokines and chemokine receptors on CAR T-cells impedes their homing to the tumor site, limiting efficacy. Self-driving CARs coexpress chemokine receptors like CCR2, CCR4, and CXCR4, guiding T-cells within the transformed niche (D). In solid tumors, CAR T-cell infiltration is further limited. Cancer-associated fibroblasts (CAFs) create a thick physical barrier, with a dense extracellular matrix (ECM) confining CAR T-cells outside the tumor mass. ECM-targeted CARs increase CAR T-cell penetration within the stroma, targeting CAF-restricted antigens or releasing ECM-degrading enzymes (E). Lack of nutrients, reactive oxygen species (ROS), hypoxia and oncometabolites, such as kynurenine (kyn) and D-2-hydroxyglutarate (D2HG), contribute to the hostile environment abating CAR T. Catalase-releasing CARs that decrease ROS accumulation, CARs overexpressing peroxisome proliferator-activated receptor γ coactivator 1-alpha (PGC1-α) enhancing mitochondrial biogenesis, and CARs releasing oncometabolites-degrading enzymes are some examples of metabolically optimized CARs (F). Created with BioRender.com.

Two clinical studies (NCT04162119, NCT04163302) are assessing the safety and efficacy of another approach based on CAR T-cells releasing PD-1-Fc fusion proteins able to block PD-L1 immunosuppressive action.[10]

Another novel strategy to bypass the immunosuppressive TME is represented by OrexiCAR T-cells engineered to locally secrete a CD47-signal regulatory protein (SIRP) α checkpoint blocker that disrupts the antiphagocytic signaling induced by tumor cells on macrophages. In a preclinical study, Dacek *et al* reported a synergistic effect of anti-CD19 CAR T-cells secreting a truncated SIRPα mimic combined with rituximab.[11]

CARs coupled with single or dual IC blockade hold promise to achieve both functional persistence of CAR T-cells and reactivate endogenous tumor-specific T-cells, leading to better clinical outcomes. Other inhibitory receptors, including LAG-3 and CTLA-4 are upregulated in T-cells after chronic stimulation. Initial evidence has shown the feasibility of knocking out these molecules even though it has yet to be determined if these modifications actually improve CAR T-cell function.[9] Unraveling the mechanisms by which inhibitory receptors affect T-cell function, along with the development of preclinical models that better recapitulate the TME are necessary to develop more effective CAR T-cell therapies taking advance of IC blockade.

Self-driving CARs to enhance trafficking to the tumor site

Fundamental to the optimization of CAR T-cell clinical outcome is the requirement for cells infused into the bloodstream to efficiently reach and remain at the tumor site. Such a challenge, well documented in solid tumors, has been extended in the last few years to hematological malignancies in which bone marrow (BM) is the primary location for leukemia initiation, maintenance, progression, and chemoresistance. Self-driving CARs typically coexpress a chemokine receptor promoting T-cell trafficking and persistence within the tumor. Cancer cells or their stroma produce chemokines supporting tumor proliferation, survival, progression, and immunosuppressive cell recruitment. The tumor chemokine signaling network can be exploited to drive CAR T-cell recruitment by engineering the expression on their surface of additional chemokine receptors, such as CCR2, CCR4, CCR8, CXCR1, CXCR2, CXCR5, CXCR6, and CX3CR1.[12] For hematological malignancies, strategies like drug-induced[13] or genetically engineered upregulation of CXCR4[14 15] have been employed to promote BM infiltration of CAR T-cells, thereby augmenting their antileukemic action. Multiple early-phase clinical trials are assessing the feasibility of this approach. CXCR5-modified EGFR.CAR T-cells are under evaluation in two phase I clinical trials (NCT04153799, NCT05060796) for the treatment of non-small cell lung cancer expressing CXCL13. CXCR2-expressing CAR T-cells directed against CD70 (NCT05353530) or Nerve Growth Factor Receptor (NGFR) (NCT01740557) are being evaluated for the treatment of glioblastoma and melanoma, respectively. CCR4-modified CD30.CAR T-cells (NCT03602157) are under investigation in lymphoma patients. Additionally, a phase I clinical study (NCT04727008) is assessing the safety and efficacy of CXCR4-expressing B-cell maturation antigen (BCMA) CAR T-cells in MM.

Overall, engineering CAR T-cells with chemokine receptors demonstrated the critical role of effector cell migration and infiltration within the tumor site to enhance their therapeutic effectiveness.[12] However, T-cell migratory patterns are complex and chemokine expression can significantly differ between tumor types, representing a major challenge for self-driving CAR T-cell clinical application, requiring tailored approach based on the specific tumor's chemokine profile. Therefore, the safety and efficacy of this strategy in clinical settings remain to be proven.

Extracellular matrix-targeted CARs to remove the physical barrier surrounding solid tumors

CAR T-cell infiltration in solid tumors might be further limited by physical barriers, such as the dense extracellular matrix (ECM) that prevents lymphocyte penetration into the parenchyma. A strategy to overcome this obstacle is targeting the tumor-associated stromal cells involved in ECM remodeling. Anti-fibroblast activation protein (FAP) CAR T-cells, which target a protease highly expressed on cancer-associated fibroblasts, are under evaluation in two clinical trials (NCT01722149 and NCT03932565).[16] Most studies are still at the preclinical level. For instance, Wagner *et al* designed a CAR targeting the cancer-specific extra domain B (EDB) splice variant of fibronectin, a protein released solely by cancer cells in a broad range of solid tumors, making it a selective tumor target antigen. Accordingly, EDB-CAR T-cells demonstrated potent antitumor activity in multiple xenograft mouse models without signs of on-target off-tumor toxicity.[17] Therefore, CARs targeting tumor-associated stromal cells which can remodel the structure of ECM, such as anti-FAP or anti-EDB, seem effective. Another promising approach involves the engineering of CARs secreting ECM-degrading enzymes, such as heparanase or hyaluronidase PH20. Engineering GD2 CAR T-cells with heparanase enhanced effector cell infiltration and antitumor activity in xenograft mouse models, without signs of toxicity in healthy tissues.[18] Similarly, the release of hyaluronidase PH20 improved CAR T-cell tumor infiltration in gastric and liver cancer mouse models.[19 20] Therefore, even if ECM-targeted CARs have not been widely tested in the clinic, the preclinical evidence suggests a high potential for this strategy to enhance the therapeutic outcome of patients with solid tumors.

Metabolically optimized CARs to survive in the TME

Targeting tumor metabolism and hypoxia to overcome their immunosuppressive effects, while enhancing CAR T-cell metabolic abilities might be another favorable optimization strategy, especially in solid tumors. Cancer cells increase Reactive Oxygen Species production in the TME

to support their maintenance and inhibit effector T-cells. Ligtenberg *et al* designed catalase-releasing CAR T-cells which demonstrated reduced oxidative stress in the TME and improved antitumor activity.[21]

Another possibility to increase CAR T-cell resistance in the TME is to enhance their mitochondrial fitness. Lontos *et al* demonstrated that a CAR engineered with peroxisome proliferator-activated receptor γ coactivator 1-alpha (PGC-1a), a central regulator of mitochondrial biogenesis, is endowed with improved antitumor activity and exhibits better exhaustion profile.[22] Also, oncometabolites, such as kynurenine (kyn) and D-2-hydroxyglutarate (D2HG), contribute to CAR T-cells failure. CAR T-cells engineered to produce kyn- or D2HG-degrading enzymes demonstrated improved expansion and antileukemic activity.[1] Even if promising, these studies are in the early stages of investigation and their true impact on the field will only be determined by clinical trials. However, multiple issues must be addressed before translating this approach to the clinic as most of the current knowledge on T-cell metabolism derives only from in vitro and in vivo studies, but its relevance to patients requires further investigation. Moreover, metabolic heterogeneity and redundancy might pose a challenge to identifying the proper metabolites or enzymes to be targeted or engineered by CAR T-cells to avoid potential toxicities. New technologies like single-cell metabolite profiling might help researchers to better address these obstacles and uncover novel metabolic interventions to enhance CAR T-cell therapeutic outcomes.

Combinatorial strategies to tackle the TME

Next-generation CARs should combine several layers of smart engineering to tackle different obstacles simultaneously. Cadilha *et al* published an attractive combined strategy equipping CAR T-cells both with dnTGF-βRII, which overcomes TGF-β-mediated immunosuppression, and CCR8, that redirects T-cells to the tumor site, demonstrating consistent antitumor efficacy in solid tumor models.[23] Recently, Ruixin *et al* presented another combined approach generating armored EGFRvIII CAR T-cells coexpressing synthetic IL-15 or IL-18 and CXCR2. IL-15/IL-18 release prevented T-cell exhaustion and enhanced CAR T-cell antitumor activity, while CXCR2 promoted their trafficking and infiltration in a breast cancer in vivo model.[24] Four clinical studies (NCT04381741, NCT03929107, NCT04833504, NCT03778346) are investigating IL-7 and CCL19-modified CAR T-cells in hematological tumors. IL-7 stimulates T-cell expansion and survival, while CCL19 induces endogenous T-cells and DCs migration to T-cell zones of lymphoid organs. IL-7/CCL19-expressing CAR T-cells have already shown efficacy in the lymphoma setting (NCT04381741) with complete remission (CR) in 4/7 patients and overall response rate in 5/7 patients, and in MM (NCT03778346) with CR of 2/2 enrolled patients.[10]

Notably, most approaches combine migratory engineering with strategies circumventing the immunosuppressive TME. The importance of CAR T migration within the tumor site has already been demonstrated by multiple preclinical studies, where increased CAR T infiltration is associated with decreased tumor progression and improved mice survival.[13 15] However, tackling only this aspect is not sufficient. Other challenges such as suboptimal proliferation, limited persistence of the therapeutic product, and the immunosuppressive role played by the TME must be addressed to enhance CAR T-cell effectiveness. Hence, while the mentioned clinical studies are in the early stages, they offer hope for achieving the most favorable therapeutic outcomes.

CONCLUSION

The development of armored CAR T-cells is a rapidly evolving field that may enable CAR T to be efficacious against a broader range of malignancies. The ongoing clinical trials will ultimately reveal whether the encouraging results observed in preclinical studies will be translated into clinical success (online supplemental table 1). Particularly, armored CARs integrating multiple of the described approaches hold great promise. Novel sophisticated T-cell engineering approaches will be the essential drivers to program CAR T-cells with more complex functionalities in tackling the microenvironment. In combination with these strategies, several further improvements are underway, such as universal hypoimmunogenic allogeneic CAR T-cell products, expedited manufacturing platforms, and non-viral transfection systems, thus extending CAR T clinical applicability to a broader patient population.

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