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Engineering and targeting potential of CAR NK cells in colorectal cancer

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Abstract
Colorectal cancer (CRC), a major global health concern, necessitates innovative treatments. Chimeric antigen receptor (CAR) T cells have shown promises, yet they grapple with challenges. The spotlight pivots to the rising heroes: CAR natural killer (NK) cells, offering advantages such as higher safety profiles, cost-effectiveness, and efficacy against solid tumors. Nevertheless, the specific mechanisms underlying CAR NK cell trafficking and their interplay within the complex tumor microenvironment require further in-depth exploration. Herein, we provide insights into the design and engineering of CAR NK cells, antigen targets in CRC, and success in overcoming resistance mechanisms with an emphasis on the potential for clinical trials.
Keywords: Chimeric antigen receptor; Natural killer cells; Colorectal cancer; Antigen targeting; Resistance mechanisms; Tumor microenvironment

Introduction

Immunotherapy stands as a beacon of hope in the realm of cancer treatment, turning immune cells into formidable warriors against the relentless invader—the tumor. Central to this transformation are chimeric antigen receptor (CAR) therapies, engineering the immune system to seek and destroy cancer cells with unparalleled precision. Earlier, we showcased nanoengineering of better performing CAR T cells,[1] mitigating the barriers of tumor microenvironment (TME)[2] and precision in targeting hematological and solid cancers.[3] However, CAR T cells present certain demerits such as; limited T cell trafficking, immunosuppressive environment, and antigen escape.[4,5] In this context, the spotlight shifts to another unsung hero of the immune system—the natural killer (NK) cells which, hitherto, we explicitly have reviewed "From Natural Basis to Design for Kill,"[6] boosting the NK cells using neoantigens[7] and recent progress in preclinical and clinical settings.[8] Exclusively, CAR NK cells offer several merits as they do not cause cytokine release syndrome,[9] demonstrate higher safety profiles via multiple killing mechanisms,[10] are more cost effective when compared to CAR T cell therapy,[11] thereby readily accessible to patients and show promise in treating solid tumors such as glioblastoma,[12] an area where CAR T cell therapy has faced challenges.[13,14]

Colorectal cancer (CRC) is the second and the fourth leading causes of cancer-related deaths worldwide and in China respectively, according to the GLOBOCAN,[15] owing to a 9.4% mortality rate and 1.9 million new cases reported in 2020 worldwide with relatively more incidence in older ages in males relative to females.[16] The very trend has recently been observed to be increasing at a faster rate among individuals of age <50 years.[17] In 2020, the highest rates were documented in Australia/New Zealand and European regions, whereas several African regions and Southern Asia reported the lowest rates.[18] The Asia-Pacific region, encompassing countries like the Republic of Korea, Singapore, and Chinese mainland, has witnessed a substantial surge in CRC incidence in the last two decades.[19] Overall, the global burden of CRC is escalating, influenced by geographic location, socioeconomic status, age, and gender, showcasing significant variations.[20]

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While the urgency to address this growing burden necessitates innovative and effective approaches for the treatment of CRC, the transition from CAR T to CAR NK is crucial for more accessible and efficient treatments. Herein, we debate on CAR NK cells with a focus on several antigen targets in CRC with strategies overcoming the resistance mechanisms and their preclinical success till the date.

CAR NK Cell Design and Engineering

Utilizing genetic engineering to modify NK cells and create CAR NK cells has emerged as a highly promising approach in the field of cancer immunotherapy. Such CAR NK cells, with their diverse killing mechanisms [Table 1], elevated safety profiles, and broad availability, have garnered significant interest for potential clinical applications.[11] CARs possess the capability to specifically target tumor-associated antigens (TAA).[21] Activation of these TAA can promote cell lysis through degranulation, supported by the presence of activating receptors like activating killer immunoglobulin receptors (KIRs), natural killer group 2 member D (NKG2D), and DNAX accessory molecule 1 (DNAM1) on NK cells.[22] For instance, the utilization of DNAM1 chimeric receptor-engineered NK cells, designed to recognize specific ligands expressed on tumor cells, represents a novel and promising avenue for anticancer immunotherapy.[23] Integrating cytokine transgenes such as interleukin (IL)-2 and IL-15 during the development of CAR NK cells holds promise for enhancing both expansion and persistence.[24] Advanced genetic manipulation techniques, including gene editing and the implementation of CAR, have significantly improved the precision, persistence, and targeting abilities of NK cells.[21] In preclinical studies, allogeneic CAR NK cells have demonstrated encouraging results, prompting their exploration in a multitude of clinical trials targeting both hematological cancers and solid tumors.[25] CAR NK cells offer advantages such as their natural ability against non-self-cells, direct and indirect killing functions, reduced risk of adverse effects, and flexibility in sourcing.[26] However, they face limitations such as limited tumor infiltration and efficacy, especially in CAR transduction, survival challenges in the immunosuppressive TME.

To improve tumor infiltration, genetic modifications have been a focal point.[27] The development of a distinct NK cell-targeting protein, referred to as NK cell–recruiting protein-conjugated antibody (NRP-body), demonstrated significant effectiveness in mouse models of pancreatic adenocarcinoma. The positive results were associated with the generation of a chemokine (C-X-C motif) ligand 16 (CXCL16) gradient facilitated by CXCL16 cleavage from the NRP-body, particularly within the pancreatic cells.[28] Moreover, CAR NK cells have been strategically engineered to express chemokine receptors, elevating their ability to migrate toward the bone marrow (BM), a critical tumor site. Notably, a recent study demonstrated efficient overexpression of the native human CXCR4 on primary CAR NK cells through genetic engineering using a fully human anti-CD19-CAR construct. This modification significantly augmented NK cell homing to the BM, showcasing enhanced migration capacity in response to recombinant stromal cell-derived factor 1 or BM stromal cells. Intriguingly, these engineered CAR NK cells retained their functional and cytolytic activity against CD19+ cells derived from various tumors, including acute lymphoblastic leukemia and chronic myeloid leukemia, distinguishing them from conventional CAR NK cells.[29] Furthermore, NK cells with modified chemokine (C-X-C motif) receptor 1 (CXCR1) exhibited heightened migration toward tumor supernatants *in vitro* and demonstrated increased infiltration into human tumors

Table 1: Different NK cell tumor targeting mechanisms and strategies for eliminating tumor cells, along with the receptors and ligands involved.

Targeting mechanism	Receptors and ligands involved	Strategy for tumor cell elimination
Distinguishing tumor cells from healthy cells	NKG2D, NKp30, NKp44, NKp46, CD16	Activation through downregulation of MHC class I in tumor cells
Granule release	Perforin and granzymes	Creation of pores in the target cell membrane and triggering apoptosis
TNF ligands expression approach	TRAIL, TNF-α, Fas-L	Inducing apoptosis in tumor cells through receptor binding
Cytokine and chemokine production	IFN-γ, TNF-α, GM-CSF	Preventing tumor cell proliferation by affecting effector molecules
Exosome production	Exosomes expressing NK cell receptors	Activating NK cells and inducing cytotoxic activity against tumor cells
Recognition during cellular stress	Upregulated NKG2D ligands (MICA, MICB, ULBP1-6)	Enhanced NK cell-mediated cytotoxicity against stressed cells
DNAM1 ligand interaction	CD155 and CD112	Activating NK cells against virus-infected and transformed cells during cellular stress
Hsp70 involvement	Extracellular Hsp70	Stimulating expression of activatory NK cell receptors
Hsp70 expression in tumors	Endogenous Hsp70	Activating NK cells and making tumor cells targets for NK cell-mediated cytotoxicity

CD: Cluster of differentiation; DNAM1: DNAX accessory molecule 1; NK: Natural killer; GM-CSF: Granulocyte-macrophage colony-stimulating factor; Hsp: Heat shock proteins; IFN-γ: Interferon-γ; MHC: major histocompatibility complex; MICA/B: MHC class I-related chain A/B; NK: Natural killer; NKG2D: Natural killer group 2 member D; TNF-α: tumor necrosis factor; TRAIL: TNF-related apoptosis-inducing ligand; ULBP1-6: UL16-binding protein 1-6.

in vivo, particularly in subcutaneous and intraperitoneal xenograft models.[30] This emphasizes the potential of engineered chemokine receptors in optimizing NK cell trafficking and infiltration into tumor sites, laying a robust foundation for further advancements in cancer immunotherapy.

Novel genetic engineering designs empower immune cells to resist the immunosuppressive TME, revealing promising prospects for enhancing cancer treatment against solid tumors. However, there are ongoing challenges related to the optimization and standardization of expansion and transfection protocols, as well as addressing the relatively short persistence of NK cells post-infusion, which necessitate further investigation and innovation.

Antigen Targets in CRC for CAR NK Cell Therapy

Solid tumors, especially CRC, characterized by diverse molecular alterations and distinct tumor antigens necessitate a targeted approach for effective therapeutic intervention. The success of CAR NK cell therapy hinges upon identifying and targeting specific tumor antigens that play pivotal roles in CRC metastasis [Table 2]. In this section, we embark on a journey to discuss the relevance of various specific tumor antigens illuminating their potential as precise targets in the pursuit of treating CRC.

Originally discovered in CRC, epithelial cell adhesion molecule (EpCAM) has shown to be highly expressed in primary tumors among various markers in gastric cancer[40] owing to its absence in tumors originating from nonepithelium tissues such as sarcomas and lymphomas. EpCAM also triggers oncogenesis via modulating endothelial mesenchymal transition correlated genes.[41] Primarily EpCAM is localized to membranes, however, it is found in cytoplasm and nucleus due to the catalytic activity of regulated intramembrane proteolysis[42] into epidermal growth factor receptor intracellular domain. Thus, EpCAM-mediated cancer progression may be linked to its subcellular distribution and high expression in membranes. The abundant expression of EpCAM on membranes, and its subcellular redistribution makes it a promising candidate for CAR NK therapy. Another promising biomarker—carcinoembryonic antigen, in CRC is characterized by a ninefold higher expression CRC cell lines compared to noncancerous cell lines, making it a suitable target.[43] Li and Mohammadi[44] in

Table 2: Summary of NK cell activating receptors and potential ligands for CAR engineering.

Antigen target	CAR NK design	Targeting mechanism	Clinical potential	Reference
Soluble E-cadherin	Multiantigen CAR	Disruption of EMT process	Potential modulation of EMT in CRC, aiding in inhibiting metastasis and improving diagnostics	[31]
BCMA and GPRC5D	Dual targeting CAR	Improved NK cell potency and persistence	Potential "off-the-shelf" cell therapy for effective treatment of malignancies	[9]
EpCAM	Second Gen CAR	Specific recognition of EpCAM-positive CRC cells, cytokine release (IFN-γ, perforin, granzyme B), cytotoxicity	A promising strategy for treating CRC enhanced therapeutic efficacy in combination with regorafenib	[32]
EpCAM and EGFRvIII	CAR-engineered NK-92 cells	CAR NK cell cytotoxicity against 3D patient-derived colon organoids	Sensitive *in vitro* platform to evaluate CAR efficacy and tumor specificity, specifically targeting EpCAM and EGFRvIII	[33]
EpCAM	iPSC-derived	cDNA encoding anti-EpCAM CAR inserted into adeno-associated virus integration site 1	Promising potential as a source for generating anti-EpCAM CAR NK cells	[34]
NKG2D	The extracellular domain of NKG2D fused to DAP12	Augmented cytolytic activity against solid tumor cell lines, significant therapeutic benefit in mice with solid tumors	Promising therapeutic potential in treating metastatic CRC patients	[35]
CEA	Anti-CEA-CAR NK-92MI cells	Recognition and lysis of high and moderate CEA-expressing tumor cell lines	Potential therapeutic approach for CEA-expressing tumors	[36]
NKG2D ligands	NKX101 expressing NKG2D activating chimeric receptor	Increased *in vitro* cytotoxicity, enhanced cytokine release	Potential for regional delivery and improved tumor control	[37]
NKG2D ligands	CAR NKG2D NK cells	Enhanced *in vitro* and *in vivo* cytotoxicity	Promising tumor reduction in locoregional delivery	[38]
HER2	CAR NK expressing immune cells	Targeting HER2 overexpression in tumors	Potentially effective in HER2-positive gastric cancers, antitumor competence in immunotherapy	[39]

BCMA: B-cell maturation antigen; CAR: Chimeric antigen receptor; cDNA: Complementary DNA; CEA: Carcinoembryonic antigen; CRC: Colorectal cancer; EMT: Epithelial–mesenchymal transition; EpCAM: Epithelial cell adhesion molecule; EGFR: Epidermal growth factor receptor; GPRC5D: G protein-coupled receptor 5D; HER2: Human epidermal growth factor receptor 2; IFN-γ: Interferon-γ; iPSC: induced pluripotent stem cell; NK: Natural killer; NKG2D: Natural killer group 2 member D.

2023 investigated statistically significant difference of carcinoembryonic antigen (CEA) in 250 CRC patients. CEA enhances metastasis by triggering Kupffer cells to secrete cytokines, subsequently promoting the expression of adhesion molecules on endothelial cells.[45] The results of diagnostic biopsy and examination of adjacent preexistent rectal mucosa showed higher co-expression of CEA and EpCAM.[46] Soluble E-cadherin is another antigen revealed to be elevated in CRC during epithelial–mesenchymal transition (EMT).[31] Therefore, CAR NK therapy could potentially be customized in various ways to target CRC cells (as depicted in Figure 1), for instance, expressing soluble E-cadherin, disrupting EMT and inhibiting cancer progression.

Leveraging CAR NK cells targeting tumor specific antigens such as, EpCAM, CEA, soluble E-cadherin, and various others upregulated during EMT, hold immense promise for precise CRC treatment. Advancements in CAR NK therapy, fine-tuned for these antigens, are key to optimizing CRC treatment, marking a transformative phase in cancer therapeutics.

Overcoming Resistance Mechanisms

CAR NK therapy, promising against solid tumors, grapples with resistance hurdles such as genetic alterations, antigen loss, immune escape mechanisms, and various others (as shown in Figure 2) that demand solutions for enhanced efficacy. Understanding these hurdles is key to unlocking the full potential of CAR NK therapy.

CAR expression demonstrated enhanced cytokine secretion but not degranulation in short-term TME co-cultures. In long-term tumor co-cultures, they outperformed control NKs, leading to significantly reduced viable tumor burden. *TIGIT* knockout CAR NK cells also showed rapid proliferation and superior tumor control compared to control, without altering key receptor expression.[47] Thus, *TIGIT* knockout CAR NKs demonstrated enhanced cytokine secretion, improved proliferation, and improved controlled growth of tumors without affecting surface expression of various antigens, for example, T cell immunoglobulin and mucin-domain containing protein 3, NKG2A, programmed cell death protein 1, and NKG2D.[48] Highly immunosuppressive TME also has a resistance mechanism that impairs endogenous as well as therapeutic immune cells.[49] In this regard, CAR NK cells are armed with IL-15 thereby stimulating NK cell survival, enhancing cytotoxicity and persistence in TME. Additionally, Gonzalez *et al*[50] showed that a combination of IL-15 and IL-21 prompts a synergistic effect, which further enhances the NK cell activity. Another resistance mechanism involves the role of A disintegrin

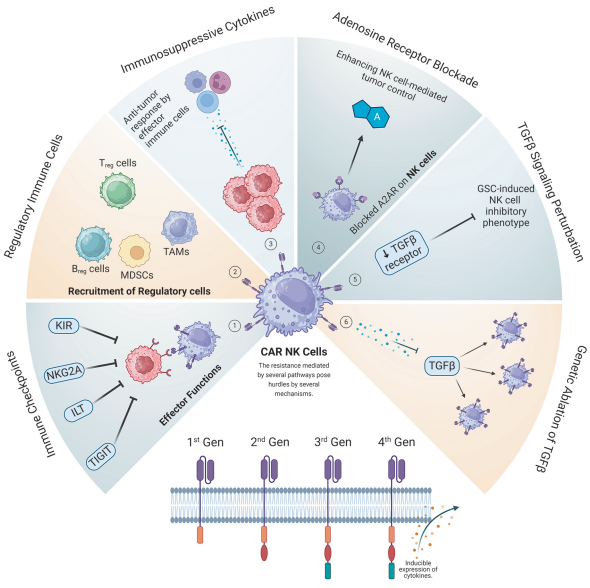

Figure 1: Mechanisms of resistance in CRC targeting NK cells. Resistance mechanisms impacting the efficacy of NK cell-based immunotherapies in CRC involve both intrinsic and extrinsic factors. (1) NK-intrinsic immune checkpoints, including KIR, NKG2A, ILT, and TIGIT pathways inhibit the effector functions of NK cells via interacting with specific tumor cell molecules, dampening the immune response. Additionally, (2) regulatory immune cell compartments, (3) immunosuppressive cytokines, and (4) targeted blockade of A2AR and (5 and 6) transforming growth factor β signaling further contribute to the intricate landscape of resistance in CRC. Advancements in CAR design has led to fourth generation CARs incorporating constitutive or inducible expression of transgenic products, such as cytokines, chemokines, or receptors, further enhancing therapeutic potential. A2AR: A2A adenosine receptor; CAR: Chimeric antigen receptor; CRC: Colorectal cancer; ILT: Immunoglobulin-like transcript; KIRs: Killer immunoglobulin receptors; MDSCs: Myeloid-derived suppressor cells; NK: Natural killer; NKG2D: Natural killer group 2 member D; TAMs: Tumour-associated macrophages; TGFβ: Transforming growth factor β; TIGIT: T-cell immunoglobulin and ITIM domain.

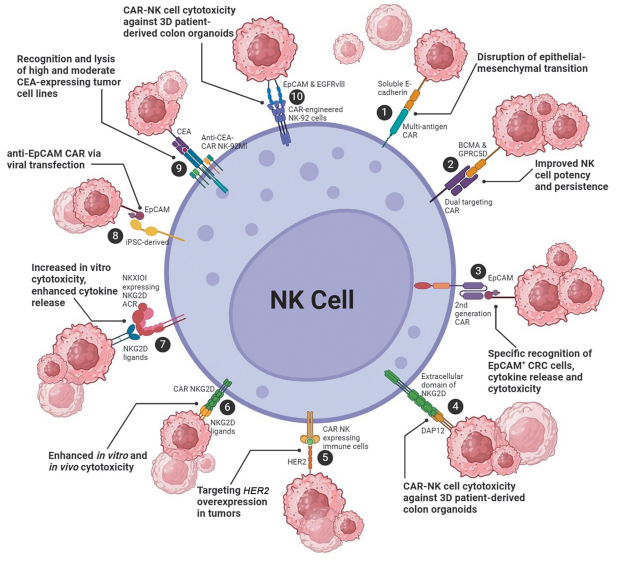

Figure 2: CAR NK cell targeting strategies for tumor antigens. CAR NK cell designs are tailored for distinct targeting mechanisms. For instance, multiantigen CAR disrupts the EMT process by targeting soluble E-cadherin. Dual targeting CAR focuses on BCMA and GPRC5D, enhancing NK cell potency and persistence. The second Gen CAR specifically recognizes EpCAM-positive CRC cells, inducing cytokine release (IFN-γ, perforin, granzyme B) and cytotoxicity. CAR-engineered NK-92 cells targeting EpCAM and EGFRvIII exhibit cytotoxicity against patient-derived colon organoids. iPSC-derived CAR NK cells with anti-EpCAM CAR targeting offer potential therapeutic benefits. Augmented cytolytic activity against solid tumor cell lines is achieved by fusing the NKG2D extracellular domain to DAP12. Additionally, anti-CEA-CAR NK-92MI cells recognize and lyse CEA-expressing tumor cell lines. CAR NK cells expressing NKG2D or NK cell expressed gene 101 (NKX101) with NKG2D activating chimeric receptor exhibit increased *in vitro* and *in vivo* cytotoxicity. Finally, CAR NK cells targeting HER2 overexpression in tumors demonstrate effective antitumor activity. BCMA: B-cell maturation antigen; CAR: Chimeric antigen receptor; CEA: Carcinoembryonic antigen; CRC: Colorectal cancer; EMT: Epithelial–mesenchymal transition; EpCAM: Epithelial cell adhesion molecule; GPRC5D: G protein-coupled receptor 5D; HER2: Human epidermal growth factor receptor 2; IFN-γ: Interferon-γ; iPSC: Induced pluripotent stem cell; NK: Natural killer; NKG2D: Natural killer group 2 member D.

and metalloproteinase 17 (ADAM17) in dampening NK cell activity by cleaving cluster of differentiation 16a (CD16a) ectodomain from the NK cell surface which prevents NK cell attachment to antibody-coated target cells, thereby diminishing CD16a signaling and reducing antibody-dependent cell-mediated cytotoxicity (ADCC) activity. For this problem, Guo *et al*[51] utilized clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-Cas9 ribonucleoproteins to disrupt ADAM17 which increased surface expression of CD16a and improved ADCC activity. The edited NK cells were further engineered to express CAR constructs, demonstrating enhanced cytotoxicity against tumor cells in the presence of specific antibodies.

Expression of CD25 by regulatory T (Treg) cells is a pivotal immunosuppressive element in solid TME especially in CRC.[52] In solution to this, Dehbashi *et al*[53] designed and developed a CAR NK cell targeting CD25 to overcome this immune escape mechanism. By engineering NK-92 cells to express an anti-CD25 CAR construct, the CAR NK cells were designed to specifically detect and lyse target cells with CD25 expression, potentially addressing the resistance posed by Treg cells in the TME. However, future investigations are warranted to validate and optimize this approach. NK cells express both an NK cell-optimized anti-CD19 CAR for direct targeting and a high-affinity, noncleavable CD16 to enhance antibody-dependent cellular cytotoxicity in leukemia and lymphoma.[54] However, this innovative approach seems promising in solid TME in future investigations. Tumor cells escape T cell detection via antigen processing and presentation defects, leading to T cell resistance. Sole T cell immunotherapy often falls short of achieving lasting tumor control due to this evasion. However, the use of programmed cell death ligand 1 (PD-L1) CAR-engineered NK cells to overcome this resistance has shown potential. These engineered cells target and eliminate the resistant tumor cell populations that escape T cell killing by upregulating PD-L1, thereby presenting a synergistic antitumor activity when combined with T cell-based immunotherapy.[55] Although a study involving dual targeting B-cell maturation antigen (BCMA)/G protein-coupled receptor 5D (GPRC5D) CAR NK cells focuses on multiple myeloma,[56] the dual targeting CAR NK approach can inspire similar strategies in solid TME, potentially overcoming antigenic escape and improving treatment efficacy in solid cancers. The promising results pave the way for investigating this strategy in a broader spectrum of cancers with challenging TME.

CAR NK cells utilize immune evasion pathways by precisely targeting distinct biomolecules present on the surface of cancerous cells. An abundance of sialic acid-containing glycans in the glycocalyx of malignant cells contributes to immune evasion, presenting a resistance mechanism against immune cell-mediated cytolysis.[57] In this regard, Antillon *et al*[58] incorporated CAR ligands into tumor cell glycans through the use of a non-natural sialic acid which increased the susceptibility of tumor cells to the cytolytic activity of CAR NK cells, thereby overcoming the immune evasion associated with sialic acid-containing glycans. The long noncoding RNA (lncRNA) ELF1 antisense RNA 1 (ELFN1-AS1) in CRC attenuates the activity of NK cells by down-regulating NKG2D and granzyme B (GZMB) via the growth differentiation factor 15/Jun kinase (GDF15/JNK) pathway, enhancing the ability of CRC cells to escape NK cell surveillance both *in vitro* and *in vivo*.[59] Targeting ELFN1-AS1 in CRC cells could be a potential therapeutic strategy to restore NK cell cytotoxicity and improve the immune surveillance against CRC, making ELFN1-AS1 a promising therapeutic target for CRC. Pleckstrin-2 (PLEK2) has been found in various cancers, especially CRC;[60] thereby a promising therapeutic focus, however, the exact role remains to be elucidated until, Mao *et al*[61] found upregulation of PLEK2 in NK and CD8+ T cell-induced resistance cancer cells. Moreover, they found that PLEK2 upregulates matrix metalloproteinase 1 (MT1-MMP) via the phosphatidylinositol 3-kinase-AKT-SP1 pathway signaling pathway, resulting in the shedding of MHC class I-related chain A (MICA) which ultimately induces immune escape of tumor cells from NK cell surveillance. Thus, PLEK2 knockout increased the sensitivity of gastric cancer (GC) cells to NK cell killing, promoted NK cell infiltration and inhibited intra-peritoneal metastasis of GC cells in mouse xenograft models.

Notably, NK cell engagers have been designed to target multiple activating receptors, which demonstrate enhanced NK cell activation and tumor cell lysis compared to traditional therapies. Trispecific NK engager therapy molecules,[62] targeting NKG2D, CD16A, and tumor antigens, exhibit greater NK cell activation and are in phase I/II trials for various solid tumors. Similarly, antibody-based NK cell engagers (ANKET) mobilize NK cells through NKp46 and CD16, leading to improved NK cell activation and tumor cell lysis *in vitro* and controlling tumor growth in preclinical models.[63] NKp46-ANKET molecules targeting CD19 or CD20 induce tumor cell killing in pediatric leukemia.[64] SAR443579, targeting CD123, shows promise in phase I/II trials for leukemia treatment.[65] Another version targeting NKp46 and CD20, with IL-2 peptide variant (IL-2v), is being developed for B cell malignancies.[66] Flexible NK (FLEX-NK) platform generates high-affinity NK cell engagers, such as CYT-338 targeting CD38, showing superior NK cell effector activities against multiple myeloma. Additionally, FLEX-NK antibody targeting glypican 3 exhibits improved inhibition of hepatocellular carcinoma growth.[67] Lastly, NK cell engagers targeting epidermal growth factor receptor (EGFR) or BCMA, and CD16A, composed of B7-H6, demonstrate efficacy against tumors *in vitro*[68] and in preclinical models.[69] Overall, these findings suggest that NK cell engagers offer a promising approach for the treatment of CRC, showing efficacy and safety in preclinical and early clinical studies compared to conventional therapies.

Unraveling resistance mechanisms like genetic alterations, antigen loss, and immune escape is vital for optimizing efficacy. Among these, genetic approaches appear of great promise, such as employing CRISPR-Cas9 technology for *TIGIT* knockout and ADAM17 disruption to showcase enhanced cytokine secretion, proliferation, and controlled tumor growth showcases a potent genetic approach. This modification, as mentioned earlier in this review, resulted in improved CAR NK cell performance, demonstrating

the potential of genetic interventions in enhancing antitumor responses. Moreover, the use of CRISPR-Cas9 to disrupt ADAM17 not only directly addresses a resistance mechanism but also illustrates the potential of genetic approaches in optimizing CAR NK cell functionality. While other mechanisms show promise, the versatility and specificity offered by genetic modifications make them particularly attractive for future investigations.

Translation in Preclinical and Clinical Studies

The results of preclinical research using CAR NK cells for cancer immunotherapy in numerous solid tumors, including CRC, glioma, breast cancer, ovarian cancer, and neuroblastoma, show the antitumor effects and effectiveness of these cells[70] thus making them enable to enter the clinical trials. Currently, a successful trial (NCT03056339) has demonstrated a promising response to treat CD19-positive cancers with CAR NK cells without the development of major toxic effects.[71] Among the 11 participants, a favorable response was observed in 73% (n = 8). Within this group of responders, seven individuals attained complete remission and one participant achieved remission of the Richter's transformation component but continued to have persistent non-Hodgkin's lymphoma. Furthermore, the CAR NK cells introduced showed expansion and persistence at subdued levels for a duration of at least 12 months.

Since high expression of EpCAM (CD326) is one of the most prevalent changes in solid tumors of epithelial origin, including CRC (NCT03013712), preclinical/clinical research is developing an immune-mediated therapeutic intervention to also target EpCAM-positive cells.[33,72] In a recent preclinical study, the effect of NK-92 modified with a second-generation CAR, targeting EpCAM, in the control/eradication of the CRC line HCT-8-Luc in a subcutaneous xenograft non-obese diabetic/severe combined immunodeficiency mice model was examined. The results showed that CAR NK-92 cells significantly inhibited tumor growth when compared to the control NK-92 cell line.[35] Preclinical phases are essential for recreating the distinct tumor phenotype. The organoid culture technology enables the long-term extracellular 3D matrix growth of gastrointestinal stem cells. CRC organoid was used in a study by Schnalzger *et al*[33] to assess the effectiveness of EpCAM CAR NK-92 and FRIZZLED CAR NK-92. Useful for assessing CAR-engineered lymphocytes is this 3D platform.

Enhancing NK antitumor activity may include inducing overexpression of activating receptors. In an effort to achieve this, Xiao *et al*[35] showed in 2019 that intraperitoneal injection of short-lived PB-derived CAR NK cells, produced by RNA electroporation with a construct coding for NKG2D extracellular domain combined to DAP10-associated protein 12 (DAP12) signaling moiety (NKG2D CAR NK), significantly decreased tumor burden and progression in xenograft mice created with human CRC cell lines. Additionally, three patients with resistant metastatic CRC were successfully treated with a local infusion of NKG2D CAR NK (one in an autologous context and two in a haploidentical environment) in the associated pilot clinical trial (NCT03415100). Two patients in particular saw a reduction in the amount of cancer cells in their ascites fluid, while another patient's liver metastasis showed a full metabolic response. These encouraging findings suggest that targeted CAR NK cell therapy for solid tumors may be a promising therapeutic approach. To determine if the treatment outcome will remain effective over time, more analyses are required. Another clinical trial (NCT05213195), which just got underway and is evaluating the outcomes of intraperitoneal and intravenous NKG2D-CAR NK infusion in patients with refractory metastatic CRC, will also provide additional insightful results in this regard. Preclinical studies analyzing the antitumor activity of allogeneic healthy donors' NK cells modified with a chimeric NKG2D receptor fused to co-stimulatory (OX40) and signaling (CD3) domains (to enhance their intrinsic activity) and equipped with membrane-bound IL-15 (to enhance *in vivo* persistence) have produced additional interesting results aimed at increasing NKG2D-mediated killing activity of NK cells against CRC liver metastases.[37,38] Regorafenib, a multikinase inhibitor having activity against many protein kinases involved in oncogenesis[73,74] and successfully utilized to treat resistant metastatic CRC, may also be used in combination with other treatments to increase the antitumor response.[75]

Since CEA (tumor antigen) is largely expressed in CRC tissues and little expressed in healthy adult tissues (lung cells and gastrointestinal–epithelial cells), it is thought to be another protein that can be used to guide targeted therapy (NCT02349724).[76] The ability of anti-CEA CAR NK-92MI, an IL-2 independent derived NK-92 cell line, to detect and eradicate CEA-expressing tumor cells at high and moderate levels has been demonstrated in a preclinical investigation.[77] Since chemotherapy commonly causes CEA to upregulate, anti-CEA-modified NK cells may be a secondary rescue line of treatment for CRC that is resistant to other forms of therapy. However, since clinical outcomes obtained by targeting CEA with CAR T cells have shown high toxicity, further investigation of CEA cell-mediated targeting will be necessary (NCT01212887). This is because CAR T-induced cytokine storm upon recognition of antigen on tumor tissues may also be related to nontumor tissues.[78]

Mucin 1 (MUC-1) and human epidermal growth factor receptor 2 (HER2) appear to be viable targets for cell-mediated therapy within the larger group of molecules being investigated as CAR targets for CRC treatment. A growing body of research indicates that MUC-1, a highly glycosylated protein that is highly expressed in CRC cells, is a potent target for a variety of immunotherapy techniques, including the creation of altered NK cells that are armed with anti-MUC-1 CAR. In this vein, a phase I/II clinical trial (NCT02839954), aims to examine the efficacy and safety of anti-MUC-1 CAR NK cell immunotherapy in individuals with MUC-1+ relapsed or refractory solid malignancies.[79] This clinical trial uses anti-MUC1 CAR NK cells for patients with solid tumors, such as CRC, however, the status is unknown. Additionally, patients are being enrolled in phase I clinical investigation (NCT04319757) to examine the security and preliminary effectiveness of anti-HER2 oNK cells

(ACE1702) against solid cancers that express the human *HER2* gene.[80] Starting from September 2021, an early phase I clinical trial is currently recruiting participant in Guangdong, China and aims to evaluate clinical safety and feasibility of NKG2D CAR T administrated in nine enrollments (NCT05248048).

The EGFR family member HER2 is overexpressed in gastric adenocarcinomas, breast cancer, and CRC, and is associated with disease stage and decreased survival.[39,81–83] Additionally, a clinical translation of a product developed from NK-92 for the treatment of solid tumors that express HER2 is being studied (NCT04319757). It has recently been demonstrated that a modified subpopulation of NK-92, which expresses functional endogenous CD16 and has been further modified by conjugation with Trastuzumab, an anti-HER2 antibody, displayed enhanced cytotoxicity against HER2-positive targets both *in vivo* and *in vitro*. In comparison to other created goods, the lack of cell manipulation via viral vector or transposon methods, which could cause viral insertion mutation or imprecise chromosomal insertion, respectively, might be advantageous.[84,85]

Then, in CRC, evaluation of NK cells in the TME and peripheral blood led to the conclusion that lower NK cell frequencies were associated with an increased risk of disease occurrence and progression, as well as a bad prognosis.[84] However, HER2-CAR NK-92 cells effectively cleared HER2-positive GC cells through advanced levels of *in vitro* cytokine production. Small tumor xenografts could be eliminated by effector cells *in vivo*, whereas bigger gastric tumors were not appreciably impacted by HER2-CAR NK-92 cells.[86] Nevertheless, after being infused with apatinib, a tyrosine kinase inhibitor that only inhibits the vascular endothelial growth factor receptor-2 (VEGFR-2), NK cells' penetration into large tumor xenografts and their therapeutic potential were enhanced.[86]

Furthermore, the effective CRISPR/Cas9 genome-editing technologies that have recently been developed have given rise to new options to make NK cells more susceptible to NK surveillance. Cellular immunotherapy for CRC by Gao *et al*[84] showed that upregulating CXCR2 and IL-2 by CRISPR-Cas9 boosted NK-92 cell antitumor activities and greatly increased survival time.

The absence of trafficking and penetration of these cells into the tumor tissue is one of the major difficulties in CAR cell therapy for solid tumors, as was previously highlighted. To address this issue, chemokine receptors connected to chemokines produced by tumor cells are typically used, chemotactically attracting CAR cells to the tumor site. In this context, CXCR1 and NKG2D were expressed on electroporated CAR NK cells *in vitro*, and these cells increased TME trafficking and migration in mice bearing established peritoneal ovarian cancer xenografts. Interestingly, CXCR1 expression does not affect CAR NK cell cytotoxicity.[30] It has been established that extracellular matrix (ECM) scaffold is necessary for efficient function (attachment and crawling) of anti-EpCAM CAR NK cells and that culture of colon organoids in suspension or scaffold is important for effective organoid death by CAR NK cells. As a result, more research must be done on how CAR cells interact with the ECM.[87] The FRIZZLED (Wnt receptor) and EGFR variant III (EGFRvIII) tumor antigens are two significant high-expression tumor antigens in CRC, and research has demonstrated that anti-EGFRvIII CAR NK cells have the ability to kill colon organoids with little off-target damage.[88] One difficulty in choosing FRIZZLED as an antigen tumor in CAR NK cell therapy is that anti-FRIZZLED CAR NK cells can kill organoids independent of the expression of the FRIZZLED receptor, which causes mucosal toxicity.[33,89]

The advancements in CAR NK cell therapy underscore its potential as a transformative approach for treating CRC, showcasing the ongoing research and development in this field. For a detailed account of the targets and their respective advancements, refer to Table 3. Nonetheless, challenges such as optimizing the trafficking of CAR NK cells to tumor tissues and selecting optimal tumor antigens to minimize off-target effects remain areas that warrant

Table 3: Clinical progress in targeting antigens using CAR NK cell therapy for CRC.

Antigen target	CAR NK design	Clinical potential	Status	NCT number
EpCAM	Second Gen CAR	A promising strategy for treating CRC enhanced therapeutic efficacy in combination with regorafenib	Ongoing	NCT03013712
NKG2D	NKG2D CAR	Significant therapeutic benefit in solid tumor models; Promising results in patients with metastatic CRC	Ongoing	NCT03415100
NKG2D	NKG2D-CAR NK cells	Promising therapeutic potential in treating metastatic CRC patients	Recruiting	NCT05213195
CEA	Anti-CEA CAR NK-92MI	Potential therapeutic approach for CEA-expressing tumors	Not yet recruiting	NCT02349724
MUC-1	Anti-MUC1 CAR NK cells	Investigating efficacy and safety in MUC-1 + relapsed or refractory CRC	Recruiting	NCT02839954
HER2	Anti-HER2 oNK cells	Assessing safety and preliminary effectiveness against solid cancers expressing HER2	Not yet recruiting	NCT04319757

CAR: Chimeric antigen receptor; CEA: Carcinoembryonic antigen; CRC: Colorectal cancer; EpCAM: Epithelial cell adhesion molecule; HER2: Human epidermal growth factor receptor 2; MUC-1: Mucin 1; NK: Natural killer; NKG2D: Natural killer group 2 member D.

continued research and development in this promising therapeutic approach.

Current Challenges in Clinical Transformation

Although there is promising evidence from preclinical and early clinical studies regarding the potential of CAR NK cell therapies, there exist several challenges that need addressing for their practical application. A significant hurdle is the limited persistence of CAR NK cells, affecting their long-term effectiveness.[90] However, the introduced CAR NK cells displayed expansion and a lasting presence, though at moderate levels, for at least 12 months.[71] These values showed an increase and persisted in the peripheral blood for up to a year post-infusion, irrespective of the administered dose level, with no observed correlation between the administered cell dose and the CAR NK copy number beyond the 14th day after infusion.

Another issue is the absence of a specific transporting pathway for CAR NK cells, hindering their infiltration and trafficking into tumor sites.[91] The immunosuppressive TME poses a significant obstacle to CAR NK cell therapy, impairing their function.[92] Additionally, the low efficiency of lentivirus transduction in CAR NK cells limits their overall effectiveness.[93] Obtaining high-purity activated NK cells for clinical use is also challenging.[94] Addressing these challenges is crucial for enhancing the acceptance and efficacy of CAR NK cell therapy for CRC in clinical settings.

While CAR NK therapy has shown promise in various clinical trials, addressing the limited lifetime of CAR NK cells in circulation and the potential side effects on normal tissues and graft-*vs*.-host disease is essential.[95] Furthermore, the lack of comprehensive clinical data, with only a few patients treated in clinical trials, makes it challenging to draw definitive conclusions about the efficacy and safety. The manufacturing process for CAR NK cells is complex, requiring specialized facilities and expertise, limiting scalability and increasing therapy costs. The antigen heterogeneity of CRC presents a challenge for developing CAR NK cells effective against diverse antigens. CRC patients often exhibit systemic immunosuppression, impacting CAR NK cell function and persistence. Overcoming this immunosuppression is critical for ensuring therapy efficacy. Navigating regulatory pathways for CAR NK cell therapy approval and commercialization poses a significant challenge, especially given the evolving regulatory frameworks for advanced cell therapies.

Conclusion and Future Prospects

In conclusion, the field of CAR NK cell immunotherapy for CRC is rapidly evolving, showcasing immense potential in targeting specific tumor antigens and advancing toward clinical applications. Antigen targets such as EpCAM, CEA, and soluble E-cadherin have demonstrated promise in precise CRC treatment. These targets, highlighted by the extensive preclinical research, provide a foundation for potential clinical translation. The utilization of CAR NK cells, armed with antitumor activity and engineered for enhanced targeting mechanisms, presents a transformative phase in cancer therapeutics, not only for CRC but also for various other malignancies. However, several challenges lie ahead. Optimizing trafficking and penetration of CAR NK cells into tumor tissues is a significant hurdle that needs to be effectively addressed to maximize therapeutic efficacy. Additionally, the selection of appropriate tumor antigens is crucial to minimize off-target effects and enhance the specificity of CAR NK cell therapy. Further research is imperative to fine-tune these aspects and bring CAR NK cell immunotherapy for CRC to its full potential.

In the coming years, concerted efforts should focus on conducting rigorous clinical trials, leveraging the preclinical success, and addressing the aforementioned challenges. Collaborations between researchers, clinicians, and industry partners are pivotal to driving this promising therapeutic approach forward and potentially revolutionizing the treatment landscape for CRC and other solid tumors.

Conflicts of interest

None.

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