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Next-Generation Chimeric Antigen Receptors for T and Natural Killer Cell Therapies against Cancer

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SUMMARY

Adoptive cellular therapy using chimeric antigen receptor (CAR) T cells has led to a paradigm shift in the treatment of various hematologic malignancies. However, the broad application of this approach for myeloid malignancies and solid cancers has been limited by the paucity and heterogeneity of target antigen expression, and lack of bona-fide tumor-specific antigens that can be targeted without cross-reactivity against normal tissues. This may lead to unwanted on-target off-tumor toxicities that could undermine the desired anti-tumor effect. Recent advances in synthetic biology and genetic engineering have enabled reprogramming of immune effector cells to enhance their selectivity towards tumors, thus mitigating on-target off-tumor adverse effects. In this review, we outline the current strategies being explored to improve CAR selectivity towards tumor cells with a focus on natural killer (NK) cells, and the progress made in translating these strategies to the clinic.

Keywords

Anti-tumor immunotherapy; chimeric antigen receptor (CAR); synthetic immune cell engineering; T cells; natural killer (NK) cells

1. Introduction

In recent decades, the field of adoptive cellular therapy has undergone substantial developments in various clinical settings[1]. Notably, chimeric antigen receptor (CAR) T cells have emerged as a groundbreaking therapeutic modality for patients with certain types of relapsed and refractory hematologic malignancies[2–6]. The advent of CAR engineering resulted from extensive research endeavors primarily focused on developing “living drugs” capable of specifically targeting and eliminating tumors. The initial work in the field of adoptive cell therapy consisted of isolating tumor-infiltrating lymphocytes (TILs) with

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COMPETING INTERESTS

Y.L., H.R., K.R. and The University of Texas MD Anderson Cancer Center have an institutional financial conflict of interest with Takeda Pharmaceutical and Affimed GmbH. K.R. participates on the Scientific Advisory Board for GemoAb, AvengeBio, Virogin Biotech, GSK, Bayer, Navan Technologies, and Caribou Biosciences. K.R. is the scientific founder of Syena. The remaining authors declare that they have no competing interests.

natural specificity against mutated proteins on tumor cells, expanding them *ex vivo* and re-infusing them to patients. While this strategy showed promising responses in certain types of cancers such as melanoma[7], its broad applicability has been limited by the complexity of harvesting TILs and their poor expansion *in vitro*[8]. The emergence of sophisticated genetic engineering techniques allowed the modification of T cells to express a T-cell receptor (TCR) against certain tumor antigens. As such, T cells have been engineered to recognize a variety of tumor antigens such as NY-ESO-1[9,10], PRAME[11,12], and selected MAGE-A family members[10,13], to treat patients with various cancers such as sarcoma and multiple myeloma[9,14,15].

As TCR T cell therapy is restricted by specific human leukocyte antigens (HLA) alleles, and as cancer cells commonly evade TCR recognition by downregulation of their major histocompatibility complex (MHC) proteins, the emergence of CAR-based therapy constituted a major advancement in the field of adoptive cell therapy due to its MHC-independent mechanism of antigen recognition[16]. In fact, CAR T cell therapy has resulted in remarkable responses in some hematologic cancers and its application has quickly evolved to its current Food and Drug Administration (FDA) approval for B-lymphoid malignancies and multiple myeloma.

However, challenges associated with the autologous nature of these products have limited their widespread implementation. The manufacturing process for CAR T cell therapies is cumbersome and costly, resulting in lengthy collection to administration times, thus posing a challenge for patients who, due to rapidly progressing disease, are in urgent need of treatment[17]. Moreover, patient-derived T cells may be limited in number or compromised in function, especially in patients who have been heavily treated prior to CAR T administration[18,19]. These limitations sparked a growing interest in alternative allogeneic cell sources that are off-the-shelf and available for point-of-care use. One approach focuses on developing allogeneic CAR T cells through genome editing to abrogate the endogenous expression of αTCR and/or MHC class I complexes, thus eliminating T cell alloreactivity and reducing the risk of graft-versus-host disease (GvHD)[20,21]. These ‘universal’ CAR T cells can be manufactured in large scale from healthy donor sources and administered to patients more safely. The first off-the-shelf CAR T cell product to be investigated in clinical trials was UCART19 for the treatment of patients with B-cell acute lymphoblastic leukemia (ALL)[22,23]. While early results with allogeneic CAR T cells are promising, challenges remain, including allo-rejection of the infused product by the recipient immune system, technical difficulties with achieving 100% editing efficiency and thus the risk of GvHD, and potential risks related to gene editing such as off-target editing, genotoxicity and acquisition of chromosomal abnormalities[24]. Alternative immune effector cells, such as natural killer (NK) cells[25] and invariant NK T (iNKT)/NKT cells[26], are actively being explored as vehicles for CAR engineering due to their high cytotoxic potential and low risk of GvHD in the allogeneic setting. Of these, NK cells have been one of the most extensively explored alternative immune cells for adoptive cell therapy.

As with any targeted approach, the broad application of CAR T cell and CAR NK cell therapy has been limited by the paucity of targetable tumor-specific antigens (TSAs). Indeed, many tumor antigens are either inherently expressed at low levels or eventually

downregulated as a mechanism of tumor escape from the targeted CAR cell therapy. Moreover, tumor antigens are often also expressed on normal healthy tissues, which might lead to life-threatening on-target off-tumor side-effects of CAR cell therapy[19,27–30]. To overcome these challenges, extensive translational research has been conducted to design the next generation of CAR immune cells with high potency and tumor selectivity.

In this review, we highlight the current approaches to CAR engineering, with a focus on NK cells, including strategies to enhance their on-target anti-tumor selectivity, and discuss the advances achieved in translating these innovations to the clinic.

2. The modular design of a CAR

Antigen recognition by a CAR is achieved via its extracellular domain, which conventionally employs the binding domain of a single-chain variable fragment (scFv), derived from a monoclonal antibody (mAb), to specifically recognize a tumor antigen. The CAR then transmits an activation signal to the carrier cell, resulting in a potent and targeted cytotoxicity. The CAR molecule consists of three parts: an extracellular domain, a transmembrane domain, and an intracellular domain[6]. The transmembrane domain is linked by a hinge region to an extracellular domain and is commonly derived from IgG, CD8, or CD28. The transmembrane fraction guides the CAR molecule to the cellular membrane. The intracellular region of the CAR molecule contains activating signaling molecules such as CD28, CD27, 4–1BB, DAP10, or CD3ζ, either individually or in various combinations, and serves to deliver stimulatory signals upon antigen ligation. CAR design has evolved significantly over the past decades to encompass four CAR generations: (i) first generation consisting of a single stimulatory domain (CD3ζ only), (ii) second generation containing a costimulatory domain along with CD3ζ, (iii) third generation containing more than one costimulatory domain in addition to CD3ζ, and (iv) fourth generation incorporating a transgenic protein such as a cytokine with constitutive or inducible expression (referred to as TRUCKs for “T cells redirected for antigen unrestricted cytokine-initiated killing”)[31,32]. The CAR transgene is introduced into the effector cells through DNA plasmid transfection or viral-based transduction, leading to CAR expression on the plasma membrane.

3. Current limitations of CAR T cell therapies

CAR T cells have led to impressive outcomes in patients with lymphoid malignancies and multiple myeloma[6], with six FDA-approved products currently available for eight indications[33–43]. Despite the remarkable clinical success of CAR T cell therapies in hematologic malignancies, their expanded clinical use has been limited by several factors. In addition to the arduous, time-consuming, and costly manufacturing process of autologous products, CAR T cell therapy has a unique toxicity profile, characterized by cytokine release syndrome (CRS) and immune effector cell-associated neurotoxicity syndrome (ICANS)[19,44]. While more research is needed to determine the risk factors predisposing patients to CAR T cell toxicity, certain associated factors have been established[44,45]. Other major limitations of CAR T cell therapy pertain to the target antigens themselves, including on-target off-tumor toxicity due to the expression of the target antigens on normal tissues. Hence, identifying an

ideal target antigen with the right balance of sensitivity and selectivity for CAR-based cell therapy has been a major challenge for cancers beyond lymphoid malignancies.

CAR NK cells offer several advantages, including a broad range of donor cell source for manufacturing, lower risk of toxicities, and multiple mechanisms of cytotoxicity, making them a promising approach for cell-based immunotherapy. Indeed, CAR NK therapy circumvents the requirement for autologous NK cells due to their reduced risk of alloreactivity and GvHD[46]. Thus, existing NK cell sources such as NK-92 cell lines, umbilical cord blood (CB), peripheral blood (PB) and induced pluripotent stem cells (iPSCs) can be leveraged for the large-scale production of "off-the-shelf" CAR NK cells. The second major advantage of CAR NK cell therapy is their excellent safety profile with a lower incidence of CRS and neurotoxicity compared to CAR T cell therapy. This difference could be attributed to the distinct profile of cytokines secreted by CAR NK cells, with lower production of cytokines classically associated with CRS such as interleukin (IL)-1β, IL-2 and IL-6[47]. Thirdly, in addition to the targeted cytotoxicity mediated by the CAR, NK cells possess multiple intrinsic mechanisms for tumor recognition as described in the next section. Thus, CAR NK cells could theoretically overcome the challenge of tumor escape through downregulation of the target antigen reported with CAR T cell therapy.

4. NK cell biology

NK cells are part of the innate immune system and unlike T cells, exert their cytotoxicity in an HLA-independent manner and without the need for prior priming[48]. In fact, multiple preclinical and clinical studies have confirmed the role of NK cells in tumor immunosurveillance and control of metastases[49–51]. NK cells are characterized by the distinct expression of CD56 and the absence of TCR and CD3 expression[52]. They are broadly categorized based on the relative expression of the cell surface receptors CD56 and CD16 into two classes: $CD56^{bright}$ $CD16^{low/-}$ NK cells, characterized by immunomodulatory and cytokine-producing properties, and $CD56^{dim}$ $CD16^{+}$ NK cells that are generally cytotoxic[53]. Notably, high-parameter cytometry and single-cell proteo-genomics have revealed much greater phenotypic and functional heterogeneity of NK cells than previously appreciated and have enabled the identification of diverse NK cell subpopulations extending far beyond the two known subsets. The CD16 receptor on NK cells binds to the Fc receptor on antibody-coated cells to exert potent antibody-dependent cell cytotoxicity (ADCC)[52]. Additionally, NK cells can be activated through an intricate interplay between activating and inhibitory germline-encoded receptors present on their cell surface[54]. These receptors convey signals of either activation (via immunoreceptor tyrosine-based activation motifs or ITAMs) or inhibition (via immunoreceptor tyrosine-based inhibition motifs or ITIMs)[55]. Notably, cancer cells downregulate MHC class I expression or upregulate stress-induced molecules like MICA/MICB, thereby disengaging inhibitory killer Ig-like receptors (KIRs; the "missing-self" phenomenon) or engaging activating receptors such as NKG2D, respectively. Consequently, NK cells become activated, directing their cytotoxic efforts towards the target cells in a finely orchestrated manner[56]. Furthermore, NK cells interact with other immune cells through the production of cytokines and chemokines. As such, they have been shown to impact the function of T and B lymphocytes, dendritic

cells (DCs), macrophages, and neutrophils[57]. These diverse roles highlight the complex biological functions of NK cells and underscore their promise for immunotherapy.

While our classical comprehension of NK cells as part of the innate immune system depicts them as fast-acting and short-lived without antigen specificity, exciting discoveries in recent years have defied this convention. In fact, multiple research groups have reported that under specific circumstances, NK cells undergo clonal expansion in response to antigen stimulation to give rise to long-lasting memory cells[58]. Adaptive NK cells were first studied in murine models of cytomegalovirus (MCMV) infection, revealing that NK cells bearing virus-specific receptors such as Ly49H, Ly49L, and NKR-P1A exhibit rapid proliferation, cytokine production, and clonal expansion upon MCMV reactivation, reminiscent of the memory-like properties typically attributed to adaptive immune cells[59]. Moreover, Wayne Yokoyama's group showed that NK cells in mice acquire memory-like features in response to stimulation with inflammatory cytokines[60]. Similarly, human adaptive NK cells have been identified[58] and studied most extensively in the context of human CMV infection[61,62]. Cytokine-induced memory of human NK cells has also been investigated. In fact, human NK cells that were activated with IL-12, IL-15, and IL-18 and rested for one to three weeks, were shown to exhibit robust anti-tumor response characterized by augmented interferon (IFN)γ production and proliferation in response to cytokines or exposure to K562 leukemia cells[63,64]. Numerous other research groups have reported analogous memory-like functionality of NK cells in various immunological contexts, challenging the classical delineation of innate and adaptive immunity[64,65].

5. Clinical progress of CAR NK cell therapy

CAR NK cell immunotherapy is emerging as an attractive therapeutic option for cancer, with encouraging clinical responses reported in multiple phase I/II trials (Table 1). Given the promising clinical activity and safety of NK cell therapy, it is imperative to define the best NK cell source and CAR design for adoptive cell therapy.

5.1. NK cell sources for clinical use

Current NK cell therapy platforms rely on various sources of cells for therapeutic applications. These include PB, CB, cell lines, hematopoietic stem and progenitor cells (HSPCs), and iPSCs[66]. Despite their potential for generating scalable and clinically significant NK cell doses for CAR NK cell therapy, each of these sources has distinct characteristics, presenting both unique advantages and challenges.

PB-derived NK cells can be obtained through apheresis from healthy donors and have been extensively studied and widely used in clinical trials of CAR NK cell therapy (e.g., NCT00995137, NCT01974479, NCT05020678, NCT04623944). CB is another valuable source of NK cells for clinical use. The ease of collecting CB units and the ability to cryopreserve them offer unique advantages of this source for NK immunotherapy. Our group has focused on the use of CB-NK cells for CAR engineering, demonstrating the ability to generate over a hundred dose of CAR NK cells from a single CB unit[67,68].

NK-92 is an immortalized NK lymphoma cell line that has received Investigational New Drug approval by the FDA for clinical testing. NK-92 cells offer a homogeneous and abundant cell source[69] for CAR engineering, however, their cancerous origin necessitates irradiation prior to administration that could limit their *in vivo* proliferation and persistence. Additionally, without extra engineering steps, cell lines like NK-92 may lack certain functional capabilities, such as the ability to mediate ADCC due to the absence of CD16 expression[70].

HSPCs and iPSCs present exciting prospects for NK cell therapy, as they are characterized by clonal growth and high expansion capacity. Differentiation of these stem cells into NK cells allows for the manufacturing of large numbers of homogeneous NK cell products[71]. However, challenges exist, such as concerns with epigenetic memory of their cellular origin with iPSC-derived NK cells[72]. Ongoing research is focused on optimizing the use of these cell sources and determining their efficacy and safety in clinical applications.

5.2. Clinical experience with CAR NK cell therapy

In recent years, CAR NK cell therapy has emerged as a promising approach for the immunotherapy of cancer. The clinical safety of administering CAR NK cells was demonstrated in a phase I study in 2018 by a group based in China (NCT02944162)[73]. The trial used NK-92 cells engineered to express a third-generation CD33-directed CAR construct incorporating CD28 and 4–1BB co-stimulatory domains for the treatment of acute myeloid leukemia (AML)[73]. While the study reported an excellent safety profile in three patients with relapsed/refractory disease, no durable responses were achieved[73]. This was mainly attributed to the limited *in vivo* persistence of the irradiated CAR NK-92 cells[73].

Our group investigated the use of cytokine armoring to enhance the *in vivo* persistence and proliferation of CAR NK cells[67]. In a first-in-human phase I/II clinical trial, we reported the excellent safety and promising activity of IL-15 armored CB-derived CAR19 NK cells in patients with relapsed/refractory B-lymphoid malignancies (NCT03056339)[67]. Notably, CAR19 NK cells were detectable up to one year post-infusion, and patients who responded to treatment exhibited substantially higher blood peak copy numbers of CAR19 NK cells[67]. These results support the incorporation of cytokine armoring to enhance the persistence and clinical activity of NK cells.

iPSC-derived NK cells offer another attractive platform for CAR engineering. FT596 is an engineered iPSC-derived NK cell product that incorporates three genes encoding: (i) a high-affinity non-cleavable Fc receptor (hnCD16) that has been modified to include the 158V variant in combination with an S197P amino acid substitution to prevent cleavage by ADAM17[74], (ii) a membrane-bound IL-15/IL-15 receptor (IL-15R) fusion protein, and (iii) an anti-CD19 CAR[75]. Interim clinical results reported as of June 2021 demonstrated the safety of FT596 with no dose-limiting toxicities[76]. A total of 20 patients underwent dose escalation treatment, with ten patients receiving FT596 alone (Regimen A) and ten patients receiving FT596 cells combined with rituximab (Regimen B)[76]. Of the evaluable patients, the overall response rate (ORR) following the first treatment cycle was 5 of 8 patients (62%) in Regimen A and 4 of 9 patients (44%) in Regimen B[76]. Longer follow-up data will help elucidate the durability of the response and the overall efficacy of this platform.

Similarly, preliminary results from a phase I study (NCT05020678) of off-the-shelf allogeneic CAR19-engineered PB-NK cells expressing a membrane-bound form of IL-15 (NKX019) were recently reported in a press release[77]. This therapy achieved a complete response rate of 70% (seven of ten patients) in patients with relapsed/refractory non-Hodgkin lymphoma and durable responses of greater than six months in multiple patients[77]. These encouraging results collectively support the promise of NK cells as alternative immune effectors for CAR cell therapy, with over 45 trials currently registered on clinicatrials.gov (Table 1).

6. The application of CAR engineering for solid tumors

A major challenge in the translation of CAR T cell or CAR NK cell therapies from hematologic malignancies to solid tumors is the identification of target antigens that are widely and homogeneously expressed at high levels on tumor cells while having virtually no expression on normal tissues. TSAs, such as EGFRvIII in glioblastoma[78], are considered as ideal targets due to their exclusive expression on tumor cells. However, they also present unique challenges[79] primarily due to their heterogeneous levels of expression on tumor cells. Thus, the generation of CARs targeting TSAs would require screening individual patients for target antigen expression and manufacturing a custom product, which is costly and time-consuming[79]. Due to the paucity of known TSAs, other antigens that have higher expression on tumor cells but are not exclusive to tumor cells (referred to as tumor-associated antigens (TAAs), have been explored. While a myriad of TAAs have been investigated for CAR T cell therapy including HER-2[80], mesothelin (MSLN)[81], CEA[82], etc., their clinical success has been limited by concerns related to on-target off-tumor targeting of normal tissues, among others.

The extent and severity of on-target off-tumor toxicity depend on a variety of factors: (i) CAR T or CAR NK cell accessibility to healthy tissues that express the target antigen; (ii) the type of tissue and its physiological activity; (iii) the expression level and cellular localization of the antigen; and (iv) the potency of the CAR-engineered cells. For instance, due to the expression of CD19 on healthy B cells, B cell aplasia has been observed in clinical trials using CAR T cells targeting CD19[83–86]. Though B cell aplasia is manageable by supplementation with intravenous immunoglobulins (IVIG), long-term B cell depletion and its associated hypogammaglobulinemia increase the risk of severe infections[87–89], and are associated with a decreased response to vaccinations (NCT04724642, NCT04410900)[90–92]. Moreover, ICANS toxicity in CAR19 T cell-treated patients may represent an on-target off-tumor side-effect due to CAR-mediated cytotoxicity targeting low levels of CD19 expressed on brain mural cells[93]. Similarly, cross-reactivity of CAR-modified immune cells against BCMA-expressing neurons and astrocytes is thought to contribute to the neurocognitive and hypokinetic movement disorders seen after infusion of BCMA CAR T cells[94]. Treatment of patients with CAR T cells recognizing carbonic anhydrase 9 (CA-IX)[95,96], HER-2[80], ERBB-2[97], or MSLN[81] has been associated with severe side-effects including acute respiratory failure and organ damage, most likely from the expression of these antigens at differing levels on epithelial cells in the lungs and the bile ducts, respectively.

On-target off-tumor toxicity has also been associated with CAR-mediated antigen recognition of a mimotope, which mimics the structure of the targeted epitope but belongs to a distinct antigen expressed on normal cells[98]. Notably, preclinical murine models may not adequately predict the off-tumor antigen binding and toxicity potential of a given CAR, and more research is needed to develop dedicated *in vivo* models for the study of on-target off-tumor toxicity[99]. Some strategies have been devised to curb the deleterious on-target off-tumor toxicities in patients treated with CAR immune therapy, such as the administration of immunosuppressive regimens or activation of safety switches including the inducible Caspase nine suicide (iC9) gene system as used in our iC9/CAR19/IL-15 NK cell clinical trial[67]. However, these strategies are often merely reactive rather than preventative, and may also compromise the therapeutic benefit of the engineered cells[100–103].

Lastly, two important CAR-mediated on-target off-tumor effects are fratricide and trogocytosis. Fratricide occurs when CAR-expressing immune cells kill their sibling cells that also endogenously express the cognate antigen. Examples include CAR T cells targeting CD7[104], CD38[105,106] or CD70[107], antigens that are expressed on normal or activated T cells in addition to cancer cells. This may then result in manufacturing challenges, poor *in vivo* persistence of the infused product or prolonged immunodeficiency through targeting of normal recipient T cells. Trogocytosis is also an important mediator of fratricide. Trogocytosis corresponds to the receptor-mediated transfer of cognate antigen from tumor cells to the receptor-expressing immune cells[108], and contributes to tumor escape and poor responses after CAR T and CAR NK cell therapy by causing antigen loss, NK cell exhaustion and fratricide[109–111]. Our studies on clinical samples from patients with lymphoid malignancies who received anti-CD19 CAR NK cell treatment confirmed a direct correlation between elevated CD19 levels on CAR NK cells secondary to trogocytosis and reduced CD19 expression on tumor cells, associated with a greater likelihood of relapse[109]. Taken together, these data indicate the need for innovative and rationally designed CARs to increase tumor specificity while preventing on-target off-tumor toxicities.

6.1. Exploration of novel targeting approaches

To improve CAR-mediated on-target anti-tumor activity, research and clinical investigations are now focusing on adapting CAR technology to other cancers, while also enhancing potency and/or safety (Table 1 & 2). As scFvs have perceivable limitations such as promoting self-aggregation of the CAR molecule which can in turn lead to premature CAR activation and exhaustion of the transduced immune effector (Fig. 1A)[112–114], alternative binding domains have been explored. These include nanobodies (NBs), recombinant antigen-specific scFvs derived from the heavy chain (V_{HH}) of a mAb. An NB has a similar antigen-binding affinity to a conventional or full-length mAb, but due to its smaller size, has greater solubility and more stable physiochemical properties (Fig. 1B)[115]. Using NBs to generate a CAR could be advantageous due to their enhanced stability, making them more amenable to additional modifications such as multi-targeting[116]. NB-CAR NK-92 cells targeting CD38 demonstrated targeted cytotoxicity against patient-derived CD38-expressing multiple myeloma cells, but the *in vivo* efficacy of this approach remains to be validated[117]. Similarly, CD7-targeted NB-based CAR NK-92 cells mediated strong cytotoxicity *in vitro* against T-cell leukemia cell lines and primary tumor cells, and *in vivo* in T-ALL xenograft

mouse models[118]. These and other studies support the further exploration of NBs as the targeting domain of CAR constructs for NK cell immunotherapy.

An alternative targeting strategy beyond scFvs and NBs is the use of natural receptors and ligands for antigen targeting. To design a natural receptor-based (NRB)-CAR, the ectodomain of the receptor of interest, and often its transmembrane domain and/or signaling endodomain, are incorporated into the CAR construct (Fig. 1C). A similar approach is also applied for natural ligand-based (NLB)-CARs to target the corresponding receptor (Fig. 1D). Natural receptors and ligands used as targeting domains in CAR NK cells include NKG2D (recognizing stress ligands such as MICA, MICB, and ULBP)[119], DNAM-1 (targeting PVR/CD155 and Nectin-2/CD112 ligands)[120], PD-1 (targeting PD-L1)[121], and CD27 (targeting CD70; NCT05092451; NCT05703854), among others (Table 1).

Approaches to fine-tune the binding affinity of the CAR extracellular domain in order to preserve or enhance the recognition of their cognate antigen while minimizing off-tumor recognition have also been explored. An example was the use of an optimized-affinity anti-CD38 CAR capable of targeting cancer cells while sparing CD38-low expressing normal cell populations (Fig. 2A)[122]. In this study, NK cells transduced with this optimized-affinity anti-CD38 CAR successfully killed primary AML blasts with minimal fratricide against CD38 low-expressing NK cells[123]. Strategies to optimize the binding of CARs to low antigen expressing targets have also been explored. Recently, innovative synapse-tuned CAR NK cells have been devised by incorporating a PDZ binding motif (PDZbm), important for cell polarization and synapse formation, within the CAR construct (Fig. 2B)[124]. This modification enhanced the strength of the synapse and the polarization of CAR T and CAR NK cells, resulting in improved effector cell function both *in vitro* and *in vivo*[124].

Other emerging approaches to broaden the scope of antigen binding include the use of vaccines to promote antigen display on DCs and thus increase the sensitivity of CAR cells to tumors expressing antigens at low levels (CARVac; Fig. 2C). In one study, an antigen known as claudin 6 (CLDN6), which is not expressed in normal tissues yet is often present at low levels in tumors[125], was targeted by a CARVac[126]. The transient expression of this antigen on DCs was amplified by CLDN6 mRNA vaccine, leading to CAR T cell activation and expansion despite low CLDN6 expression on tumor cells[126]. This strategy has been investigated in a clinical trial for the treatment of relapsed/refractory testicular, ovarian, and endometrial cancer as well as soft-tissue sarcoma (NCT04503278), with an ORR of 43% and disease control rate of 86% (CT002 - BNT211) (Table 2). It would be very interesting to apply the CARVac strategy with NK cells, given the strong cross-talk between NK cells and DCs[127].

Engineering NK cells to express a TCR offers the opportunity to combine the intrinsic, anti-tumor effector functions of NK cells with the TCR's ability for the recognition of intracellular antigens (Fig. 2D)[128–130]. In a recent study, NK-92 cells were engineered to express both a TCR specific to the E7 protein of HPV16 and a CAR targeting TROP2[130]. This combination strategy led to a significant increase in NK cell activation and anti-tumor activity against HPV-driven cancers[130]. Lastly, in an innovative effort to target oncogenic drivers by CAR therapy, an intracellular unmutated oncogenic driver peptide (QYNPIRTTF)

that is commonly expressed in neuroblastoma and presented by HLA was identified and targeted using a peptide-centric CAR that recognizes this TSA (Fig. 2E)[131].

6.2. Dual-targeting strategies to improve tumor recognition and reduce toxicity

Dual antigen-targeting CARs have been investigated to overcome antigen escape, and to refocus CAR specificity towards tumor cells and away from normal cells. Targeting two or more antigens with CAR T or CAR NK cells can be achieved in different ways: (i) co-administration of CAR products with different antigen specificities, (ii) engineering a cell product with two or more CAR viral vectors, (iii) introduction of a bicistronic construct encoding for two CARs, or (iv) introduction of a tandem CAR with two different scFvs linked to the same signaling endodomain, whereby antigen recognition by either scFv leads to CAR signal transduction (Fig. 2F)[132,133]. Dual-targeting CAR T cells have been used in clinical trials for patients with B cell malignancies (targeting CD19/CD20[134] and CD19/CD22)[135] and in multiple myeloma targeting BCMA and CD38 (ChiCTR1800018143[136]; Table 1). However, the net clinical benefit of dual-targeting strategies requires further evaluation, with some suggestion that dual-targeting CAR T cells may not sustain their antigen specificity and potency against both antigens[135].

Dual-targeting strategies have also been explored with NK cells (Table 2). Notably, in preclinical studies with the previously discussed iPSC NK cell product FT596, dual-targeting against lymphoma was achieved by engineering the cells to express a CAR against CD19 and by combining them with a CD20 mAb that mediated ADCC by binding to hnCD16[137]. Similarly, FT596 cells engineered to express anti-BCMA CAR and co-administered with anti-CD38 antibodies were shown to mediate strong activity against multiple myeloma in multiple xenogeneic mouse models[138]. Dual-targeting has also been successfully achieved in preclinical studies with NK-92 cells directed against both CD19 and BCMA[139], or CD19 and CD138[140].

Dual-targeting strategies may also be an attractive strategy to address the inherent challenge of tumor heterogeneity in solid tumors. As such, dual-targeting CAR NK cells against PD-L1 and ErbB2 were highly effective against solid tumor cell lines expressing both antigens and maintained their cytotoxicity even when one antigen was lost or became inaccessible[141]. Dual-specificity CAR NK cells that simultaneously recognize two distinct antigens with a shared epitope have also been investigated. For instance, CAR NK cells targeting a shared epitope of EGFR and its mutant form EGFRvIII were shown to be superior to single targeting CAR NK cells in glioblastoma xenografts[142]. Thus, dual-targeting and dual-specificity CAR NK cells could provide a promising approach to counteract the immune escape mechanisms employed by cancer cells.

Multi-antigen targeting can also be achieved through “target-switchable” CARs, allowing for the recognition of multiple tumor antigens without re-engineering the cell product. One such platform is referred to as split, universal, and programmable (SUPRA) CAR (Fig. 2G), which includes a leucine zipper extracellular domain linked to a conventional CAR signaling endodomain (zipCAR)[143]. An antigen-recognition module that binds to the leucine zipper can then activate zipCAR expressing T cells upon target engagement[143]. Other novel receptor design platforms that combine a CAR backbone with a versatile

extracellular domain that binds a chemical or a genetic tag linked to a tumor-specific scFv include peptide neoepitope (PNE)-targeting CARs[144–146], anti-Tag CARs (e.g., fluorescein isothiocyanate CAR)[147], SpyCatcher CAR[148], fusion protein CAR[149], and Fab-based adaptor CAR (AdCAR)[150]. Bispecific antibody-binding adaptor CARs[151] and "split CARs" that require recognition of both targeted TAAs for full activation (Fig. 2H)[152–154] are other dual-targeting approaches shown to increase tumor specificity.

As enhancing on-target activity carries the risk of increasing off-tumor toxicity, extensive research has focused on approaches for CARs to discern tumor vs. healthy tissue targets. These next-generation CARs incorporate novel features such as the capacity to sense signals from the tumor milieu, regulation of CAR expression and activation after drug administration, etc. These designs have been evaluated in mouse models and some are also currently being assessed in clinical trials (Table 1 & 2).

6.3. Inducing CAR activation by cues from the solid tumor microenvironment (TME)

Because the tumor microenvironment (TME) has unique characteristics that are not present on healthy tissues but are essential for tumor growth, targeting its elements [e.g., extracellular matrix (ECM), stroma, vasculature, low pH, immunosuppressive metabolites, etc.] might be an attractive approach to enhance the activity of cell therapies against solid tumors (Fig. 3A)[155–157].

To divert CAR T or CAR NK cell activity away from normal tissues, CARs that rely on sensing certain cues within the TME have been investigated. One such approach is the use of hypoxia-sensing CARs that only activate the CAR response under hypoxic conditions, which is a hallmark of the solid TME[158]. For example, the promoter of HypoxiCARs contains a hypoxia-response element (HRE), such that the expression of hypoxia-inducible factor 1α (HIF-1-α) that normally increases under low oxygen conditions is mandatory for CAR expression (Fig. 3B)[159,160]. Another design fused the CAR molecule to the oxygen-dependent degradation domain (ODD) of HIF-1-α, thus, promoting the degradation of CAR molecule under normoxic conditions via ubiquitination[160]. This CAR T cell system demonstrated high anti-tumor activity in mouse models of solid tumors, with the CAR T cells being exclusively present in the tumor and absent in non-tumor sites[160].

Another interesting approach leverages the abundance of proteases in the TME to limit the off-target toxicity of CARs (Fig. 3C). Here, the CAR is modified to express an inhibitory or masking peptide that hinders its ability to bind to its cognate antigen under normal conditions. The inhibitory peptide in a 'masked CAR' is susceptible to protease cleavage, such that and in the protease-rich TME, it is cleaved to unmask the CAR antigen binding domain[161]. The masking approach does not alter the CAR activity, since anti-EGFR masked CAR T cells showed similar *in vivo* activity to control CAR cells[161].

The use of protease-sensitive CARs has also been tested with NK cells. In a preclinical study in glioblastoma, NK cells were engineered to express a dual-targeting GD2-NKG2D CAR that, in response to proteases in the TME, locally released an antibody fragment to block the immunosuppressive purinergic signaling mediated by CD73[162]. By reducing adenosine levels in the glioblastoma TME, this combinatorial strategy addresses key drivers

of glioblastoma resistance to CAR NK cell therapy[162]. Because normal tissues also express a wide variety of proteases, the safety of this approach requires further evaluation.

Finally, as the TME often contains chemokines and cytokines, modulation of chemokine signaling to improve the trafficking and localization of CAR T and CAR NK cells to tumor sites have also been investigated (Fig. 3D)[163]. For example, in a preclinical study, EGFRvIII-directed CAR NK cells that also expressed CXCR4 had greater chemotaxis towards glioblastoma cells secreting CXCL12/SDF-1α, resulting in superior tumor control and improved survival in xenograft models[164]. Similarly, forced expression of the chemokine receptor CXCR1, which is activated by IL-8 (secreted by multiple solid tumors), enhanced the migration and activity of intravenously administered NKG2D CAR NK cells in peritoneal ovarian cancer xenografts[165].

6.4. OFF-switch CARs

Alternative strategies to reduce CAR-mediated on-target off-tumor activity consist of regulating the expression and activation of the CAR molecule through drug administration. The more classical models of regulatory CARs employ an inducible caspase “suicide switch” that can be pharmacologically activated leading to the elimination of the CAR T or CAR NK cells[100]. Our group demonstrated efficient elimination of NK cells expressing a CAR and a suicide switch based on iC9 upon its pharmacologic activation using the small molecule dimerizer AP1903 or Rimiducid both preclinically[68] and clinically[67]. Another suicide system that has been used in CAR NK cells is the herpes simplex virus (HSV) thymidine kinase (HSV TK), which converts ganciclovir into a toxic product[166].

Other strategies to control CAR expression incorporate a reversible OFF-switch. For example, CARs engineered with a C2H2 zinc finger degron motif can be induced to interact with an E3 ubiquitin ligase by lenalidomide, leading to CAR proteasomal degradation (Fig. 3E)[167]. Another drug-controlled system termed signal neutralization by an inhibitable protease (SNIP) incorporates the hepatitis C virus (HCV) NS3 protease (NS3p) together with an NS3p cleavage site at the intracellular end of the transmembrane domain of a CAR to maintain the CAR in an inactive state[168]. The CAR can in turn be activated upon exposure to an NS3p inhibitor to prevent its proteolytic cleavage (Fig. 3F)[168].

6.5. ON-switch CARs

Similar tactics have been used to create ON-switches that control CAR induction and activation by drugs or other chemical or physical factors. For instance, a doxycycline inducible CAR was engineered where the tet response element 3G (TRE3G) was fused to the CAR vector, so that administration of doxycycline induced a conformational change in TRE3G to enable CAR expression[169]. An inducible system shown to enhance CAR NK cell activation and cytokine production combined the MyD88/CD40 signaling endodomain with anti-CD123 or anti-BCMA-CAR. Mimicking toll-like receptor (TLR) activation in DCs and as a potent costimulatory moiety in T cells, the inducible MyD88/CD40 moiety could be activated with Rimiducid to enhance CAR NK cell function and synergize with IL-15 signaling[170]. It will be important to validate the benefit of these novel approaches in reducing on-target off-tumor activity without comprising anti-tumor potency in the clinic.

6.6. Synthetic circuits and logic-gating strategies

To further regulate CAR activity, other strategies were developed that required recognition and sequential signaling of multiple antigens for activation. One such strategy that has been widely investigated is the design of synthetic Notch (SynNotch) receptors (Fig. 3G)[171]. A SynNotch receptor is designed to recognize a tumor antigen of interest, which, upon ligand binding, induces cleavage of an orthogonal transcription factor that in turn induces expression of a second CAR directed towards another tumor antigen[172]. As such, CAR activity towards a second cognate antigen is only initiated in the presence of the first tumor antigen, thus requiring an 'AND' logic of both antigens to be present in order to induce CAR activation[173,174]. The potential benefits of this strategy were demonstrated in preclinical models of human mesothelioma, ovarian cancer, and glioblastoma through controlling tumor growth, preventing CAR-mediated tonic signaling and maintaining a long-lived memory phenotype[173,175]. The SynNotch system was also employed to enable an inducible autocrine circuit to drive IL-2 expression in CAR T cells upon engagement with tumor antigens, resulting in more efficient CAR T cell infiltration into solid tumors and enhanced anti-tumor activity[176].

The SynNotch system has also been used to increase the secretion of granzyme B (GZMB) and perforin (PRF1) by NK cells and to regulate their intracellular pools by coupling them with pSHP inhibition[177]. Similarly, NK cells engineered to express a logic-gated GPC3–SynNotch-inducible CD147-CAR were shown to have increased specificity against hepatocellular carcinoma and reduced toxicity in preclinical models, as both antigens were required for the full targeted activity of these CAR NK cells[178]. However, the immunogenicity potential of the SynNotch receptor, a non-human artificial protein, as well as the high level of background signaling due to ligand-independent receptor activity pose safety concerns[171,179]. To overcome some of these limitations, a SynNotch system that uses humanized domains with tunable sensing and optimized transcriptional response with the ability to achieve the intended programmed gene regulation (referred to as SNIPR) was recently described[179].

An alternative approach to prevent CAR activity against undesirable targets is the use of a "NOT" logic gating strategy. This approach uses an inhibitory CAR (iCAR) directed against a self-antigen expressed on healthy cells linked to the signaling endodomain of a checkpoint molecule (e.g., PD-1 and CTLA-4)[180]. Recognition of the self-antigen on a heathy cell by the iCAR results in inhibition of CAR T or CAR NK cell activity (Fig. 3H)[180]. To overcome self-recognition of trogocytic antigen-expressing (TROG+) NK cells by the activating CAR (aCAR), and the resultant fratricide and exhaustion, our research group combined the activity of two CARs — an iCAR directed against a self-antigen expressed on NK cells and an aCAR against a tumor antigen[109]. This strategy resulted in CAR NK cells receiving a 'don't kill me' signal when interacting with their TROG+ NK siblings, while preserving the function of their aCAR against the tumor target[109]. By combining the activity of these two CARs, we were able to reduce NK cell exhaustion and fratricide and improve their anti-tumor activity *in vivo*[109].

7. Conclusions and future research

CAR-based cell therapy that combines targeted precision medicine with immunotherapy using living cells has proven therapeutic activity in patients with certain hematologic malignancies. Much progress has been made in designing the next generation of CARs to enhance the effector function, proliferation, persistence, and safety of immune cells. Indeed, we are currently witnessing a burst of innovative cell therapy approaches being explored both preclinically and in the clinic. However, considerable effort is still needed to replicate the success observed with CAR cell therapies in B-lymphoid malignancies in other malignancies. The future will likely focus on developing multi-pronged approaches that leverage our ever-growing scientific and clinical knowledge of tumor immunology and immunotherapy with novel engineering strategies to improve the safety, potency and feasibility of this therapeutic platform.

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DATA AVAILABILITY STATEMENT

Data sharing not applicable – no new data generated

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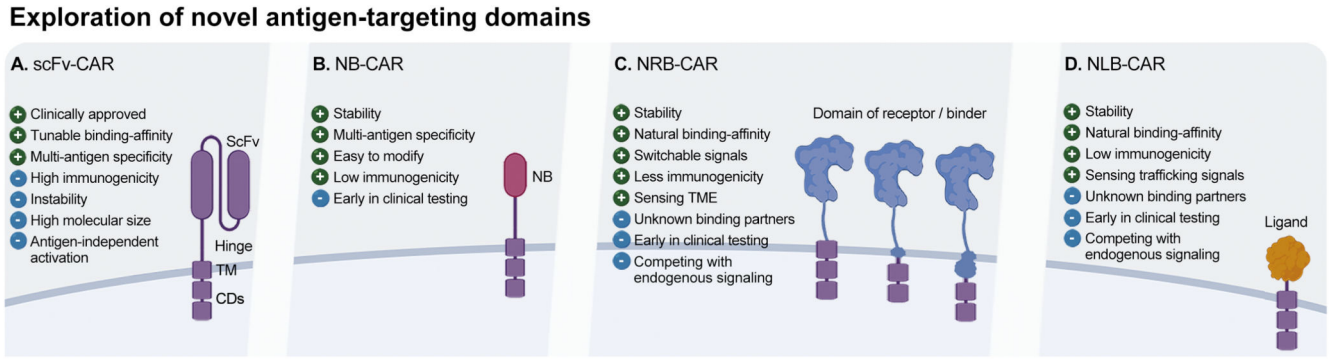

Figure 1.
Strategies to explore novel antigen-targeting domains.

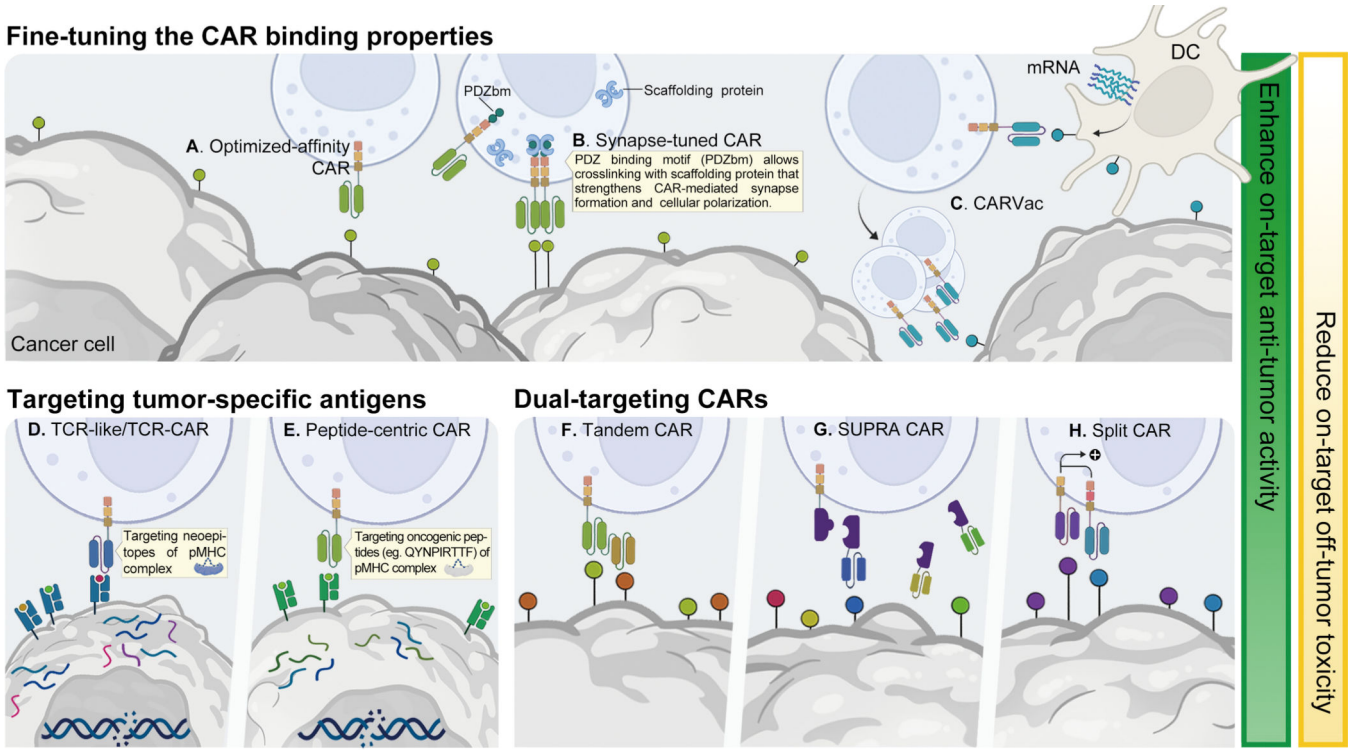

Figure 2.
Strategies to advance CAR NK cell mediated on-target anti-tumor activity.

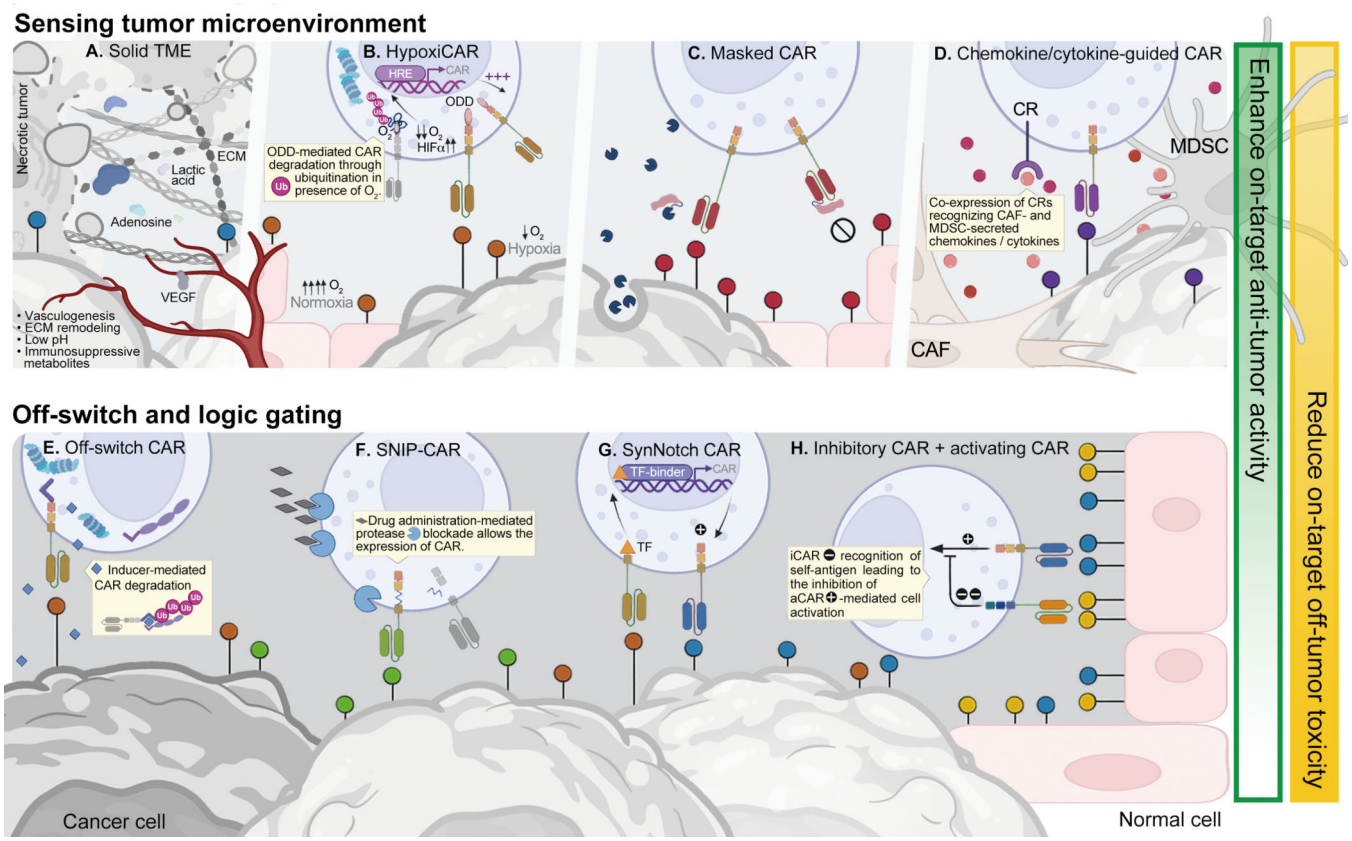

Figure 3.
Strategies to overcome CAR NK cell-mediated on-target off-tumor toxicity.

Table 1.

Current clinical trials of CAR NK cell-based anti-tumor therapy.

CAR strategy	NK cell source	Target antigen	Cancer	Phase	Status	NCT No.
scFv-CAR	Undisclosed	CD33	AML	I	Recruiting	NCT05008575
	NK-92	CD33	AML	I/II	Unknown	NCT02944162
	NK-92	ROBO1	Pancreatic cancer	I/II	Unknown	NCT03941457
	NK-92[&]	ROBO1	Solid tumors	I/II	Unknown	NCT03931720
	NK-92	ROBO1	Solid tumors	I/II	Unknown	NCT03940820
	iPSC-NK	PSMA	Prostate cancer	Early I	Recruiting	NCT03692663
	NK-92	PD-L1	Gastric cancer/Head and neck cancer	II	Recruiting	NCT04847466
	NK-92	PD-L1	Pancreatic cancer	II	Recruiting	NCT04390399
	NK-92	PD-L1	Solid tumors	I	Active, not recruiting	NCT04050709
	NK-92	PD-L1	Solid tumors	II	Active, not recruiting	NCT03228667
	HSC-NK	MUC1	Solid tumors	I/II	Unknown	NCT02839954
	iPSC-NK	Mesothelin	Ovarian cancer	Early I	Unknown	NCT03692637
	NK-92	HER-2	Glioblastoma	I	Recruiting	NCT03383978
	Primary NK	CLDN6	Solid tumors	I/II	Recruiting	NCT05410717
	NK-92	CD7	Leukemia/Lymphoma	I/II	Unknown	NCT02742727
	CB- NK	CD5	Hematologic malignancies	I/II	Not yet recruiting	NCT05110742
	iPSC-NK	CD22	B cell malignancies	Early I	Unknown	NCT03692767
	CB-NK	CD19	B cell malignancies	I/II	Completed	NCT03056339
	CB-NK	CD19	B cell malignancies	I	Recruiting	NCT04796675
	CB-NK	CD19	B cell malignancies	I	Recruiting	NCT04796688
	Primary NK	CD19	B cell malignancies	I	Completed	NCT00995137
	Primary NK	CD19	B cell malignancies	I	Recruiting	NCT05410041
	Primary NK	CD19	B cell malignancies	I	Recruiting	NCT05020678
	Primary NK	CD19	B cell malignancies	I	Recruiting	NCT05379647
	Primary NK	CD19	B cell malignancies	I	Suspended	NCT01974479
	NK-92	CD19	B cell malignancies	I/II	Unknown	NCT02892695
	iPSC-NK	CD19	B cell malignancies	I	Recruiting	NCT05336409
	iPSC-NK	CD19	B cell malignancies	Early I	Unknown	NCT03690310
	iPSC-NK	CD19	B cell malignancies	Early I	Unknown	NCT03824951
	CB-NK	CD19	NHL	I	Not yet recruiting	NCT04639739
	CB-NK	CD19	NHL	II	Recruiting	NCT05020015
	CB-NK	CD19	NHL	I	Recruiting	NCT05472558
	Primary NK	CD19	NHL	I	Recruiting	NCT04887012
	NK-92	BCMA	MM	I/II	Unknown	NCT03940833
	CB-NK	BCMA	MM	I	Recruiting	NCT05008536
	Undisclosed	5T4	Solid tumors	I	Recruiting	NCT05194709

CAR strategy	NK cell source	Target antigen	Cancer	Phase	Status	NCT No.
NRB-CAR	Undisclosed	5T4	Solid tumors	I	Recruiting	NCT05137275
	NK-92	DLL3	SCLC	I	Recruiting	NCT05507593
	CB-NK	CD70	RCC/Mesothelioma/ Osteosarcoma	I/II	Recruiting	NCT05703854
	CB-NK	CD70	Hematologic malignancies	I/II	Recruiting	NCT05092451
	CB-NK	NKG2D ligand	AML	I	Terminated	NCT05247957
	Primary NK	NKG2D ligand	AML	I	Recruiting	NCT04623944
	Undisclosed	NKG2D ligand	Colorectal cancer	I	Recruiting	NCT05213195
	Primary NK	NKG2D ligand	Solid tumors	I	Unknown	NCT03415100
	NK-92	NKG2D ligand	Solid tumors	I	Recruiting	NCT05528341
	NK-92	PD-L1	NSCLC	I	Recruiting	NCT03656705
Dual-targeting CAR	iPSC-NK	CD19/CD22	B cell malignancies	Early I	Unknown	NCT03824951
	iPSC-NK	CD19/CD22	B cell malignancies	Early I	Unknown	NCT03824964
	CB-NK	CD33/CLL1	AML	I	Recruiting	NCT05215015
Dual-targeting CAR (CAR+hnCD16)	iPSC-NK	CD19/CD20	B cell malignancies	I	Recruiting	NCT04245722
	iPSC-NK	BCMA/CD38	MM	I	Recruiting	NCT05182073

CB: cord blood; scFv: singe-chain variable fragment; NRB: natural receptor-based; hnCD16: high affinity non-cleavable CD16; HSC: hematopoietic stem cell; iPSC: induced pluripotent stem cell; AML: acute myeloid leukemia; MM: multiple myeloma; NSCLC: Non-small cell lung cancer; NHL: non-hodgkin's lymphoma; SCLC: small cell lung cancer; RCC: renal cell carcinoma.

&: Combination therapy with anti-ROBO1 CAR T cells

Table 2.

Current clinical trials of CAR T cell-based anti-tumor therapy using “next-generation” CAR strategies.

CAR strategy	Target antigen	Cancer type	Phase	Status	NCT No.
NB-CAR	CD7	Hematologic malignancies	I	Unknown	NCT04004637
NB-CAR+ tandem CAR	CD19/CD20	B cell malignancies	I	Unknown	NCT03881761
	BCMA	MM	I/II	Unknown	NCT03090659
	CD70	Various *1	I/II	Suspended	NCT02830724
	Fc	Various *2	NA	Terminated	NCT02840110
	Fc (HER2)	Solid tumors	I	Terminated	NCT03680560
	Fc (CD20)	B cell malignancies	I	Completed	NCT02776813
	Fc (CD20)	B cell malignancies	I	Terminated	NCT03189836
	Fc (BCMA)	MM	I	Terminated	NCT03266692
NRB-CAR	NKG2D ligand	Various *3	I	Not yet recruiting	NCT05302037
	NKG2D ligand	Solid tumors	I	Unknown	NCT04107142
	NKG2D ligand	Solid tumors	I	Recruiting	NCT05131763
	NKG2D ligand	Hematologic malignancies	I	Completed	NCT03612739
	NKG2D ligand	Hematologic malignancies	I	Withdrawn	NCT03018405
	NKG2D ligand	Hematologic malignancies	I/II	Unknown	NCT05248048
	NKG2D ligand	Colorectal cancer	Early I	Recruiting	NCT03310008
	NKG2D ligand	Colorectal cancer	I	Unknown	NCT03692429
	NKG2D ligand	Colorectal cancer	I	Recruiting	NCT04991948
	NKG2D ligand	Colorectal cancer	I	Recruiting	NCT03370198
	NKG2D ligand	Colorectal cancer	I	Unknown	NCT03466320
	NKG2D ligand	AML	I/II	Completed	NCT04167696
	NKG2D ligand	AML	I	Recruiting	NCT05131763
	PSMA	Prostate cancer	I	Recruiting	NCT04768608
NRB-CAR + OFF-switch	PD-L1	GBM	I	Unknown	NCT02937844
	BCMA/TACI	MM	I/II	Terminated	NCT03287804
	BCMA/TACI	MM	I	Recruiting	NCT05020444
	BCMA/TACI	MM	Early I	Not yet recruiting	NCT04657861
	ErbB family	HNSCC	I	Recruiting	NCT01818323
NLB-CAR	GMR	Hematologic malignancies	I/II	Recruiting	jRCT2033210029
	ICAM-I	Thyroid cancer	I	Recruiting	NCT04420754
	IL-13Rα2	GBM	I	Active, not recruiting	NCT02208362
	IL-13Rα2	GBM	I	Recruiting	NCT04003649
	IL-13Rα2	GBM	I	Recruiting	NCT04510051
	IL-13Rα2	GBM	I	Recruiting	NCT04661384
	MMP-2	GBM	I	Recruiting	NCT04214392
CARVac	CLDN6	Solid tumors	I/II	Recruiting	NCT04503278
Dual-targeting CAR	BCMA/CS1	MM	Early I	Unknown	NCT04156269
	BCMA/GPRC5D	MM	I	Not yet recruiting	NCT05325801
	CD123/CD33	Hematologic malignancies	Early I	Unknown	NCT04156256

CAR strategy	Target antigen	Cancer type	Phase	Status	NCT No.
	CD123/CLL1	AML	II/III	Unknown	NCT03631576
	CD19/BCMA	MM	I	Recruiting	NCT05412329
	CD19/BCMA	MM	I	Recruiting	NCT04162353
	CD19/BCMA	MM	I/II	Recruiting	NCT03455972
	CD19/BCMA	MM	I	Unknown	NCT03767725
	CD19/BCMA	MM	Early I	Recruiting	NCT04236011
	CD19/BCMA	MM	I/II	Recruiting	NCT04714827
	CD19/BCMA	MM	I	Unknown	NCT04194931
	CD19/BCMA	MM	Early I	Unknown	NCT04182581
	CD19/BCMA	MM	Early I	Unknown	NCT04412889
	CD19/BCMA	MM	I	Unknown	NCT03706547
	CD19/BCMA	MM	Early I	Not yet recruiting	NCT04617704
	CD19/CD20	NHL	I	Recruiting	NCT05421663
	CD19/CD20	NHL	I/II	Unknown	NCT04553393
	CD19/CD20	NHL	I	Unknown	NCT04693676
	CD19/CD20	NHL	I	Unknown	NCT04655677
	CD19/CD20	NHL	I	Unknown	NCT04696432
	CD19/CD20	NHL	I/II	Recruiting	NCT04697940
	CD19/CD20	NHL	Early I	Suspended	NCT04697290
	CD19/CD20	ALL	I	Recruiting	NCT04049383
	CD19/CD20	B cell malignancies	I	Completed	NCT03019055
	CD19/CD20	B cell malignancies	I	Completed	NCT04260932
	CD19/CD20	B cell malignancies	I	Completed	NCT04260945
	CD19/CD20	B cell malignancies	I/II	Recruiting	NCT04186520
	CD19/CD20	B cell malignancies	I/II	Completed	NCT03097770
	CD19/CD20	B cell malignancies	Early I	Unknown	NCT04156178
Dual-targeting CAR	CD19/CD20	B cell malignancies	I	Terminated	NCT04160195
	CD19/CD20	B cell malignancies	I/II	Unknown	NCT03207178
	CD19/CD20	DLBCL	I	Recruiting	NCT04215016
	CD19/CD20	DLBCL	I	Unknown	NCT04486872
	CD19/CD20	DLBCL	I/II	Unknown	NCT02737085
	CD19/CD20	Hematologic malignancies	I	Unknown	NCT03271515
	CD19/CD20	Hematologic malignancies	Early I	Unknown	NCT04700319
	CD19/CD20/CD22	Hematologic malignancies	I/II	Unknown	NCT03398967
	CD19/CD20/CD22	Hematologic malignancies	I	Recruiting	NCT05418088
	CD19/CD20/CD22	B cell malignancies	I	Recruiting	NCT05318963
	CD19/CD20/CD22	B cell malignancies	I	Recruiting	NCT05094206
	CD19/CD20/CD22	B cell malignancies	I	Recruiting	NCT05388695
	CD19/CD22	B cell malignancies	I	Recruiting	NCT04204161
	CD19/CD22	B cell malignancies	Early I	Unknown	NCT03825731
	CD19/CD22	B cell malignancies	I	Unknown	NCT03463928
	CD19/CD22	B cell malignancies	I	Recruiting	NCT04007029

CAR strategy	Target antigen	Cancer type	Phase	Status	NCT No.
	CD19/CD22	B cell malignancies	I	Withdrawn	NCT04094766
	CD19/CD22	B cell malignancies	I	Recruiting	NCT03233854
	CD19/CD22	B cell malignancies	I/II	Recruiting	NCT04715217
	CD19/CD22	B cell malignancies	I/II	Recruiting	NCT04782193
	CD19/CD22	B cell malignancies	I	Terminated	NCT03593109
	CD19/CD22	B cell malignancies	I/II	Suspended	NCT03098355
	CD19/CD22	B cell malignancies	I/II	Recruiting	NCT04788472
	CD19/CD22	B cell malignancies	I	Recruiting	NCT03448393
	CD19/CD22	DLBCL	I/II	Active, not recruiting	NCT03287817
	CD19/CD22	HNL	II	Recruiting	NCT04539444
	CD19/CD22	NHL	Early I	Recruiting	NCT04303247
	CD19/CD22	NHL	I/II	Not yet recruiting	NCT04626908
	CD19/CD22	Hematologic malignancies	I	Recruiting	NCT03330691
	CD19/CD22	Hematologic malignancies	I/II	Not yet recruiting	NCT04029038
	CD19/CD22	Hematologic malignancies	I	Active, not recruiting	NCT02443831
	CD19/CD22	Hematologic malignancies	I/II	Unknown	NCT03185494
	CD19/CD22	ALL	I/II	Recruiting	NCT04714593
	CD19/CD22	ALL	I/II	Recruiting	NCT04499573
	CD19/CD22	ALL	I	Recruiting	NCT03919526
	CD19/CD22	ALL	I/II	Recruiting	NCT04781634
	CD19/CD22	ALL	I/II	Completed	NCT03289455
	CD19/CD22	ALL	I	Unknown	NCT04303520
	CD19/CD22	ALL	I	Not yet recruiting	NCT05223686
	CD19/CD22	ALL	I	Withdrawn	NCT05168748
	CD19/CD22	ALL	I/II	Recruiting	NCT04740203
	CD19/CD22	ALL	Early I	Recruiting	NCT04626765
	CD19/CD22	ALL	I	Unknown	ChiCTR-OIB-17013670
	CD19/CD22	ALL	I	Unknown	ChiCTR1800015575
	CD19/CD22	ALL	I	Active	EUDRA CT 2016–004680-39
	CD19/CD22	ALL	Early I	Active, not recruiting	NCT04034446
	CD19/CD22	ALL	I/II	Recruiting	NCT03614858
Dual-targeting CAR	CD19/PD-L1	Hematologic malignancies	I	Unknown	NCT03932955
	CD19/PD-L1	B cell malignancies	I	Recruiting	NCT04850560
	CD20/CD22	Hematologic malignancies	Early I	Recruiting	NCT04283006
	CD33/CLL1	Hematologic malignancies	Early I	Recruiting	NCT03795779
	CD33/CLL1	AML	Early I	Not yet recruiting	NCT05016063
	CD33/CLL1	AML	I	Recruiting	NCT05248685
	CD38/BCMA	MM	I	Recruiting	ChiCTR1800018143
	c-Met/PD-L1	Hepatocellular Carcinoma	Early I	Unknown	NCT03672305
	EGFR/B7H3	Lung cancer / TNBC	Early I	Recruiting	NCT05341492

CAR strategy	Target antigen	Cancer type	Phase	Status	NCT No.
	HER2/PD-L1	Solid tumors	Early I	Recruiting	NCT04684459
	CD33/CD123/ CLL-1	AML	I/II	Recruiting	NCT04010877
	CD44v6/GD/Her2	Breast cancer	I/II	Recruiting	NCT04430595
	CD10/CD20/CD22	ALL	Early I	Recruiting	NCT03407859
	Multiple[$1]	MM	I/II	Recruiting	NCT03196414
	Multiple[$2]	ALL	I/II	Recruiting	NCT04430530
	Multiple[$3]	AML	I/II	Unknown	NCT03222674
	Multiple[$4]	Lung cancer	I	Recruiting	NCT03198052
	Multiple[$5]	B cell malignancies	I/II	Recruiting	NCT04429438
Switchable CAR	CD19	B cell malignancies	I	Recruiting	NCT04450069
	BCMA	MM	Early I	Not yet recruiting	NCT04727008
	CD19	DLBCL	I	Recruiting	NCT04381741
	CD19	B cell malignancies	NA	Completed	NCT04833504
	CD19	B cell malignancies	II	Unknown	NCT03929107
CRs-CAR	CD30	Hematologic malignancies	I	Recruiting	NCT03602157
	EGFR	NSCLC	I	Recruiting	NCT04153799
	EGFR	NSCLC	Early I	Recruiting	NCT05060796
	PSMA[#]	Prostate cancer	I	Not yet recruiting	NCT04227275
	PSMA[#]	Prostate cancer	I	Recruiting	NCT03089203
	BCMA	MM	I	Active, not recruiting	NCT03070327
	CD19	MCL	II	Recruiting	NCT04484012
OFF-Switch CAR	CD19	Hematologic malignancies	I	Active, not recruiting	NCT03085173
	CD22	Hematologic malignancies	I	Active, not recruiting	NCT03244306

NB: nanobody; NRB: natural receptor-based; NL: natural ligand-based; AML: acute myeloid leukemia; MM: multiple myeloma; HNSCC: Head and neck squamous cell carcinoma; GBM: glioblastoma

[*1]: Pancreatic cancer, renal cell carcinoma, breast cancer, melanoma, ovarian cancer

[*2]: B cell lymphoma, multiple myeloma, solid tumors

[*3]: Relapsed and refractory malignancies

MM: multiple myeloma; NHL: non-hodgkin's lymphoma; DLBCL: diffuse large B-cell lymphoma; ALL: acute lymphocytic leukemia

CRs: chemokine/cytokine receptor; AML: acute myeloid leukemia; MM: multiple myeloma; DLBCL: diffuse large B-cell lymphoma; ALL: acute lymphocytic leukemia; TNBC: triple negative breast cancer; MCL: mantle cell lymphoma; NSCLC: non-small cell lung cancer

[$1]: CD138, BCMA, CD19, and/or other antigens

[$2]: CD22, CD123, CD38, CD10, and/or CD20

[$3]: CD33, CD38, CD123, CD56, MUC1, and/or CLL1

[$4]: GPC3, Mesothelin, Claudin18.2, GUCY2C, B7-H3, PSCA, PSMA, MUC1, TGFβ, HER-2, Lewis-Y, AXL, and/or EGFR

[$5]: CD19, CD20, CD22, CD70, CD13, CD79b, GD2 and/or PSMA

[#]: TGFβ-resistant by modifying CAR T cell with a dominant negative TGFβ receptor (TGFβRdn)