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REVIEW ARTICLE OPEN

CAR-T and CAR-NK as cellular cancer immunotherapy for solid tumors

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In the past decade, chimeric antigen receptor (CAR)-T cell therapy has emerged as a promising immunotherapeutic approach for combating cancers, demonstrating remarkable efficacy in relapsed/refractory hematological malignancies in both pediatric and adult patients. CAR-natural killer (CAR-NK) cell complements CAR-T cell therapy by offering several distinct advantages. CAR-NK cells do not require HLA compatibility and exhibit low safety concerns. Moreover, CAR-NK cells are conducive to "off-the-shelf" therapeutics, providing significant logistic advantages over CAR-T cells. Both CAR-T and CAR-NK cells have shown consistent and promising results in hematological malignancies. However, their efficacy against solid tumors remains limited due to various obstacles including limited tumor trafficking and infiltration, as well as an immuno-suppressive tumor microenvironment. In this review, we discuss the recent advances and current challenges of CAR-T and CAR-NK cell immunotherapies, with a specific focus on the obstacles to their application in solid tumors. We also analyze in depth the advantages and drawbacks of CAR-NK cells compared to CAR-T cells and highlight CAR-NK CAR optimization. Finally, we explore future perspectives of these adoptive immunotherapies, highlighting the increasing contribution of cutting-edge biotechnological tools in shaping the next generation of cellular immunotherapy.

Keywords: CAR-T; CAR-NK; Cell therapy; Cancer immunotherapy; Solid tumor

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INTRODUCTION

Immunotherapy, which aims to stimulate the immune system to eradicate cancer, has recently revolutionized cancer treatment and constitutes the fourth cornerstone of cancer therapy alongside surgery, radiation, and chemotherapy [1, 2]. Current cancer immunotherapy research encompasses a broad range of approaches, including antibodies, vaccines, cytokines, oncolytic viruses, bi-specific molecules, and cellular therapies [3, 4]. Among these, immune checkpoint inhibitors (ICIs) and adoptive cell therapy (ACT) have emerged as the most successful immunotherapy strategies for cancer treatment [5–9]. ICIs, which block immune checkpoints, have achieved significant tumor regression and changed standards of care for a variety of solid tumor malignancies, including melanoma, lung cancer, and head and neck cancer [5, 10, 11]. However, primary and acquired resistance remains common due to the scarcity of anti-tumor T cells and impaired memory T cells.

Unlike small molecule drugs or antibodies, cells have the potential to sense and dynamically respond to diseases [12]. Cellular immunotherapy, which involves the transfer of modified immune cells back into the patient, has rapidly grown in clinical investigation and achieved significant success in hematologic malignancies, though its use in solid tumor malignancies is still in its early stages [2, 13].

In this review, we explore the potential of CAR-T and CAR-NK cells, along with the current limitations of these treatment modalities against cancer, especially solid tumors. We present an overview of the engineering strategies implemented in recent years to address the main challenges limiting the CAR-T and CAR-NK cell effectiveness, showcasing the broad versatility of cellular immunotherapy. Finally, we highlight the potential advantages and limitations of CAR-NK cells over CAR-T cells and outline the future perspectives of these cancer therapies.

CAR-T CELL CANCER THERAPY

T cells, as a key component of the adaptive immune system, can be divided into different subgroups based on function, co-receptor expression (CD4 or CD8), trafficking, metabolism, and lifespan, playing a crucial role in fighting cancer due to their potent ability to recognize and eliminate cancerous cells [14]. Consequently, T cells have emerged as primary candidates for

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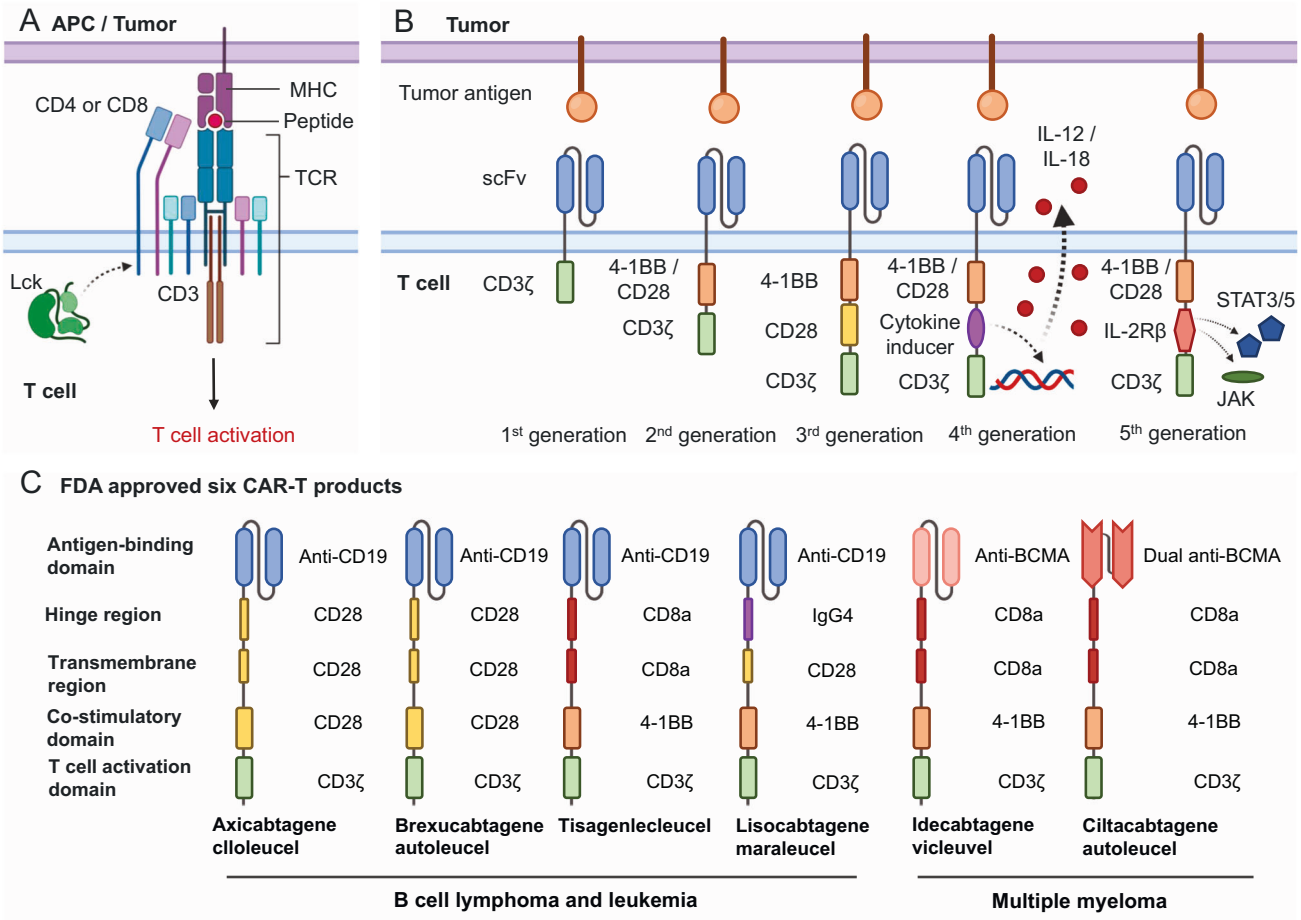

Fig. 1 Chimeric antigen receptor (CAR) construct. **A** When a T cell encounters antigen-presenting cells or tumor cells, T cell receptor (TCR) and CD3 complex, together with CD4 or CD8, will recognize MHC complex-presented peptides, then trigger TCR signaling cascade, leading to T cell activation. **B** The first-generation CAR majorly involves two parts: scFv at the extracellular part and CD3ζ chain at the intracellular part. ScFv domain can recognize tumor surface antigens, and then the CD3ζ chain will directly activate T cells. The second-generation CAR incorporated a co-stimulation domain, either 4-1BB or CD28 signaling domain. The third-generation CAR has both 4-1BB and CD28 signaling domains. **C** Differences of antigen-binding domain, hinge region, transmembrane region, co-stimulatory domain, and T cell activation domain of six FDA-approved CAR-T products. Those products are used to treat B-cell lymphoma, B-cell leukemia, and multiple myeloma. APC antigen-presenting cell, MHC major histocompatibility complex, TCR-T cell receptor, scFv single-chain variable fragment

cancer immunotherapy. However, in immunocompetent patients, tumors often undergo "cancer immunoediting", enabling them to evade cellular immunity and establish a microenvironment that facilitates tumor outgrowth [15]. Therefore, effective ACT needs to overcome the tumor immune escape mechanisms, including T cell dysfunction [16–18] and the absence of tumor-associated antigens (TAA), such as lineage antigens in hematological malignancies. This can be achieved by expanding and activating T cells ex vivo or genetically engineering them to boost their cancer-fighting capabilities.

Currently, there are three major modalities of autologous T cell immunotherapy: tumor-infiltrating lymphocyte (TIL) therapy, genetically engineered T cell receptors T (TCR-T) cell therapy, and CAR-T cell therapy [19]. TIL therapy, which involves expanding a heterogeneous population of endogenous T cells with a pool of native TCRs from a harvested tumor, has been approved by the FDA for the treatment of advanced melanoma (FDA news release on 2/16/2024). This strategy is particularly promising for "hot tumors", characterized by a tumor microenvironment (TME) enriched with TILs, indicating a preexisting immune response [20]. TCR-T cell therapy involves expanding T cells with genetically encoded TCRs directed toward specific antigen targets. In contrast, CAR-T cell therapy involves expanding genetically engineered T cells equipped with synthetic receptors (CARs) that recognize specific antigens on cancer cells [21]. Additionally, more sophisticated engineered T cells, such as synthetic TCR and antigen receptor (STAR-T) [22] and HLA-independent T cell receptors (HIT) [23], are also on the horizon.

The recognition and killing of cancer cells by T cells rely on canonical TCR signaling. Initially, T cells are primed by peptide antigens presented by major histocompatibility complex (MHC), which activates a series of downstream signals to facilitate cancer killing [16] (Fig. 1A). In contrast, CARs were first proposed and invented by Eshhar et al. to enable T cell cytotoxicity independent of MHC restriction, allowing for target-specific cytotoxicity and broader therapeutic applicability [24]. The first generation of CARs consists of an extracellular single-chain variable fragment (scFv) that recognizes specific antigens, linked via a hinge and transmembrane (TM) domain to a C-terminal CD3ζ activation domain (Fig. 1B). These antibody-based antigen receptors redirect the inherent cytotoxic nature of T cells to kill cancer cells in an antigen-specific manner.

However, the first generation of CAR-T cells, which relied solely on the CD3 ζ-chain to simulate TCR signaling, exhibited limited proliferation, engraftment, and cytotoxicity, resulting in unsatisfactory clinical efficacy. Subsequent modifications to CAR molecules led to the development of second and third-generation CAR-T cells, which included one (second-generation) or two (third-

generation) intracellular co-stimulatory (ICOS) domains upstream of CD3ζ (Fig. 1B) [25]. These enhancements improved CAR-T cell cytotoxicity, cytokine production, and proliferation in response to target stimulation.

Currently, there are six FDA-approved CAR agents targeting CD19 or BCMA for hematological malignancies [26]. All approved CAR products employ a second-generation CAR construct, consisting of an antigen-binding domain, a hinge region, a TM region, a co-stimulatory domain (CD28 or 4-1BB), and a T cell activation domain (Fig. 1C). These CAR-T cell therapies have achieved durable remission in many patients with hematological malignancies. In pivotal trials, CD19-CAR therapy outperformed the standard of care (SOC) as a second-line treatment for large B-cell lymphoma and has shown high effectiveness as a first-line therapy. BCMA-CAR therapy has demonstrated high response rates in multiple myeloma [27]. Recently, ongoing clinical trials conducted by major manufacturer pharmaceutical companies are comparing the efficacy of CAR-T cells to SOCs, aiming to introduce them to earlier lines of treatment for multiple myeloma. Such success has ushered in the era of cell therapy for hematological diseases.

Challenges in CAR-T cell therapy for solid tumors

Substantial attention and efforts have been devoted in recent years to improve CAR-T cell therapy for solid tumors. As of June 2024, data from ClinicalTrials.gov indicate a total number of 405 CAR-T cell clinical trials targeting solid tumors with varying statuses (29 completed, 30 active/not recruiting, 179 actively recruiting or enrolling by invitation, 90 with unknown status, 39 suspended/terminated/withdrawn, and 38 not yet recruiting). Despite showing signs of activity in solid tumors, outcomes from clinical trials have been disappointing to date, with no consistently high rates of durable responses observed [26].

The exact reasons why CAR-T cells have underperformed in solid tumors remain unclear, primarily due to insufficient biological information to evaluate key aspects of therapy. Most clinical studies evaluating CAR-T cells in solid tumors have predominantly reported generic tumor response parameters and the presence/persistence of CAR-T cells in peripheral blood (PB) [28, 29]. However, pivotal details regarding CAR-T cell infiltration, phenotype, and interactions with the TME are largely lacking, as they require post-infusion bioptic data.

As an illustrative example, the application of CAR-T cells in patients affected by high-grade glioma can be cited. Currently, eleven single report or Phase I clinical trials have been performed [30–40], including ~130 patients. However, post-infusion pathological data have only been obtained for 11 patients, always following clinical-driven surgical indications or post-mortem. These data suggest several important findings: (1) CAR-T cells patchy infiltrate the tumor when administrated intravenously [38], (2) their persistence appears to be limited [30, 31, 38], and (3) the therapy administration is followed by an upregulation of immunosuppressive signals in TME [38]. Furthermore, both pathological data and liquid biopsy samples acquired in these trials have highlighted the well-known ability of glioblastoma in antigen escaping. However, the non-systematic acquisition of the pathological data precludes speculation on the mechanistic series events that allow tumor immunoescape [41].

Without this, we rely solely on reasonable considerations obtained from solid tumor biology or inferences coming from mouse models. In fact, solid cancers present unique challenges for CAR-T therapy. First, unlike B-cell malignancies, which possess several lineage-specific epitopes, cells within most of the solid tumors are heterogeneous [42–44]. Additionally, even when a tumor antigen is shared with healthy cells, in hematological tumors it can be feasible to sacrifice the entire antigen-positive lineage to eradicate the cancer. This is because the intrinsic characteristic of the bone marrow tissue, that can be ablated, cytoreducted, stimulated with growth factors, and even transplanted allowing the survival of the patient. In contrast, most solid tumors originate from tissues where a widespread autoimmune cross-reaction would be life-threatening. For this reason, identifying tumor-specific antigens that can be targeted by CAR-T cells has proven difficult. Although antigens that are overexpressed in tumors or TAA have been chosen, there are non-negligible levels of antigen expression in healthy tissues, raising concerns about toxicity issues [43, 45]. Second, fibrotic tumor stroma of solid tumor, which is comprised of extracellular matrix (ECM) and cancer-associated fibroblasts (CAFs), along with the abnormal vasculature at the tumor site, create physical barriers that impede CAR-T cell infiltration and penetration into the tumor [46–48]. Additionally, the immuno-suppressive TME of solid tumor characterized by numerous suppressive immune cells such as regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs), as well as immuno-suppressive ligands and agents such as programmed cell death 1 ligand 1 (PD-L1), TGF-β, and adenosine, further hinder CAR-T cell cytotoxic activity, proliferation, and persistence in combating solid tumors [43, 49] (Fig. 2).

Engineering strategies to improve CAR-T cell therapy for solid tumor

Combating antigen heterogeneity in solid tumor. Numerous strategies have been explored to address the antigen heterogeneity of solid tumors with the primary focus on achieving multi-target targeting (Fig. 2). One approach involves combinatorial strategies, such as sequential or combination treatments involving different CAR-T cell products. By concurrently targeting multiple antigens, this strategy aims to mitigate antigen escape phenomena commonly associated with CAR-T cell therapy. This strategy has already been demonstrated to be clinically safe and feasible in diffuse large B-cell lymphoma [50, 51] and may represent a promising approach also in solid tumors. Another innovative avenue is the development of multi-target CAR-T cells, achieved through the incorporation of two distinct CAR constructs into T cells or the utilization of bi-specific or Tandem (TanCAR) CAR-T cells. For instance, Muhammad et al. synthesized a novel TanCAR, comprising a tandem arrangement of IL13 and EphA2 single-chain variable fragments (scFv), demonstrating that the IL13-anti-EphA2 TanCAR exhibited markedly stronger anti-tumor efficacy compared to single CAR-T cells, both in vitro and in vivo [52].

Additional modification of CAR-T cells has been explored to enhance their targeting capabilities. One such strategy involves enabling CAR-T cells to secrete bi-specific T cell engagers (BiTEs) which engage with additional targets, thereby augmenting their anti-tumor efficacy. For example, Wing et al. designed folate receptor alpha (FR-α) targeting T cell engager CAR-T cells (anti-EGFR BiTEs) and showed that anti-EGFR BiTEs increased the anti-tumor efficacy of anti-EGFR CAR-T cells in a mouse model of glioblastoma [53]. Moreover, in a recent clinical trial, CARv3-TEAM-E T cells were evaluated in glioblastoma expressing the epidermal growth factor receptor variant III (EGFRv3). These cells were designed to recognize both this tumor-specific antigen, as well as the wild-type EGFR protein, through the secretion of a T cell-engaging antibody molecule (TEAM) [40]. In this way, TEAM-E T cells provide a potential solution to overcome the loss of EGFR variant III observed in glioblastoma after target immunotherapies like peptide vaccination [54] or CAR-T cells [38]. This Phase 1 trial has shown the feasibility and relative safety of the intracranial injection of the TEAM-E T cells. All of the three patients enrolled in the trial have shown a promising initial radiological response, albeit transient.

Furthermore, universal CARs are developed to realize multiple antigen targeting. Adapter elements serve as key components to enable the targeting of multiple antigens with a common CAR-T cell population. Noteworthy examples include avidin-linked CAR-T cells, leveraging the binding affinity between avidin and biotin to recognize multiple targets [55]. Similarly, approaches involving

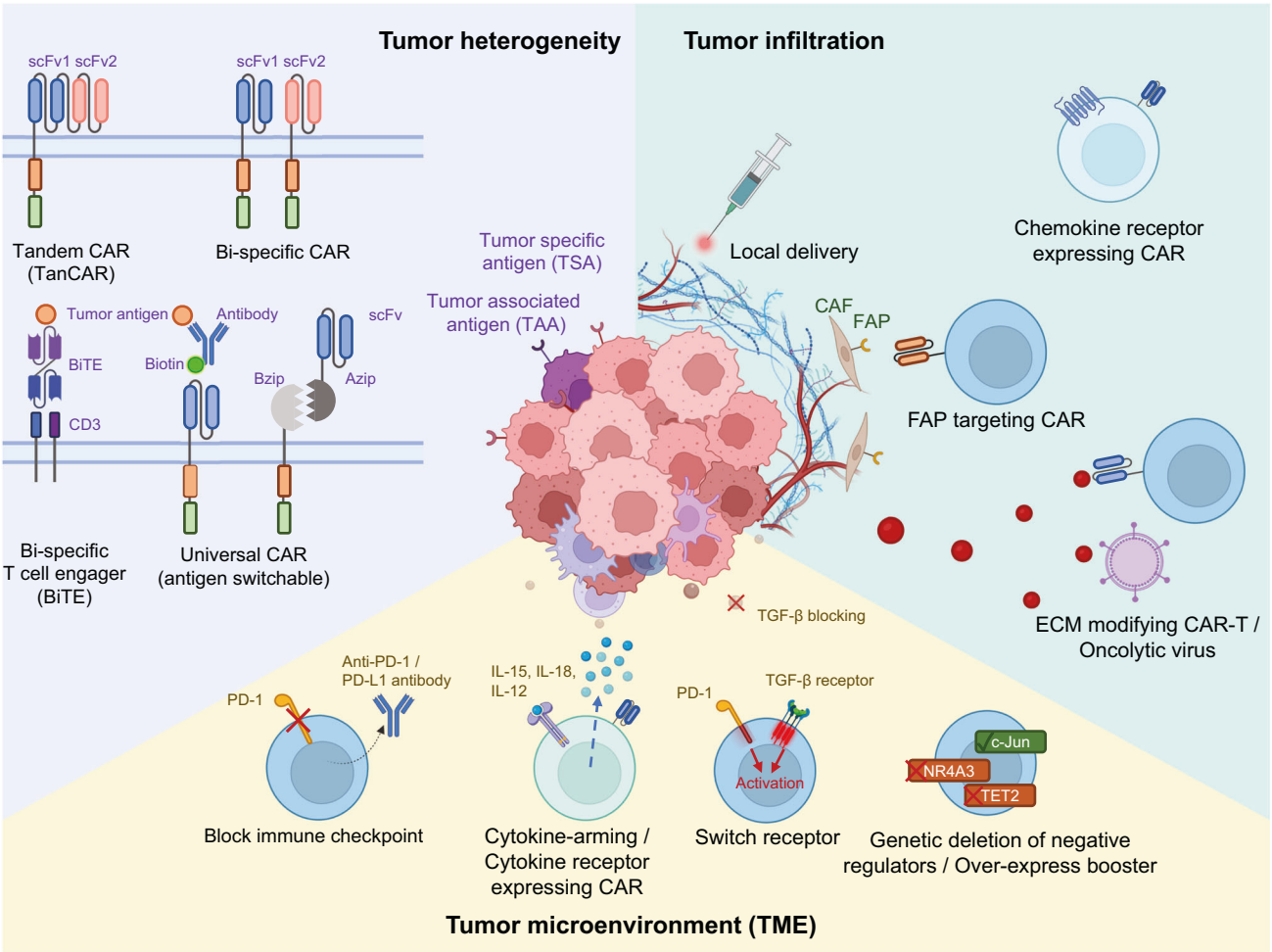

Fig. 2 Challenges of CAR-T faces and engineering strategies to overcome solid tumor. Treating solid tumors poses three major challenges for CAR-T cells: tumor heterogeneity, tumor infiltration, and inhibitory tumor microenvironment (TME). There are two types of tumor antigens, tumor-specific antigen (TSA) which only expresses in tumor cells, and tumor-associated antigen (TAA) which can be expressed in normal cells as well. Meanwhile, tumor cells may express tumor antigens at different levels as well. To overcome heterogeneity, efforts have been made to engineer CARs with two different scFvs (TanCAR) or express two different CARs recognizing different antigens (Bi-specific CAR). Bi-specific T cell engager can link tumor antigen and CD3 complex to initiate T cell tumor killing. Solid tumor also has physical barriers and extracellular matrix that limit CAR-T cell infiltration. To overcome this challenge, researchers have tried local delivery of CAR-T cells, engineering chemokine receptor-expressing CAR, FAP-targeting CAR, or EMC modifying CAR-T or oncolytic virus. Immune suppressive TME is another big challenge which consists of inhibitory immune cell infiltration, immune suppressive factors such as TGF-β, as well as chemical environment alterations. Researchers have tried to block immune checkpoints such as PD-1, using genetic deletion, administration of anti-PD-1/PD-L1 antibodies, or T cell autocrine antibodies. Efforts have also been made to engineer cytokine-arming or cytokine receptor-expressing CAR, replace inhibitory domains with activation domains in PD-1 or TGF-β as switch receptor, and delete T cell internal negative regulators or over-express T cell function boosters

the engineering of CAR-T cells with Fcγ receptors, enabling recognition of the common Fc domain of antibodies, have been investigated to achieve multi-antigen targeting [56]. These strategies represent promising advancements in the field of CAR-T cell therapy, offering innovative solutions to overcome challenges associated with tumor heterogeneity and antigen escape.

Lastly, CAR-T cell therapy can be combined with vaccination against the target antigen recognized by the CAR. Once expressed by antigen-presenting cells, the target is able not only to promote CAR-T cell expansion and maturation in a memory phenotype [57] but also to engage the host immune cells to mount a response against different antigens of the same target [58].

This mechanism, known as epitope spreading, confers robustness to the immunotherapy while simultaneously addressing pre-treatment antigen heterogeneity and antigen loss induced by the treatment. Evaluated in Phase I/II clinical trial in advanced solid tumors, the mRNA CAR-T cell-amplifying vaccine showed no alteration of the safety profile of the CLDN-6 specific CAR-T cells [59]. Furthermore, despite this first publication being incomplete due to an amendment to the protocol, this trial has shown encouraging results in terms of disease control in a group of refractory tumors.

Increasing trafficking and infiltration into solid tumors. To combat cancer cells, CAR-T cells are requisite to contact and stimulated by its cognate antigens on tumors. Leveraging various cytokine and chemokine receptors capable of mediating immune cell trafficking, researchers have endeavored to engineer CAR-T cells to improve their trafficking and infiltration into solid tumors [60, 61] (Fig. 2). Jin et al. discovered significant amounts of IL-8, CXCL1, and CXCL2 were released from tumors. They engineered CARs modified with IL-8 receptors, CXCR1, and CXCR2, demonstrating that these modified CARs could be guided to migrate into tumors, inducing an

enhanced anti-tumor response in solid tumors [62]. Moreover, the activation of CC-chemokine ligand 17 (CCL17) and CCL22, secreted by Reed–Sternberg cells of Hodgkin lymphoma (HL), activated their receptor CC-chemokine receptor 4 (CCR4) expressed T helper cells and Tregs [63]. Savoldo's group showed that CD30 CAR-T cells co-expressing CCR4 (CCR4.CD30.CAR-T cells) exhibited improved tumor homing and anti-lymphoma activity compared to CD30 CAR-T cells lacking CCR4 expression [64]. Later on, data from a clinical trial (NCT03602157) further showed CCR4.CD30. CAR-T cells' promising efficacy in patients with relapsed/refractory (r/r) classical HL, and displayed enhanced trafficking to the skin, rendering them more effective in CD30+ cutaneous T cell lymphomas [65]. Similarly, modifying CAR-T cells with the CCL2 cognate receptor CCR2b resulted in a 10-fold increase in infiltration of anti-GD2 CAR-T cells into neuroblastoma xenograft tumors and a 12-fold increase in infiltration into mesothelioma xenografts compared to unmodified CAR-T cells [66, 67]. Li et al. enhanced CAR-T cells by co-expressing IL-7 and CCR2b, demonstrating improved CAR-T cell survival and infiltration in glioblastoma and melanoma [68]. CCR2b-anti MSLN CAR-T cells exhibited enhanced migration and infiltration into tumor tissue, displaying superior anti-tumor efficacy, particularly in non-small-cell lung carcinoma [69]. In recent years, novel cytokine and chemokine receptors have been investigated in preclinical studies to improve CAR-T cell infiltration into tumors. For instance, CLDN18.2-specific IL-7 and CCL21 co-expressing CLDN18.2-specific cytokines (7×21 CAR-T cells) enhanced proliferation and chemotaxis of CAR-T cells across various cancer models such as PANC02 (pancreatic cancer), E0771-A2 (breast cancer), and Hepa1-6-A2 (liver cancer) [70]. Additionally, IL-7 and CCL19 expressing anti-GM2 CAR-T cells exhibited abundant CAR-T cell infiltration and strong therapeutic effects in GM2-positive solid cancers in xenograft models [71]. Trinh et al. designed a CX3CR1 overexpressing (NKG2D) CAR-T expression and found that CAR-T cells infiltrated tumors at higher rates than control-activated T cells or IL-15-overexpressing NKG2D CAR-T cells in a liver cancer model [72].

In clinical settings, regional delivery of CAR-T cells into tumors at various anatomical sites, including the brain [32], breast [73], pleura, and liver [74] has emerged as an alternative delivery strategy to enhance CAR-T cell localization in tumors. Prapa et al. compared intracerebral versus intravenous anti-GD2 CAR-T injections to treat glioblastoma, revealing that the intracerebral route significantly increased survival time in a dose-dependent manner without side effects [75]. Clinical data from the Phase 1b HITM-SIR trial demonstrated the safety and efficacy of CAR-T cell hepatic artery infusions therapy, which was not associated with severe cytokine release syndrome (CRS) or neurotoxicity [76, 77].

However, solid TMEs present physical barriers that CAR-T cells must overcome to penetrate tumors. One such barrier is the protease fibroblast activation protein (FAP), expressed by many tumor-associated stromal fibroblasts, which plays a crucial role in remodeling the tumor ECM. Targeting FAP-expressing stromal cells with CAR-T cells has been explored to facilitate immune cell infiltration into tumors. Nevertheless, results from studies are contradictory. While some studies reported limited effects on tumor progression and adverse effects on bone marrow stromal cells [78], others demonstrated decreased tumor growth without severe toxicities when combined with vaccines [79].

Apart from FAP targeting, engineering CAR-T cells to secrete ECM-modifying enzymes is another approach to enhance CAR-T cell penetration into solid tumors. For instance, anti-GD2 CAR-T cells engineered to degrade heparin sulfate proteoglycans in the ECM through heparinase expression exhibited improved tumor infiltration and prolonged survival compared to CAR-T cells lacking heparinase expression [80]. Other ECM-degrading enzymes are also under exploration for their potential role in CAR-T cell therapy. Recently, Wang et al. constructed a recombinant oncolytic vaccinia virus encoding hyaluronidase to degrade hyaluronic acid, which often impedes intratumoral dissemination of anti-tumor drugs. Their findings demonstrated increased intratumoral dissemination of chemo drugs, immune cell infiltration, and activation of $CD8^+$ T cells [81]. While ECM modification presents an exciting frontier in CAR-T cell therapy for solid tumors, caution is warranted due to the complicated and currently unpredictable effects of ECM-modifying enzymes. Clinical trials have shown mixed results, indicating the need for further investigation into the efficacy and toxicity profile of these approaches. Additionally, there are concerns regarding potential thromboembolic events associated with ECM modification, as evidenced by the administration of low molecular weight heparin supplementation to mitigate risks in clinical trials [82, 83].

In summary, while significant strides have been made in enhancing CAR-T cell trafficking and infiltration into solid tumors, ongoing research is essential to further understand the complexities of the TME and optimize therapeutic strategies for improved efficacy and safety in clinical settings.

Blocking CAR-T cell dysfunction. Once located in the tumor, CAR-T cells must contend with direct inhibitory signals present in the TME. Multiple inhibitory signals, including the expression of PD-1 and other inhibitory receptors, have been identified as mechanisms of CAR-T cell dysfunction [84]. Interfering with checkpoint signal pathways is a common approach to alleviate CAR-T cell dysfunction and restore their anti-tumor efficacy [85, 86] (Fig. 2). A well-known inhibitory pathway is the PD-1–PD-L1 axis. PD-1 is an immune checkpoint receptor expressed in T cells. When PD-1 is bound by its ligand PD-L1 expressed on tumor cells as well as other cell types, it induces T cells to adopt an exhausted phenotype [87]. Combinational therapy of PD-1 antibody checkpoint blockade and CAR-T cells showed increased efficacy of CAR-T cell therapy in both preclinical and clinical settings [88]. Various groups have demonstrated increased efficacy of CAR-T cell therapy with the coadministration of antibodies that inhibit the PD-1 pathway in preclinical models [89, 90]. Jaspers et al. found that anti-PD-1 blockade increased memory phenotype, reduced exhaustion, and induced durable responses of anti-DLL3 CAR-T cells in multiple SCLC models [91]. Similarly, a Phase I clinical showed that anti-mesothelin CAR-T cells showed therapeutic effects in patients with malignant pleural disease, in combination with the anti-PD-1 agent pembrolizumab [92]. Instead of using CAR-T cells in combination with established ICIs, researchers also employed genetic engineering strategies to disrupt checkpoint pathways. Wang et al. generated PD-1 and TCR deficient mesothelin-specific CAR-T (MPTK-CAR-T) cells using clustered regularly interspaced short palindromic repeats (CRISPR) technology and evaluated them in a dose-escalation clinical study [93]. No dose-limiting toxicity or unexpected adverse events were observed in any of the 15 patients. The best overall response was stable disease (2/15 patients) [93]. Agarwal et al. found that CRISPR-mediated deletion of *CTLA4* permitted unopposed CD28 signaling and maintenance of CAR expression on the T cell surface under the condition of high antigen load [94]. CTLA4 deficiency in CAR-T cells improved proliferation and anti-tumor efficacy in preclinical models of leukemia and myeloma, which rescued the function of T cells from patients with leukemia that previously failed CAR-T cell treatment [94]. 4-1BB-based CAR-T cells armed with autocrine PD-L1 scFv antibody effectively reversed exhaustion and enhanced the anti-tumor immune response in solid tumors and hematologic malignancies by blocking the PD-1/PD-L1 signaling [95]. A similar study showed that mesothelin-targeting CAR-T cells secreting single-chain trimeric 4-1BB ligand fused to anti-PD-1 scFv (αPD1-41BBL) exhibited reduced inhibitory receptor upregulation; enhanced persistence, proliferation, and memory status; and augmented anti-solid tumor efficacy [96].

In the TME, various immuno-suppressive molecules, such as adenosine and TGF-β, produced by immuno-suppressive immune cells like Tregs and MDSCs, impose inhibitory roles on CAR-T cells

[97]. CAR-T cells have been engineered to counteract the actions of adenosine, with CRISPR-mediated deletion of the adenosine A2A receptor enhancing CAR-T cell efficacy in several studies [65, 98]. Similarly, reducing adenosine levels through overexpression of adenosine deaminase has induced stemness and enhanced CAR-T functionality [99]. TGF-β inhibition via CRISPR or small molecule blockers such as LY2157299 has been shown to promote the long-term efficacy of CAR-T cells against solid tumors [93, 100]. Inactivation of TGF-β signaling in CAR-T cells through dominant-negative TGF-β receptor II has achieved optimistic preclinical and clinical results against solid tumors [101].

Another effective method to modify inhibitory signals in CAR-T cells is to express decoy or switch cytokine receptors, which converts inhibitory signals present in the TME into pro-inflammatory signals. For instance, switch receptors with chimeric signaling domains can convert TGF-β signals or PD-1/PD-L1 inhibitor signals through the engagement of modified receptors to signal through co-stimulatory domains such as 4-1BB or IL-12 [102, 103]. Similarly, cytokine receptors containing the extracellular domain of the IL-4 receptor fused with the endodomain of the IL-7 receptor can translate inhibitory IL-4 signals into activating IL-7 signals in T cells and CAR-T cells [104]. Another approach involves fusing the IL-4 receptor extracellular domain with the shared β-subunit of the IL-2 and IL-15 receptors, thereby converting inhibitory IL-4 signals into homeostatic IL-7, IL-2, or IL-15 signals [105]. These strategies represent innovative approaches to mitigate inhibitory signals present in the TME, thereby enhancing CAR-T cell efficacy in combating solid tumors.

Enhancing CAR-T cell anti-tumor function with immunostimulatory signals. An alternative strategy to enable CAR-T cells to overcome inhibitory signals in the TME involves engineering CAR-T cells with immunostimulatory signals to achieve enhanced anti-tumor functions (Fig. 2). The limited success of early iteration CAR-T cell designs lacking co-stimulation underscores the importance of incorporating a co-stimulatory domain. Currently, all CAR-T cells in clinical use contain either a CD28 or 4-1BB co-stimulatory domain [27]. Preclinical investigations are exploring the utility of including additional co-stimulatory molecules such as ICOS, OX40, and CD27, or various combinations of multiple co-stimulatory domains in third- and fourth-generation CAR constructs [106–109]. ICOS and 4-1BB co-stimulation have been shown to dramatically enhance CAR-T cell persistence [110]. Moreno-Cortes et al. generated ICOS.OX40 tandem co-stimulated anti-ROR1 CAR-T cells, demonstrating enhanced in vitro and in vivo cytotoxicity and prolonged persistence [111].

Augmenting CAR-T cells to secrete stimulatory cytokines represents another approach to enhancing CAR-T cell function. These "armored" CAR-T cells redirected for universal cytokine-mediated killing (TRUCKS) encode not only a CAR but also a cytokine, interleukin, pro-inflammatory ligand, or chemokine to counteract the immuno-suppressive microenvironment prevalent in most solid tumors [112–115]. Cytokines such as IL-12, IL-18, or IL-15 are commonly used to empower CAR-T cells with better in vivo proliferation, survival, and persistence. For example, IL-12 signaling facilitates CAR-T cell-mediated IFNγ production, which is necessary for tumor cell killing, and intratumoral IL-12 delivery enhances CAR-T cell immunotherapy in preclinical models of glioblastoma [116]. Lee et al. engineered membrane-bound IL-12 (mbIL12) in CAR-T cells, leading to increased proliferative capacity, better survival, and greater cytotoxicity compared to unarmored CAR-T cells, promoting durable anti-tumor responses in mice [117]. IL-18 augmented IFN-gamma secretion and proliferation of T cells activated by the endogenous TCR, enhancing anti-tumor activity [118]. Furthermore, IL-15 plays a significant role in T cell proliferation and persistence [45, 119, 120]. CAR-T cells armored with several other cytokines, such as IL-10 or IL-4/IL-15-based inverted cytokine receptors, have also shown significantly enhanced efficacy against solid tumors in preclinical models [121, 122].

Enhancing CAR-T cell function with endogenous genetic regulators. Moreover, with the advancement of CRISPR screens and single-cell sequencing technologies, various previously unknown or understudied T cell genetic regulators, such as suppressors and boosters, have been discovered recently [123, 124]. Genetic deletion of T cell negative regulators or overexpression of T cell boosters are powerful strategies to enhance the fundamental properties of T cells and thereby CAR-T cell anti-tumor functions. For instance, knockout of *Regnase-1* promotes TCF-1 expression to enhance CAR-T cell expansion and memory-like cell formation, reduce exhaustion, and support long-term CAR-T cell persistence [125]. Dong et al. discovered that RNA helicase *DHX37* knockout enhanced the efficacy of $CD8^+$ T cells against tumors by regulating NF-κB [126]. *RASA2* ablation enhanced MAPK signaling and prolonged survival and cytolytic activity of CAR-T cells in mice xenografted with tumors [127]. Ye et al. identified proline metabolism (PRODH2) as a means to enhance CAR-T therapy through a genome-scale gain-of-function CRISPR screen in CD8 T cells [128]. A genome-scale open reading frame screen identified lymphotoxin-β receptor (LTBR) as a synthetic driver of T cell proliferation [129]. These regulators have shown promising efficacy in improving T cell fitness and anti-tumor efficacy and are generally universal across different types of CARs because they are fundamental genes in T cells [125–129]. Their therapeutic values await clinical testing.

In summary, engineering CAR-T cells with enhanced anti-tumor functions through co-stimulatory domains, cytokine secretion, and modulation of T cell regulators presents a promising avenue for improving CAR-T therapy against solid tumors. Continued preclinical investigations and future clinical trials will be crucial for translating these approaches into effective clinical treatments.

Improving the clinical safety of CAR-T cells

Despite the clinical success of CAR-T therapy in hematological malignancies and the perspective to become a turning point also in solid tumors, this therapy still remains burdened by life-threatening side effects, such as CRS and neurotoxicity [130]. CRS is a systemic condition manifesting with constitutional symptoms, fever, hypotension, and organ dysfunction in the most severe cases [131]. It is provoked by a mass release of cytokines, with a crucial pathogenic role played by IL-1 and IL-6 [132], by CAR-T cells and TME cells upon cancer cell recognition by CAR-T cells [133]. CAR-T neurotoxicity, also known as immune effector cell-associated neurotoxicity syndrome (ICANS), is a toxic encephalopathy clinically closely linked to CRS in terms of pathogenesis [130]. The prevalence of CRS in the registration trials resulted as extremely high, inducing the conclusion that to a certain extent, all patients treated with CAR-T develop a CRS, while around half of the patients develop neurotoxicity [134]. It is worth mentioning that common experimental designs used to study in vivo CAR technology fail to adequately recapitulate this risk. This is demonstrated by the fact that CRS was first described in the clinical setting, without any anticipation by the preclinical studies [135–137]. While the assessment of CRS in mice requires the collection of straightforward parameters, including body temperature, weight, serum cytokines [132], and serum amyloid A3 [138], the detection of ICANS needs a more complex experimental evaluation, for which a consensus has not yet been achieved. In a milestone paper in which the crucial role of IL-1 and IL-6 in CRS and ICANS was highlighted, Norelli et al. evaluated histopathological signs of meningeal inflammation and defined lethal neurotoxicity as death preceded by motor deficit or seizures, in the absence of CRS criteria [132]. A more clinically detailed definition was provided by Faulhaber et al. who subjected the mice to a daily neurophenotype scoring and an open-field test, highlighting a correlation between ICANS and brain capillary obstruction [139]. Furthermore, a recent

paper by Vinnakota et al., using a fully murine CAR-T cell model, described the activation of microglia after CAR-T injection. The described neuroinflammation can be reduced by interfering with the TGFβ-activated kinase1-NFκB-p38 MAPK pathway, ameliorating the neurocognitive deficits [140].

The current therapy for ICANS consists of a high-dose steroid course [141], which at the same time reduces the clinical effectiveness of CAR-T cells [142]. Therefore, an in-depth understanding of the pathophysiology of this manifestation would be highly beneficial to propose targeted therapy able to spare the CAR-T anti-cancer activity.

Another active study field is aimed at reducing the risk of off-target toxic activity, which could prove crucial for application in solid tumors. Among these, synNotch-regulated CAR enables T cells to express the CAR only in the presence of a specific priming antigen, increasing the specificity of the response while simultaneously preserving CAR-T cells from the tonic signaling provided by a constantly expressed CAR [143]. Similarly, by splitting the CD3ζ and co-stimulatory domains into CARs with different specificities, T cells can be engineered to be activated only in the concomitant presence of the two targets [144]. At the same time, also inhibitory CARs can prevent CAR-T cells from off-target activation [145]. The combination of both split activatory signaling (AND) and inhibitory CARs (NOT) allows for the integration of Boolean logic gates into cancer immunotherapy, enhancing the precision of targeting cancer cells.

Worth mentioning is a synthetic biology solution that can mitigate both on-target (CRS and ICANS) and off-target toxicity, consisting of switchable CAR-T cells that can be modulated to transition from inactive (OFF) to active (ON) state, or vice versa, through the administration of regulator molecules. These constructs can be grossly divided into two families, the OFF-switcher [146–148] and the ON-switcher [149–152]. The latter offers the additional advantage of protecting CAR-T cells from exhaustion induced by tonic activation [151].

CAR-NK CELL THERAPY

NK cells are innate lymphoid cells that play a critical role against tumors and viral infection [153]. NK cells recognize stressed cells [154] and exert a rapid and robust cytotoxic activity together with the production of inflammatory cytokines [155]. The activation of NK cells is regulated by a sophisticated integration of multiple germline-encoded receptors that provide either "kill" or "do not kill" signals [156]. The equilibrium between these opposing signals dictates NK cell responsiveness and is finely tuned during an education process aimed at achieving functional maturation and self-tolerance [157]. NK cells detect MHC class I (MHC-I) molecules through a variety of MHC-I-specific inhibitory receptors, including inhibitory killer cell immunoglobulin-like receptors (KIRs). The absence or low expression of MHC-I on the cell surface, which is frequently observed in tumor cells, can trigger NK-induced killing, known as "missing self" recognition [158]. Moreover, NK cells recognize ligands that are upregulated on the cell surface of stressed cells, known as "induced self" recognition. Besides "missing self" and "induced self" responses, antibody-dependent cellular cytotoxicity (ADCC) is another critical mechanism of NK-mediated recognition and killing of cancer cells. NK cells can mediate IgG opsonized cytotoxicity through CD16 (FCγRIII), which binds to the Fc portion of IgG antibodies [159] and induces cytotoxicity [160].

Upon activation, NK cells execute multiple functions to eliminate or constrain the growth of cancer cells. Interestingly, for their first killing events, NK cells eliminate target cells by forming lytic immunological synapses and secrete pre-assembled cytolytic granules containing granzyme B and perforin, leading to the apoptosis of the target cells [161, 162]. Later, NK cells switch to the use of death receptors, such as Fas ligand and TNF-related apoptosis-inducing ligand (TRAIL), to induce target cell death [163]. Moreover, NK cells can produce several pro-inflammatory cytokines, including IFN-γ and TNF-α, to limit the growth of target cells and orchestrate the function of other innate and adaptive immune cells [164].

NK cells were proposed as cellular immunotherapy for cancer over 30 years ago due to their diverse mechanisms for recognizing and eliminating cancer cells [165]. Furthermore, since NK cells do not recognize targets presented by the HLA system, NK cells-based immunotherapy can be used in an allogenic setting without the risk of graft-versus-host disease (GvHD) [166] making possible the development of a universal, "off-the-shelf" immunotherapy. These promising characteristics have recently sparked significant interest in CAR-NK cells, leading to exponential growth in technological advances and preclinical results. Leveraging decades of experience in T cell engineering, CAR-NK cells have undergone a significantly accelerated development toward clinical applications [167].

Challenges in CAR-NK cell therapy for solid tumor

While the current clinical experience with CAR-NK cells mostly comes from hematological malignancies (as summarized in Table 1), insights into the biological characteristics of NK cells and the preclinical findings can help predict potential pitfalls in their application against solid tumors. Similar to CAR-T cells, the application of CAR-NK cells in solid tumors faces challenges related to trafficking toward the tumor. Despite playing a crucial role in orchestrating the anti-tumor responses [168], NK cells are often limited in their presence within several solid tumors [169], indicating difficulties in their ability to reach, infiltrate, and persist within TME. Additionally, infiltrating NK cells in solid tumors often display a dysfunctional phenotype [170–174], partly due to the detrimental factors such as hypoxia [175] and soluble inhibitory factors [176–178] present in the TME. Finally, NK cell-based immunotherapy must contend with the short half-life [179] and short-term anti-cancer activity [180]. Although the persistence of CAR-NK cells is a challenge shared with their application in hematological malignancies, it becomes even more crucial in the setting of solid tumors.

Strategies to improve CAR-NK cell therapy for solid tumors (Fig. 3)

Increasing NK trafficking and infiltration to solid tumors. Clinical evidence suggests that the presence of activated NK cells infiltrating solid tumors correlates with positive prognostic outcomes [181–183], despite as aforementioned their low ability to infiltrate and persist [169]. For this reason, in the first clinical study evaluating CAR-NK cells in solid tumors, the cells were locally administered in the colon cancer metastatic sites [184]. The three patients treated showed signs of local response, including a drastic reduction of cells in the ascites and a complete metabolic response of the liver metastasis. However, since the higher risk of adverse events and the logistic difficulties related to locoregional delivery compared to intravenous injection, various strategies have been tested to enhance NK cell migration.

Genetic engineering CAR-NK cells to express the chemokine receptor CXCR4 has resulted in increased migration toward CXCL12/SDF-1α and enhanced in vivo efficacy against CXCL12/SDF-1α-secreting glioblastoma model U-87 [185]. Overexpression of CXCR1 in CAR-NK cells has shown increased migration toward the pro-inflammatory cytokine IL-8 gradient, resulting in increased infiltration in peritoneal ovarian cancer xenografts [186]. Furthermore, CXCR2-transduced NK cells have demonstrated increased migration along CXCR2 ligands or renal cell carcinoma tumor supernatants [185]. Similar results can be achieved by inhibiting the G protein-coupled receptor CXCR3, involved in the mobilization of NK cells from the bone marrow to the PB, thereby increasing NK infiltration in a multiple myeloma model [187]. An

Table 1. Representative clinical trials evaluating CAR-NK cell therapy

Clinical trials evaluating CAR-NK cells						
Trial	**NK source**	**CAR construct**	**Target**	**Disease**	**Outcome**	**Safety**
NCT02944162 [236] ($n = 3$)	NK-92	CD28-4-1BB-CD3ζ	CD33	AML	No sustained responses	• 2 CRS (Grade 1) • No GvHD • No neurotoxicity
NCT03415100 [184] ($n = 3$)	Peripheral blood	NKG2D.CD8.DAP12	NKG2D-ligands	Colorectal cancer metastasis	Local response observed in all of the three patients (two patients with peritoneal carcinomatosis display ascites reduction and decrease in cancer cell number, the patient with liver metastasis achieved metabolic CR)	• 1 CRS (Grade 1) • No neurotoxicity • No GvHD • No serious adverse events (Grade ≥ 3)
NCT04245722 [300] ($n = 20$)	iPSC	NKG2D-2B4-CD3ζ-IL15RF-hnCD16	CD19	R/R B-cell tumors (B-NHL/CLL)	17 efficacy-evaluable patients Objective response = 9/17	• 2 CRS (Grades 1 and 2) • No neurotoxicity • No GvHD • No dose-limiting-toxicity
NCT04023071 [288] ($n = 13$)	iPSC	(Not entirely reported) hnCD16	CD20	R/R B-cell tumors (B-NHL/CLL)	Objective response = 8/13 CR = 7/13	• No CRS • No neurotoxicity • No GvHD • No CAR-NK cells-related Grade ≥ 3 adverse events • No dose-limiting-toxicity
NCT03056339 Dose-escalation phase [216] ($n = 11$) Expansion phase [217] ($n = 26$)	Cord blood	CD28-CD3ζ.iCasp9-IL-15	CD19	R/R B-cell tumors (B-NHL/CLL; ALL; LL)	Day 100 OR = 48.6% 1-year-OS = 68%	• 1 CRS (Grade 1) • No neurotoxicity • No GvHD • Reversible Grades 3 or 4 hematological toxicity (likely induced by the lymphodepleting chemotherapy)

hnCD16a high-affinity non-cleavable variant of CD16a, *NHL* non-Hodgkin lymphoma, *CLL* chronic lymphocytic leukemia, *AML* acute myeloid leukemia, *ALL* acute lymphoblastic leukemia, *LL* lymphoplasmacytic lymphoma, *OR* overall response, *OS* overall survival

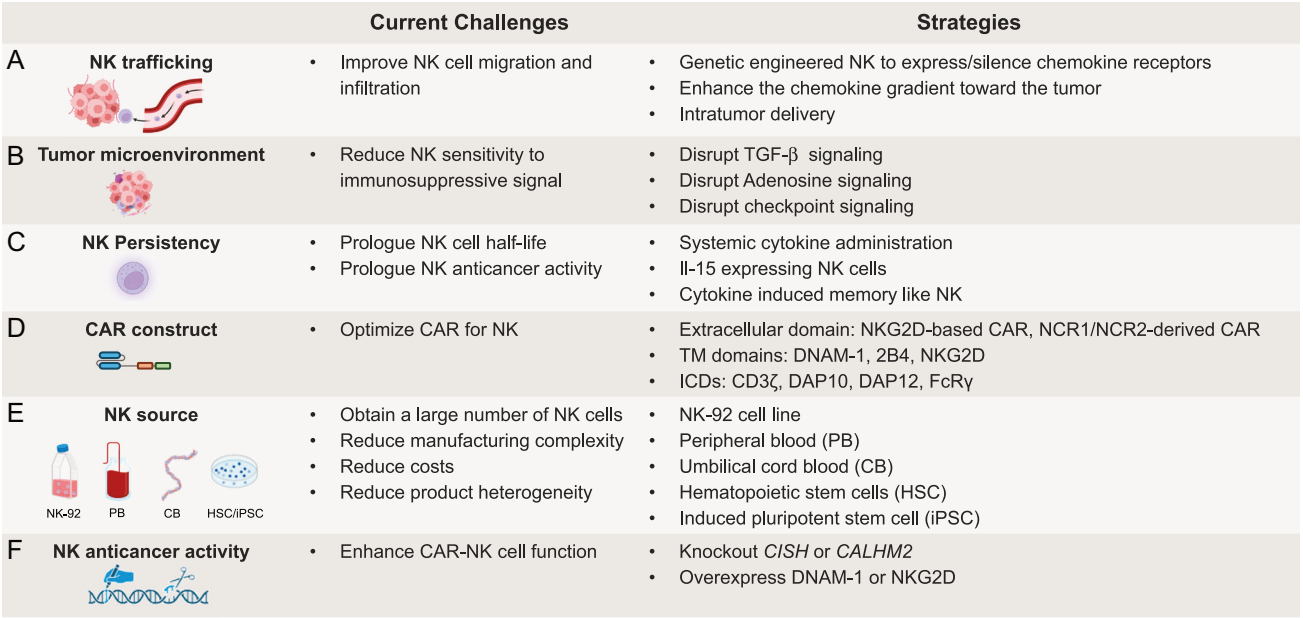

Fig. 3 Current challenges and ongoing strategies to improve CAR-NK cell therapy for solid tumors. CAR-NK cells face common obstacles with CAR-T in effectively targeting solid tumors, including **A** cell trafficking and **B** the immuno-suppressive effects of the tumor microenvironment Potential strategies to overcome these shared challenges are closely linked to understanding NK biology in the context of solid tumors. NK cells are known for their short half-life and time-limited anti-cancer activity (**C**), and overcoming these biological characteristics may represent a turning point for the application to solid tumors. Indeed, the reduced availability of direct contact with cancer cells in solid tumors compared to hematological ones makes the generation of long-surviving and long-acting NK cells crucial. CAR-NK cells have benefited from the translation of well-consolidated designs optimized for CAR-T. In recent years, increased attention has been focused on different strategies to optimize CAR specifically for NK cells (**D**). Transmembrane (TM) and intracellular co-stimulatory domains (ICDs). **E** Different sources have been explored to obtain NK cells for clinical applications, including the immortalized NK-92 line, peripheral blood (PB), cord blood (CB), hematopoietic stem cells (HSC), and induced pluripotent stem cells (iPSC). Each of these sources encompasses advantages and limitations. The challenge of obtaining CAR-NK cells with the optimal balance between pharmacoeconomic sustainability (cost and manufacturing complexity) and reproducible efficacy (homogeneous and effective product) is a critical step to fully harness their potential as an off-the-shelf therapy. **F** Gene editing strategies aimed at boosting NK cell anti-cancer activity have begun to be explored in both hematological [249] and solid tumor models [250, 251]. Based on the swift advances observed in this field for CAR-T cells, it is likely that the biotechnological solutions to boost NK cell activity will exponentially increase in the coming years

alternative strategy proposed by Lee et al. is the NK cell-recruiting protein-conjugated antibody [188]. This molecule releases CXCL16 once cleaved by furin expressed on the surface of pancreatic cancer cells, creating a gradient that enhances NK migration toward the tumor in vivo.

Overcoming TME immunosuppression. Unlike dispersed hematologic malignancies, solid tumors, especially late-stage ones, exhibit an inhibitory TME due to hypoxia, a low pH, presence of suppressive cytokines, lactate, prostaglandins, and others that negatively impact NK cell function [189]. TGF-β dampens various aspects of NK cell anti-tumor function, including cytokine secretion, degranulation, metabolism, and mTOR signaling [190–192]. Therefore, pharmacological inhibition of TGF-β [193] or engineering the TGF-β receptor on NK cells can potentially overcome TGF-β-mediated inhibition and enhance NK anti-cancer activity [194–196]. With this rationale, Prof. Weathers's group started a Phase I clinical trial evaluating the use of NK cells genetically depleted for the TGF-β receptor 2 and the endogenous glucocorticoid receptor NR3C1 (ClinicalTrials.gov Identifier: NCT04991870). If this trial shows positive results, this strategy can also be implemented in CAR-NK to improve the detrimental effect of TME.

NK anti-tumor function is also affected by extracellular adenosine, an element of hypoxia-driven purinergic signaling [197]. Adenosine is generated in high concentrations in the TME from ATP and ADP by the sequential activity of CD39/CD73 ectonucleotidases, providing a broad immuno-suppressive effect on both T cells and NK cells [198, 199]. Specifically, CD39-expressing Tregs are capable of inhibiting the anti-tumor immunity of NK cells both in vivo and in vitro [200]. Different strategies can be pursued to prevent the detrimental role of adenosine on NK cells, including ectonucleotidase or A2 receptor inhibitors [201]. Furthermore, lymphocytes can be engineered to suppress the expression of adenosine receptors [202–204].

As described for CAR-T, CAR-NK can also benefit from the concomitant use of checkpoint inhibitors. Both CTLA4 and PD-1 are expressed by activated NK cells [205, 206] and NK anti-cancer activity can be enhanced by disrupting these signaling pathways [207, 208]. Furthermore, additional checkpoint inhibitors, including NKG2A, TIGIT, and TIM3, have been shown to negatively impact NK function [209, 210] and conversely, their inhibition may improve NK anti-cancer activity [211–213].

Increasing NK persistency. One of the primary challenges in the clinical application of NK cells for immunotherapy is their short half-life post-infusion, typically limited to a few weeks [214, 215]. Currently, the most successful strategy in the clinical setting involves NK cells encoding IL-15, which have demonstrated in clinical settings, the persistence for over 1 year after infusion [216]. These data were reported in the dose-escalation phase of the trial evaluating CAR-NK cells in B-cell tumors, enforced by the observation that responder patients display a longer persistence of the injected cells [217].

Once these data were integrated with the expansion phase (37 patients in total), the CD19/IL-15 CAR-NK demonstrated an overall response rate of 48.6 and 68% of the patients survived for at least 1 year. This indicates an effectiveness comparable to the current

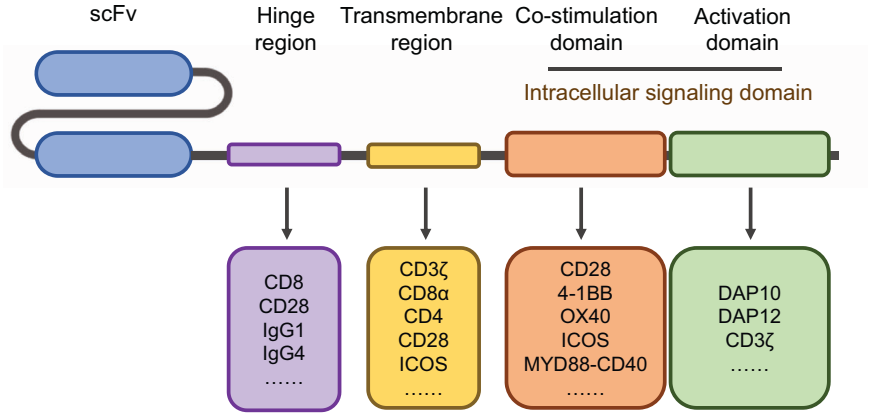

Fig. 4 Blueprint of CAR-NK construct design and optimization. Like CAR-T, CAR-NK cells follow a modular design comprising an antigen-binding domain, a hinge, a transmembrane domain, and an intracellular signaling domain. Design and optimization strategies can also be specific to each domain. In the hinge region, options such as CD8, CD28, IgG1, and IgG4 have demonstrated some efficacy in CAR-T cells and merit consideration. Additionally, for the transmembrane domains, while CD3ζ, CD8α, CD4, CD28, and ICOS are commonly employed in CAR-T cells, receptors specific to NK activation, such as CD16, NKp44, NKp46, NKG2D, 2B4, and DNAM-1, should be explored. When it comes to co-stimulation domains, candidates like CD28, 4-1BB, OX40, ICOS, and MYD88-CD40 warrant investigation. Furthermore, for the activation domain of the intracellular signaling module, besides CD3ζ, NK-specific activating receptors, DAP10, and DAP12, offer promising avenues for experimentation

CAR-T cell therapies [27], but notably no major toxicity events such as severe CRS, neurotoxicity, or GvHD were observed [217].

Furthermore, the Rezvani group has also provided mechanistic insights by integrating the post-infusion transcriptomic profile obtained from the clinical trial with a preclinical model of non-curative lymphoma [218]. Armoring CAR-NK with IL-15 appears to ameliorate the loss of metabolic fitness observed after cell infusion, enhancing in this way the tumor control in vivo.

The inclusion of IL-15 into the CAR construct represents a promising strategy also in other hematological malignancies [219], and solid tumors, where these cells have shown a long persistence in vivo in a preclinical model of pancreatic cancer [220].

Another promising option for allogeneic cell therapy is harnessing memory NK cells. This concept arises from evidence suggesting that NK cells exhibit features of adaptive memory under specific circumstances [221]. Like memory B and T cells, memory NK cells possess the ability to mount an enhanced response upon rechallenge to the same stimulus encountered previously and can be maintained by a long-lived/self-maintaining population [222]. Cytokine-induced memory-like (CIML) NK cells are generated through ex vivo priming with IL-12, IL-15, and IL-18. These cells exhibit prolonged persistence and demonstrate higher anti-cancer activity after a resting period, encouraging investigation in clinical settings. Indeed, CIML NK cells have shown encouraging results in clinical settings [223–225] and represent a promising strategy as a platform for CAR-NK technology [226].

Optimizing CAR constructs for NK signaling (Fig. 4). Currently, most CAR-NK cell studies and clinical trials simply adapt CAR constructs that were originally designed for CAR-T cells. While these constructs can also function in NK cells, they are not optimized for NK signaling. Given the variety of activating receptors and adapter protein domains that contribute to NK fine-tuned activation, NK CAR constructs could have more variations and different combinations in the choice of extracellular, TM, and intracellular signaling domains. This approach could lead to the development of more effective and tailored CAR-NK cells for cancer immunotherapy.

Extracellular scFv domains: Single and tumor-specific targets are challenging to find due to the expression of most so-called TAAs on solid tumors are often expressed at lower levels in healthy tissue. Moreover, targeting a single antigen might lead to immune escape, as often observed in CAR-T therapy. Notably, NK-activating receptors naturally have less specificity and can target multiple cancer antigens. NKG2D, for example, can recognize up to eight stress-induced molecules, including MICA/B and RAET1/ULBP family, on the surface of distressed cells [227]. Therefore, certain CAR constructs exploit the natural tumor recognition of NK receptors. NKG2D-based CAR-NK cells have been shown to be effective against multiple myeloma cells in a preclinical setting [228] and in a clinical study with metastatic colorectal cancer patients [184]. Similarly, NCR1-derived CAR or NCR2-derived CAR constructs are tested to improve cancer immunotherapy [229, 230]. These approaches leverage the broader tumor recognition capabilities of NK cells to potentially overcome the limitations associated with targeting single antigens, benefiting both CAR-T and CAR-NK cell therapy.

Transmembrane domains and hinge: The TM domain serves to connect the extracellular domain of the CAR to the intracellular activation signaling domains and dock the CAR construct to the cell membrane. Therefore, they are important for signal transduction CAR dimerization and signal transduction. In CAR-NK cells, the most commonly used TM domains are adapted from CAR-T cell constructs, such as CD3ζ, CD8, and CD28. However, the TM domains of several NK-activating receptors, such as CD16, NKp44, NKp46, NKG2D, 2B4, and DNAM-1, possess charged amino acids capable of directly interacting with signaling adapters and follow the natural orientation (N to C terminal, except for NKG2D). Consequently, these TM domains are actively explored in the NK CAR screen studies [231]. Interestingly, CAR constructs containing TM domains from NK-activating receptors, such as DNAM-1, 2B4, and NKG2D, typically yield higher cytotoxicity.

Intracellular co-stimulatory domains: Once the signals from activating receptors surpass those provided by inhibitory receptors [232], NK cells are activated through signaling adapter proteins. These proteins, in addition to CD3ζ, include DAP10, DAP12, and FcRγ [233]. The incorporation of these activated signaling adapters, either individually or in combination, to optimize CAR design for NK cells has begun to be explored in recent years [234]. Li et al. identified a tailored NK CAR construct containing the NKG2D TM domain, the 2B4 co-stimulatory domain, and the CD3ζ signaling domain, which mediated stronger antigen-specific cytotoxicity than T CAR and other constructs

[231]. Moreover, CAR constructs including other NK-specific signaling domains, such as DAP10 and DAP12, could also increase NK anti-tumor efficacy [231]. These findings underscore the importance of optimizing CAR constructs specifically for NK cells to enhance their therapeutic potential. However, there is a lack of systematic research on NK CAR constructs, and the full potential of NK CAR-mediated signaling has yet to be fully explored.

Novel expansion methods and sources of NK cells. To date, various sources have been explored to obtain NK cells for clinical applications, including the immortalized cell line NK-92, umbilical cord blood (CB), CB-derived $CD34^+$ hematopoietic stem and progenitor cells, and induced pluripotent stem cells (iPSCs). Each of them presents advantages and disadvantages in terms of the linked manufacturing processes, including source collection, NK isolation/differentiation, genetic modification, and expansion into large amounts.

Immortalized NK cell lines: NK-92 cells, derived from a patient with non-HL, offer the potential for unlimited ex vivo expansion with minimal manufacturing effort [235]. Moreover, due to their homogeneous nature and lower resistance to transduction, NK-92 cells serve as an ideal prototype for developing CAR-based immunotherapies [236]. Consequently, NK-92 cells were the first NK cell line to gain authorization from the FDA for clinical trials and the platform for the first-in-man trial involving CAR-NK [236]. An interim report of this Phase I clinical trial evaluating CD33-CAR NK-92 cells in three patients with relapsed or refractory acute myeloid leukemia reported no significant adverse event but also no durable disease control [236].

Indeed, due to their immortalized nature, NK-92 cells require irradiation before clinical use, resulting in a subsequent reduction in persistence and clinical efficacy [237]. Furthermore, NK-92 cells lack ADCC due to the absence of CD16 expression [238]. Therefore, the development of genetically engineered NK-92-derived products is essential for enhancing their in vivo anti-tumor function.

Peripheral blood (PB)-derived NK cells: Primary NK cells can be isolated and expanded from PB, where they commonly represent 5–15% of the lymphocytes [239]. To obtain the required amount of cells for clinical applications, this protocol requires the apheresis of a healthy donor and has been the main source of non-genetically modified NK cells in recent years, obtained through both negative selection after CD3/CD19 depletion or positive selection [240].

Umbilical cord blood (CB)-derived NK cells: Unlike PB, CB lymphocytes contain up to 30% NK cells and a higher percentage of $CD56^{bright}$ subset, making CB a rich source of therapeutic effector NK cells [241]. Furthermore, CB is abundant in HPCs, making it an ideal substrate for the in vitro differentiation of therapeutic NK cells with desired phenotypes [242]. However, CB-tderived NK cells show weaker cytotoxic activity against K562 leukemia cells and produce less IFN-γ compared to PB-derived NK cells, possibly due to their higher expression of inhibitory receptors such as NKG2A [241, 242]. Further exploration is needed to understand the regulators guiding CB HPC differentiation into effector NK cells with enhanced in vivo anti-tumor efficacy. The therapeutic potential of CB-derived NK cells requires systematic evaluation in more clinical trials.

Hematopoietic stem cells (HSC)-derived NK cells: To date, most adoptive NK cell therapies have utilized PB NK cells, CB NK cells, or NK-92 cells. However, each of these cell sources presents significant limitations, as discussed earlier. Due to the issues of clinical efficacy and donor heterogeneity, considerable attention is now directed toward stem cell-derived NK cells, offering standardized "off-the-shelf" therapies for patients regardless of their HLA haplotype. NK cells expanded and differentiated from $CD34^+$ HSC offer the potential for unlimited quantities and are more amenable to genetic manipulation [243]. Although this method inherits many logistical advantages from using CB as a source for NK expansion, it requires more than 40 days of expansion time due to the relatively low absolute number of $CD34^+$ cells retrieved from CB [155]. Overall, these sources have undergone testing in Phase I clinical trials for acute myeloid leukemia, showing promising results [243].

Induced pluripotent stem cell (iPSC)-derived NK cells: The iPSC-based methodology involves reprogramming adult somatic cells into pluripotent cells, followed by differentiation into NK cells and subsequent expansion to generate the final products [244]. iPSCs can be derived from easily accessible cells such as fibroblasts and blood cells, offering the potential to generate a large number of homogeneous NK cells from a single clone. This approach addresses the variability associated with editing a bulk NK cell population, making iPSCs-derived NK particularly suitable for gene-edited products [245–247]. Additionally, iPSC-derived NK cells have been shown to exhibit more potent cytotoxicity than PB NK cells [231]. Currently, iPSC-derived NK cells with multiple genes modified are being actively evaluated in clinical trials [248].

Alternative strategies for enhancing CAR-NK cell function. To date, only a small number of genes have been identified where knockout or perturbation has a strong effect on NK cell's anti-tumor efficacy. For example, the deletion of *Cytokine-inducible SH2-containing protein* (*CISH*) in iPSC or CB-derived NK cells enhances in vivo persistence and anti-tumor response [218, 249]. It has also been shown that overexpressing DNAM-1 and/or NKG2D in NK cells enhances effectiveness against patient-derived sarcoma specimens in vitro [250]. The complex interaction among various mechanisms can interfere with NK activity in the setting of solid tumors, posing challenges for reductionist approaches to exploring these issues. On the other hand, this complex scenario is particularly suitable for unbiased functional genetic screens using in vivo models that can recapitulate the interrogated phenotype. We have recently conducted a study in which an in vivo CRISPR screen and orthogonal single-cell profiling of infiltrating NK cells across different tumor models have convergently identified the novel NK suppressor *Calcium homeostasis modulator family member 2* (*CALHM2*). Knockdown of this calcium channel resulted in improving CAR-NK anti-cancer efficacy both in murine and human NK cells [251]. Finally, CAR-NK cells have been shown to acquire the target antigen on their surface through a trygocytosis process [252]. This event not only reduces the presence of the tumor antigen in the target cells but also induces a fratricide depletion of the therapeutic cells. To prevent this process and enhance cell activity, CAR-NK cells can be engineered to express a second inhibitory KIR-based CAR, able to recognize a "do not kill me" signal like the NK-expressed CS1 antigen [253]. This strategy has been demonstrated to improve in vivo the CAR-NK cell anti-cancer activity against B-cell lymphoma [252].

CAR-T VS CAR-NK CELLS

Advantages of CAR-NK cells over CAR-T cells (Fig. 5)

One of the major limitations of the broader applicability of CAR-T therapy is linked to the necessity to use donor-derived cells to avoid both GvHD and the rejection of the infused cells, known as host-versus-graft (HvG) reaction. Since patients are usually heavily treated before CAR-T therapy, harvesting sufficient healthy autologous T cells from patients can significantly delay treatment and pose challenges for CAR-T manufacturing. The lengthy and cumbersome process of CAR-T manufacturing makes many patients either ineligible for the treatment or experience disease progression after being enrolled in the treatment process [254].

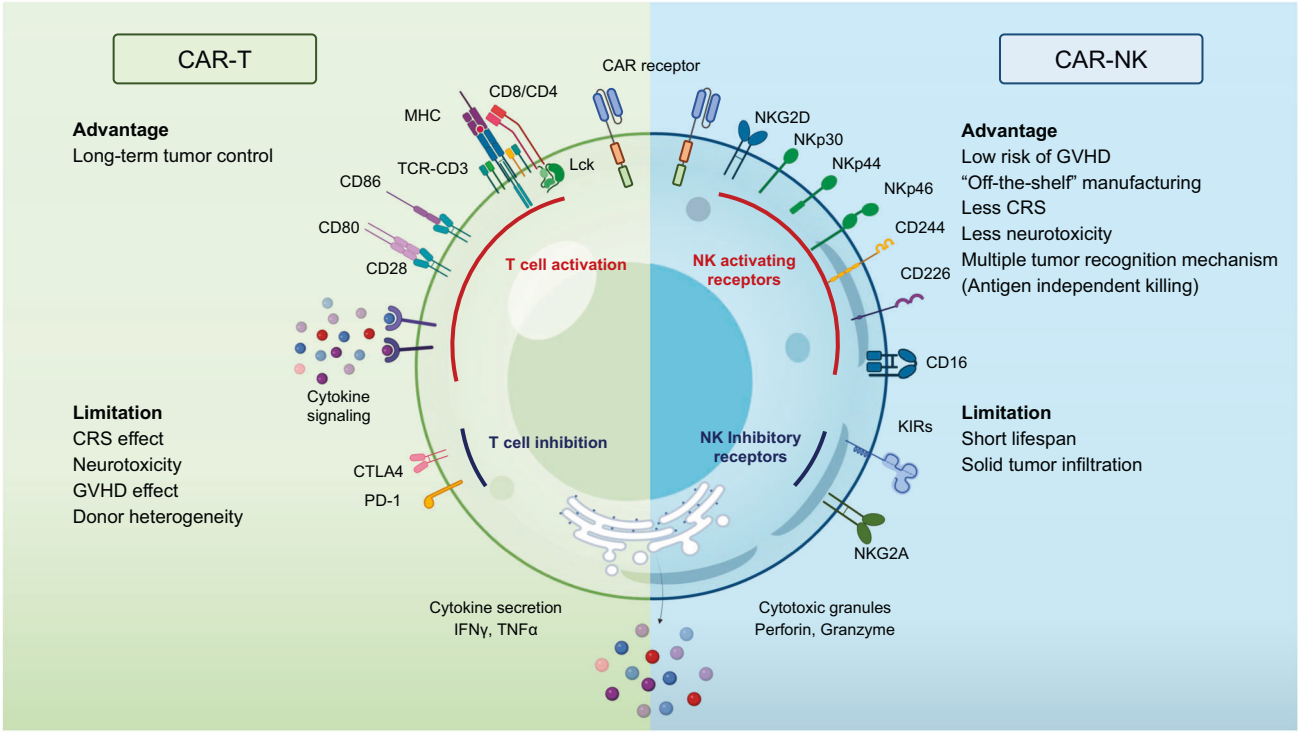

Fig. 5 Advantages and limitations of CAR-T and CAR-NK cells. Both CAR-T and CAR-NK cells exhibit distinct advantages and limitations. CAR-T cells offer long-term tumor control capabilities but are associated with risks such as cytokine release syndrome, neurotoxicity, GvHD effects, and donor heterogeneity. On the other hand, CAR-NK cells present several advantages, including a lower risk of GvHD, "off-the-shelf" manufacturing feasibility, reduced incidence of cytokine release syndrome and neurotoxicity, as well as antigen-independent killing abilities. However, they do have drawbacks such as a shorter lifespan and potentially limited tumor infiltration capabilities

It must be mentioned that for hematological tumors previously treated with allogeneic hematopoietic transplantation, CAR-T cells derived from the same donor can be used without evidence of GvHD [255]. However, the necessity to produce a patient-specific product deeply impacts the possibility of scaling up the manufacturing process to reduce the cost, which is estimated in the USA between $370,000 and $530,000 per single treatment [256]. Therefore, the possibility of using an "off-the-shelf" allogeneic product currently represents a priority in this field.

Different strategies can be pursued to reduce and limit the risk of CAR-T cells-induced GvHD. This adverse event mainly depends on the expression of functional αβTCR, therefore CAR-T cells can be genetically modified to not express the TCRα chain [257–260] and used as an allogeneic product. Since CAR-NK cells act in an HLA-unrestricted manner, these cells have the advantage of not inducing GvHD in an allogenic setting and therefore do not require any additional gene editing to be used as an "off-the-shelf" therapy. Existing NK-92 cell lines or primary NK cells sourced from allogeneic donors can be utilized and manufactured through mass production, as demonstrated by Rezvani's group which has proposed a protocol able to obtain more than a hundred CAR-NK cell doses from one CB unit [261].

On the contrary, both allogenic TCRα chain$^-$ CAR-T and CAR-NK cells remain vulnerable to HvG reaction when administered in immunocompetent patients. In hematological malignancies, the possibility of disrupting the recipient's immune system as part of the therapeutic process has allowed different options to avoid rejection. Among these, knocking out the common lymphocyte antigen CD52 [262] on CAR cells and administering them together with the lymphodepleting anti-CD56 antibody alemtuzumab is a viable strategy [263]. However, in conditions like solid tumors, the necessity to preserve also the host immune system implies pursuing different approaches. Gene editing approaches aimed to make invisible these cells to the immune system must contend with the robustness of immunosurveillance. Indeed, while the knockout of β2-microglobulin can counteract the HLA-dependent recognition of recipient T cells [262], it provides a "missing self" activating signal to the NK cells. This issue could be avoided by overexpressing the non-classical HLA-E or G [262, 264] but this requires an additional gene editing step.

Safety profile with reduced CRS and neurotoxicity. CAR-T cell activation often leads to the release of inflammatory cytokines, resulting in CRS and neurotoxicity [254, 265]. However, CAR-NK cells release different profiles of cytokines. Indeed, as consistently demonstrated by a long series of clinical trials with parental NK cells, patients who received allogenic CAR-NK cell therapy did not develop CRS or neurotoxicity and did not exhibit increased levels of pro-inflammatory cytokines, such as IL-1β, IL-6, and IL-10 [214, 266–268]. Despite the current extremely limited clinical experience with CAR-NK compared to CAR-T cells [184, 217, 236], these clinical data so far confirm what has been observed for parental NK cells and have shown that, in contrast to CAR-T, CAR-NK cells have been rarely associated with CRS and not associated with neurotoxicity [217, 269]. This suggests that CAR-NK cells may offer a safer alternative to CAR-T cells about these serious adverse events.

Multiple tumor recognition mechanisms. Since CAR-NK cells can recognize and eliminate cancer cells in both antigen-dependent and antigen-independent manners [270] this therapy may present significant advantages in the application for solid tumors compared to CAR-T. As previously mentioned, different strategies are currently under evaluation to allow CAR-T to overcome the heterogeneity and antigen escape of solid tumors. In contrast, NK cells naturally possess antigen-independent mechanisms that may prove beneficial for tumors with fine subclonal heterogeneity [271]. Additionally, NK cells can mediate ADCC through CD16,

which binds to the Fc domain of IgG bound on cancer cells. This further enhances their potential effectiveness against solid tumors.

Limitations of CAR-NK cells

Despite CAR-NK's promising features, several significant limitations exist that hinder its successful application in treating solid tumors. Many of these limitations parallel those associated with CAR-T therapy, such as poor tumor infiltration [272, 273] and detrimental interactions with the TME [189]. Moreover, NK cells have specific limitations unique to their biology and function.

Resistance to viral transduction. One of the main limitations of CAR-NK cell application consists of the resistance of NK cells to viral transduction [274] compared to T cells [275]. In a recent clinical trial using a retrovirus vector to generate CAR-NK cells a median transduction efficiency of 72.4% was observed [217]. However, the wide range of variability in these data (from 22.7% to 91.1%) represents a significant limitation that could affect the statistical power of a controlled Phase II or Phase III clinical trial. This result was obtained by stimulating NK cells with IL-2 and feeder cells the day before the transduction, taking advantage of the retrovirus's preference for infecting actively replicating cells [276]. Among the six CAR-T cell products currently approved by FDA, four adopt a lentivirus vector (Carvykti™, Kymriah™, Breyanzi®, and Abecma®), and two a γ-retroviruses one (Yescarta™ and Tecartus™). Despite there are currently no clinical data indicating that one strategy is superior or safer than the other, at least for the current generation of viral vectors, it must be noted that γ-retroviruses preferentially insert near enhancers and promoters, implying a higher risk of insertional mutagenesis compared to lentivirus vectors [277]. This phenomenon dramatically emerged in clinical settings when children affected by X-linked severe combined immunodeficiency treated with γ-retrovirus-edited HSCs developed leukemia [278–280]. For this reason, different strategies have been explored to optimize also a lentivirus-based transduction protocol for NK cells. These strategies must address the antiviral response that lentivirus triggers in NK cells, which induces their apoptosis [281]. Among them, worthy of mention is the exposure to cytokine combinations like IL-2, IL-12, and IL-21 [281–283] or the pretreatment with the TBK1-inhibitor BX759, able to interfere with the signal downstream the pattern recognition receptors [281, 284]. The results from these studies have demonstrated the possibility of increasing lentivirus transduction efficiency in human primary NK cells to 25–40% [281, 284], therefore further research and innovative solutions are needed to optimize this process in NK cells.

Short lifespan and poor persistence. NK cells have a short half-life [285] and exhibit poor persistence once transferred from ex vivo conditions with supraphysiological cytokine exposure to in vivo conditions [286]. This aspect could potentially imply the necessity for multiple infusions. For CAR-T cells, a second infusion is not associated with major toxicity risks [287], but this strategy has only started to be explored for CAR-NK cells. Current evidence seems to indicate that multiple administrations of CAR-NK are not associated with serious adverse events. However, these data come from the interim reports of three Phase I clinical trials, performed in solid tumors [184] and hematological malignancies [236, 288], that together enrolled a total of 19 patients. Furthermore, it must be mentioned that in the first study, the cell product only transiently expressed the CAR, as it was obtained by electroporating NK cells with NKG2D CAR mRNA and was locally injected into the metastatic sites. Therefore, further studies are needed for the safety evaluation of this strategy. Furthermore, this approach would potentially impact the cost-effectiveness of the CAR therapy, therefore there is an active exploration into reprogramming CAR-NK cells to possess memory or memory-like properties, aiming for long-term tumor control. This avenue of research holds promise for addressing the limitations associated with NK cell persistence in CAR-NK cell therapy.

FUTURE PERSPECTIVES

FDA has recently issued a warning on approved CAR-T cell products due to the observation of T cell malignancies in a small number of CAR-T-treated patients. Despite the overall low rate of such events (22 cases reported among over 27,000 treated patients reported to date), the recurrent reports across 5 of the 6 approved CAR-T cell therapy products have led the FDA to add a class-wide boxed warning to these therapies [289]. In three of these cases, cancer cells have been demonstrated to contain the CAR [290], indicating a malignant transformation of the infused therapy as the pivotal cause. This malignant transformation appears to be a rare event, however, since the current perspective of a broad expansion of clinical indication do CAR therapy, encompassing cancers in earlier therapeutic stages and other conditions, including autoimmune diseases [291], and HIV [292], the assessment of rare adverse events will become even more pertinent. At the moment we are writing this review, the mechanistic events leading to the cancerous transformation of CAR-T cells are under investigation, and no firm conclusions have been drawn on the direct involvement of the virus vector in this process [290]. However, the FDA warning represents an opportunity to review all the critical steps of CAR manufacturing, including vector safety, and evaluate potential solutions.

A proposed strategy to address these safety concerns includes the incorporation of a suicide gene, like caspase 9 [64] or the herpes simplex virus tyrosine kinase [293] to induce apoptosis in the event of toxicity or malignant transformation. Furthermore, alternative delivery systems are under investigation in clinical trials, including CRISPR/Cas9 and transposon/transposase-based approaches [294–297]. Both strategies present advantages and disadvantages, for which an in-depth discussion can be found in a dedicated review [298], however, it is evident that these technologies are living in an exponential development phase that will likely surpass the retro/lentivirus-based approaches in terms of safety, quality of the product (e.g., efficiency of transgene delivery), logistic feasibility and costs. For example, in our laboratory, we have developed a transposon/transposase-based system, named MAJESTIC (mRNA Adeno-associated virus-Sleeping Beauty Joint Engineering of Atable Therapeutic Immune Cells). By making as transient the Sleeping Beauty transposase component, this system avoids repeated transposon mobilization, further reducing the risk of insertional mutagenesis [299].

The contribution of innovative biotechnological tools to the improvement of CAR immunotherapies has only begun to show its potential. For example, many of the solutions to overcome the limitations of CAR-T and NK cells summarized in this review have been achieved through the application of high-throughput CRISPR technology [123, 124, 126, 128, 129, 251]. Furthermore, data derived from ongoing clinical trials will allow for a deeper understanding of the limitations of CAR therapies, enabling the design of more precise readouts for functional genetic screens. This dynamic and virtuous exchange between biotechnology development and data from clinical trials represents the keystone for evolving CAR-T and CAR-NK therapies toward the next generations of cancer treatment, ultimately leading to improved patient outcomes.

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AUTHOR CONTRIBUTIONS

SC and LP designed the structure of this review. LP drafted the sections on CAR-T cells. LY and GS drafted the sections on CAR-NK cells. LZ and GS generated the figures with input from LP and LY. All authors edited the manuscript text and/or figures. SC secured funding and supervised the work.

COMPETING INTERESTS

The authors declare no competing interests related to this study. SC is also a (co) founder of EvolveImmune, Cellinfinity, NumericGlobal, MagicTime, and Chen Consulting, all unrelated to this study.

ADDITIONAL INFORMATION

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